INFLAMMATORY MEDIATORS: THEIR ROLES DURING PREGNANCY AND PARTURITION

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ABSTRACT

Pregnancy and parturition necessitate profound alterations in the maternal immune response likely effected by paracrine interactions between inflammatory mediators, hormones and various local factors. However, the nature of these interactions and how they effect the recognition and maintenance of pregnancy and initiation of parturition are not well understood. The aim of this thesis was to investigate the role of inflammatory mediators, specifically cytokines, prostaglandin E_2 (PGE_2), nitric oxide (NO), metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs) during pregnancy and parturition.

Part 1.

This investigated the roles of inflammatory mediators in the initiation and maintenance of pregnancy. The role of seminal plasma, specifically its component PGs (PGE_2 and 19-hydroxy PG), in the initial adaptation of the maternal immune response to pregnancy was studied in the non-pregnant cervix, peripheral blood and a monocyte cell line. PGs stimulated release of IL-10, thus favouring development of a T-helper (Th)-2 type, pregnancy favourable immune response. They also stimulated release of the neutrophil chemotactic factor IL-8, which may be involved in mediating post-coital cervical leukocytosis. Furthermore, peripheral blood from pregnant women, specifically the mononuclear cell ($CD14^+$) fraction, released significantly more monocyte chemotactic peptide-1 (MCP-1), which also favours a Th-2 type immune response, than that from non-pregnant women. Release of IL-8 and regulated on activation and normally T-cell expressed and secreted (RANTES) was comparable between groups. The concentrations of MCP-1, IL-8 and RANTES in the fluid compartments within the first trimester uterus were also examined. The chemokines were differentially distributed with MCP-1 and IL-8 being present in amniotic fluid, maternal serum and extra-embryonic coelom with highest levels of both in the latter compartment. RANTES was only detectable in peripheral serum. These findings support the hypothesis that inflammatory mediators are important in the initiation and maintenance of pregnancy and may play a role in early placental and fetal development.

Part 2.

This examined the involvement and regulation of inflammatory mediators in cervical ripening and parturition. Cervical ripening involves tissue remodelling mediated by inflammatory mediators, infiltrating cells, MMPs and TIMPs. However their regulation in the cervix are not well understood. In the non-pregnant cervix, IL-8 release was stimulated by PGE_2 and NO and inhibited by dexamethasone and progesterone. In

addition, the anti-inflammatory factor secretory leukocyte protease inhibitor was stimulated by progesterone and inhibited by PGE_2 and PGE_2 release was stimulated by NO. In addition, MMP-2 and -9 and TIMPs-1 and -4 were released by the non-pregnant cervix but this secretion was not affected by *in vitro* administration of PGE_2 or NO. Withdrawal of progesterone, which is essential for pregnancy maintenance, also initiates cervical ripening by unknown mechanisms. *In vivo* administration of the anti-gestogen mifepristone increased protein expression of MMP-1, -8 and -9, CD45 (leukocyte common antigen), neutrophil elastase and CD68 (monocyte marker) but not MMP-2, TIMP-1, -2 and -4 in the first trimester cervix as detected by immunohistochemistry. To summarise the cervical studies, the non-pregnant and pregnant cervix are capable of releasing a wide range of inflammatory mediators, MMPs and TIMPs, co-ordination of which may mediate cervical ripening. Next, the release and regulation of inflammatory mediators at the materno-fetal interface were investigated. MCP-1, IL-8, RANTES and IL-10 were released by third trimester amnion, chorion, decidua and placenta with secretion of MCP-1, IL-8 and IL-10 but not RANTES being stimulated by PGE_2 in a perfused placental cotyledon system. Secretory leukocyte protease inhibitor was released predominantly by decidua and was present in increasing concentrations within amniotic fluid during pregnancy and labour. Cytokines may play a role in the inflammatory process of parturition, PGE_2 may play an important immunomodulatory role within the placenta at term and secretory leukocyte protease inhibitor might act both to limit the pro-inflammatory cascades ongoing during parturition and to protect against microbial invasion. To summarise this section, inflammatory mediators are important in the initiation and regulation of cervical ripening and parturition with complex interactions occurring within the cervix and at the feto-maternal interface.

DECLARATION

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Dr Fiona Charlotte Denison

PUBLICATIONS

The following publications have arisen from work undertaken in this thesis:

Ledingham, M.A., Denison, F.C., Riley, S.C. and Norman, J.E. (1999) Matrix metalloproteinases -2 and -9 and their inhibitors (TIMPs) are produced by the human uterine cervix but their secretion is not regulated by nitric oxide donors. Human Reproduction. (Accepted)

Denison, F.C., Riley, S.C., Calder, A.A. and Kelly, R.W. (1999) Production of secretory leukocyte protease inhibitor increases within the uterus with the onset of labour: Characterisation of sites of synthesis in the uterus. Journal of Endocrinology. (In Press)

Denison, F.C., Calder, A.A., and Kelly, R.W. (1999) The action of PGE_2 on the human cervix: stimulation of IL-8 and inhibition of leukocyte protease. American Journal of Obstetrics and Gynaecology 180, 614-620

Denison, F.C., Grant, V., Calder, A.A. and Kelly, R.W. (1999) Seminal plasma components stimulate interleukin-8 and interleukin-10 release. Molecular Human Reproduction 5, 220-226

Denison, F.C., Kelly, R.W., Calder, A.A. and Riley, S.C. (1998) Cytokine secretion by human fetal membranes, decidua and placenta at term. Human Reproduction 13, 3560-3565

Denison, F.C., Riley S.C., Wathen N.C., Chard T., Calder, A.A., and Kelly, R.W. (1998) Differential concentrations of MCP-1 and IL-8 within the fluid compartments present during the first trimester of pregnancy. Human Reproduction, 13, 2292-2295

Denison, F.C., Kelly, R.W. and Calder, A.A. (1997) Differential secretion of chemokines from peripheral blood in pregnant compared with non-pregnant women. Journal of Reproductive Immunology, 34, 225-240

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ABBREVIATIONS

AA	Arachidonic acid
ABC - HRP	Avidin - Biotin - Complex, conjugated to horse radish peroxidase
ACTH	Adrenocorticotrophin
AF	Amniotic fluid
AP-1	Activator protein-1
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanine monophosphate
CD	Cellular differentiation marker
CMV	Cytomegalovirus
COX	Cyclo-oxygenase
CRH	Corticotrophin releasing hormone
DAB	3, 3' - diaminobenzidine
DAR	Donkey anti-rabbit
DARC	Duffy antigen receptor for chemokines
DHEAS	Dehydroepiandrosterone
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E_2	Oestradiol
ECM	Extra-cellular matrix
EDGF	Epidermal derived growth factor
EECF	Extra-embryonic coelomic fluid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
Fab	Fragment antibody binding
FAD	Flavine adenine dinucleotide
FCS	Fetal calf serum
FMN	Flavine mononucleotide
G	Guanine
GAGs	Glycosaminoglycans
GDP	Guanine diphosphate
GTP	Guanine triphosphate

GM-CSF	Granulocyte macrophage colony stimulating factor
GNRH	Gonadotrophin releasing hormone
GPI	Glycosylphosphatidylinositol
hCG	Human chorionic gonadotrophin
HIV	Human immunodeficiency virus
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IP_3	Inositol 1,4,5-triphosphate
IP-10	Immune protein-10
LBP	Lipopolysaccharide binding protein
LHRH	Luteinising hormone releasing hormone
Li	Lithium
LPS	Lipopolysaccharide
MACS	Magnetic cell sorter
MCP-1	Monocyte chemotactic peptide-1
M-CSF	Monocyte colony stimulating factor
MDC	Monocyte derived chemotaxin
MDNCF	Monocyte derived neutrophil chemotactic factor
MHC	Major histocompatibility complex
MIP	Monocyte inhibitory peptide
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MS	Maternal serum
mw	Molecular weight
NADPH	Nicotinamide adenine diphosphate
NAF	Neutrophil activating factor
NAP-2	Neutrophil activating peptide-2
NBF	Neutral buffered formalin
NCF	Neutrophil chemotactic factor
NFκB	Nuclear factor kappa-beta
NK	Natural killer
NP	Non-pregnant
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide

NOS	Nitric oxide synthase
NSB	Non-specific binding
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PG	Prostaglandin
PGDH	Prostaglandin Dehydrogenase
PGHS	Prostaglandin H endoperoxidase synthase (synonym: cyclooxygenase)
PGI_2	Prostacyclin
PLA_2	Phospholipase A_2
PMA	Phorbol 12-myristate 13-acetate
PLSD	Protected least squared difference
RANTES	Regulated on activation and normally T-cell expressed and presumably secreted
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Medium
RU486	Mifepristone
SLPI	Secretory leukocyte protease inhibitor
SNP	Sodium nitroprusside
TBS	Tris buffered saline
TGF	Transforming growth factor
Th-1	T helper cell type 1
Th-2	T helper cell type 2
TIL	Term in labour
TIMP	Tissue inhibitor of metalloproteinases
7-TMB	Seven transmembrane
TNF	Tumour necrosis factor
TNIL	Term not in labour
TXA_2	Thromboxane A_2
VCAM-1	Vascular cell adhesion molecule-1
VIP	Vasoactive intestinal peptide

CHAPTER 1

LITERATURE REVIEW

1.1 PREGNANCY, PARTURITION AND INFLAMMATION

Many reproductive processes depend on the timely activation and regulation of inflammatory processes. Inflammation is the physiological response to most types of tissue injury, infection, immunological stimulation and foreign or altered host antigen. Systemically, it is manifested by release of acute phase proteins by the liver and elevation of the body temperature. At the local level, it is characterised by an initial vasodilation and increased vascular permeability, followed by directional accumulation and extravasation of effector cell into the surrounding tissue (neutrophils and monocytes then B- and T-cells). A wide range of inflammatory mediators are involved in co-ordinating these events including cytokines, chemokines, anaphylotoxins of the complement cascade, kinins of the coagulation cascade, prostaglandins, leukotrienes and other fatty acid or lipid mediators.

The initiation, recognition and maintenance of pregnancy, control of cervical ripening and stimulation and regulation of parturition are processes in which inflammatory mediators, by interacting with hormones and various local factors, are thought to have a pivotal role. The nature and regulation of these interactions are not well understood. However, it is vitally important that these mechanisms, which operate in normal pregnancy, are better understood. Until this is achieved, therapeutic options available to treat pathophysiological situations such as implantation failure, recurrent miscarriage and preterm labour will remain sub-optimal.

The ensuing literature review will initially cover some of the cytokines, chemokines and other inflammatory mediators, which are likely involved in generating and regulating inflammatory and immunological responses during pregnancy. The current knowledge as to the roles of inflammatory mediators in the specific processes of the initiation and maintenance of pregnancy, control of cervical ripening and parturition will then be considered.

1.2 CYTOKINES

Cytokines are soluble proteins or glycoproteins that act as humoral regulators at nano- to pico-molar concentrations under normal or pathological conditions to modulate functional

activities of individual cells or tissues. In addition, they mediate interactions between cells directly and regulate process occurring in the extracellular environment by autocrine, paracrine and juxtacrine means. Cytokines differ from classical hormones in they have many sites of production, a high degree of biological redundancy and pleiotropy, act on relatively selective cellular targets and are rarely found in the circulation. They bind to specific receptors on the surface of the target cell, which are coupled to intra-cellular signalling systems and second messenger pathways, thus effecting their action.

The rapidly expanding cytokine family is diverse and can be subdivided into many divisions including the interleukins (IL-1 to IL-18, to date), colony stimulating factors (GM-CSF, C-CSF, M-CSF), interferons (IFN-α, -β and -γ) and growth factors (platelet derived growth factors, PDGF; epidermal derived growth factors, EDGF; transforming growth factors, TGF). Alternatively, cytokines can be classified according to their principal function into pro-inflammatory, anti-inflammatory and immunomodulatory mediators. Due to huge number of different cytokines, only those that are studied in this thesis or which have relevance to reproduction will be discussed.

The pleiotrophic functions of cytokines necessitate that their release is tightly regulated. This occurs at many levels.

Gene expression

Although a few cytokines (IL-6) are constitutively expressed, the majority are expressed and synthesised only after stimulation by agents such as infection and inflammation. The synthesised cytokines may then be stored in granules (PDGF), bound to membrane proteins (tumour necrosis factor α, TNF-α; IL-1) or complexed to extra-cellular matrix (ECM) or membrane receptors (IL-8).

Processing

Many cytokines require enzymatic cleavage (IL-1, TNF-α) to be activated although the *in vivo* mechanism remains to be established for some (transforming growth factor-β, TGF-β).

Sequestration

Certain cytokines are sequestered, binding to molecules like heparin sulphate, decorin and cellular differentiation (CD) 44 in ECM (IL-1α), stromal cells or endothelial cells (IL-8).

Other cytokines are bound to soluble binding molecules that may sequester, transport or activate them.

Receptor modulation

The effect of cytokines can be antagonised at the level of the receptor by natural receptor inhibitors (IL-1α receptor antagonist) or by down- or up-regulation of receptor expression.

1.21 PRO-INFLAMMATORY

Interleukin-12

Background

Cytokine natural killer cell stimulatory cell factor or cytotoxic lymphocyte maturation factor was initially discovered in 1989 as a stimulator of natural killer cell proliferation and augmentor of cytotoxic lymphocyte responses. It was subsequently renamed interleukin-12 (IL-12; Gubler, et al., 1991).

Gene and protein structure

IL-12 is unusual in that it is a 70 kDa heterodimer composed of a p35 and a p40 subunit encoded by 3p12-q13.2 and 5q31-q33, respectively (Snijders, et al., 1996). The production of each subunit is regulated independently, mainly at the mRNA level, by transcription factors such as nuclear factor kappa-beta (NFκB; Murphy, et al., 1995) with the amount of bioactive IL-12 being determined by the level of p35 expression.

Receptors

The IL-12 receptor is a 110 kDa protein which is expressed on natural killer (NK) cells and activated peripheral blood mononuclear cells (1000 - 9000 per cell) and $CD4^+$ and $CD8^+$ T lymphocytes (Desai, et al., 1992).

Sources

IL-12 is released by a wide range of cells including monocytes and macrophages (Blotta, et al., 1997), B-cells, polymorphonuclear cells (Cassatella, et al., 1995) and Langerhan cells (Kang, et al., 1996). Release of IL-12 is stimulated by GM-CSF, IFN-γ (Trinchieri and Gerosa, 1996), HIV surface glycoprotein 120 (Fantuzzi, et al., 1996), class II MHC

(Koch, et al., 1996), interaction with CD40 - CD40 ligand (Kato, et al., 1966) and lipopolysaccharide (LPS) (Hilkens, et al., 1996) and inhibited by corticosteroids (Blotta, et al., 1997), monocyte chemotactic peptide-1 (MCP-1; Chensue, et al., 1996), IL-10 (D'Andrea, et al., 1993), TGF-β (Hunter, et al., 1995), IL-4 (Koch, et al., 1996) and prostaglandin E_2 (PGE_2; Kelly, et al., 1997; Kraan, et al., 1995).

Biological Function

IL-12 favours a T helper-1 (Th-1) type of immune response (Gazzinelli, et al., 1994), described in detail later, by stimulating IFN-γ synthesis (Gerosa, et al., 1996) and inducing cytotoxic T-cell differentiation (Kang, et al., 1996). It also effects differentiation and activation of NK cells and cytotoxic T-cells via stat 4 (Thierfelder, et al., 1996), inhibits IL-13 release, immunoglobulin E (IgE) class switching (Morris, et al., 1994) and apoptosis (Clerici, et al., 1994).

Other pro-inflammatory cytokines

IL-1 and TNF-α are important cytokines involved in initiating many inflammatory reactions (Brannstrom, et al., 1993).

The IL-1 system comprises the two ligands, IL-1α and IL-1β, which share 26 % homology, receptors (type 1 and type 2) and the IL-1 receptor antagonist. Secreted by most cells, IL-1 release is stimulated many factors including TNF-α, IFN-γ, bacteria and LPS. In addition to its potent pro-inflammatory effects, it stimulates T-helper cells, immunoglobulin production, pituitary adrenocorticotrophic releasing hormone (ACTH) release, astro- and microglial cell proliferation, is prothrombotic and activates neutrophils.

TNF-α is secreted by many different cell types including monocytes, macrophages, T-cells, neutrophils and NK cells with its release being stimulated by many factors including IL-6, TGF-β, bacteria and LPS. Via binding to its cognate receptors (type 1 and type 2), TNF-α induces cytolysis, cytostasis, coagulation, neutrophil chemotaxis, angiogenesis and stimulates release of collagenases and PGE_2.

1.22 ANTI-INFLAMMATORY

Interleukin-10 (IL-10)

Background

The cDNA encoding a cytokine synthesis inhibitory factor was first identified from a library of activated murine T cell clones in 1989. The human homologue was subsequently cloned, expressed and renamed interleukin-10 (For reviews see: Trotta and Windsor, 1995; Yssel and de Waal Malefyt, 1995).

Gene and protein structure

The human IL-10 gene (4.7 kBa), contains five exons, four introns and binding sites for a wide number of transcription factors including glucocorticoids and cyclic adenine monophosphate (cAMP). The mature encoded IL-10 protein contains 160 amino acids and exists as a dimer in solution (Trotta and Windsor, 1995).

Receptors

The human IL-10 receptor, which is structurally related to the IFN-γ receptor, is a 61 kDa protein encoded by chromosome 11 (Tan, et al., 1993). It is expressed by cells of haemopoietic origin including monocytes, B-cells, NK cells and T-cells.

Sources

IL-10 is released by a wide range of different cell types including T lymphocytes (Yssel and de Waal Malefyt, 1995), eosinophils (Nakajima, et al., 1996), Langerhan (Chang, et al., 1995), dendritic (deSaintVis, et al., 1998) and microglial cells (Ehrlich, et al., 1998). Its release is regulated locally at the paracrine and autocrine level (Cohen, et al., 1997) by many factors including IL-12 (Peritt, et al., 1995), IL-8 (Lu, et al., 1995), PGE_2 (Kraan, et al., 1995) and glucocorticoids.

Biological Functions

IL-10, as its initial name cytokine synthesis inhibitory factor suggests, is a potent immunosuppressive and immunomodulatory cytokine. It has profound effects on T cell function favouring development of a T helper-2 (Th-2) type of immune response (Mosmann and Moore, 1991), suppressing T-cell proliferation (Ayala, et al., 1994), downregulating expression of the T-cell co-stimulatory ligand B7-1 (Iglesias, et al., 1997)

and inducing anergy in T-cell clones (Groux, et al., 1996; Groux, et al., 1997). In addition, IL-10 induces apoptosis (Estaquier, et al., 1997) and is important in post-operative immunosuppression (Klava, et al., 1997) and transplantation tolerance (Bromberg, 1995).

1.23 IMMUNOMODULATORY CYTOKINES

Interleukin-6

Interleukin-6 (IL-6) is a pleiotrophic, immunomodulatory cytokine released by many cells. Its receptors are expressed predominately on peripheral monocytes, macrophages, T- and B-lymphocytes. IL-6 has been described as a "physiological regulator of the acute phase reaction" and in addition, is a potent haemopoietic agent, B- and T cell differentiation factor, neurotroph and stimulator of pituitary ACTH.

1.3 CHEMOKINES

Chemokines, previously referred to as members of the intercrines or small cytokine family (scy family), are a subset of cytokines that are specific in their ability to induce chemotaxis and activation of different cell types (Baggiolini, 1998; Baggiolini, et al., 1997; Mantovani, et al., 1996). The chemokine family comprises small heparin binding glycoproteins (8 - 10 kDa), which demonstrate 20 - 50 % sequence homologies at the amino acid sequence and have common gene structures. In addition, they are identified by the presence of four conserved cysteine residues that are involved in intramolecular disulphide bonds giving them comparable secondary structures. Chemokines can be divided structurally, by the positioning of the first two amino-terminal cysteine residues, into one of four subfamilies (Figure 1). The C-X-C (cysteine - X amino acid - cysteine) or α-chemokines are characterised by separation of the cysteine pair by a non-conserved amino acid. In contrast, the two cysteine amino acids are adjacent in C-C (cysteine – cysteine) or β-chemokines. Recently two further classes of chemokines have been identified. The C or γ subclass (Kelner, et al., 1994) have a single cysteine at the N-terminal. To date, the sole member of this class is lymphotactin, which is chemotactic for T and B lymphocytes but not neutrophils and monocytes. The final subfamily is the fractalkines, characterised by the C-X-X-X-C (Bazan, et al., 1997) motif.

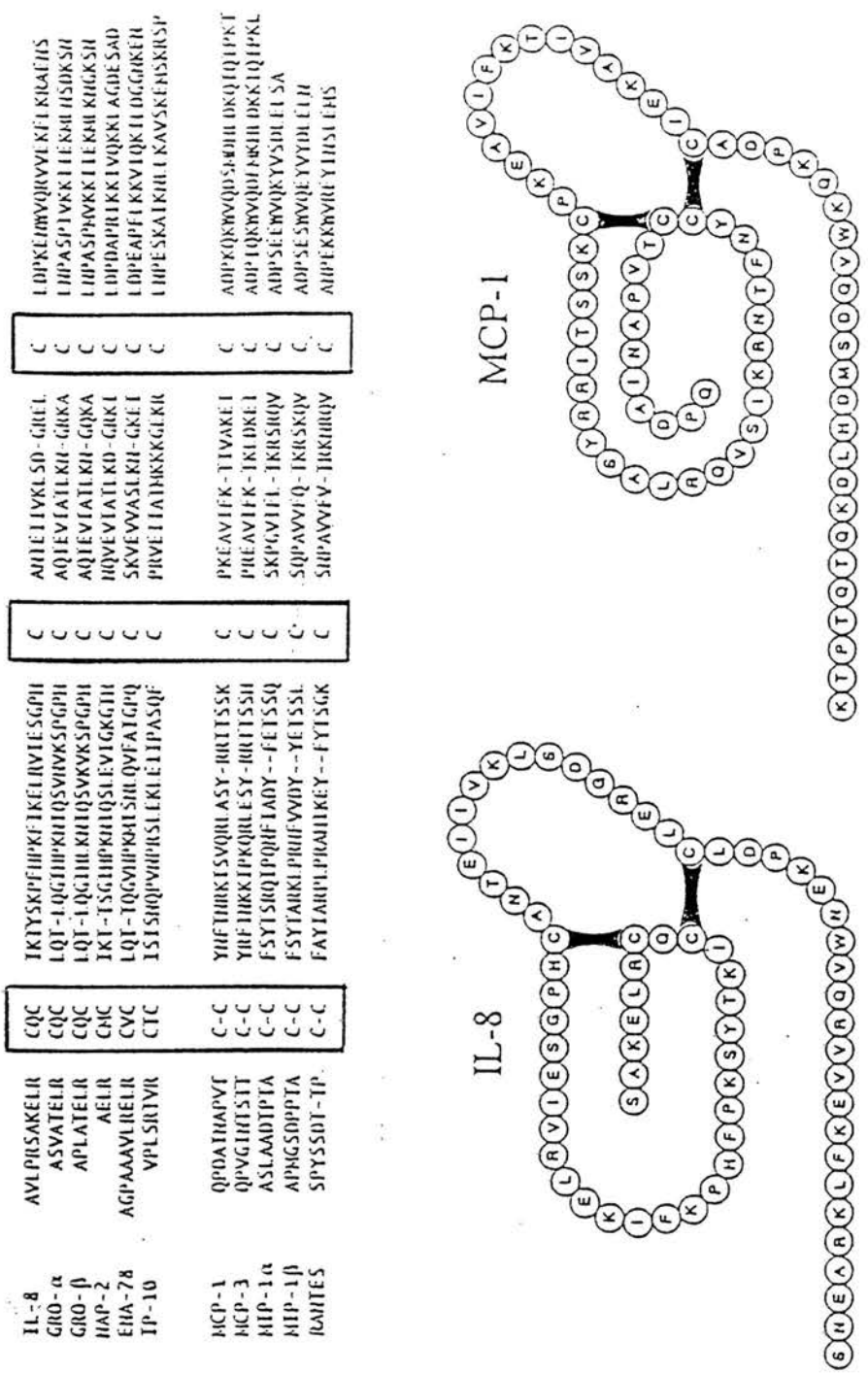


Figure 1

Amino acid sequences and two-loop structure of representative C-X-C and C-C chemokines. Sequences are aligned according to the four highly conserved cysteine residues, shown in boxes. The secondary structures for IL-8 and MCP-1 are also illustrated with their disulphide bonds. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp and Y, Tyr *(Modified from Baggiolini et al., 1994)*

1.31 LEUKOCYTE CHEMOTAXIS

The key property of chemokines is their ability to induce leukocyte chemotaxis. Leukocyte migration is a complex multistep process (Dunon, et al., 1996; Fowman, et al., 1997) and is thought to occur in a number of sequential steps.

1. Leukocytes constitutively express the cell adhesion molecule, L-Selectin, which contains the carbohydrate sialyl ligand, Lewis X. At inflammatory sites, pro-inflammatory cytokines such as TNF-α upregulate expression of E- and P-Selectin on vascular endothelium. Leukocytes then bind transiently to E- and P-Selectin on the activated endothelium via L-Selectin. The binding is weak permitting rolling of leukocytes along the endothelial wall.

2. High local concentrations of cytokines and chemokines induce shedding of L-Selectin from the leukocyte surface and upregulation and activation of β1 and β2 integrin expression. The integrins bind strongly to their counterligands, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), expressed on vascular endothelium, thus arresting leukocyte rolling. Leukocytes subsequently flatten and adhere tightly to the endothelial surface.

3. Within tissue stroma, chemokines adhere to glycosaminoglycans (GAGs), such as heparin and chondroitin sulphate (Koopman and Krangel, 1997; Middleton, et al., 1997; Witt and Lander, 1994). This creates a transendothelial chemotactic gradient facilitating leukocyte extravasation from the vasculature and subsequent migration to the site of high chemokine concentration within the stroma.

1.32 CHEMOKINE RECEPTORS

Chemokines act via seven-transmembrane binding (7-TMB) receptors, which are linked, to guanine nucleotide binding (G) proteins. The 7-TMB receptors form part of the rhodopsin or serpentine superfamily of receptors which mediate many biological functions including neurotransmission, hormone action and light perception (Strader, et al., 1994). The receptors (339 - 373 amino acids) comprise an extra-cellular N-terminal domain connected to the intra-cellular C-terminal domain by seven hydrophobic α-helical

transmembrane spanning regions, which form three intracellular and three extracellular transmembrane loops. The N-terminus contains many aspartate and glutamate residues whereas the C-tail is rich in serines and threonines. Afterreceptor activation, the latter become phosphorylated and bind arrestin thus desensitising the receptor (Lefkowitz, 1994). The receptors are coupled to G-proteins via their 2nd and 3rd intra-cellular loops. G-proteins exist in an inactive form bound to guanine diphosphate (GDP) and are activated after binding guanine triphosphate (GTP). Both monomeric and heterotrimeric (α, β and γ) forms exist but it is the latter that is involved in chemokine signal transduction.

The interaction and subsequent receptor activation is best described for interleukin-8 (IL-8), although it is likely that other chemokines will operate in a similar manner. Receptor activation can be considered in a series of sequential steps. The ordered domain of monomeric IL-8 binds to the extracellular domains of the 7-TMB (Ratharathanam, et al., 1994). This induces a conformational change in the receptor partly exposing the transmembrane domains which bind to the N-terminal domain of IL-8, inducing receptor and then G-protein activation (Murphy, 1996). A variety of downstream effectors can then be activated including adenylcyclase, phospholipase A_2 (PLA_2), C and D and tyrosine kinase. There is no evidence that an individual chemokine receptors can discriminate between two separate chemokines to generate distinct signal transduction pathways. Chemokine signals can however be sorted functionally by cell type-dependant (Baggiolini and Dahinden, 1994) and concentration dependant signal-sorting (Laudanna, et al., 1996).

Chemokine receptors can be subdivided into four main classes:

1. C-X-C chemokine receptors

C-X-C receptors demonstrate 36 - 77 % homology (Baggiolini, et al., 1997) and usually bind a specific C-X-C chemokine with high affinity thereby affording them a relatively selective function. Four different C-X-C receptors have been identified, C-X-C receptor 1-4 (R1-R4).

2. C-C chemokine receptors

C-C receptors demonstrate 46 - 89 % homology (Baggiolini, et al., 1997) but unlike C-X-C chemokines are relatively non-specific, with each receptor binding a wide range of different C-C chemokines. To date, five C-C chemokine receptors have been identified (C-C 1-5) with each capable of binding at least three different C-C chemokines.

3. Duffy antigen/receptor for chemokines (DARC)

DARC (338 amino acids) has <20 % homology with C-X-C and C-C receptors and, although it binds both C-X-C and C-C chemokines, is not capable of signal transduction. It was initially identified as the invasin for the malaria parasite *Plasmodium vivax* (Miller, et al., 1976). DARC present on all erythrocytes and some non-erythroid cells including endothelium of post-capillary venules, Purkinje cells of the brain (Horuk, et al., 1996) and some B and T cells. The function of DARC is not certain but it has been proposed as a sink for chemokines within the blood (Darbonne, et al., 1991) and more recently as a vehicle for chemokine presentation (Murphy, 1996).

4. Viral chemokine receptors

Many viral receptors are homologous to chemokine receptors; for example the cytomegalovirus receptor (CMV) US28 recognises MCP-1, regulated on activation and normally T-cell expressed and presumably secreted (RANTES), monocyte inhibitory peptide-1α (MIP-1α) and MIP-1β (Gao and Murphy, 1994) and C-C R3 and R5 and C-C R4 (fusin) act as HIV receptor cofactors with CD4 for HIV infection into macrophages (Deng, et al., 1996) and T cells (Feng, et al., 1996), respectively. In addition, certain viral infections induce expression of chemokine receptors, such as the induction of IL-8 receptors by CMV infection (Marayama, et al., 1994) and C-C receptors by HIV (Schmidtmayerova, et al., 1996). Further evidence supporting the link between chemokines and viral infectivity comes from humans, homozygous for an inactivating mutation of the C-C R5 gene who are resistant to HIV infection (Samson, et al., 1996).

1.33 α- OR C-X-C CHEMOKINES

α-Chemokines can be subdivided into those containing the ELR (glutamic acid - leucine - arginine) motif between the NH_2 terminus and the first cysteine which are chemotactic for neutrophils, such as interleukin-8 (IL-8; Clark-Lewis, et al., 1991) and those without this sequence which generally act on lymphocytes, such as immune protein-10 (IP-10).

Specific C-X-C chemokines

Interleukin-8 (IL-8)

Background

The cDNA for IL-8 was initially cloned in 1987 from a differential screen of stimulated peripheral blood mononuclear cells and named 3-10C (Schmid and Weissman, 1987). It was subsequently identified as a soluble factor in culture supernatants of peripheral blood monocytes after LPS stimulation and classified as a chemokine (Lindley, et al., 1993). IL-8 has many synonyms including neutrophil activating factor (NAF; Waltz, et al., 1987), monocyte derived neutrophil chemotactic factor (MDNCF; Yoshimura, et al., 1987), monocyte derived chemotaxin (MDC; Kownatzki, et al., 1991) and neutrophil chemotactic factor (NCF).

Gene and protein structure

The IL-8 gene is 5.25 kBa in length, contains four exons (Mukaida, et al., 1989) and encodes a mRNA transcript of approximately 1.8 kBa (Schmid and Weissman, 1987). Many regulatory nuclear transcription factors such as NFκB, activator protein-1 (AP-1), AP-2 and the glucocorticoid response element, can potentially bind to the 5' flanking region of the IL-8 gene (Mukaida, et al., 1989). The IL-8 protein is a 99 amino acid non-glycosylated peptide which is cleaved enzymatically on release from the cells generating the mature 8 kDa (72 amino acid) protein (Lindley and Aschauer, 1988). N-terminal proteolytic cleavage generates both shorter (69 amino acids) and longer (79 amino acid) variants including the 77 amino acid endothelial form with the shorter variants being more biologically active (Gimbrone, et al., 1989). The principal structural features of IL-8 are the Cys - Cys bridges and the ELR motif. The latter is essential for functional activation of the neutrophil receptor (Clark-Lewis, et al., 1991) and its angiogenic properties (Strieter, et al., 1992; Strieter, et al., 1995). On crystallisation or in concentrated solution, IL-8 exists as a non-covalent homodimer which is stabilised by side chain interactions and hydrogen bonds to form two parallel α-helixes lying on an anti-parallel β-pleated sheet (Baldwin, et al., 1990). However, it likely that *in vivo* IL-8 acts as a monomer (Clore, et al., 1992; Rajarathnam, et al., 1994).

Receptors

There are two forms of IL-8 receptor (Cerretti, et al., 1993), type I (interleukin receptor A; C-X-C R1) and type II (interleukin receptor B; C-X-C R2). Both receptors are dimeric G-

protein coupled glycoproteins (59 kDa and 67 kDa subunits) encoded by the gene 2q35 (Morris, et al., 1992). They are present on the surface of neutrophils (20,000 - 75,000 receptor sites per cell; Besmer, et al., 1989) and T-lymphocytes (300 receptor sites per cell). Binding of IL-8 to the specific type 1 receptor (Kd for IL-8 = 0.80 - 0.40 nM) is thought to mediate neutrophil chemotaxis, degranulation and generation of superoxide radicles. Type II receptor (Kd for IL-8 = 0.3 - 5 nM) is non-specific and recognises chemokines related to IL-8 such as neutrophil activating peptide - 2 (NAP-2) and MIP-2. IL-8 also binds to the DARC receptor (Besmer, et al., 1989).

Sources

IL-8 is released by a wide range of different cell types including monocytes (Yoshimura, et al., 1987), macrophages, endothelial cells (Gimbrone, et al., 1989), lymphocytes, fibroblasts (Larsen, et al., 1989), epithelial cells, neutrophils (Fujishima, et al., 1993), mesangial and microglial cells (Ehrlich, et al., 1998). Its synthesis is induced by many factors including inflammatory cytokines such as IL-1 (DeMarco, et al., 1991; Kaplanski, et al., 1994) and TNF-α (Goldstein, et al., 1996), LPS, hypoxia (Karakurum, et al., 1994), sheer stress, neutrophil adherence to endothelium, dsDNA and viral and bacterial infection. Its synthesis is inhibited by dexamethasone, progesterone (Kelly, et al., 1992), IL-10 (Wang, et al., 1994), TGF-β (Smith, et al., 1996) and IL-4.

Biological Functions

The principal function of IL-8 is in inducing the adhesion, chemotaxis, activation and degranulation of neutrophils (Baggiolini, et al., 1989; Colditz, et al., 1989) with the chemokine having a role in all stages. IL-8 facilitates neutrophil adhesion by inducing shedding of L-selectin from the neutrophil surface and inducing translocation of the CD11-CD18 integrin heterodimer to the neutrophil surface, which then binds to endothelial ICAM-1. The chemokine induces neutrophil chemotaxis directly by binding to vascular endothelium (Roit, 1992), thus promoting neutrophil adherence to vascular endothelium and subsequent emigration, and indirectly by binding to the ECM creating a diffusion gradient. In addition, IL-8 induced neutrophil extravasation is facilitated by the presence of the vasodilator PGE_2 which, by raising the threshold for infiltration from 10^{-11} mol to 10^{-14} mol IL-8 (Colditz, 1990), increases the number of accumulating neutrophils and the associated volume of plasma exudation (Rampart, et al., 1989). Finally, IL-8 causes activation of extravasated neutrophils within tissue. Structural changes in the neutrophil membrane (Wymann, et al., 1987) precede exocytosis of intra-cellular granules and stimulation of the respiratory burst (Baggiolini and Wymann, 1990). Two types of

intra-cellular granules are released, the specific granules containing matrix metalloproteinase-8 (MMP-8; neutrophil collagenase), lysozyme, B_{12}-binding proteins and lactoferrin and the azurophilic granules containing elastase, myeloperoxidase and lysozyme. The respiratory burst, generated by the trans-membrane NADPH-oxidase electron transport system, results in formation of cytotoxic hydrogen peroxide and superoxide radicals (Baggiolini, et al., 1992).

IL-8 is also chemotactic for T-cells (Larsen, et al., 1989), smooth muscle cells (Baggiolini, et al., 1992), eosinophils and basophils, inducing histamine release from the latter cell type. The chemokine also acts as a mitogenic and chemotactic agent in keratinocytes (Larsen, et al., 1989), an angiogenic factor (Koch, et al., 1992) and has been implicated in tumour growth and metastasis (Luca, et al., 1997).

1.34 β- OR C-C CHEMOKINES

β-chemokines map to human chromosome 17q11-32.

Specific C-C chemokines

Monocyte Chemotactic Peptide – 1 (MCP-1)

Background

In the early 1980's, it was reported that human tumour culture supernatants contained chemotactic activity for mononuclear cells (Bottazzi, et al., 1983). This activity was subsequently identified as monocyte chemotactic factor, monocyte chemotactic and activating factor, chemotactic factor or monocyte chemotactic peptide-1 (Rollins, et al., 1988).

Gene and protein structure

The MCP-1 gene (Mehrabian, et al., 1991) is analogous to the mouse competence gene JE (Yoshimura, et al., 1989) containing three exons, two introns (Chang, et al., 1989) and binding sites for a variety of transcription factors including NFκB and AP-1. The protein is a 76 amino acid basic heparin binding glycoprotein (12 kDa) which displays varying degrees of glycosylation (Jiang, et al., 1990). The tertiary structure of MCP-1 is similar

to that of IL-8 with both capable of existing as monomers and dimers (Gronenborn and Clore, 1991).

Receptors

MCP-1 binds to C-C R-2 and C-C R-4 receptors with the carboxy terminal of the receptors thought to be critical for chemotaxis, signalling and receptor internalisation (Aral, et al., 1997).

Sources

MCP-1 is released by a wide range of different cell types including monocytes, pericytes (Chensue, et al., 1995), fibroblasts (Rolfe, et al., 1992), vascular smooth muscle (Wang, et al., 1991), endothelial (Hub and Rot, 1998), retinal pigment (Elner, et al., 1997) and synovial cells (DeMarco, et al., 1991) cells. MCP-1 is induced by a wide range of factors including LPS, IL-1, TNF-α (Goldstein, et al., 1996) and the membrane attack component of complement (Kilgore, et al., 1996) and inhibited by progesterone (Kelly, et al., 1997).

Biological Function

MCP-1, in addition to being a monocyte chemotaxin, is also chemotactic for T-cells (Cai, et al., 1995), basophils, mast cells (Feliciani, et al., 1995), NK cells and IL-2 activated NK cells (Allavena, et al., 1994). A number of other functions have also been ascribed to MCP-1 including that of an angiogenic agent, tumour suppressive agent (Rollins and Sunday, 1991), mitogen for vascular smooth muscle (Porreca, et al., 1997), regulator of cytokine expression (Jiang, et al., 1992) and immunomodulator, favouring development of a Th-2 type of immune response (Chensue, et al., 1996).

Regulated on Activation and Normally T-cell Expressed and Secreted (RANTES)

Background

The cDNA was first identified during a general genetic screen of T-cells (Schall, et al., 1988) with the protein subsequently given the acronym RANTES, based on functional predictions from the gene and protein.

Gene and protein structure

The RANTES gene has three exons and two introns and encodes a mRNA transcript of approximately 1.2 kBa in length. Unlike MCP-1 and IL-8, the transcriptional regulation of RANTES depends on the cell in which it is being expressed with transcription in T-cells, fibroblasts and endothelial and epithelial cells being developmentally regulated (Ortiz, et al., 1996), an immediate early gene response (Rathanaswami, et al., 1993) and the result of cytokine stimulation (Crane, et al., 1998), respectively. RANTES protein comprises a highly basic 91 amino acid glycosylated polypeptide which likely exists as a dimer at physiological pH (Fairbrother and Skelton, 1996).

Receptors

RANTES binds to C-C R1, R3, R4 and R5 receptors, which are expressed on monocytes, eosinophils, basophils and T-cells. It also binds to the DARC antigen and the CMV US28 receptor.

Sources

RANTES is released by many cell types including T-cells (Wingett, et al., 1996), monocytes, basophils, mesangial cells, fibroblasts, endothelium (Hub and Rot, 1998), epithelium (Stellato, et al., 1995), airway smooth muscle cells (John, et al., 1997) and eosinophils.

Biological Function

RANTES is a potent chemotactic and activating agent for $CD4^+$ and $CD45RO^+$ (memory) T-cells, eosinophils (Alam, et al., 1993) and basophils, is chemo- and haptotactic for monocytes and chemotactic for NK cells, inducing histamine release from the latter cell type (Kuna, et al., 1992). It also mediates adhesion of $CD4^+$ T cells to ICAM-1 and VCAM-1 via upregulation of $\beta 1$ and $\beta 2$ integrin expression. Recently, RANTES has been demonstrated to inhibit HIV replication by competing for binding to C-C R5 receptor. RANTES expression is induced by many factors including TNF-α and IL-1α and inhibited by TGF-β, IL-6 and IFN-γ.

1.4 OTHER INFLAMMATORY MEDIATORS

1.41 PROSTAGLANDINS

Nomenclature

Prostaglandins were first identified in 1936 by Von Euler in prostatic extracts (Von Euler, 1936). Structurally, they comprise a family of C_{20} carboxylic acids with a cyclopentane ring from which two aliphatic side chains linked to a carboxyl and methyl group protrude (Mitchell, 1992). Prostaglandins are divided into the A, B, C, D, E, F, J, G and H series with the latter two being unstable endoperoxide intermediates involved in prostaglandin synthesis. The PGE and PGF series can be further classified into three subgroups, E_1, E_2 and E_3 and $F_{1\alpha}$, $F_{2\alpha}$ and $F_{3\alpha}$, respectively with the subscript denoting the number of side chain double bonds. The two series differ in that the PGEs have a C-9 keto-oxygen and a C-11 hydroxyl group in the cyclopentane ring whereas the PGFs have hydroxyl groups at both positions (Willis and Smith, 1994).

Synthesis

Prostaglandins are produced by *de novo* synthesis from the fatty acid precursor arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid, AA) which is released from membrane bound phospholipids (phosphatidylcholine and phosphatidylethanolamine), after cell signalling, principally by PLA_2. Phospholipase C, in conjunction with diacylglycerol lipase, also releases a small amount of arachidonic acid. Subsequent synthesis is by the multi-enzyme prostaglandin H endoperoxide synthase/cyclooxygenase (PGHS/COX) complex. Arachidonic acid initially undergoes double oxidation (cyclooxygenation) to produce PGG_2, which then rapidly undergoes peroxidase conversion to PGH_2. The endoperoxides are subsequently modified by synthases or isomerases to generate specific prostanoids, the most common being PGE_2, $PGF_{2\alpha}$, PGD_2, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2).

Cyclooxygenase enzymes

Two isoforms of the cyclooxygenase enzyme have been identified, COX-1 and more recently COX-2 (Goppelt-Streube, 1995; Kujubu, et al., 1991). Although the isoforms share 60 % homology at the amino acid level, they are products of different genes and have marked differences in their regulation and subcellular expression. The constitutively

expressed COX-1 is thought to play a housekeeping role in prostaglandin production (O'Banion, et al., 1992; Simmons, et al., 1991) and is associated with the endoplasmic reticulum (Morita, et al., 1995). In contrast, COX-2 is dynamically regulated, in part via NFκB and localised to the nuclear envelope (Morita, et al., 1995). COX-2 is a typical inducible gene with many binding sites in the 5' untranslated TATA box and the presence of binding sequences (AUUUA) for ribonucleases in the 3' untranslated end (Malter, 1989). The expression of its 4.1 kBa mRNA is tightly regulated at the level of transcription, being stimulated by inflammatory mediators (LPS, Phorbol 12 - Myristate 13 - Acetate, PMA), growth factors (PDGF), cytokines (IL-1, TNF-α) and hormones (human chorionic gonadotrophin; (hCG; Han, et al., 1996) and inhibited by glucocorticoids (Masferrer and Seibert, 1994) and IL-1 receptor antagonist (Porreca, et al., 1996). Further regulation occurs pre-translation by modulation of mRNA stability and post-translation by rapid degradation of the mRNA by ribonucleases.

Metabolism

The principle site of prostaglandin metabolism is the lung with prostaglandins being metabolised by the progesterone stimulated prostaglandin dehydrogenases (PGDH-1 and PGDH-2) and Δ^{13}-reductase to the inactive 13-14-dihydro-15-keto metabolites. The majority of circulating prostaglandins are metabolised with one passage through the lung with only a small amount of catabolism occurring locally within tissues. However during pregnancy, uterine PGDH localised predominately in fetal membrane trophoblast, also contributes significantly to prostaglandin metabolism (Cheung, et al., 1990).

Receptors

Prostaglandin receptors are members of the 7-TMB class of receptors. The physiological response elicited by a specific receptor is unique and due in part to utilisation of different intracellular 2^{nd} messenger systems such as phosphatidylinositol and adenylate cyclase (Pierce, et al., 1995). Five classes of receptor have been identified, DP, EP, FP, IP and TP with the receptors preferentially binding PGD, PGE, PGF, PGI and TXA, respectively (Coleman, et al., 1990). The EP and TP families are further divided into subfamilies with four EP receptor isoforms, EP-1, -2, -3 and -4 being identified so far. In general, EP-1 and EP-3 elicit stimulatory responses whereas EP-2 and EP-4 evoke inhibitory effects (Coleman, et al., 1994).

Biological Effects

Prostaglandins are powerful mediators, which exert diverse effects in many biological systems. Their general effects which include pro- (TXA_2) and anti- (PGEs, PGI_2) coagulant properties, inducing vasodilation (PGEs and PGI_2) and vasoconstriction (PGFs and TXA_2), bronchodilation (PGEs) and bronchoconstriction (PGFs) and regulation of transmembrane ion flow are well established. More recently, it has been established that prostaglandins possess powerful immunomodulatory properties and are capable of both promoting and inhibiting immune responses via their interactions with and regulation of local cytokine release. Many of these have already been discussed and those of direct relevance to pregnancy and parturition will be covered later.

1.42 NITRIC OXIDE

Nitric oxide is a potent locally acting mediator which is synthesised from arginine by nitric oxide synthase (NOS) which generates nitric oxide and citrulline. Alternatively, arginine can be metabolised by arginase to give urea and ornithine. Three different isoforms of NOS have been identified, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Endothelial NOS and nNOS, encoded by chromosomes 12 (150 kBa, 29 exons) and 7 (21 - 22 kBa, 26 exons) respectively, are constitutively expressed and calcium/calmodulin dependant (Mayer and Hemmens, 1997). In contrast, iNOS, encoded by chromosome 17 (37 kBa, 26 exons), is rapidly induced and calcium/calmodulin independent (Mayer and Hemmens, 1997). All three isoforms are composed of a C-terminal reductase, which has binding sites for FAD, FMN and NADPH and an N-terminal oxygenase that binds haem and tetrahydrobiopterin, the latter being a cofactor for the NAPDH dependant oxygenation of nitric oxide.

Nitric oxide is synthesised by a wide range of different cell types including macrophages (Imai, et al., 1993), cardiac muscle, astroglia, hepatocytes, osteoclasts (Sunyer, et al., 1996), endothelium (Berman, et al., 1998), myometrium (Thomson, et al., 1997), fetal membranes (Ticconi, et al., 1996) and cervix. Release of nitric oxide is regulated by many hormones and inflammatory mediators, with the latter thought to act via $NF\kappa B$ (DeVera, et al., 1997). Nitric oxide is stimulated by LPS, IFN-γ (Corraliza, et al., 1995; Haddad, et al., 1997), TNF-α, IL-1α (Nusing, et al., 1996; Sunyer, et al., 1996), hypoxia (Palmer, et al., 1998) and oestrogen (Hunt, et al., 1997; Kleinert, et al., 1998) and inhibited by TGF-β (Alleva, et al., 1994) and dexamethasone (O'Connor and

Moncada, 1991). Modolell (Modolell, et al., 1995) proposed that the NOS and arginase were reciprocally regulated with Th-1 cytokines favouring NOS and Th-2 cytokines arginase. The effects of many factors on nitric oxide production however frequently conflict, with PGE_2, IL-8, IL-10 and progesterone being capable of stimulating (Benbernou, et al., 1997; Sunyer, et al., 1996) and inhibiting nitric oxide release (Alleva, et al., 1994; Modolell, et al., 1995). Therefore, the proposed regulatory control exerted by Th-1/-2 cytokines on the NOS/arginase balance is controversial. Control of nitric oxide synthesis is thus highly complicated and may be influenced by many factors including intra-tissue differences, experimental procedures and the concentration of nitric oxide within the system being studied (Swierkosz, et al., 1995).

Unlike cytokines and prostaglandins, nitric oxide does not act as a classical intracellular messenger by binding to soluble or membrane bound receptors. Instead, it is freely diffusible and its biological effects are determined by its chemical reactivity and site of synthesis. Many, but not all of the physiological effects of nitric oxide are mediated by its binding to the haem group of soluble guanylate cyclase, thereby stimulating cyclic monophosphate (cGMP) formation by several hundred fold. Nitric oxide is a potent mediator of platelet aggregation, vascular permeability and smooth muscle relaxation (Buhimschi, et al., 1997) and proliferation. It is an important mediator of the immune system stimulating IL-8 (Corriveau, et al., 1998; Cuthbertson, et al., 1998) and MMP (Maeda, et al., 1998) release via activating the cysteine shift and inhibiting IL-1β and TNF-α secretion. In addition, nitric oxide regulates the release of pituitary luteinising hormone releasing hormone (LHRH; Moretto, et al., 1993) and placental corticotrophin releasing hormone (CRH) release (Clifton and Challis, 1997) and can paradoxically both stimulate (Brockhaus and Brune, 1998) and inhibit apoptosis (Kim, et al., 1997) depending on other environmental factors. Nitric oxide also exerts opposing effects on COX-2, stimulating and inhibiting the enzyme *in vitro* and *in vivo* (Salvemini, et al., 1994; Vane, et al., 1994). Whether a positive or negative feedback exists between nitric oxide and COX-2 is not certain (Tetsuka, et al., 1994).

1.43 MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are a family of zinc and calcium dependent enzymes that are responsible for degradation of the ECM. They can be subdivided, by nature of their specificity for the ECM and location into the collagenases, gelatinases, stromelysins, membrane-type MMPs and a miscellaneous group. Seventeen MMPs have so far been characterised (Table 1).

MMPs are secreted as inactive proMMPs or zymogens, which are activated by proteolytic cleavage to give the active enzyme (Hulboy, et al., 1997). At the amino terminus is the 'pre' domain, which signals for cellular export. Cleavage and removal of the 'pro' domain, which is adjacent to the 'pre' domain, by other MMPs including membrane bound MMPs (Sato, et al., 1994), serine proteases (plasminogen activator and kallikreins; Espey, 1992) and mast cell proteases (Suzuki, et al., 1995) converts proMMPs to the active enzyme. 'Pro' domain cleavage, otherwise known as the cysteine switch mechanism, involves disengagement of a cysteine in the conserved 'pro' domain sequence PRCGVPDV. This uncovers a zinc ion, which is held by co-ordinate bonding to three histidines in the active site (amino acid consensus sequence HEXGHXXGXXHS) in the adjacent catalytic domain. Finally, binding of water to the uncovered zinc in the active site completes MMP activation.

In addition to the three fundamental domains, all MMPs apart from MMP-7 possess other regions that confer diversity in their association with ECM and substrate. For example; haemopexin-like domains (all MMPs) mediate association to the ECM and MMP inhibitors (Baragi, et al., 1994), fibronectin domains (MMP-2 and MMP-9) facilitate gelatin binding, Mt-MMPs contain a transmembrane domain that localises the enzyme to the plasma membrane and Mt-MMPs and MMP-11 possess a region which binds furins, which are protein-processing enzymes.

Expression of proMMP genes is subject to control during transcription, translation, by changes in mRNA stability, at the activation of the pro-enzymes by proteolytic cleavage and by the association and dissociation of MMPs with their specific inhibitors. MMP release can be stimulated by cytokines (IL-1, TNF-α and IL-6), ECM components, MMPs, LPS, nitric oxide and PGE_2 and inhibited by SLPI, cytokines (IL-4, IL-10, TGF-β and IFN-γ) oestrogen and progesterone (Hulboy, et al., 1997).

Group	Name	MMP numbers	MW (latent) active	Substrate specificity
Collagenases	Interstitial collagenase	MMP-1	(52000) 42000 (56000) 46,000	Collagens I, II, III, VII, gelatins, TNF-α
	Neutrophil collagenase	MMP-8	(75000) 65000	Collagens I, II, III
	Collagenase 3	MMP-13	(53700)	Collagen I
Gelatinases	72 kDa Gelatinase	MMP-2	(72000) 62000	Gelatins, collagens I, IV, V, VII, elastin, fibronectin, TNF-α
	92 kDa Gelatinase	MMP-9	(92000) 84000	Gelatins, collagens IV, V, elastin, TNF-α
Stromelysins	Stromelysin	MMP-3	(56000) 45000 (59000) 28000	Proteoglycans, laminin, fibronectin, collagen III, IV, V, gelatins, TNF-α, MMP-1, -8, and -9
	Matrilysin	MMP-7	(28000) 19000	Proteoglycans, laminin, fibronectin, gelatin, collagen IV, elastin, TNF-α, MMP-1 and -9
	Stromelysin 2	MMP-10	(60000) 55000	Proteoglycans, fibronectin, collagen III, IV, V, gelatins, MMP-8
	Stromelysin 3	MMP-11		Laminin, fibronectin
Membrane type MMPs	Mt-MMP-1	MMP-14	(63000)	MMP-2
	Mt-MMP-2	MMP-15	?	?
	Mt-MMP-3	MMP-16	?	MMP-2
	Mt-MMP-4	MMP-17	?	?
Other	Metalloelastase	MMP-12	?	Elastin, fibronectin, collagen IV

Table 1

Matrix metalloproteinases - nomenclature and substrate specificity

The principal inhibitors of MMP function are the liver derived, serum borne α_2-macroglobulin and the natural inhibitors, the tissue inhibitors of metalloproteinase release (TIMPs). TIMPs are arranged in two domains by disulphide bonds with each TIMP inhibiting the activity of most MMPs by non-covalent 1 : 1 stoichiometric binding to the active site of the MMP. There is no apparent selectivity of TIMPs for particular MMPs with this residing in their specific distribution within tissues. Four TIMPs have so far been identified (Table 2).

Inhibitor	Relative Molecular Mass	Glycosylation	Extracellular Location
TIMP-1	28 000	Glycosylated	Soluble in ECM and body fluids
TIMP-2	21 000	Not glycosylated	Soluble in ECM and body fluids
TIMP-3	24 000	Not glycosylated	Bound to ECM
TIMP-4	22 000	?	?

Table 2
Tissue inhibitors of matrix metalloproteinases - nomenclature and substrate specificity

The ratio of MMPs to TIMPs determines whether tissue remodelling or deposition occurs. In addition to inhibiting MMP activity, TIMPs stimulate progesterone production by various cells (Boujrad, et al., 1995) and stimulate proliferation of various cell types (Edwards, et al., 1996).

1.44 SECRETORY LEUKOCYTE PROTEASE INHIBITOR

Gene and protein structure

Secretory leukocyte protease inhibitor is a member of the chelonianin family of proteinase inhibitors which also includes elafin (Francart, et al., 1997). The majority of its protease inhibitor action activity resides in the carboxy domain (Meckelein, et al., 1991). The function of the amino domain is less well defined but it may have a role in stabilising binding of secretory leukocyte protease inhibitor to elastase (Ying, et al., 1994) and in mediating the anti-bacterial activity of secretory leukocyte protease inhibitor (Hiemstra, et al., 1996).

Receptors

Secretory leukocyte protease inhibitor binds to a high affinity, surface receptor on monocytes (equivalent to 7,000 receptors per monocyte; Kd = 55 ± 5 kDa) of molecular weight 55 ± 5 kDa. In other sites, such as epithelial surfaces, secretory leukocyte protease inhibitor receptor has not been characterised.

Sources

Secretory leukocyte protease inhibitor is released by a wide range of cells and tissues including neutrophils, intact (Asano, et al., 1995) and remodelled epithelial (Marchand, et al., 1995) surfaces and endometrium (Badinga, et al., 1994). In addition, secretory leukocyte protease inhibitor is present in serum (Reed, et al., 1998), seminal plasma (Ohlsson, et al., 1995), peritoneal fluid (Bergenfeldt and Ohlsson, 1993) and in ECM associated with elastin fibres (Kramps, et al., 1989; Rudolphus, et al., 1994). Release of secretory leukocyte protease inhibitor is stimulated by corticosteroids (Abbinantenissen, et al., 1995; Abe, et al., 1997), trypsin (Abbinantenissen, et al., 1993), cytokines (Jin, et al., 1998; Sallenave, et al., 1994) and PMA (Maruyama, et al., 1994). Secretory leukocyte protease inhibitor is inhibited by elastase (Marchand, et al., 1997) and IFN-γ (Jin, et al., 1997).

Biological Function

Although initially described as a potent inhibitor of neutrophil elastase and cathepsin-G (Thompson and Ohlsson, 1986), secretory leukocyte protease inhibitor also inhibits the activity of mast cell kinases (Pemberton, et al., 1998; Walter, et al., 1996) and chymotrypsin (Franzke, et al., 1996), prolongs clotting (Masuda, et al., 1995) and has anti-bacterial (Hiemstra, et al., 1996), anti-fungal (Tomee, et al., 1997) and anti-viral (Shine, et al., 1997) activity including against HIV (Shugars, et al., 1996) and anti-coagulant properties (Masuda, et al., 1995). Recently secretory leukocyte protease inhibitor has also been shown to suppress the LPS-induced activation of NFκB, production of nitric oxide and TNF-α (Jin, et al., 1997) and inhibit release of MMPs via inhibition of COX-2 (Zhang, et al., 1996).

1.45 Th-1 AND Th-2 IMMUNE MODULATION

In 1988, Mosmann and colleagues (Mosmann and Coffman, 1989) proposed the hypothesis that T-helper cells (CD4+) could be divided into Th-1 and Th-2 subtypes

according to their profile of cytokine release and immunological functions. Th-1 type cytokines (IL-2, IL-12, IFN-γ) are pro-inflammatory and stimulate a cell mediated type of immune response whereas Th-2 type cytokines (IL-10, IL-4, IL-5 and IL-6) tend to be immunosuppressive and favour humoral immune responses. The original concept has now been expanded since cells other than T cells have been found to secrete Th-1 and Th-2 type cytokines (Mosmann and Sad, 1996). It is now appreciated that cellular cytokine secretion is likely a continuum, with "pure" Th-1 and Th-2 type release representing the two extremes. In addition, other regulatory T-cell subsets have recently been described with Th-0 cells secreting both patterns of cytokines (Mosmann and Coffman, 1989), Th-3 releasing high amounts of TGF-β (Chen, et al., 1994) and Tr-1 or regulatory T-cells (Groux, et al., 1997).

The generation of a Th-1 or Th-2 immune response is tightly controlled with a variety of factors being implicated. There is mutual repression between Th-1 and Th-2 responses with IL-12 stimulating Th-1 cytokines and inhibiting Th-2 (Trinchieri, 1993) and T cell derived IL-10 effecting the opposite (Mosmann and Moore, 1991). Chemokines, by virtue of their specific chemotactic properties, preferentially attract Th-1 (monocyte inhibitory peptide-1, MIP-1α; MIP-1β and RANTES) or Th-2 (MCP-1; (Chensue, et al., 1996) or both T cell subsets (stromal cell-derived factor-α; Siveke and Hamann, 1998). In addition, MIP-1α and MCP-1 drive uncommitted Th-0 cells to differentiate into Th-1 and Th-2 secreting T helper cell populations, respectively (Karpus and Kennedy, 1997). Prostaglandins are also important modulators of humoral and cell mediated immune responses with PGE_2 being recently shown to favour a Th-2 tilt (downregulation of IFN-γ, IL-12 and IL-2 and upregulation of IL-10) to the immune response (Fedyk, et al., 1997; Hilkens, et al., 1995; Kelly, et al., 1997; Kraan, et al., 1995; Snijdewint, et al., 1993). Other inflammatory mediators have contradictory effects on the Th-1/Th-2 balance. In mice, nitric oxide favours a Th-2 response (Bauer, et al., 1997) but in humans, it has been shown to prevent over-expansion of Th-1 cells (TaylorRobinson, 1997), induce a Th-2 response (Benbernou, et al., 1997) and have an equal effect on both T cell subtypes depending on the system studied (Bauer, et al., 1997).

Th-1/Th-2 shift during pregnancy

Wegmann proposed the hypothesis that a Th-1/Th-2 paradigm exists during pregnancy (Wegmann, et al., 1993). He postulated that a Th-2 cytokine profile would confer local immunosuppression at the materno-fetal interface thus favouring successful pregnancy. In contrast, a Th-1 response would be detrimental to pregnancy and be associated with a

poor reproductive outcome. Considerable experimental and clinical evidence now supports these postulates. Both the peri-implantation endometrium (Lim, et al., 1998) and fertilised ovum (Kelemen, et al., 1998) induce and secrete a predominant Th-2 type cytokine profile. This is supported by high levels of circulating progesterone, which favours a Th-2 type cytokine response (Piccinni, et al., 1995) and $CD4^+$ and $CD8^+$ T-cells which, during successful pregnancy, preferentially secrete Th-2 cytokines (Reinhard, et al., 1998). In contrast, the Th-1 cytokines TNF-α, IFN-γ and IL-2 have adverse effects on trophoblast proliferation and trophoblast development (Raghupathy, 1997). This is manifested clinically in women with a history of recurrent spontaneous miscarriage who demonstrate a Th-1 bias in their immune response locally within the uterus (Haynes and Smith, 1997; Hill, et al., 1995) and peripherally in circulating T-cell clones (Piccinni and Romagnani, 1996; Raghupathy, 1997). Furthermore, there is evidence that conditions such as rheumatoid arthritis, associated with a Th-1 response remit during pregnancy and those such as systemic lupus erythematosis, mediated by a Th-2 response, are exacerbated (Wilder, 1998). Within the uterus, many cells are capable of secreting Th-2 type cytokines including NK cells (Chaouat, et al., 1997), T helper cells, macrophages, trophoblast and endometrial cells (Kelly and Critchley, 1997).

1.5 PARTURITION

In many animal species, such as sheep, the mechanisms and mediators involved in the initiation and maintenance of parturition are well characterised. However, in humans the precise factors involved are not well understood and knowledge of the initiating agents has progressed little since Hippocrates proposed in about 430 BC that:

> "When the child is grown big and the mother can no longer provide him with enough nourishment he becomes agitated, breaks through the membranes and incontinently passes out into the external world free from any bonds."

James Young Simpson subsequently refuted the various theories of parturition which existed at that time such as:

> "the supposed origin of the act of labour in certain states of vital development or physical expansion of the fundus, body or cervix uteri, in some supposed conditions of the foetus, liquor amnii, or placenta etc., " and suggested that "the loosening or decadence of the membranes, or membranes and placenta from the interior of the uterus, constituted the determining cause of parturition; and that this loosening or decadence was itself the result of the effete degeneration of the structure of the decidua towards the full term pregnancy."

Although many theories, some of which are described below, have been proposed for parturition, none fully accounts for the unique and highly complex process that cumulates in emptying of the uterus and delivery of the neonate.

1.51 THEORIES OF PARTURITION

Progesterone withdrawal

High levels of progesterone within the utero-placental unit are essential for initiation and maintenance of a successful pregnancy. Progesterone acts locally within the uterus during pregnancy to suppress uterine contractions (Beck, et al., 1978) and decrease gap junction formation (Ambrus and Rao, 1994). In many species such as sheep, a relative fall in progesterone and rise in oestrogen levels, peripherally and locally within the uterus, heralds the onset of parturition. In term rat myometrium, a significant decrease in nuclear

progesterone receptors and increase in oestrogen receptors correlates with an increase in gap junction formation and initiation of parturition (Nathanielsz, 1991). Similarly, a pre-partum rise in oestrogen in monkeys at term induces gap junction formation, synthesis of oxytocin and prostaglandins and alteration of myometrial activity from contractures to parturient contractions (Saito, et al., 1985).

Csapo, therefore postulated that a similar "progesterone block" might operate at term in humans (Csapo, 1956) and, in 1975 hypothesised the "see-saw" theory of pregnancy (Csapo, 1975). He proposed that pregnancy maintenance was dependent on the balance between factors favouring pregnancy, such as progesterone and those promoting its termination, such as $PGF_{2\alpha}$. To date, however, there is no good experimental evidence to support progesterone withdrawal as the initiating factor for human parturition. A study which examined peripheral levels of progesterone, dehydroepiandrosterone, (DHEAS; an oestrogen precursor), oestrogen and oestradiol in women with favourable (Modified Bishops Score >4) and unfavourable cervices (Modified Bishops Score <4) prior to the induction of term labour found that concentrations of DHEAS were significantly elevated only in those with ripe cervices (Zuidema, et al., 1986). This study and others (Anderson, et al., 1985) did not demonstrate a pre-parturient fall in peripheral progesterone concentrations. Furthermore, it has been demonstrated that peripheral progesterone levels are lower in women with dysfunctional compared with normal spontaneous labour at term, the conclusion being that "high" circulating progesterone was necessary for normal parturition. In contrast, an increased salivary oestriol/progesterone ratio has been demonstrated in the three weeks prior to the onset of spontaneous labour but not in women who ultimately delivered post-dates.

The situation is no less confusing within the term uterus with evidence for local changes in the progesterone : oestrogen ratio (Romero, et al., 1988), and their metabolism (Milewich, et al., 1977) being conflicting. In addition, although the number of nuclear progesterone and oestrogen receptors within the uterus decreases during pregnancy to low (Khan-Dawood and Dawood, 1984; Padayachi, et al., 1987; Perrot-Applanat, et al., 1994) or undetectable levels at term (Padayachi, et al., 1987), there is no evidence that any further alteration in receptor number (Khan-Dawood and Dawood, 1984) or isotype occurs at parturition. It may be however, that formation of a local anti-gestogen (e.g. TGF-β), reduction in affinity of progesterone binding for its receptors or sequestration by a binding protein may effect a local fall in progesterone concentration within the feto-placental unit thus permitting parturition. Indirect evidence supporting a local fall in progesterone at

term comes from studies demonstrating a rise in concentrations of IL-8 (Sennstrom, et al., 1997) and gap junctions (Chow and Lye, 1994) within the uterus at parturition. These factors are normally inhibited by progesterone (Ambrus and Rao, 1994; Kelly, et al., 1994).

Inflammatory reaction

More recently, parturition has been likened to an inflammatory reaction (Liggins, 1981) with pro-inflammatory cytokines such as IL-1, IL-6 and IL-8 acting as initiators and propagators at the local level (Steinborn, et al., 1998). Concentrations of IL-1 and IL-6 rise in peripheral serum (Arntzen, et al., 1997), in the uterus (Ammala, et al., 1997) and amniotic fluid at the onset of labour with levels increasing with the strength of uterine contractions (Arntzen, et al., 1997), the degree of leukocyte invasion (Halgunset, et al., 1994) and cervical dilatation (Steinborn, et al., 1996). In addition, IL-1 and IL-6 can potentially further propagate the inflammatory reaction by stimulating release of IL-8 and uterine prostaglandins (Kelly, 1994). However, the criticism that has been raised about all studies conducted on cytokines and parturition, is whether changes in cytokine levels are the cause or effect of the parturient process.

Prostaglandins, particularly the PGE and PGF series, are thought to have an important role in parturition. They are synthesised by amnion, chorion and decidua and act within the uterus to change membrane structure (Gibb, 1998) and contract the myometrium. It is generally accepted (MacDonald and Casey, 1993) that concentrations of PGE_2 and $PGF_{2\alpha}$ rise in amniotic fluid prior to the onset of uterine contractions (Romero, et al., 1996) and increase further with the onset of labour (Gibb, 1998). The expression of the inducible PGHS-2 isoenzyme (Mijovic, et al., 1998) increases within chorion and amnion and expression of PGDH decreases in chorion at term. Alteration in the ratio of these enzymes, which may in part be due to the regulatory effect of glucocorticoids, may permit prostaglandins synthesised by the fetal membranes to gain access to the myometrium and cervix. In addition, a factor present in amniotic fluid at term is thought to have a permissive effect on the release of prostanoids from the fetal membranes (Brennand, et al., 1998).

Recently, nitric oxide has also been implicated in parturition although whether it acts to promote (Chwalisz and Garfield, 1998) or inhibit the process is controversial (Nanno, et al., 1998). Experimental and clinical evidence suggests that nitric oxide plays an important role in animals during pregnancy by maintaining uterine quiescence and

placental perfusion. In rats at term, uterine nitric oxide expression is significantly down regulated prior to parturition, thus permitting uterine contractions. However, in humans although there is a significant fall in peripheral levels of nitric oxide (Nanno, et al., 1998), studies in the uterus have failed to demonstrate changes in levels of nitric oxide or nitric oxide synthase (Thomson, et al., 1997) during human parturition.

Many other factors have been implicated in initiating and maintaining parturition. Concentrations of gravidin, a phospholipase A_2 inhibitor (Baumann, et al., 1998) and activin A, an immunomodulatory agent which stimulates PGE_2 release (Petraglia, et al., 1993) also increase (Petraglia, et al., 1997) prior to parturition. In addition, the expression of certain endothelial cell adhesion molecules (endothelial leukocyte adhesion molecule-1 and VCAM-1) increase within the lower uterine segment (Winkler, et al., 1998). These factors could further promote a pro-inflammatory parturient response.

Placental clock

Concentrations of maternal plasma corticotrophin releasing hormone (CRH), which is secreted mainly by the placenta, rise during pregnancy and are elevated at the time of preterm labour (Campbell, et al., 1987). McLean and colleagues demonstrated that maternal plasma levels of CRH were elevated in the first trimester in those women destined to deliver prematurely and were lower in those who delivered post-dates. In addition, high circulating concentrations of corticotrophin releasing hormone binding protein (CRH-BP) effectively blocked the bioactivity of CRH until the final three weeks before parturition when the rise in plasma CRH was accompanied by a fall in CRH-BP. They suggest the existence of "a placental clock which triggers the onset of parturition after a predetermined length of gestation and that the maternal plasma CRH is an indicator of the rate of progress towards this event".

Supportive of this hypothesis is the observation that levels of CRH-type 1 receptor (CRH-R1) mRNA and protein increase within the lower uterine segment with the onset of labour. Challis and colleagues (Challis, 1998) have suggested that the rise in placental CRH may contribute to relaxation of the lower uterine segment via CRH-R1 and to stimulation of fundal myometrial activity either directly via binding to CRH-R2 and inducing calcium mobilisation or indirectly by stimulating fundal prostaglandin production.

Maturation of the fetal hypothalamo-pituitary-adrenal axis

A number of theories have been proposed in which the fetal adrenal interacts with steroid hormone production by the placenta to trigger parturition.

One hypothesis propounded by Wilson et al (1996) (Wilson and Parsons, 1996) is that increased levels of oestriol at term stimulate placental 11β-hydroxysteroid dehydrogenase, which converts cortisol to inactive cortisone thus lowering exposure of the fetus to maternal cortisol. Negative feedback to the fetal pituitary releases corticotrophin, which stimulates fetal adrenal DHEAS synthesis. DHEAS is then converted to oestriol in the placenta after being 16 α-hydroxylated in the fetal liver. The rise in placental oestriol release could then stimulate release of oxytocin, prostaglandins and gap junctions, leading to uterine contractions and cervical dilatation.

An alternative model suggests that cortisol blockade of progesterone acts to initiates parturition (Karalis, et al., 1996). Karalis et al., (1996) suggest that the huge rise in fetal cortisol at term competes with progesterone for glucocorticoid-receptor binding thus blocking progesterone inhibition of the placental CRH gene. The rise in placental CRH augments fetal corticotrophin, which then stimulates fetal adrenal cortisol and DHEAS production. Parturition would then be initiated by an increase in placental oestriol as described above.

Tensegrity and parturition

Tensegrity theory and frameworks (structures with cables, bars and struts) have been employed in the field of continuum mechanics for over 50 years (Djouadi, et al., 1998). It is only recently, however that such models have been applied to biological systems such as angiogenesis (Vailhe, et al., 1997). Tensegrity theory proposes that living cells and nuclei are hard-wired to respond immediately to mechanical stresses transmitted over cell surface receptors that physically couple the cytoskeleton to the extracellular environmental matrix (for example integrins; Maniotis, et al., 1997) or to other cells (for example cadherins, selectins and cell adhesion molecules (Potard, et al., 1997). Many cell adhesion molecules, that are activated by cell binding to growth factors and ECM, associate with cytoskeletal scaffolds within focal adhesion complexes. Mechanical signals may therefore be integrated with other environmental signals and transduced into a biochemical response through force–dependant changes in scaffold geometry or molecular mechanics.

It has been proposed that during pregnancy, the increase in fetal size leads to stretch induced uterine growth via progesterone mediated myometrial hypertrophy. At term, a local reduction in progesterone would result in fetal growth not being translated into myometrial growth thus leading to an increase in myometrial wall tension (Lye, 1998). By applying the theory of tensegrity, this increased tension on the ECM could be transmitted to the nucleus via the surface focal adhesion complexes, leading to alterations in gene expression and transcription of pro-parturient factors such as IL-8 and contraction associated proteins including connexin-43 and the oxytocin receptor (Lye, 1998).

1.52 CERVIX

Background

Prior to the 1940s, clinicians and scientists assumed that the cervix was synonymous with the muscular uterus. However, in 1947, Danforth (Danforth, 1947) described the cervix as a fibrous organ consisting mainly of collagen and elastin, embedded in a proteoglycan ECM. This was poorly received by the scientific community and "provoked little response except for sporadic criticism and implication that (the) findings were due either to artefact or faulty technique" (Danforth, 1980). However, this initial description was subsequently confirmed in 1954 (Danforth, 1954) when it was established that the cervix was a fibrous organ which contained less that 15 % poorly organised peripherally located smooth muscle. The cervix is delineated from the uterine corpus by an ill-defined fibromuscular junction, which lies near the internal os.

Structural components

Collagen

The basic tropocollagen molecule (MW 300,000) is composed of three parallel polypeptide chains, which are tightly coiled into a superhelix (Uldbjerg, et al., 1983). These spontaneously form fibrils through alignment of charged and hydrophobic amino acids. Every third residue in tropocollagen is glycine with proline and 3- and 4-hydroxyproline constituting a further 20 – 23 % (Kokenyesi, 1991). The cervix is composed of collagen types I (70 %) and III (30 %), in which the superhelixes form parallel striated fibrils (Minamoto, et al., 1987), and a small amount of collagen type IV (Leppert and Yu, 1991). The majority of cervical collagen is likely produced by the

fibroblasts although the smooth muscle cells may contribute a small component. It is arranged in an inner and outer longitudinal layer separated by circularly arranged fibres. Collagen types II, IV and V are mainly found in cartilage and basement membrane, respectively.

Collagen mRNA is translated on ribosomes as prepro α chains, which contain a signal peptide required for the passage of chains through the endoplasmic reticulum. The signal peptide is cleaved and the prepro α chains assemble into procollagen. Procollagen undergoes further post-translational modification within the cell and in the extracellular space, after secretion via Golgi-derived vesicle molecules, to produce mature tropocollagen. Mature collagen fibrils are then formed by aggregation of tropocollagen molecules which form intermolecular cross bridges between their lysine and hydroxylysine residues (Fietzek and Kihn, 1976).

Elastin

Elastin comprises about 1.5 % of the connective tissue fraction of the cervix (Leppert, et al., 1983). It comprises one-third glycine residues with a higher proportion of valine and alanine and a lower proportion of hydroxyproline and proline than collagen. Elastin is the main component of elastin fibrils which extend from the external os to the periphery and the upwards to the internal os underlying smooth muscle cells (Leppert, et al., 1986). The fibrils are cross-linked by desmosine bonds between lysine and allysine. Elastin contains regions known as "oiled coils", which are capable of stretching to 2.5 times their original length (Smith, et al., 1983), and these are thought to be responsible for the elastic recoil of the cervix during ripening and parturition (Christiansen, et al., 1991).

Proteoglycan Matrix

The proteoglycan matrix consists of glycoproteins comprising heteropolysaccharide glycosaminoglycan (GAG) chains attached to a protein core backbone (Kokenyesi, 1991). Glycosaminoglycans are highly hydrophilic sulphated polysaccharides composed of repeating polysaccharide subunits. The principal GAGs within the cervix are heparin, heparan sulphate, keratan sulphate, chondroitin - 4 and - 6 sulphate, dermatan sulphate and decorin, the most abundant GAG (Uldbjerg, et al., 1983) formed by attachment of any GAG to a small core protein. Hyaluronic acid is the exception to the general description of GAGs being a much larger molecule (80×10^6) which is neither sulphated nor attached to a core protein. The proteoglycan matrix forms a scaffold to which collagen and elastin are attached, thus conferring structure and rigidity to the cervical stroma. The

strength of the collagen attachment is dependant on the nature of the GAG side chains with those containing iduronic acid, such as dermatan sulphate and heparan sulphate, having increased conformational flexibility and collagen binding compared with those without such as chondroitin sulphate. Finally, the uterine cervix contains a high percentage of water (80 % in non-pregnant) with hyaluronic acid possessing a particularly high affinity for it.

Smooth muscle

Cervical tissue comprises 10 - 15 % smooth muscle fibres, which are peripherally located. There is a wide variation in amount and distribution of these fibres between different individuals and they are not thought to contribute significantly to either the structure or function of the cervix.

Structure of cervix during pregnancy and parturition

The structure of the cervix alters throughout pregnancy. However, the most profound changes occur at parturition during cervical ripening when a marked loosening of the tightly ordered collagen bundles and stromal remodelling occurs. Unfortunately, studies which have aimed to quantify this structural remodelling, have often yielded conflicting results. There are many reasons for this. The techniques utilised between studies are different thus making interstudy comparisons difficult. For example, the amount of hydroxyproline is frequently used to estimate the amount of collagen within the cervix. This biochemical method detects both polymerised and depolymerised collagen fibrils despite the latter likely contributing little to the structural rigidity of the tissue (Uldbjerg, et al., 1983). In contrast, the alternative Picrosirius red staining technique stains only orientated polymerised fibrils with dissociated collagen being unstained (Junqueira, et al., 1979). In addition, the methods use to calculate concentrations of collagen or GAGs are not standardised with dry weight (Cabrol, et al., 1980), dry defatted weight (Kitamura, et al., 1980) and wet tissue weight (Uldbjerg, et al., 1983) all being used as the denominator. More importantly, due to the heterogeneous nature of the cervix and the rapid remodelling that occurs during labour, the precise site and timing of the sample can affect the tissues obtained and therefore the subsequent analysis. With these provisos, the following structural changes occur in the cervix during pregnancy and at term.

The absolute concentration (as measured by extractable hydroxyproline) of collagen decreases by about 70 % during pregnancy but its solubility (by pepsin digestion) doubles indicating increased turnover (Ekman-Ordeberg, 1998). As labour approaches, the

collagen fibrils become looser, more widely scattered and exhibit less crosslinking. During cervical ripening, active degradation of collagen by collagenases generates irregular fibres surrounded by amorphous conglomerations of partially depolymerised collagen with the amount of intact collagen being inversely proportional to the degree of cervical ripeness (Ekman-Ordeberg, et al., 1986). In contrast, the concentration of elastin within the uterus increases eight-fold during pregnancy with levels rapidly returning to the non-pregnant state post partum (Gunja Smith and Woessner, 1985).

Measurement of cervical GAGs is controversial with some studies demonstrating an increase (up to two and a half fold (Danforth, et al., 1974; Owiny, et al., 1987; Rath, et al., 1994), and others a decrease (Ekman-Ordeberg, 1998) in total concentration throughout pregnancy and parturition. Other studies have however reported an increase in GAG concentration up to the onset of parturition followed by a precipitous fall during labour (Rath, et al., 1991; Von Maillot, et al., 1979) or almost no difference between the composition of non-pregnant and intra-partum cervices (Junqueira, et al., 1979). The relative proportions of different proteoglycans alters at parturition with an increase in high molecular weight proteoglycans such as versican (10 - 15 %; Ekman-Ordeberg, 1998) and chondroitin sulphate and a decrease in lower molecular weight proteoglycans such as decorin. This is thought to contribute to collagen fibril disintegration. Whether other proteoglycans such as hyaluronic acid increase or decrease at parturition remain however a matter of debate.

The pregnant and parturient cervix contains a higher proportion of water than the non-pregnant cervix with reported values being 78 – 86 % compared with 74 %, respectively.

Other changes during cervical ripening

The neuronal innervation of the cervix alters during cervical ripening with an increase in neurones releasing vasoactive intestinal peptide (VIP) and PHM-27, thus favouring vasodilation and a decrease those receptive to cGRP thereby reducing pain nocioception. Expression of HLA-DR and S-100 also fall suggesting that the immune status of the cervix might alter during parturition. The concentrations of oestrogen and progesterone receptors decrease at term. The level of their mRNA however, remains constant suggesting that there is increased turnover of the respective receptor. Finally, the concentrations of mRNA and receptor for both insulin growth factor-1 (IGF-1) and endocervical oxytocin increase prior to parturition (Ekman-Ordeberg, 1998).

Mechanism of cervical ripening

Liggins proposed in 1981 that cervical ripening resembled an inflammatory reaction (Liggins, 1981). This hypothesis was in part based on histological studies, which had demonstrated that cervical ripening was accompanied by marked infiltration of the cervical stroma by neutrophils, macrophages and mast cells emigrating from cervical venules (Junqueira, et al., 1980). It is only recently, however that the local mediators involved in this process have begun to be unravelled.

The profound collagen degradation occurring during cervical ripening is thought to be mediated by collagenases. Studies have demonstrated that they increase significantly within the cervix and lower uterine segment (frequently assumed to reflect cervical events) during the latter stages of cervical ripening (Osmers, et al., 1992; Rajabi, et al., 1988; Rechberger and Woessner, 1993) and labour (13 - 23 fold). The two principal collagenases, MMP-8 and MMP-1 are secreted by different cell types and have distinct collagen specificity's. MMP-8 is released in granules from neutrophils and cleaves collagen type I twenty times faster than type III whereas MMP-1 is a fibroblast product and degrades collagen type III fifteen times more effectively than type I (Hasty, et al., 1987). Due to its enzymatic specificity and cellular origin it likely that MMP-8 is the predominant collagenolytic enzyme involved in cervical remodelling. This hypothesis is supported by studies demonstrating release of neutrophil derived MMP-8 (Hasty, et al., 1990) and an increase in leukocyte elastase (Uldbjerg, et al., 1983) activity during cervical ripening. In contrast, in-situ hybridisation studies have failed to demonstrate an increase in MMP-1 release by cervical fibroblasts during cervical ripening (Osmers, et al., 1992; Rath, et al., 1991).

Inflammatory mediators and cervical ripening

A variety of inflammatory mediators have been implicated in cervical ripening. Prostaglandins, particularly the PGE_2 series have been used for over 30 years to ripen the cervix (Calder and Embrey, 1973) and they are still the most widely used and effective cervical ripening agents in current clinical practice. However, the mechanism by which prostaglandins effect cervical remodelling is not well understood. Prostaglandin E_2, PGI_2 and $PGF_{2\alpha}$ are released by the cervix with concentrations increasing within the cervix (Ellwood, et al., 1980) and cervical mucus at term. In addition, cervical tissue expresses PGE_2 and $PGF_{2\alpha}$ receptors (Greer, 1992), with the former being predominately of the EP2 subtype in baboons (Smith, et al., 1998). Administration of PGE_2 reduces cervical collagen concentration but it is not clear whether this is a direct consequence of collagen

breakdown with some studies demonstrating an increase in collagenase activity (Szalay, et al., 1989) and others no difference (Rath, et al., 1987). Alternatively, prostaglandins might act by modifying the composition of GAGs such as chondroitin sulphate thus altering the interaction of collagen with ECM and its hydration (Calder and Greer, 1991). Finally, it has been suggested that PGE_2 might act primarily as a vasodilator and synergise with chemotactic agents to facilitate leukocyte infiltration into the cervix thereby effecting cervical ripening (Kelly, 1994).

The potent neutrophil chemoattractant IL-8 is released by cervix and cervical fibroblasts *in vitro* and *in vivo* in both humans (Barclay, et al., 1993) and animals. In rabbits (El Maradny, et al., 1994) and guinea pigs (Chwalisz, et al., 1994), its intra-cervical application induces cervical ripening, which is characterised by neutrophil infiltration and stromal oedema that is morphologically indistinguishable from the physiological process. Studies in humans have demonstrated a significant increase in IL-8 concentration within the cervix (Sennstrom, et al., 1997) and lower uterine segment at term (Winkler, et al., 1998) with levels correlating with MMP-8 and MMP-9 concentrations (Osmers, et al., 1995). In addition, the synergism between the progesterone regulated (Ito, et al., 1994) IL-8 and mediators such as DHEAS (Kanayama, et al., 1998; Ogawa, et al., 1998), IL-1 and hyaluronic acid (Kobayashi and Terao, 1997), which are also implicated in cervical remodelling, further supports a role of IL-8 in cervical ripening. Other cytokines such as IL-1β and TNF-α have also been shown to induce cervical ripening in rabbits (El Maradny, et al., 1996) and guinea pigs (Chwalisz, et al., 1994) however the remodelling which they effect less closely resembles the physiological process.

Finally, nitric oxide has recently been proposed as a mediator of cervical ripening (Chwalisz and Garfield, 1998). In the rat there is down regulation of cervical iNOS expression during pregnancy and upregulation at the time of parturition (Ali, et al., 1997) and in guinea pigs, intra-cervical application of sodium nitroprusside (SNP), a nitric oxide donor, induces cervical ripening which is morphologically identical to the physiological process (Chwalisz, et al., 1997). The role of nitric oxide in inducing cervical ripening in humans is less clear. Nitric oxide has been demonstrated to induce clinically effective cervical softening in primiparous women, comparable to the cervical state of parous women, prior to termination of pregnancy (Thomson, et al., 1997). Conversely, there are studies documenting its successful use in arresting preterm labour with premature cervical dilatation (Lees, et al., 1994; Rowlands, et al., 1996). However, this effect may be due to the tocolytic properties of nitric oxide and not the consequence of a direct cervical

effect. The mechanism by which nitric oxide may effect cervical ripening is not known but given that it interacts with prostaglandins, MMPs and cytokines it is likely that, in addition to acting as a vasodilator, it will participate in pro-inflammatory remodelling cascades.

1.52 MYOMETRIAL CONTRACTIONS

The onset of regular uterine contractions is an essential component of parturition since, in conjunction with progressive cervical dilatation, this leads to delivery of the fetus. Any discussion of parturition is therefore incomplete unless the structure and physiology of the myometrial "powerhouse" is considered.

Structure

Myometrium is composed of smooth muscle fibres, comprising actin, myosin and intermediate filaments, which are embedded in a collagenous ECM. Myosin consists of two heavy (20 kDa) and two light chains (15 kDa). These are arranged functionally into the head, which contains the actin binding complex and the ATPase enzyme involved in contraction and relaxation, and the tail which participates in formation of the myosin filaments and transmission of muscle tension. Actin monomers (45 kDa) are polymerised into long thin filaments that form cross-bridges with the myosin heads to generate the contractile forces during uterine contractions. Finally, intermediate filaments, by acting as a scaffold to link proteinaceous dense bodies with individual muscle fibres, help to co-ordinate uterine contractions. Transmission and integration of myometrial contractions is further facilitated by the connective tissue and arrangement of the fibres into random bundles (Huszar and Roberts, 1982).

Physiology of Myometrial contractions

In myometrium, the actin-myosin interaction is regulated by enzymatic phosphorylation (contraction) or dephosphorylation (relaxation) of myosin light chains (Adelstein and Eisenberg, 1980). Myosin light chain phosphorylation depends on the activity of myosin light chain kinase (MLCK) whose activity is tightly regulated.

MLCK is activated by binding to a hydrophilic domain of calmodulin and calcium. The calcium originates either by release from intra-cellular calcium stores within the sarcoplasmic reticulum or from entry into the cell via calcium channels. Release of

calcium by the sarcoplasmic reticulum is promoted by inositol 1,4,5-triphosphate (IP_3), generated by phospholipase C mediated hydrolysis of phosphatidyl inositol, which opens sarcoplasmic calcium channels. Extra-cellular calcium enters the cell through voltage gated membrane channels which open with cellular depolarisation. The depolarisation is facilitated by gap junctions, composed of connexins, which have low electrical resistance (Lowenstein, 1981) and enhance metabolic coupling between cells (Larsen, 1983). In addition, oxytocin can further increase intra-cellular calcium by stimulating its release from the sarcoplasmic reticulum (Egarter and Husselein, 1992), favouring ingress through the voltage gated channels (Ciray, et al., 1998) and inhibiting the ATPase pump which normally pumps calcium out of the cell. Myometrial contractions and phosphorylation of MLCK correlate closely with intra-cellular calcium concentrations with levels of at least 10^{-6}M required to generate a contraction (Huszar and Naftolin, 1984).

MLCK is inhibited by protein kinase A which both phosphorylates MLCK, decreasing its affinity for the myosin light chains and generates cAMP, which facilitates calcium uptake into the sarcoplasmic reticulum, thus reducing free calcium. Intracellular calcium is further reduced by cellular repolarisation which closes the voltage gated calcium channels and activation of membrane bound calcium/sodium ion 'pumps' which pump calcium out of the cell. Finally, actin-myosin relaxation can also occur by the action of myosin light chain phosphatase, which cleaves phosphate from the myosin light chain resulting in dissociation of the actin-myosin complex.

Uterine contractions and parturition

In many pregnant animals such as sheep and non-human primates (Nathanielsz, 1991), a pattern of myometrial activity termed contractures have been described. These are an intrinsic property of uterine muscle (Lye and Freiag, 1988) and consist of prolonged, low amplitude increases in intrauterine pressure. Prior to parturition, contractures switch to uterine contractions, which are shorter, more regular and generate greater intrauterine pressures. The precise timing of this switch differs between species and is dependant on a circadian rhythm (Honnebier, et al., 1991) and photoperiodicity (Nathanielsz and Honnebier, 1992), generally occurring at night.

Human myometrium is also capable of exhibiting uncoordinated activity, termed Braxton-Hicks contractions (Braxton-Hicks, 1872), from early pregnancy. Whether contractures precede contractions is however less certain due to difficulty in achieving accurate intra-uterine pressure recordings. The majority of studies that have been performed utilise

external tocography, which although being a reasonable reflection of the intra-uterine environment (Buhimschi, et al., 1998), is inherently inaccurate. Longitudinal studies have demonstrated that uterine activity increases in frequency, amplitude and duration from 20 - 24 weeks (0 - 1 contraction/hour) to 37 - 40 weeks (5 - 10 contractions/hour) when it is maximal nocturnally (Moore, et al., 1994) originating from the right upper quadrant of the uterus (Fallenstein, et al., 1997). Contraction pattern is influenced by rest, coitus (Moore, et al., 1994) and medical contact (Bennett, et al., 1995) but whether parity has an independent effect is more controversial (Dickinson, et al., 1997; Fallenstein, et al., 1997).

At parturition, the myometrium functions as a syncytium. This is facilitated by the expression of low resistance gap junctions within the myometrium which increase in number (Geimonen, et al., 1998) and size (Ciray, et al., 1995) at the onset of labour. The concentration of gap junctions (250 per cell) correlates with the frequency of uterine contractions and the stage of cervical dilatation (Garfield, 1981). Gap junctions are composed of six identical connexin proteins which are arranged in a hexameric array (Revel and Karnovsky, 1967). The predominant connexin present in myometrium is connexin-43 but recently the presence of connexin-40 and -45 have also been identified (Kilarski, et al., 1998).

The role of oxytocin in augmenting parturition and uterine contractions has been more controversial with early studies failing to demonstrate a change in levels at parturition (Dawood, et al., 1978). More recent studies, employing frequent sampling, however demonstrated that oxytocin was released in a pulsatile manner with the frequency and amplitude of pulses increasing significantly throughout labour (Fuchs, et al., 1991). In addition, the concentration, but not the affinity (Fuchs, et al., 1984), of the G-protein coupled oxytocin receptor and its mRNA increases throughout pregnancy with levels of the latter reaching 300 times that of the non-pregnant state during the first stage of labour (Kimura, et al., 1996). In addition to increasing intracellular calcium, thus favouring uterine contractions as previously described, oxytocin depolarises myometrial membranes (Ciray, et al., 1998) and may increase the number of gap junctions (Ambrus and Rao, 1994), although this is controversial (Ciray, et al., 1995).

1.54 OTHER INTRA-UTERINE COMPONENTS

Placenta

The placenta is composed of cytotrophoblast and syncytiotrophoblast. Placenta has many functions including mediating fetal gaseous exchange, excreting waste products, elaborating many hormones and cytokines and contributing to the materno-fetal immunological interface. At parturition, the placenta secretes hormones such as oestriol, and there is increased release of pro-inflammatory mediators (IL-1, IL-6; Mitchell, et al., 1993).

Fetal membranes

Fetal membranes consist of amnion and chorion. The avascular amnion comprises an inner epithelial layer, basement membrane, compact, fibroblast and an outer reticular layer, which underlies the chorion. Chorion is composed of a fibroblast and reticular layer, basement membrane and its principal constituent, the trophoblast, which is adjacent and adherent to decidua (Chard and Lilford, 1997). Fetal membranes release many inflammatory mediators including cytokines, prostaglandins, MMPs and TIMPs. Although amnion derived prostaglandins increase at labour (Bennett, et al., 1992) (Skinner and Challis, 1985) whether significant quantities gain access to the myometrium is not certain due to the high concentrations of PGDH in the chorion (Cheung and Challis, 1989).

Decidua

Under the influence of progesterone, endometrial stromal cells enlarge, proliferate and store glycogen converting the endometrial lining of the non-pregnant uterus into the decidua of pregnancy. During the first half of pregnancy, decidual stroma comprises mainly large granular lymphocytes and trophoblast associated macrophages with some B- and T-lymphocytes (Chard and Lilford, 1997). As pregnancy progresses, these cells decline in number resulting in term decidua being much less distinct and lined with flattened epithelium. Decidua is divided into three regions in relation to the developing fetus. Decidua basalis underlies the conceptus, is adjacent to the chorion and is involved in formation of the maternal side of the placenta. The decidua capsularis lies over the surface of the conceptus and the decidua parietalis lines the remainder of the uterus (Ritchie, 1995). Decidua synthesises many cytokines (IL-1, IL-6), hormones (prolactin, relaxin) and prostaglandins which, at parturition, likely interact in a paracrine manner with those released by fetal membranes (Dudley, et al., 1992).

Amniotic fluid

In early pregnancy, amniotic fluid is mainly a transudate of fetal serum via fetal skin and the umbilical cord. At term, there are contributions from the amniotic membrane, fetal urine and excretion of lung fluids. The primary function of amniotic fluid is to protect the fetus from external trauma, however it also acts as a reservoir for waste products and inflammatory mediators. Prior to and during labour, concentrations of cytokines (IL-1, IL-8; Halgunset, et al., 1994; Laham, et al., 1993) and prostaglandins (Romero, et al., 1994) increase significantly within amniotic fluid.

SUMMARY

To summarise, the literature review has considered some of the cytokines, chemokines and inflammatory mediators including prostaglandins, nitric oxide, MMPs and TIMPs likely involved in the control and regulation of reproductive processes. Current knowledge as to their roles in the specific processes of the initiation and maintenance of pregnancy, parturition and control of cervical ripening and was then considered.

HYPOTHESIS

It is therefore **hypothesised** that inflammatory mediators play a vital role in the initiation and maintenance of pregnancy, the control of cervical ripening and parturition.

AIMS

The specific **aims** of this thesis were therefore to :

1. Investigate the roles of inflammatory mediators in the initiation and maintenance of pregnancy

2. Examine the roles of inflammatory mediators in cervical ripening and parturition

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 MATERIALS

The sources of all the reagents used for the experiments described in all the following chapters are detailed below (Table 3). For ease of reference, they have been subdivided into experimental categories.

Table 3

Source of reagents used for experiments

TISSUE COLLECTION MATERIALS	SOURCE
Li-Heparin blood collecting tubes	Monovette, Sarstedt, Amsterdam, Holland
RPMI 1640 Culture Medium	Sigma-Aldrich Company Ltd., Poole, UK
10 % Neutral Buffered Formalin (NBF)	See Appendix 1 for recipe
Tissue-Tek O.C.T Compound (Tissue Collecting Medium)	Tissue-Tek, Miles Inc., Slough, UK
Peel-A-Way Disposal Embedding Moulds	Polysciences Inc., Warrington, USA
Nunc Cryotubes	Gibco, Paisley, UK
Isopentane	Merck Ltd. (BDH), Lutterworth, UK

TISSUE CULTURE MATERIALS - GENERAL	SOURCE
24 well culture plates	Costar Ltd., High Wycombe, UK
RPMI 1640 culture medium	Sigma-Aldrich Company Ltd., Poole, UK
Fetal calf serum (FCS)	Sigma-Aldrich Company Ltd., Poole, UK
Phosphate buffered saline (PBS)	Gibco, Paisley, UK
Penicillin/Streptomycin	Gibco, Paisley, UK
Gentamicin	Sigma-Aldrich Company Ltd., Poole, UK

TISSUE CULTURE MATERIALS - GENERAL cont.	SOURCE
Complete medium	See Appendix 1 for recipe
Phorbol 12-Myristate 13-Acetate	Sigma-Aldrich Company Ltd., Poole, UK
Prostaglandin E_2	Sigma-Aldrich Company Ltd., Poole, UK
19-hydroxy PGE	Cayman Chemicals, Ann Arbor, Michigan, USA
Progesterone	Sigma-Aldrich Company Ltd., Poole, UK
Dexamethasone	Sigma-Aldrich Company Ltd., Poole, UK
Medroxy progesterone acetate	Sigma-Aldrich Company Ltd., Poole, UK
Oestrogen	Sigma-Aldrich Company Ltd., Poole, UK
Mifepristone	Roussel-Uclaf, Paris, France
Lipopolysaccharide	Sigma-Aldrich Company Ltd., Poole, UK
Spermine NONOate	Sigma-Aldrich Company Ltd., Poole, UK
Interleukin-1α	R & D Systems, Oxford, UK
Interleukin-1β	R & D Systems, Oxford, UK
Tumour necrosis factor-α	Sigma-Aldrich Company Ltd., Poole, UK
Insulin/Transferrin/Selenium	Gibco, Paisley, UK
Trypsin	Sigma-Aldrich Company Ltd., Poole, UK
DNAse	Sigma-Aldrich Company Ltd., Poole, UK

PBMC/CD 14 CELL SEPARATION	SOURCE
Lymphocyte separation medium	Sigma, Density 1.077, Poole, UK
Cell separation buffer	See Appendix 1 for details
30 mm filter	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS anti-$CD14^+$ Microbeads	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS separation column	Miltenyi Biotec, Bergisch Gladbach, Germany

CELL/SAMPLE PREPARATION	SOURCE
Cervical fibroblasts	
Steeping buffer	See appendix 1 for details
Dispase I (neutral protease; grade I)	Boehringer Mannheim, Lewes, UK
Tissue culture flasks	Corning, Costar, High Wycombe, UK
Amnion and chorio-decidua cell preparation	
Absorbent capillary matting	The Fyba Pot Company Ltd, Khottingley, UK
Transport solution	See appendix 1 for details
Digestion medium	See appendix 1 for details
0.171 mm nylon mesh	Lockertex, Warrington, UK
Placental perfusion	
Kreb's Solution	Gibco, Paisley, UK

Placental perfusion cont.	SOURCE
Dextran	Sigma-Aldrich Company Ltd., Poole, UK
Noradrenalin	Sigma-Aldrich Company Ltd., Poole, UK
Seminal plasma fraction	
Ethanol	Hayman, Witham, UK
ENV^+ extraction columns	International Sorbent Technology Ltd., YstradMynach, Hengoed, UK
Hexafluoropropanol	Sigma-Aldrich Company Ltd., Poole, UK
Chloroform	Merk, Poole, UK

CELL LINES	SOURCE
U937 cells	European Cell Culture Collection, Salisbury, UK

ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA) - GENERAL	SOURCE
Plates (96 well)	Nunc Maxi Sorp, Gibco, Paisley, UK
Coating solution	See Appendix 1 for recipe
Wash buffer	See Appendix 1 for recipe
ELISA assay buffer	See Appendix 1 for recipe
Streptavidin peroxidase	Boehringer Mannheim, Lewes, UK
Substrate	See Appendix 1 for recipe
Sulphuric acid (H_2SO_4)	Merck Ltd. (BDH), Lutterworth, UK

CYTOKINE ASSAYS	SOURCE
MCP-1 ELISA	
MCP-1 capture antibody	Toray Industries Inc., Tokyo, Japan
MCP-1 standard	Toray Industries Inc., Tokyo, Japan
Peroxidase labelled Fab fragment to recombinant MCP-1	Fab fragment from Toray Industries Inc., Tokyo, Japan. Peroxidation undertaken in house.
IL-8 ELISA	
IL-8 capture antibody	R&D Systems, Oxford, UK
IL-8 recombinant standard	Toray Industries Inc., Tokyo, Japan
IL-8 detection antibody	R&D Systems, Oxford, UK
Amplex red substrate	See Appendix I for recipe
RANTES ELISA	
RANTES coating antibody	R&D Systems, Oxford, UK
RANTES recombinant standard	R&D Systems, Oxford, UK
RANTES detection antibody	R&D Systems, Oxford, UK
IL-10 ELISA	
IL-10 coating antibody	Pharmingen, San Diego, USA
IL-10 recombinant standard	Pharmingen, San Diego, USA
IL-10 detection antibody	Pharmingen, San Diego, USA
Polyperoxidase	CLB Laboratories, Amsterdam, Holland

Secretory leukocyte protease inhibitor ELISA	SOURCE
Recombinant secretory leukocyte protease inhibitor	R&D Systems, Oxford, UK
Polyclonal anti-secretory leukocyte protease inhibitor	R&D Systems, Oxford, UK
Anti-sheep/goat IgG-peroxidase Fab fragments	Boehringer Mannheim, Lewes, UK
Commercial secretory leukocyte protease inhibitor assay kit	R&D Systems, Oxford, UK

PROSTAGLANDIN ASSAYS	SOURCE
PGE_2 ELISA	
Methyloximating solution	See Appendix 1 for recipe
Plates (96 well)	Costar amine-binding plates, Paisley, UK
Donkey anti-rabbit (DAR) serum	Scottish Antibody Production Unit, Carluke, UK
Blocking solution	See Appendix 1 for recipe
Synthetic standards	Applied Therapeutics, Paisley, UK
Dimethylformamide (DMF)	Sigma-Aldrich Company Ltd., Poole, UK
Tributylamine	Sigma-Aldrich Company Ltd., Poole, UK
Butylchloroformate	Sigma-Aldrich Company Ltd., Poole, UK
Biocytin	Sigma-Aldrich Company Ltd., Poole, UK

19-hydroxy PGE ELISA	SOURCE
Plates (96 well)	Costar amine-binding plates, Paisley, UK
Standards	Purified in house
Acetonitrile	Merk, Poole, UK
Methanol	Merk, Poole, UK
Methyl formate	Merk, Poole, UK
Tributylamine	Sigma-Aldrich Company Ltd., Poole, UK
Butylchloroformate	Sigma-Aldrich Company Ltd., Poole, UK
Pro-Gly-Tyr-Biotin	Synthesised in house

IMMUNOHISTOCHEMISTRY GENERAL	SOURCE
Histoclear	National Diagnostics, Atlanta, Georgia, USA
Hydrogen peroxide (H_2O_2)	Merck Ltd. (BDH), Lutterworth, UK
Methanol	Merck Ltd. (BDH), Lutterworth, UK
ABC-HRP and Elite complexes	Vectastain kits, Vector laboratories, Peterborough, UK
3, 3'-diaminobenzidine (DAB) substrate	Vector SK-4100, Vector Laboratories, Peterborough, UK
Harris's haematoxylin	Pioneer Research Chemicals Ltd., Colchester, UK
Microscope glass slides	Merck Ltd. (BDH), Lutterworth, UK
Glass coverslips	Merck Ltd. (BDH), Lutterworth, UK

SPECIFIC IMMUNOHISTOCHEMISTRY	SOURCE
Cytokeratin	
Monoclonal human anti-mouse pan cytokeratin antibody	Sigma-Aldrich Company Ltd., Poole, UK
Vimentin	
Monoclonal human anti-mouse vimentin antibody	Sigma-Aldrich Company Ltd., Poole, UK
MMP-1	
Human anti-rabbit monoclonal antibody	Triple Point Biologics, Insight Biotechnology, Wembley, UK
MMP-2	
Human anti-mouse monoclonal antibody	Calbiochem, Nottingham, UK
MMP-8	
Human anti-rabbit monoclonal antibody	Triple Point Biologics, Insight Biotechnology, Wembley, UK
MMP-9	
Human anti-mouse monoclonal antibody	Triple Point Biologics, Insight Biotechnology, Wembley, UK
TIMP-1	
Human anti-rabbit monoclonal antibody	Triple Point Biologics, Insight Biotechnology, Wembley, UK
TIMP-2	
Human anti-rabbit monoclonal antibody	Triple Point Biologics, Insight Biotechnology, Wembley, UK

SPECIFIC IMMUNOHISTOCHEMISTRY cont.	SOURCE
TIMP-3	
Human anti-rabbit monoclonal antibody	Triple Point Biologics, Insight Biotechnology, Wembley, UK
TIMP-4	
Human anti-rabbit monoclonal antibody	Chemicon, Harrow, UK
CD45 (leukocyte common antigen)	
Human anti-mouse monoclonal antibody	Dako, Cambridge, UK
CD68	
Human anti-mouse monoclonal antibody	Dako, Cambridge, UK
Neutrophil elastase	
Human anti-mouse monoclonal antibody	Dako, Cambridge, UK
Secretory leukocyte protease inhibitor	
Normal donkey serum	Scottish Antibody Production Unit, Carluke, UK
Polyclonal SLPI primary a/b (as for ELISA)	R&D Systems, Oxford, UK
Anti-sheep/goat Fab secondary antibody (as for ELISA)	R&D Systems, Oxford, UK

ZYMOGRAPHY	SOURCE
Sample application buffer	See Appendix 1 for recipe
Running tank buffer	See Appendix 1 for recipe
Resolving gel buffer (Stock A)	See Appendix 1 for recipe

ZYMOGRAPHY	SOURCE
Stacking gel buffer (Stock B)	See Appendix 1 for recipe
Stock wash buffer X 10	See Appendix 1 for recipe
Resolving gel	See Appendix 1 for recipe
Stacking gel	See Appendix 1 for recipe
Triton-X-100 Wash	See Appendix 1 for recipe
Digestion buffer	See Appendix 1 for recipe
Destaining solution	See Appendix 1 for recipe
Staining solution	See Appendix 1 for recipe
Butanol	Sigma-Aldrich Company Ltd., Poole, UK

REVERSE ZYMOGRAPHY	SOURCE
Wash buffer/Rinse buffer	See Appendix 1 for recipe
Incubation buffer/Digestion buffer	See Appendix 1 for recipe
Resolving gel (Lower gel)	See Appendix 1 for recipe
Stacking gel (Upper gel)	See Appendix 1 for recipe
All other reagents/solutions as for Zymography	

WESTERN BLOT	SOURCE
Dialysis cellulose membrane (D 9277, 10 mm)	Sigma-Aldrich Company Ltd., Poole, UK
Homogenisation/Extraction buffer	See Appendix 1 for recipe
Sample application buffer	See Appendix 1 for recipe
Stock wash buffer	See Appendix 1 for recipe

WESTERN BLOT cont.	SOURCE
Membrane blocking buffer	See Appendix 1 for recipe
Transfer/Blotting buffer	See Appendix 1 for recipe
Resolving gel (Lower gel)	See Appendix 1 for recipe
Stacking gel (Upper gel)	See Appendix 1 for recipe
Primary antibody solution	See Appendix 1 for recipe

2.2 METHODS

2.21 SAMPLE COLLECTION

2.211 PERIPHERAL BLOOD

Peripheral blood was obtained by sterile venepuncture in Li-heparin tubes from:

Non-pregnant women

The women had regular menstrual cycles, were not using any hormonal form of contraception and were at various stages of the menstrual cycle. Preliminary studies demonstrated that results were not influenced by stage of the menstrual cycle.

Pregnant women

1. *First trimester*
   Healthy pregnant women during the first trimester of pregnancy (8 - 12 weeks).

2. *Term not in labour*
   Healthy women with uncomplicated pregnancies at term (>37 weeks) who were not in labour.

Labour was defined as the onset of regular uterine activity leading to progressive cervical effacement and dilatation of greater than 3 cm.

3. *Term in labour*
   Healthy women with uncomplicated pregnancies at term (>37 weeks) who were in spontaneous, active labour. Exclusion criteria included use of analgesia, oxytocics, pyrexia (37.5°C) and prolonged rupture of membranes.

Prolonged rupture of membranes was defined as rupture of membranes more than 48 hours prior to the onset of labour.

Peripheral blood was

1. Stored at 4°C until dilution 1 : 10 in Rosewell Park Memorial Institute Medium (RPMI 1640) within 20 minutes of collection.

2. Separated as described below (Section 2.221).

2.212 CERVICAL BIOPSIES

Cervical biopsies were obtained from:

Non-pregnant women

A longitudinal section of the anterior lip of the cervix was taken using a scalpel within 10 minutes of hysterectomy. Biopsies, approximately 20 - 35 mg in weight, 15 - 20 mm in length and 2 - 3 mm in diameter, were obtained from healthy, non-pregnant women (n = 50, ages 29 - 45) with regular menstrual cycles undergoing a hysterectomy for a non-malignant condition. Preliminary studies demonstrated that stage of the menstrual cycle did not affect the results.

Biopsies were divided into two pieces on a sterile surgical swab and placed immediately into:

1. RPMI 1640 culture medium at 4°C, for transport.

2. 10 % Neutral Buffered Formalin (NBF) to fix overnight at 4°C, then stored in 70 % ethanol prior to routine paraffin embedding.

Pregnant women

Mifepristone (RU486) treatment prior to termination of pregnancy

Thirty nulliparous women of less than 9 weeks amenorrhoea who were to have a therapeutic suction termination of pregnancy under general anaesthesia were recruited. Exclusion criteria included age less than 16, serious medical condition, previous cervical

surgery and inability to give informed consent. Women were randomised into one of six groups. The surgeons performing the terminations were blinded to the treatment allocation of the women.

Study Groups

Group	Interval between mifepristone treatment and termination	Number
Group 1	No treatment	6
Group 2	6 hours	6
Group 3	12 hours	6
Group 4	24 hours	6
Group 5	36 hours	6

Table 4

Details of patient numbers and treatment protocols for mifepristone (RU486) treatment prior to termination of pregnancy

Immediately prior to termination, a cervical biopsy was taken from the anterior lip of the cervix using a Shumaker punch biopsy forceps. Biopsies were fixed overnight in 10 % NBF at 4°C, then stored in 70 % ethanol prior to routine paraffin embedding.

Written, informed consent was obtained prior to tissue collection from Lothian Research Ethics Committee.

2.213 AMNION, CHORIO-DECIDUA, DECIDUA AND PLACENTA TISSUE

Amnion, chorio-decidua, decidua and placenta were collected from women at term with uncomplicated pregnancies either at caesarean section (elective caesarean section, not in labour; emergency caesarean section in spontaneous, active labour, not induced) or after spontaneous vaginal delivery as detailed below:

1. *Explant studies*

   Term elective caesarean section, n = 6

2. *Placental perfusion studies*

After spontaneous vaginal delivery, n = 8.

3. *Immunohistochemistry*

Term elective caesarean section, n = 4.

Term emergency caesarean section in spontaneous, active labour, n = 4.

Tissue was transported to the laboratory in RPMI 1640 at 4°C and processed within 10 minutes of collection. Informed and written consent was obtained from women prior to collection of all samples.

2.214 AMNIOTIC FLUID AND EXTRA-EMBRYONIC COELOMIC FLUID

First trimester

Amniotic and extra-embryonic coelomic fluid were collected by transvaginal ultrasound-guided needle aspiration between 8 - 11 weeks of gestation prior to the termination of pregnancy under general anaesthesia, as described in detail previously (Wathen, et al., 1991). Women with vaginal bleeding or uncertain menstrual dates were excluded from the study. Fetal viability and gestational age were confirmed by identification of the fetal heart and by measurement of crown-rump length, respectively by ultrasonic measurement.

Second trimester

Samples of amniotic fluid from the second trimester (n = 20; 15 - 18 weeks) were obtained from women undergoing an amniocentesis as part of the karyotype screening programme for Downs' Syndrome. All pregnancies were karyotypically normal.

Third trimester

Samples of amniotic fluid were obtained from women undergoing an elective caesarean section (n = 15; >37 weeks) or after spontaneous vaginal delivery (n = 15; >37 weeks, uncomplicated labour, not induced).

All samples were centrifuged at 1000 g for 5 minutes and stored at -20°C prior to analysis. Informed and written consent was obtained from women prior to collection of all samples.

2.215 FETAL URINE SAMPLES

Fetal urine samples, which were clear and not contaminated with blood, were collected at first micturition immediately after delivery at term by elective caesarean section from women with uncomplicated pregnancies (n = 12). Samples were centrifuged at 1000 g for 5 minutes and stored at -20°C prior to immunoassay. Informed and written consent was obtained from women prior to collection of all samples.

2.216 FETAL SERUM SAMPLES

Fetal serum was collected at term from the placental venous cord vein after spontaneous vaginal delivery (n = 5) and emergency caesarean section (spontaneous active labour, n = 5). Samples were centrifuged at 1000 g for 10 minutes. Informed and written consent was obtained from women prior to collection of all samples.

2.217 SEMINAL PLASMA

Human seminal plasma was obtained from healthy young men (ages 18 - 45) involved in the ongoing semen donor programme. Informed and written consent was obtained from men prior to collection of semen.

2.22 TISSUE CULTURE

2.221 PERIPHERAL BLOOD

Whole blood cultures

Peripheral whole blood, which had been diluted 1 : 10 in RPMI 1640, was plated out (1 ml/well) in 24 well culture plates and cultured for 24 hours at 37°C in 95 % air and 5 %

CO_2 under humid conditions with treatments added at the time of subculturing. The harvested media were frozen at -20°C until analysis.

Peripheral blood mononuclear cell preparations (PBMC)

A PBMC preparation separates out peripheral blood mononuclear cells (all leukocytes apart from neutrophils) by centrifuging whole blood with lymphocyte separation medium by density separation. Mononuclear cells form a layer at the interface between serum and the separation medium after centrifugation.

Undiluted whole blood (10 mls) was layered on top of lymphocyte separation medium (5 mls; Figure 2A and 2B). Tubes were centrifuged at 2500 rpm for 25 minutes at 25°C prior to removing the interface mononuclear cell (Figure 2C) and resuspending the cells in PBS (15 mls). The pellet containing the neutrophils and red blood cells was saved for subsequent neutrophil extraction. Mononuclear cells were then centrifuged at 1500 rpm for 40 minutes at 25°C, the supernatant removed and the pellet washed twice in PBS (centrifuged at 1500 rpm for 10 minutes at 25°C). Cells were resuspended in 10 mls complete medium (Figure 2D), counted with a haemocytometer and plated out at 1 x 10^5 cells/ml prior to culturing as described previously. Cell viability by trypan blue exclusion was >95 %. Media were stored at -20°C until analysis.

Neutrophil isolation

Sterile water (5 mls) was added (45 seconds) to the pelleted neutrophils and red blood cells to lyse the red blood cells, prior to adding double strength PBS (5 mls) to quench the reaction. Neutrophils were then pelleted by centrifugation (1000 rpm for 8 minutes), resuspended in complete medium, counted and plated out as described above. Cell viability by trypan blue exclusion was >95 %. Media were stored at -20°C until analysis.

Magnetic Cell Sorting of PBMC preparations into $CD14^+$ and $CD14^-$ cell fractions

Magnetic cell sorting (MACS) is a technique which enables a heterogeneous cell population to be sorted into a distinct cell populations. Cells are labelled with colloidal super-paramagnetic Microbeads (~ 100 nm diameter) conjugated to an antibody which recognises an epitope of a specific cell surface marker. Labelled cells are then passed through a high gradient magnetic column. Unlabelled cells pass through the column while labelled cells are retained and can be easily eluted by removing the column from

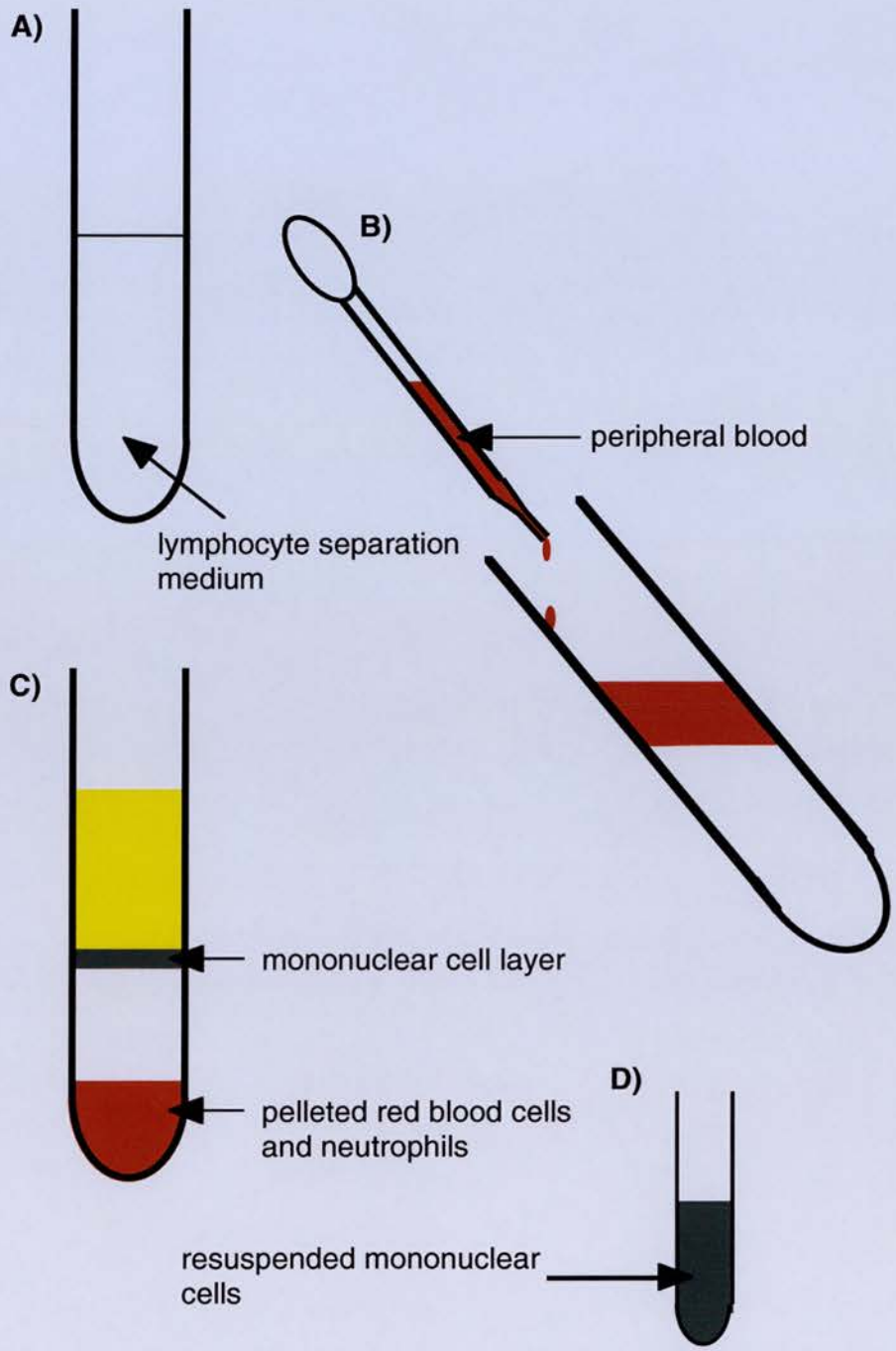


Figure 2

Peripheral blood mononuclear cell preparation. **A)** Lymphocyte separation medium, **B)** Peripheral blood layered on medium, **C)** Mononuclear and red cell/lymphocyte layer after centrifugation and **D)** Resuspended mononuclear cells.

the magnetic field. More than 10^9 cells can be processed within 15 minutes and enrichment and depletion rates of more than 100- and 1000-fold, respectively can be achieved. The magnetic tagging does not appear to affect cell viability and proliferation.

CD14 is a glycosylphosphatidylinositol (GPI) - anchored cell surface protein of myeloid lineage cells which is expressed on the surface of peripheral monocytes. It acts as a LPS receptor, binding LPS in conjunction with the soluble LPS binding protein (LBP). This initiates cellular activation via kinase cascades (Ulevitch and Tobias, 1995). CD14 can be used to isolate cells of myeloid lineage from peripheral blood.

Pelleted PBMC preparations were resuspended in separation buffer (2 mls), passed through a 30 mm filter, centrifuged at 1500 rpm for 15 minutes at 20°C then resuspended in separation buffer (100 μl). MACS colloidal super-paramagnetic Microbeads, conjugated with mouse anti-human CD14 antibodies (Mouse isotype IgG_{2a}) were then added (25 μl) and the sample incubated at 4°C for 15 - 20 minutes. The MACS separation column was pre-washed with 500 μl separation buffer, placed within the magnetic field of the MACS separator magnet, and separation buffer (700 μl) added to elute the $CD14^-$ fraction. The column was washed with separation buffer (1.5 mls), removed from the magnet and rewashed (1.2 mls) to elute the $CD14^+$ fraction. Both fractions were resuspended in 5 mls complete medium, counted and plated out at 1 x 10^5 cells/ml prior to culturing for 24 hours as described above. Cell viability by trypan blue exclusion was >95 %. Media were stored at -20°C until analysis.

2.222 CERVICAL BIOPSIES

Whole tissue explants

Cervical biopsies from non-pregnant and pregnant women were dissected into small pieces (1 - 2 mm^3, 2 - 4 mg) and cultured with treatments in quadruplicate as previously described. Biopsies for cytokine analysis were cultured in complete media and those for zymography and reverse zymography in RPMI 1640. Biopsies were weighed after 24 hours culture. Media were stored at -20°C until analysis.

Cervical Fibroblast cell preparation

Non-pregnant cervical explants were washed twice in PBS and incubated in steeping buffer for 60 minutes at 23°C. Biopsies were then incubated with Dispase I (1 U/ml in PBS) for 2 - 3 hours at 37°C with gentle agitation. Dispase is a neutral protease derived from Bacillus polymyxa which does not damage cell membranes and separates epithelial cells from the underlying stroma by cleaving the basement membrane zone region (Stenn, et al., 1989). Explants were washed twice in PBS, the ecto- and endo-cervical epithelium sheared off in sheets using a scalpel and the underlying cervical stroma scraped and dissected into small pieces 1 mm^3. The resulting cellular and tissue debri was placed into 75 mm^3 tissue culture flasks and cultured in complete medium as previously described. Cervical fibroblasts grew to confluence within 28 days and were used up to passage 6. Cells for cytokine ELISAs were subsequently grown in complete medium and those for zymography in RPMI 1640. Viability was >95 % by trypan blue exclusion and cells were >95 % vimentin positive.

2.223 AMNION, CHORION-DECIDUA, DECIDUA AND PLACENTA EXPLANT CULTURE

Discs of amnion (12 mm diameter; wet weight 15 - 20 mg) and chorio-decidua (9 mm diameter; wet weight 15 - 25 mg) were prepared using a cork borer. Decidua (wet weight 15 - 25 mg) was dissected off the myometrial aspect of the posterior uterine wall from a site away from the placental bed and pieces of villous placental tissue dissected from the middle of a central cotyledon (wet weight 15 - 30 mg). Explants were placed on absorbent capillary matting in a 24-well plate and maintained in complete medium for 24 hours at 37°C in 95 % air and 5 % CO_2 under humid conditions. The harvested media were then frozen at –20°C until analysis.

Preparation of amnion and chorion cells

Amnion and chorion were collected immediately after delivery from patients undergoing elective caesarean section at term (not in labour), transported to the laboratory in transport solution at 4°C then incubated in steeping buffer for 60 minutes at 23°C. Amnion and chorion trophoblast cells were prepared for and maintained in culture using methods adapted from Jones et al. (1989) (Jones, et al., 1989; See Appendix I for recipes and Appendix II for detailed experimental protocols). Briefly, excess decidua was scraped from the chorion laeve and amnion and chorion were minced, washed and incubated in

digestion medium with mechanical agitation for 40 minutes at 37°C. Dispersed cells were collected by passing through a 0.171 mm nylon mesh three times. Cells were washed in complete medium and viability's for all preparations were assessed as >90 % by trypan blue exclusion. Cells were cultured in 24 well plates (2 x 10^5/well; 1 ml medium/well) in complete culture medium for 2 - 4 days. Chorion fibroblasts were obtained by dilution of chorion trophoblast cells with subsequent overgrowth of fibroblasts to confluence within 28 days. Cells were used up to passage 5. Cells for cytokine ELISAs were subsequently grown in complete medium. Amnion epithelial cells, chorion trophoblast and chorion fibroblasts were characterised with the epithelial and fibroblast cell markers cytokeratin and vimentin, respectively. Amnion cell preparations were 95 % cytokeratin positive and 5 % vimentin positive, chorion trophoblast cells preparations were 95 % cytokeratin positive and 5 % vimentin positive and chorion fibroblasts were 95 % vimentin positive and 5 % cytokeratin positive.

2.224 DUAL PERFUSION OF A PLACENTAL COTYLEDON

A peripheral cotyledon was selected which was macroscopically intact with parallel chorionic artery and vein, cannulated and mounted in a perfusion chamber as described previously (Schneider and Huch, 1985; Schneider, et al., 1972), with minor modifications (Benediktsson, et al., 1997). The maternal and fetal compartments were perfused with Kreb's solution at flow rates of 10 and 6 ml/minute, and gassed with 95 % O_2/5 % CO_2 and 95 % N_2/5 % CO_2, respectively. The perfusate on the fetal side was supplemented with dextran (20 g/l; average molecular weight 74 kDa). Perfusions were started within 10 minutes of delivery. After a 40 minute equilibration period, which permitted the residual blood to be eluted and circulatory perfusion pressures to stabilise, consecutive 10 minute samples were collected from both maternal and fetal circuits. A pre-infusion sample was followed by three samples during the infusion of PGE_2 (500 pg/ml for 30 minutes) into the maternal circuit, followed by two post-infusion samples. Samples were stored at –20°C before being assayed.

2.225 PREPARATION OF SEMINAL PLASMA FRACTION

Lipid extraction of seminal plasma was performed to remove the majority of the cytotoxic polyamines such as spermine and spermidine (Allen and Roberts, 1986). Human seminal

plasma (700 mls) was treated with ethanol (1000 mls) and centrifuged at 4°C for 20 minutes at 1,500 g. The supernatant was evaporated at 23°C to a final volume of 450 mls then passed through ENV+ extraction columns which had previously been washed with 1 : 1 ethanol/hexafluoropropanol (HFP). Lipid was eluted with 1 : 1 ethanol/HFP and the liquid evaporated at 23°C to 10 mls. Chloroform (15 mls) was added to leach the residue and the chloroform layer evaporated and redissolved in ethanol (4 mls) to give the seminal plasma fraction.

2.226 U937 CELL LINE

U937 cells are a pro-monocytic cell line derived from a diffuse hystiocytic lymphoma, which is committed to the macrophage branch of the myeloid lineage. Cells were used as a model to further investigate cytokine release. Cells were maintained in complete medium and plated out at $1x10^5$ cells/ml for experiments. Studies were either performed on undifferentiated cells or on those which had received phorbol 12-myristate 13-acetate (PMA), coincident with treatments, for 24 hours to differentiate them into macrophages.

2.23 ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISAs)

This technique is used to determine the concentration of a given substance in a solution by comparison with a standard curve created from solutions of known concentrations. There are two principal types of ELISA:

1. Two site ELISA

In this technique, plates are precoated with a coating antibody (Figure 3A and 3B). The substance (X) to be measured is then added and is recognised and bound by the coating antibody by a specific epitope (Figure 3C). Next, the detection antibody, labelled with biotin, is added which binds to X by a different epitope (Figure 3D). Finally, streptavidin peroxidase is added which binds to the biotin (Figure 3E) and this is detected by addition of substrate (Figure 3F). For example: IL-8, RANTES, IL-10. Alternatively, the detection antibody may be peroxidase linked thus omitting the streptavidin peroxidase step. For example: MCP-1.

2. Competition ELISA

Plates are coated with the recombinant protein to be measured (rY) and then blocked to remove non-specific binding (Figures 4A and B). The media containing the substance Y to be measured is then added with anti-sera (Figures 4C1 and C2). The anti-sera recognises identical epitopes on the rY to that on Y and competes for binding. The plates are then washed. If the media being measured contains a high concentration of Y, then the majority of the anti-sera will have become conjugated to it and will be washed off (Figure 4D2). However, if the media contains low concentrations of Y, then anti-sera will have become conjugated and bound to the rY on the plate (Figure 4D1). The secondary antibody is then added which binds to an epitope on the anti-sera and this is then detected by adding the detection system. Wells with a lots of anti-sera bound (low levels of Y in original sample) will read high (Figure 4E1) and those with low amounts of anti-sera bound will read low (high levels of Y in origin sample; Figure 4E2). The amount of Y in the origin sample can be calculated by comparing values obtained by using a standard curve.

Detailed protocols

Monocyte chemotactic protein-1 (MCP-1)

MCP-1 was measured by ELISA using a peroxidase labelled fragment antibody binding (Fab) fragment of an antibody to recombinant MCP-1 as previously described (Ida, et al., 1994; Kelly, et al., 1997). Briefly, plates were coated overnight at 4°C with MCP-1 capture antibody (4 μg/ml in PBS; 100 μl/well) then washed once in tap water and coating solution added (100 μl/well) for 30 minutes. Plates were air dried and stored with a desiccant at 4°C. Samples and standards were added (diluted in ELISA buffer; 100 μl/well) with the highest concentration of standard being 500 pg/ml. Plates were incubated for 3 hours at 23°C on an orbital plate shaker prior to adding peroxidase-coupled detection antibody (diluted 1 : 200 in ELISA Buffer; 100 μl/well) and incubating for 45 minutes at 23°C on an orbital shaker. Finally, plates were washed and substrate added (200 μl/well) prior to quenching with 2N H_2SO_4. Absorption was read at 450 nm within 30 minutes of quenching. The intra-assay precision was 6.3 % relative standard deviation (r.s.d), inter-assay precision was 8.6 % r.s.d and the detection limit of the assay was 7.5 pg/ml.

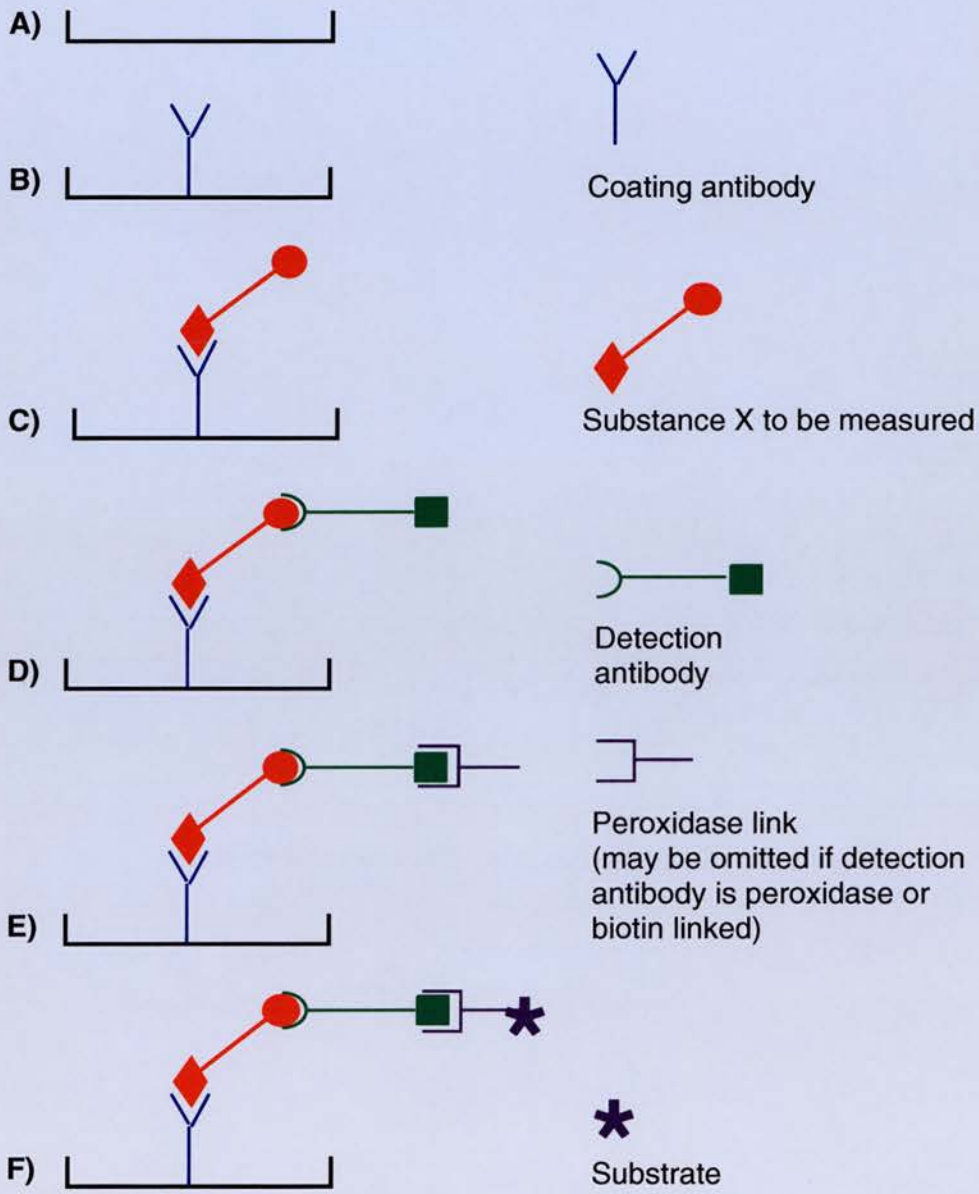


Figure 3

Two site ELISA. **A)** Uncoated ELISA plate, **B)** Plate coated with coating antibody, **C)** Substance X binding to coating antibody, **D)** Detection antibody added, **E)** Peroxidase link added, **F)** Substrate/detection system added.

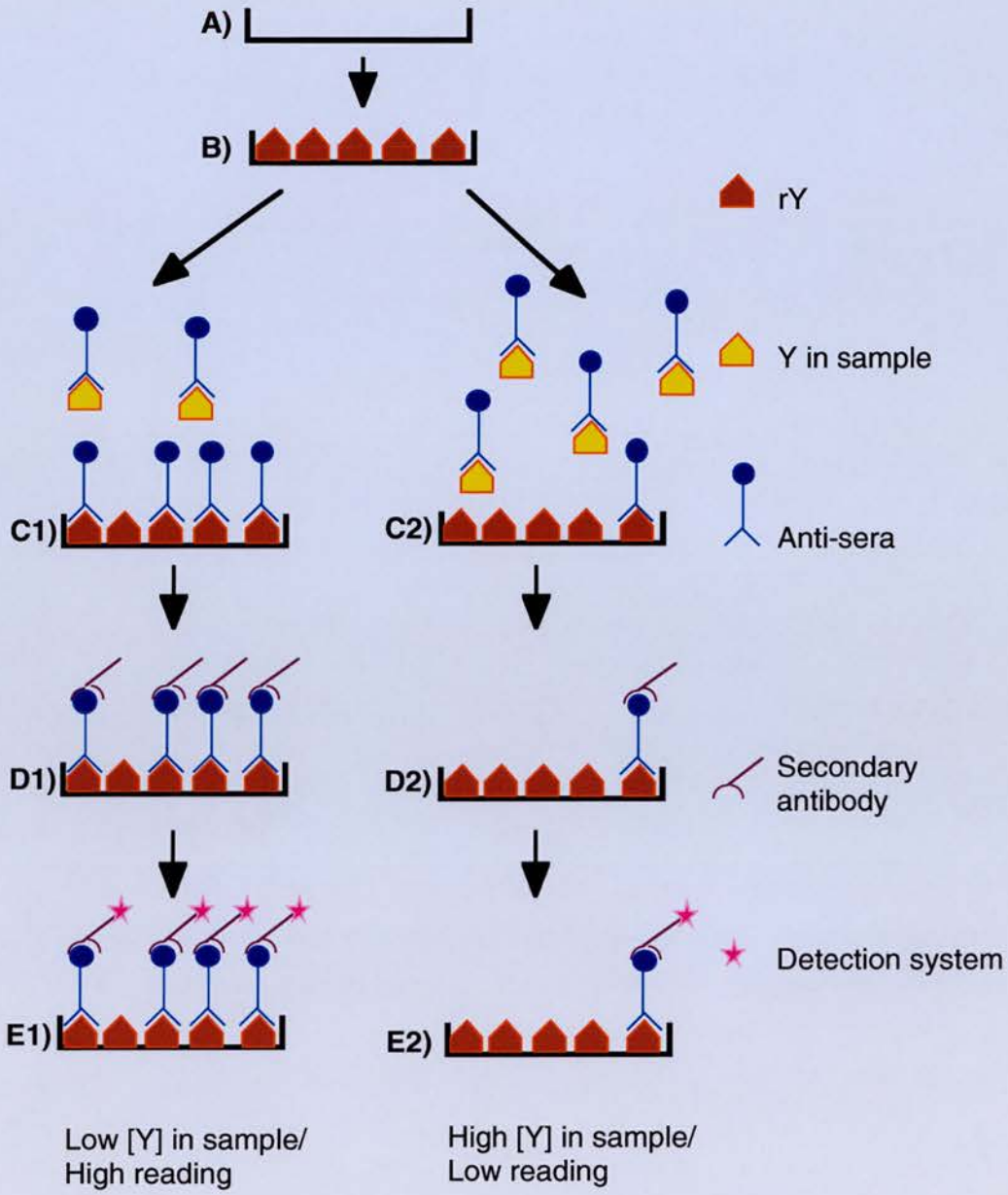


Figure 4

Competition ELISA. **A)** Uncoated ELISA plate, **B)** Plate coated with recombinant protein (rY), **C1)** Media (low concentration substance Y) and anti-sera added, **C2)** Media (high concentration substance Y) and anti-sera added, **D1)** High amounts anti-sera conjugated to secondary antibody bound to plate after washing, **D2)** Low amounts anti-sera conjugated to secondary antibody bound to plate after washing, **E1)** Detection system added, high reading, **E2)** Detection system added, low reading.

Interleukin-8 (IL-8)

IL-8 was measured by ELISA utilising matched pairs of capture and biotinylated labelled detection antibodies against IL-8. Briefly, plates were coated overnight at 4°C with capture antibody (100 μl/well), washed once in water, and coating solution (100 μl/well) added for 30 minutes at 23°C. The coating solution was removed and the plates stored as for MCP-1. Prior to use, the plates were washed once in water. Samples and standards were added (100 μl/well) with the top standard being 500 pg/ml. Plates were incubated for 3 hours at 23°C on an orbital shaker, washed as above then detection antibody (30 ng/ml in ELISA buffer) added and incubated on a shaker for 45 minutes at 23°C. After a further wash, streptavidin peroxidase was added at (0.02 U; 100 μl/well) and plates were incubated for 20 minutes at 23°C on an orbital shaker. They were then washed, substrate was added and were read as for MCP-1. The intra- and inter-assay precision's were 9.1 % r.s.d and 22.1 % r.s.d. respectively, and the detection limit of the assay was 7.5 pg/ml.

For the high sensitivity method, the following modifications were made. The top standard was 2000 pg/ml (12 standards), streptavidin multi-peroxidase (100 ng/ml, 100 μl/well) was utilised instead of streptavidin peroxidase and Amplex red substrate was added (200 μl/well). Plated were read after 30 - 40 minutes using a fluorimeter in the Amplex red setting (excitation 563, emission 587).

Assays for RANTES, IL-1β, IL-6 and TNF-α were performed as for IL-8 (normal sensitivity) with the following modifications:

Assay for RANTES

RANTES was measured by ELISA utilising matched pairs of capture and biotinylated labelled detection antibodies against RANTES. The capture antibody (coated at 2 μg/ml; 100 μl/well), standards (recombinant, top standard 500 pg/ml, 100 μl/well) and detection antibody (added at 50 μg/ml) were all obtained from R & D Systems, Oxford, UK. The intra- and inter-assay precisions for this assay were 10.4 % r.s.d and 11.2 % r.s.d respectively, and the detection limit of the assay was 7.5 pg/ml.

Interleukin-10

All dilutions were in 10 % FCS in PBS unless otherwise stated. Plates were coated with capture antibody (200 ng/ml in PBS; 100 μl/well) for 60 minutes at 23°C, then blocked (10 % FCS in PBS; 300 μl/well) for a further 60 minutes at 23°C then washed. Samples

and standards were added (100 μl/well) with the top standard being 500 pg/ml. Plates were incubated on an orbital shaker for 60 minutes, washed and detection antibody (125 ng/ml; 100 μl/well) added and incubated for 60 minutes at 23°C. After a further wash, polyperoxidase (1 ng/ml in ELISA buffer without Tween; 100 μl/well) was added for 30 minutes. Finally, plates were washed and read as for MCP-1. The intra- and inter-assay variations were 6.4 % and 10.1 % respectively, and the detection limit of the assay was 7.5 pg/ml.

Secretory leukocyte protease inhibitor assay

Secretory leukocyte protease inhibitor was measured by a competitive ELISA. Plates were coated with recombinant secretory leukocyte protease inhibitor raised against the human peptide sequence (Thompson and Ohlsson, 1986); (0.025 μg/ml in PBS/1 % carbonate buffer; 300 μl/well) for 60 minutes at 23°C, blocked with milk powder (0.1 mg/ml in distilled H_2O; 400 μl/well) for 30 minutes at 23°C then washed. ELISA assay buffer was added (250 μl/well) for the non-specific binding (NSB). Recombinant standards (highest concentration 50 ng/ml; 200 μl/well), samples (200 μl/well) and the polyclonal antibody raised in goat against recombinant human secretory leukocyte protease inhibitor (2 μg/ml in ELISA buffer; 50 μl/well) were added. Plates were incubated for 120 minutes at 23°C, washed and anti-sheep/goat IgG Fab fragments raised in donkey conjugated to peroxidase (diluted 1 : 500 in ELISA buffer; 100 μl/well) added. Finally, plates were incubated for 120 minutes at 23°C, washed, substrate added and plates read as for MCP-1. The intra- and inter- assay precisions were 9.2 % r.s.d and 10.1 % r.s.d respectively, and the detection limit of the assay was 9.8 pg/ml.

PGE_2 ELISA

Samples were treated 1 : 1 with methyloximating solution overnight at 4°C. Amine binding plates were coated with donkey anti-rabbit (DAR serum). Briefly, they were coated with rabbit IgG (1 mg/ml diluted in PBS/1 % carbonate buffer; 200 μl/well) for 16 hours at 4°C, the solution flicked out and blocking solution added (250 μl/well) for 120 minutes at 23°C. They were then washed, DAR serum added (150 μl/well), incubated for 16 hours at 4°C then washed, air dried and stored with a desiccant at 4°C. The assay used a PGE-biotin link as a pro-label. To prepare the link, 0.06 M synthetic PGE_2 was added to 320 μl dry dimethylformamide (DMF), the solution cooled to 4°C and 6 μl tributylamine and 3 μl butylchloroformate were added with stirring for 30 minutes at 4°C. Next 0.05 M biocytin (diluted in 300 μl 1 : 1 DMF/distilled H_2O) was added and the vial left at 4°C for

30 minutes then at 23°C for a further 30 minutes. Finally, the solution was ether extracted and purified by reverse phase chromatography. Rabbit anti-sera were raised against PGE_2 complexed to keyhole limpet haemocyanin and have been previously characterised (Kelly, et al., 1989). Samples (100 μl/well) and synthetic standards (highest concentration 5120 pg/ml; 100 μl/well) were added in duplicate and link ($1:1.5x10^6$; 50 μl/well) was added to all wells. Anti-sera was added (1:50,000; 50 μl/well) to all wells except the NSB. The final concentration of methyloximating solution in standards and samples was 12.5 %. Plates were incubated at 4°C for 16 hours, washed and streptavidin peroxidase (0.2 U/ml; 100 μl/well) added. The plates were then incubated for 20 minutes at 23°C on an orbital shaker, washed, substrate added and plates read as for MCP-1. The intra- and inter-assay coefficients were 7.8 % and 15.0 % respectively and the ED50 was 195 pg/ml.

19-hydroxy PGE assay

The plates and anti-sera were prepared as described above. 19-hydroxy PGE standards containing equal amounts of 19-hydroxy PGE_1 and 19-hydroxy PGE_2 were prepared as follows. Human seminal plasma (65 ml) was centrifuged at 4°C for 15 minutes at 1000 g, mixed with acetonitrile (65 ml), centrifuged at 4°C for 15 minutes at 2000 g prior to freeze-drying the supernatant overnight. The residue was leached with methanol, evaporated, added to a silica column and the prostaglandins (PGE_2 and 19-hydroxy PGE) were eluted with increasing concentrations of methanol in methyl formate. The fractions were analysed by thin layer chromatography and that corresponding to the 19-hydroxy PGE fraction was quantitated, using crystalline synthetic PGE_2 as a standard, by treatment with 0.05 M NaOH and measurement of UV absorption at 280 nm in ethanol. To prepare the link, approximately 1 mg 19-hydroxy PGE was added to 200 μl dry DMF, the solution cooled to 4°C and 3 μl tributylamine and 1.5 μl butylchloroformate were added with stirring for 30 minutes at 4°C. Finally, 12 mM Pro-Gly-Tyr-Biotin (dissolved in 300 μl 1 : 1 DMF/distilled H_2O) was added and the solution extracted as described above. Standards (5120 pg/ml to 78 pg/ml), link (1 : 20,000) and anti-sera (1 : 20,000) were diluted in ELISA buffer without Tween and the assay set up as for PGE_2. The intra-assay coefficient was 8.46 % and the ED50 was 196 pg/ml.

2.24 ZYMOGRAPHY

The technique of zymography detects activity of the latent and active forms of matrix metalloproteinases (MMPs). Samples are separated by electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel which has a substrate, in this case gelatin, incorporated within it. The gels are then incubated overnight at 37°C and the latent form of the enzyme, which is activated by the sodium dodecyl sulphate, and the active form of the enzyme digest the substrate within the gel at the site corresponding to their molecular weight. The gel is then stained with Coomassie Blue then destained. The sites of enzymatic gelatinase activity are manifest as a clear area of digestion within the gel. The zymograms may then be semi-quantitated by densitometry (See Appendix I for recipes and Appendix II for detailed experimental protocols).

Briefly, freeze dried samples, which had been reconstituted in 0.1 % SDS, were loaded (maximum loading 15 μl) into the prepared gelatin sodium dodecyl sulphate-polyacrylamide gel (4 % stacking, 7.5 % resolving gel optimal for separating 50 - 120 kDa proteins) and run at 100 V for 90 minutes. Gels were washed twice in Triton X Wash Buffer (15 minutes/wash) and twice in wash buffer (~ 2 minutes/wash). Digestion buffer was then added and gels were incubated at 37°C overnight. The following day, gels were transferred to staining solution for ~ 3 hours prior to destaining for ~ 90 minutes. The gels were then be stored in water until photographed or scanned.

2.25 REVERSE ZYMOGRAPHY

Reverse zymography detects activity of the TIMPs. In this technique, active MMP incorporated into the gelatin sodium dodecyl sulphate polyacrylamide gel binds to TIMPs within the samples. When the electrophoresed gels are incubated overnight, the MMPs within the gel become activated and digest the gelatin throughout the gel apart from where it is bound to TIMPs within the sample. After staining, the TIMPs are detected as a stained band on an otherwise clear background (See Appendix I for recipes and Appendix II for detailed experimental protocols).

Briefly, reconstituted samples (in 0.1 % SDS) were electrophoresed on a sodium dodecyl sulphate-polyacrylamide gel (5 % stacking, 12 % resolving gel optimal for separation of

10 - 40 kDa proteins) which contained gelatin and a MMP preparation from BHK-21 cells at 100 V for 90 minutes. Gels were washed twice in rinse buffer (15 minutes/wash) with gentle agitation and then for a further 150 minutes in fresh rinse buffer. The buffer was then discarded, gels were washed twice in ElgaStat H_20, transferred to digestion buffer and incubated at 37°C for 17 hours. The following day, gels were rinsed twice in ElgaStat H_20 and stained and destained as for zymography.

2.26 WESTERN BLOT

Western blotting detects the presence of proteins, separated by electrophoresis by specific antibodies after transfer to a nitro-cellulose membrane. When compared with zymography and reverse zymography, it has the advantage that it can distinguish between proteins which electrophorese to the same points on a gel, for example TIMP-1 and TIMP-4 (See Appendix I for recipes and Appendix II for detailed experimental protocols).

Briefly, samples which had been dialysed then freeze dried were reconstituted in sample buffer, boiled for 5 minutes, centrifuged at 13,000 rpm for 3 minutes at 4° C, added to the prepared gel and electrophoresed at 100 V for 120 minutes. For optimal separation, 8 % gels were used for 60 - 120 kDa proteins, 10 % gels for 40 - 70 kDa proteins and 12 % gels for 10 - 40 kDa proteins. Gels and nitro-cellulose membranes, and fibre pads and filter papers, were equilibrated in transfer buffer for 30 minutes and 10 minutes, respectively. The gel sandwich was constructed and electrophoresed with a Bio-Rad Ice Cooling Unit at 100 V for 60 minutes. Gels were then stained (60 - 120 minutes) and destained as necessary to check protein transfer.

Membranes were blocked in 5 % BSA in TTBS overnight at 4°C prior to adding the primary antibody for 3 to 4 hours (diluted in primary antibody solution). Next, membranes were washed in TTBS (1 x wash for 30 seconds; 3 x 5 minute washes), the secondary antibody was added (diluted in TTBS; 1 : 200 - 1 : 400) for 45 minutes at 23°C and the membranes rewashed (3 x 5 minute washes). Finally, Avidin Biotinylated enzyme Complex (ABC) peroxidase was added for 30 minutes at 23°C, the membranes were washed (3 x 5 minutes in TTBS) and positive staining detected by addition of DAB. Staining was quenched in distilled H_20, and membranes dried and stored in the dark.

2.27 IMMUNOHISTOCHEMISTRY

The technique of immunohistochemistry involves localisation of an antigen within a section of tissue by a specific primary antibody. A variety of detection systems can then be used to detect and visualise the specific staining.

Non-specific staining

Non-specific staining occurs when tissue other than at the site of the desired antigen stains immunopositive and may be due to a variety of causes.

1. Non-specific primary antibody binding

Problem: The primary antibody may bind non-specifically to epitopes in the tissue.

Solution: Having a good antibody is essential. The immunohistochemistry results can be compared with those obtained by Western Blotting to ensure that the results are valid.

2. Endogenous peroxidase

Problem: Tissues may contain endogenous peroxidase which may catalyse the enzymatic detection thus producing 3, 3' -diaminobenzidine (DAB) staining at the site of the endogenous enzyme.

Solution: Prior to addition of the primary antibody, tissues were exposed to dilute H_2O_2 to saturate any endogenous peroxidase thus rendering it inactive.

3. Non-specific secondary antibody binding

Problem: The secondary antibody may bind to epitopes in the original tissue mimicking those from the species it was raised against as well as to the primary antibody.

Solution: Normal serum from the species from which the secondary antibody was raised against was added prior to addition of the secondary antibody.

Controls

Positive Control: The relevant positive control included on each staining run contained the desired antigen and was therefore expected to stain positively.

Negative Control: Cells or sections of tissue being stained were included which were treated in an identical manner except the primary antibody was omitted or they received non-immune serum or immunoglobulins from the species providing the primary antibody. These sections should be expected to exhibit minimal staining.

General protocol

Immunohistochemistry was performed on cultured cells and on tissue sections. Cultured cells were fixed for 30 minutes in 10 % NBF at 23°C and then stored in 70 % ethanol. Tissue was fixed in 10 % NBF for 24 hours at 4°C and stored in ethanol 70 % ethanol prior to routine paraffin embedding.

Cultured Cell Immunohistochemistry

Cells were rehydrated through descending graded of ethanol to distilled water (2 minute washes) then washed in PBS (2 minutes). Next, cells were permeabilised with 0.05 % Triton (10 minutes), washed in PBS (3 x 2 minute washes) and microwaved in 0.01 M sodium citrate on 10 % power (3 minutes/plate). After standing for 10 minutes, plates were washed in PBS (5 minutes) and endogenous peroxide was blocked (3 % H_2O_2). Plates were washed in distilled H_20 (2 x 2 minute washes), PBS (2 minute wash) and blocked (30 minutes) prior to adding the primary antibody (diluted in blocking serum) to incubate overnight at 4°C. The following day, cells were washed in PBS (2 x 2 minute washes) and the secondary antibody was added (30 minutes). Plates were then washed in PBS (2 minutes), avidin-biotin complex added (30 minutes) and rewashed in PBS (2 minutes). The positive staining was then detected by application of the peroxidase substrate DAB, which produces a brown stain. Cells were lightly counterstained with Harris' haematoxylin, a purple non-specific nuclear stain. Following washing in distilled H_20, cells were left in distilled H_20.

Slide immunohistochemistry

Sections of tissue (5 μm) were mounted on glass slides, then dewaxed in histoclear and rehydrated as described above. Endogenous peroxidase was blocked by H_2O_2 (1 % - 3 % solution in distilled H_20), slides were washed, blocked and primary antibody added as described above. The protocol for the following day was as for the cell immunohistochemistry protocol. After detection and counterstaining, slides were dehydrated in graded ethanol (2 minute/solution) and histoclear (5 minutes) and mounted from Xylene (60 minutes) with Pertex mounting medium.

Haematoxylin and Eosin stain (H & E stain)

Tissue sections were dewaxed in histoclear (5 minutes), rehydrated in graded alcohols (absolute ethanol 20 seconds, 95 % IMS 20 seconds and 70 % IMS 20 seconds) and rinsed in tap water prior to staining in Haematoxylin (5 minutes). Next, slides were rinsed in 1 % acid alcohol (5 - 20 seconds), Scotts tap water (5 - 20 seconds) then Eosin

(5 - 20 seconds) being rinsed in tap water between each stage. Finally, slides were dehydrated in graded alcohols (70 % IMS 20 seconds, 95 % IMS 20 seconds and absolute ethanol 20 seconds) steeped in histoclear (5 minutes) and Pertex mounted. H and E staining was utilised to check the morphology of the non-pregnant (data not shown) and first trimester cervical biopsies (Figure 5).

Specific Protocols

Cytokeratin (epithelial marker)

Endogenous peroxide was blocked with 3 % H_2O_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal goat serum (150 µl normal goat serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-rabbit polyclonal primary antibody was added at 1 : 500 (in diluted in goat serum, overnight at 4°C) and secondary goat anti-rabbit added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

Vimentin (fibroblast marker)

Endogenous peroxide was blocked with 3 % H_2O_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal horse serum (150 µl normal horse serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-mouse polyclonal primary antibody was added at 1 : 10 - 1 : 25 (in diluted horse serum, overnight at 4°C) and secondary horse anti-mouse added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

MMP-1

Endogenous peroxide was blocked with 2 % H_2O_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal goat serum (150 µl normal goat serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-rabbit primary antibody was added at 1 : 1000 (in diluted goat serum, overnight at 4°C) and secondary goat anti-rabbit biotinylated IgG antibody added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

MMP-2

Endogenous peroxide was blocked with 1 % H_2O_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal horse serum (150 µl normal horse serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-mouse primary antibody was added at 1 : 50 (in diluted horse serum, overnight at 4°C) and secondary

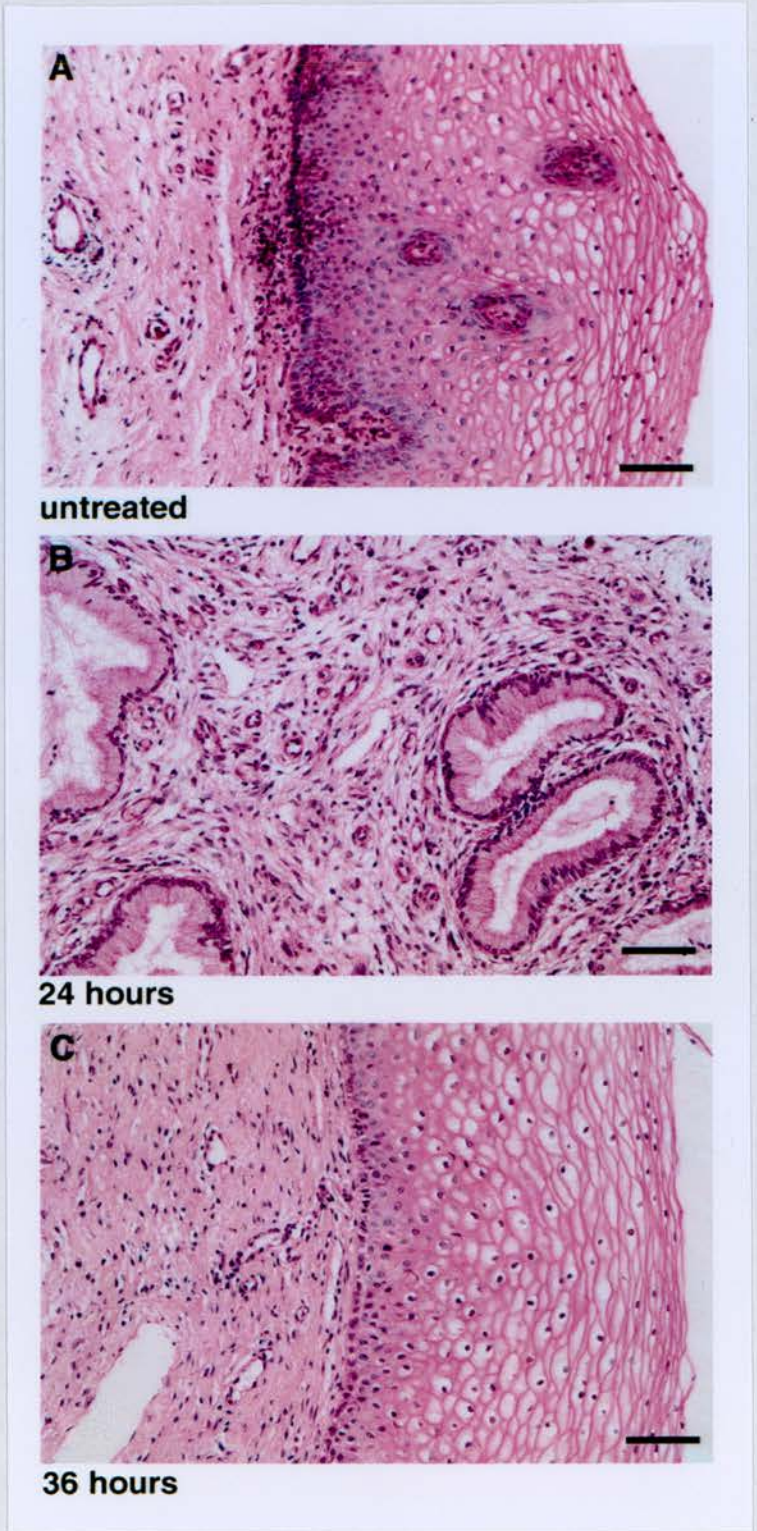


Figure 5

Haematoxylin and Eosin stained first trimester cervical biopsies after *in vivo* administration of mifepristone. **A)** untreated, **B)** 24 hours post mifepristone treatment demonstrating glandular cervical morphology and **C)** 36 hours post mifepristone. Scale bars = 100 μm.

horse anti-mouse biotinylated IgG antibody added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

MMP-8

Endogenous peroxide was blocked with 2 % H_20_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal goat serum (150 µl normal goat serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-rabbit primary antibody was added at 1 : 2000 (in diluted horse serum, overnight at 4°C) and secondary goat anti-rabbit biotinylated IgG antibody added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

MMP-9

Endogenous peroxide was blocked with 1 % H_20_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal horse serum (150 µl normal horse serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-mouse primary antibody was added at 1 : 50 (in diluted horse serum, overnight at 4°C) and secondary horse anti-mouse biotinylated IgG antibody added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

TIMP-1

Endogenous peroxide was blocked with 2 % H_20_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal goat serum (150 µl normal goat serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-rabbit primary antibody was added at 1 : 250 (in diluted goat serum, overnight at 4°C) and the secondary goat anti-rabbit biotinylated antibody added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

TIMP-2

Endogenous peroxide was blocked with 2 % H_20_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal goat serum (150 µl normal goat serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-rabbit primary antibody was added at 1 : 500 (in diluted goat serum, overnight at 4°C) and the secondary goat anti-rabbit biotinylated antibody added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

TIMP-3

Endogenous peroxide was blocked with 2 % H_2O_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal goat serum (150 µl normal goat serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-rabbit primary antibody was added at 1 : 1000 and the secondary goat anti-rabbit biotinylated antibody added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

TIMP-4

Endogenous peroxide was blocked with 2 % H_2O_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal goat serum (150 µl normal goat serum : 10 mls 0.01M PBS, 60 minutes at 23°C). The human anti-rabbit primary antibody was added at 1 : 1000 (in diluted goat serum, overnight at 4°C) and the secondary goat anti-rabbit biotinylated antibody added at 1 : 200 (diluted in 0.01M PBS, 45 minutes at 23°C).

Neutrophil elastase

Endogenous peroxide was blocked with 3 % H_2O_2 (5 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal horse serum (150 µl normal horse serum : 10 mls 0.01M PBS, 20 minutes at 23°C). The human anti-mouse primary antibody was added at 1 : 50 (diluted in 0.01M PBS, overnight at 4°C), mouse IgG was added at 1 : 800 (diluted in 0.01M PBS, overnight at 4°C) for the negative control and the secondary horse anti-mouse biotinylated IgG antibody added at 1 : 200 (diluted in 0.01M PBS, 30 minutes at 23°C).

CD45 (leukocyte common antigen)

Endogenous peroxide was blocked with 3 % H_2O_2 (5 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with normal horse serum (150 µl normal horse serum : 10 mls 0.01M PBS, 20 minutes at 23°C). The human anti-mouse primary antibody was added at 1 : 50 (diluted in 0.01M PBS, 60 minutes at 23°C), mouse IgG was added at 1 : 800 (diluted in 0.01M PBS, 60 minutes at 23°C) for the negative control and the secondary horse anti-mouse biotinylated IgG antibody added at 1 : 200 (diluted in 0.01M PBS, 30 minutes at 23°C).

CD68 (monocyte/macrophage marker)

Slides were trypsin digested (0.1 % trypsin in 0.1 % $CaCl_2$, 60 minutes at 23°C). Endogenous peroxide was blocked with 3 % H_2O_2 (10 minutes at 23°C). Non-specific

binding was eliminated by pre-blocking with normal horse serum (150 μl normal horse serum : 10 mls 0.01M PBS, 20 minutes at 23°C). The human anti-mouse primary antibody was added at 1 : 50 (diluted in 0.01M PBS, 60 minutes at 23°C), mouse IgG was added at 1 : 300 (diluted in 0.01M PBS, 60 minutes at 23°C) for the negative control and the secondary horse anti-mouse biotinylated IgG antibody added at 1 : 200 (diluted in 0.01M PBS, 30 minutes at 23°C).

Secretory leukocyte protease inhibitor

Endogenous peroxide was blocked with 1 % H_2O_2 (30 minutes at 23°C). Non-specific binding was eliminated by pre-blocking with 10 % normal donkey serum (30 minutes at 23°C) The polyclonal secretory leukocyte protease inhibitor primary antibody was applied at 1 : 400 (25 μg/ml in 10 % normal donkey serum, 60 minutes at 23°C) and the secondary anti-sheep/goat Fab fragment raised in donkey conjugated to peroxidase applied at 1 : 100 (diluted in 0.01M PBS, 90 minutes at 23°C). Term fetal membranes were used as a positive control. The primary antibody was omitted or normal donkey serum used as the negative control.

Scoring of immunohistochemistry

All sections were assessed qualitatively by two independent observers who were blinded to the identity of the slides. Slides were assessed as having -, -/+, +, ++ or +++ intensity of stromal immunostaining and specific cellular staining. Positive immunostaining was defined as +, ++ or +++ and negative immunostaining as -.

	Stromal staining	Cellular staining
-	None	None
-/+	Patchy staining in some fields	Occasional cells in some fields
+	Weak diffuse staining in most fields	Few cells staining in most fields
++	Moderate staining in all fields	Many cells staining in all fields
+++	Strong staining in all fields	Numerous cells staining in all fields

It has previously been demonstrated in endometrial sections that there is a high correlation between objectively measured immunoreactivity (measured by computerised image analysis) and subjective semi-quantitative scoring of immunostaining patterns (Wang et al., 1998)

PART 1

INFLAMMATORY MEDIATORS: INITIATION AND MAINTENANCE OF PREGNANCY

GENERAL INTRODUCTION - PART 1

The initiation and maintenance of pregnancy necessitates profound alterations in the maternal immune response to prevent rejection of the fetus. In 1953, Sir Peter Medawar observed that this posed the immunologist "some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates." He called the enigma the "riddle of the success of the fetal allograft" and envisaged that the uterus was an "immunologically privileged site", analogous to the anterior chamber of the eye (Medawar, 1953). Medawar's initial concept of the immunoprotective uterus has however, recently been challenged by the suggestion that maternal recognition of fetal antigens by non-classical MHC-1 (E and G) expressed by trophoblast might be beneficial or even necessary for the establishment of normal pregnancy (Loke and King, 1996). An alternative hypothesis was proposed by Thomas Wegmann in 1993. He suggested that successful pregnancy might depend on bi-directional cytokine interactions in the maternal-fetal relationship resulting in a Th-1 to Th-2 shift in the maternal immune response (Wegmann, et al., 1993).

However, the precise nature of these cytokine interactions during conception, initiation and maintenance of the early pregnancy are not well understood. The aim of the first part of this thesis was therefore to investigate the release and regulation of cytokines (MCP-1, IL-8, RANTES and IL-10) during these processes. The specific questions which were addressed were:

1. **Initiation and maintenance**

   Does seminal plasma have a role in biasing the maternal immune response towards a pro-pregnancy state? How does this equate with the observed post-coital pro-inflammatory cervical leukocytosis?

2. **Adaptation of maternal immune system, specifically peripheral leukocytes to pregnancy**

   Does the potential of peripheral blood to release cytokines (MCP-1, IL-8 and RANTES) alter during pregnancy? If changes do occur, do they have implications for the initiation and maintenance of pregnancy?

3. Inflammatory mediators within the first trimester uterus and the initiation and maintenance of pregnancy

Are cytokines (MCP-1, IL-8 and RANTES) present in the fluid compartments of the first trimester? Does their distribution have implications for the initiation and maintenance of the early conceptus and placentation?

CHAPTER 3

SEMINAL PLASMA COMPONENTS STIMULATE INTERLEUKIN-8 AND INHIBIT INTERLEUKIN-10 RELEASE FROM CERVIX, PERIPHERAL BLOOD AND U937 CELLS (MONOCYTE CELL LINE)

3.1 INTRODUCTION

Coitus and the deposition of semen into the female vagina precedes the inception of every naturally conceived pregnancy. However, the nature of the initial interactions between seminal plasma and spermatozoa, the principal constituents of semen, and the local immune system of the female reproductive tract remain poorly understood.

There is clearly documented evidence that a significant leukocyte infiltration, comprising predominately polymorphonuclear cells and macrophages, occurs within the cervix (Hunter, et al., 1987; Mortimer, 1983; Moyer, et al., 1970; Sinosich and Saunders, 1987) after insemination. This physiological post-coital leukocytic cell reaction develops very rapidly with large numbers of leukocytes being detected 15 minutes post insemination with cells ($>10^9$) significantly outnumbering spermatozoa ($<3 \times 10^8$) by 4 hours post-coitus (Pandaya and Cohen, 1985). A similar phenomenon has also been reported in other vaginal inseminators including rabbits (Tyler, 1977), goats (Mattner, 1968) and cattle (Howe and Black, 1963) and in intra-uterine inseminators such as mice (De, et al., 1991; Parr and Parr, 1991) and rats. The function of this leukocytic reaction is however not certain, with phagocytosis of those sperm destined not to be involved in fertilisation (Moyer, et al., 1970), prevention of infection and modulation of the local immune response all being proposed (Barratt, et al., 1990) as putative functions. In addition, the factor or factors which initiate the leukocytosis in humans are unknown and pure sperm, seminal plasma stripped of spermatozoa and a combination have all been implicated (Howe and Black, 1963; Mattner, 1968).

In contrast, it is vital that the maternal immune system does not mount a pro-inflammatory rejection response to novel antigens expressed by sperm and the conceptus during the critical processes of sperm transport, fertilisation and implantation. Clinically, there is indirect evidence that the presence of seminal plasma during coitus may play a role in preventing such adverse immune responses from developing. In women, who have conceived with minimal exposure to seminal plasma, such as those with limited sexual experience, using barrier contraceptives or undergoing *in vitro* fertilisation, there is an increased risk of implantation failure, spontaneous abortion and pre-eclampsia (Bellinge, et al., 1986; Klonoff-Cohen, et al., 1989). All of these conditions are thought to be mediated, in part by underlying immunological abnormalities.

Human seminal plasma is highly heterogeneous and contains many factors which could affect the maternal immune response. These include soluble p55 TNF-α receptor (Liabakk, et al., 1993), receptors for the Fc portion of γ-globulin, TGF-β (Nocera and Chu, 1993), spermine (Evans, et al., 1995) and complement inhibitors (Kelly, 1995). However, it is the prostaglandins PGE_2 and 19-hydroxy PGE, present in mM concentrations within semen (Kelly, et al., 1976; Templeton, et al., 1978), which are thought to be the principal effectors of immunosuppression and immunomodulation (Kelly, 1995). Seminal plasma prostaglandins are potent stimulators of cAMP and have been shown to inhibit lymphocyte proliferation and NK cell activity. In addition, *in vitro* studies in peripheral blood have demonstrated that PGE_2 modifies cytokine release from antigen presenting cells by stimulating release of the immunosuppressive, pregnancy favourable, Th-2 type cytokine IL-10 and inhibiting release of the Th-1 type cytokine IL-12 (Kelly, et al., 1997; Wegmann, et al., 1993). However, whether seminal plasma, PGE_2 or 19-hydroxy PGE_2 effect a similar cytokine shift in the human cervix or female reproductive immune system is not known.

This study therefore had two principal aims. First, to investigate whether any factors in seminal plasma could be responsible for the observed pro-inflammatory post-coital leukocytosis and second to assess if seminal plasma could effect development of a Th-2 cytokine profile in cervical explants. The cytokines studied were the pro-inflammatory IL-8, the anti-inflammatory secretory leukocyte protease inhibitor and Th-2 type cytokine IL-10. The initial findings with seminal plasma extracts on cervical explants were further characterised by examining the effect of seminal plasma, a seminal plasma extract, PGE_2 and 19-hydroxy PGE on mediator release by peripheral blood and the human pro-monocyte cell line U937.

3.2 MATERIAL AND METHODS

Tissue Collection

Pooled human seminal plasma was obtained from healthy young men involved in the ongoing semen donor programme (more than 20 men). Cervical biopsies, approximately 20 to 35 mg in weight, 15 - 20 mm in length and 2 - 3 mm in diameter, were obtained from healthy, non-pregnant women (n = 15, ages 29 - 45) with regular menstrual cycles undergoing a hysterectomy for a non-malignant condition. These were taken from the anterior lip of the cervix immediately post-hysterectomy. Peripheral blood was taken using a Li-heparin from healthy non-pregnant women (n = 6, ages 20 - 35) with normal menstrual cycles not using any hormonal form of contraception. The women were at various stages of the menstrual cycle.

Preparation of seminal plasma fraction

A seminal plasma fraction was prepared by lipid extraction as previously described (Section 2.225) to remove the majority of the polyamines such as spermine and spermidine, which may have cytotoxic effects (Allen and Roberts, 1986). PGE_2 and 19-hydroxy PGE concentrations were assayed as previously described (Section 2.23). The seminal plasma fraction contained 165 μg/ml PGE_2 and 705 μg/ml 19-hydroxy PGE.

Tissue culture

Cervical biopsies

Explants were cultured in complete medium supplemented with seminal plasma fraction diluted 1 : 2000 with treatments in quadruplicate. The harvested media were frozen at –20°C until analysis.

Peripheral blood

Peripheral blood was cultured as previously described (Section 2.221) in RPMI 1640 supplemented with whole seminal plasma (1 % to 0.0001 %), seminal plasma fraction (1 % to 0.0001 %), PGE_2 (10^{-6}M - 10^{-9}M) and 19-hydroxy PGE (10^{-6} M - 10^{-9} M). Treatments were added in quadruplicate and media were removed and stored at -20°C until analysis.

U937 cells

U937 cells were plated out as previously described at 2 x 10^5 cells/ml in complete media (section 2.226), supplemented with whole seminal plasma (1 % to 0.0001 %), seminal plasma fraction (1 % to 0.0001 %), PGE_2 (10^{-6} to 10^{-10} M) and 19-hydroxy PGE (10^{-6} to 10^{-10} M). In addition, cells were treated with 10^{-7}M PMA for the IL-10 experiments because no IL-10 was released from cells without PMA treatment. Treatments were added in quadruplicate and media were removed and stored at -20°C until analysis.

Cytokine assays

IL-8, secretory leukocyte protease inhibitor, IL-10, PGE_2 and 19-hydroxy PGE were assayed by ELISA as previously described (Section 2.23).

Statistical analysis

Statistical analysis of the data was performed using StatView 4.1 (Abacus Inc., Berkley, CA, USA). The data were normally distributed and are expressed as cytokine release as % of control (mean ± s.e.m) with a statistically significant difference defined as $p<0.05$.

Ethical Approval

Ethical approval for the collection of all samples was obtained from the Lothian Trust Ethical Committee with the informed and written consent of patients.

3.3 RESULTS

Cervical explants

The lipid extracted seminal plasma fraction significantly stimulated ($p<0.005$) IL-8 and inhibited ($p<0.005$) secretory leukocyte protease inhibitor release (Figure 6; n = 15 different women, treatments in quadruplicate). IL-10 release was below the detection limit of the assay employed.

Peripheral blood data

Whole seminal plasma significantly ($p<0.05$) inhibited IL-8 release at concentrations of greater than 0.1 %. Maximum inhibition of IL-8 release down to 8 % of control values ($p<0.005$) was achieved when seminal plasma was added at 1 %. In contrast, seminal plasma fraction significantly ($p<0.05$) stimulated IL-8 release when used at 0.001 % with a 10-fold stimulation achieved when peripheral blood was cultured with 0.1 % seminal plasma fraction ($p<0.005$; Figure 7; n = 6 separate experiments, treatments in quadruplicate). Both PGE_2 and 19-hydroxy PGE significantly stimulated ($p<0.05$; $p<0.005$ respectively) IL-8 release with maximal stimulation achieved at 10^{-6}M. 19-hydroxy PGE was significantly ($p<0.05$) more effective in stimulating IL-8 release than PGE_2 at concentrations of greater than 10^{-8}M (Figure 8; n = 6 separate experiments, treatments in quadruplicate). Secretory leukocyte protease inhibitor release was below the detection limit of the assay used.

U937 monocyte cell line

Seminal plasma and the seminal plasma fraction significantly (both $p<0.005$) stimulated IL-8 release from U937 cells cultured without PMA by comparable amounts with maximum stimulation at 0.1 % (Figure 9; n = 6 separate experiments, treatments in quadruplicate). PGE_2 significantly ($p<0.05$) stimulated IL-8 release from U937 cells with maximum stimulation at 10^{-6}M. 19-hydroxy PGE also stimulated ($p<0.005$) IL-8 release and, as demonstrated in peripheral blood, it was significantly ($p<0.05$) more effective in inducing IL-8 release than PGE_2 (Figure 10; n = 6 separate experiments, treatments in quadruplicate). Secretory leukocyte protease inhibitor release was not detectable from U937 cells.

Seminal plasma and the seminal plasma fraction significantly stimulated ($p<0.05$) IL-10 release from U937 cells with maximum stimulation at 0.1 % and 0.01 % respectively, for

seminal plasma and its fraction (Figure 11; n = 6 separate experiments, treatments in quadruplicate). The seminal plasma fraction inhibited secretion of IL-10 back to control values when added at 0.1 %. PGE_2 significantly ($p<0.05$) stimulated IL-10 release from U937 cells with maximum stimulation at 10^{-6}M. Although 19-hydroxy PGE also stimulated IL-10 release this failed to reach significance (Figure 12; n = 6 separate experiments, treatments in quadruplicate).

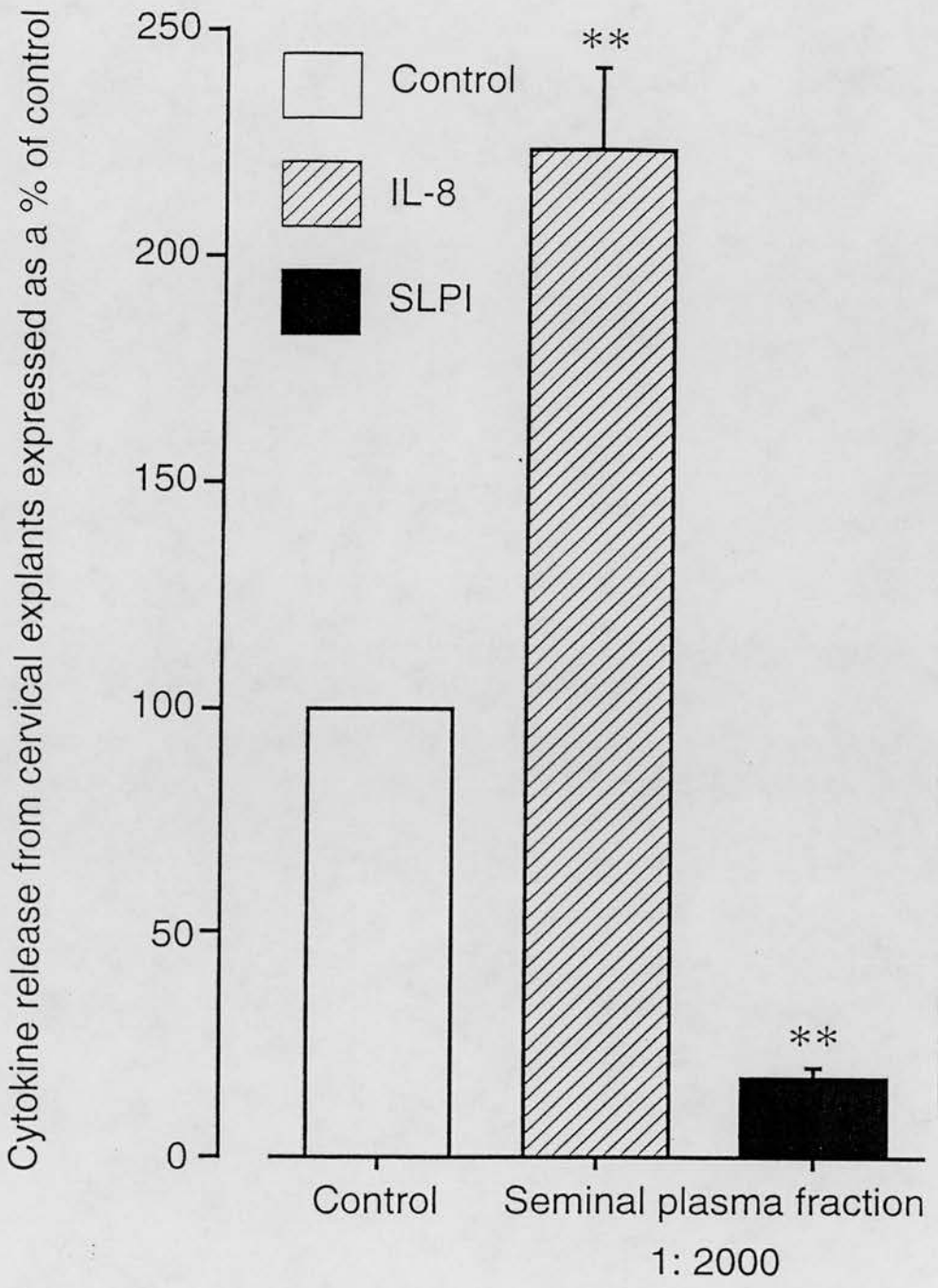


Figure 6

Effect of seminal plasma fraction on IL-8 and secretory leukocyte protease inhibitor release from non-pregnant cervical explants. Release of IL-8 release was stimulated and secretory leukocyte protease inhibitor inhibited by seminal plasma fraction at 1 : 2000. SLPI, secretory leukocyte protease inhibitor. Values are expressed as cytokine release as % of control; mean ± s.e.m. n = 15 different women, treatments in quadruplicate. Significance; **, $p<0.005$.

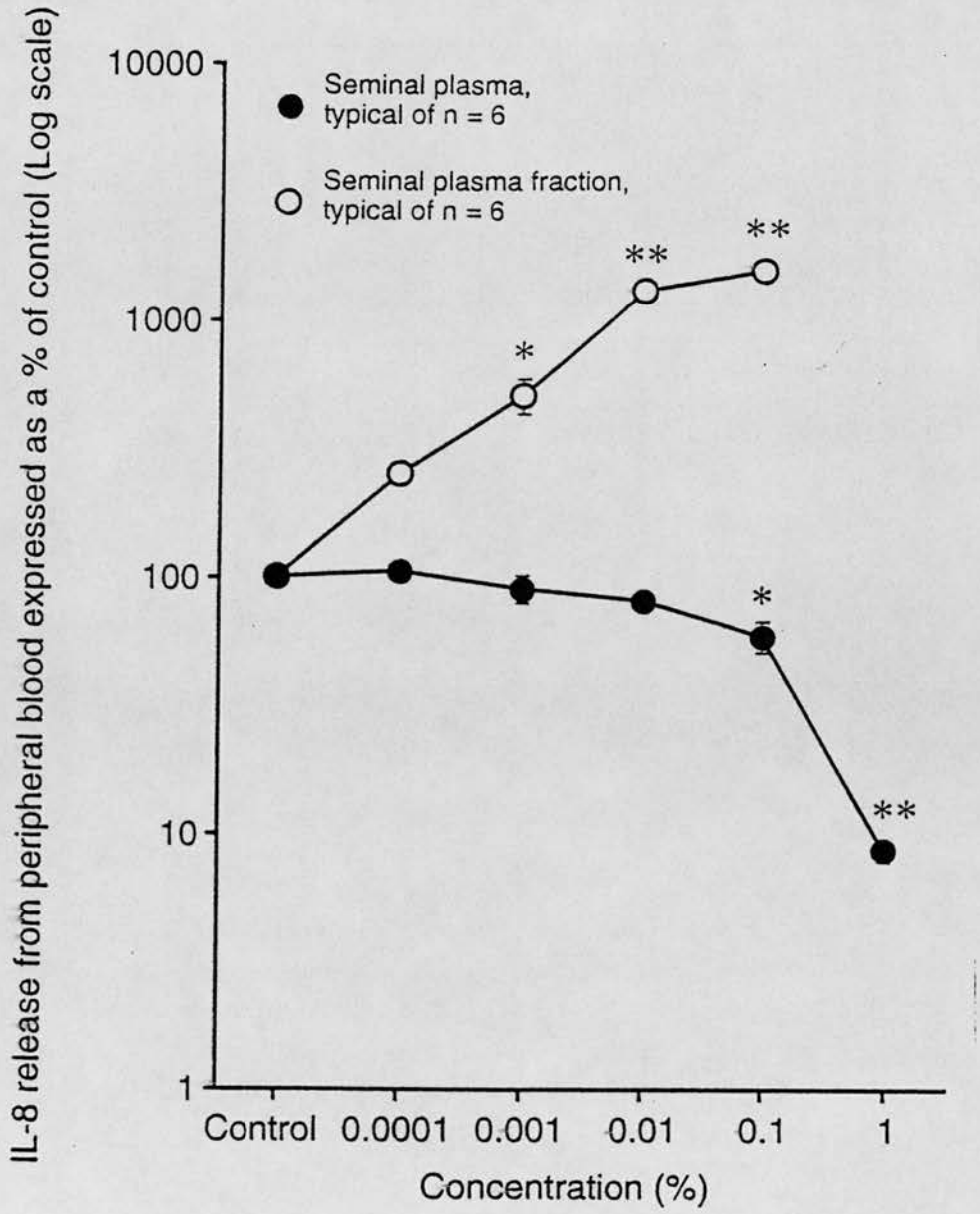


Figure 7

The effect of seminal plasma and seminal plasma fraction on IL-8 release from peripheral blood. IL-8 release was significantly stimulated by the seminal plasma fraction whereas seminal plasma significantly inhibited IL-8 release. Values are expressed as the log of IL-8 release as % of control; mean ± s.e.m. Typical of n = 6 separate experiments, treatments in quadruplicate. Significance of difference from control; *, $p<0.05$; **, $p<0.005$.

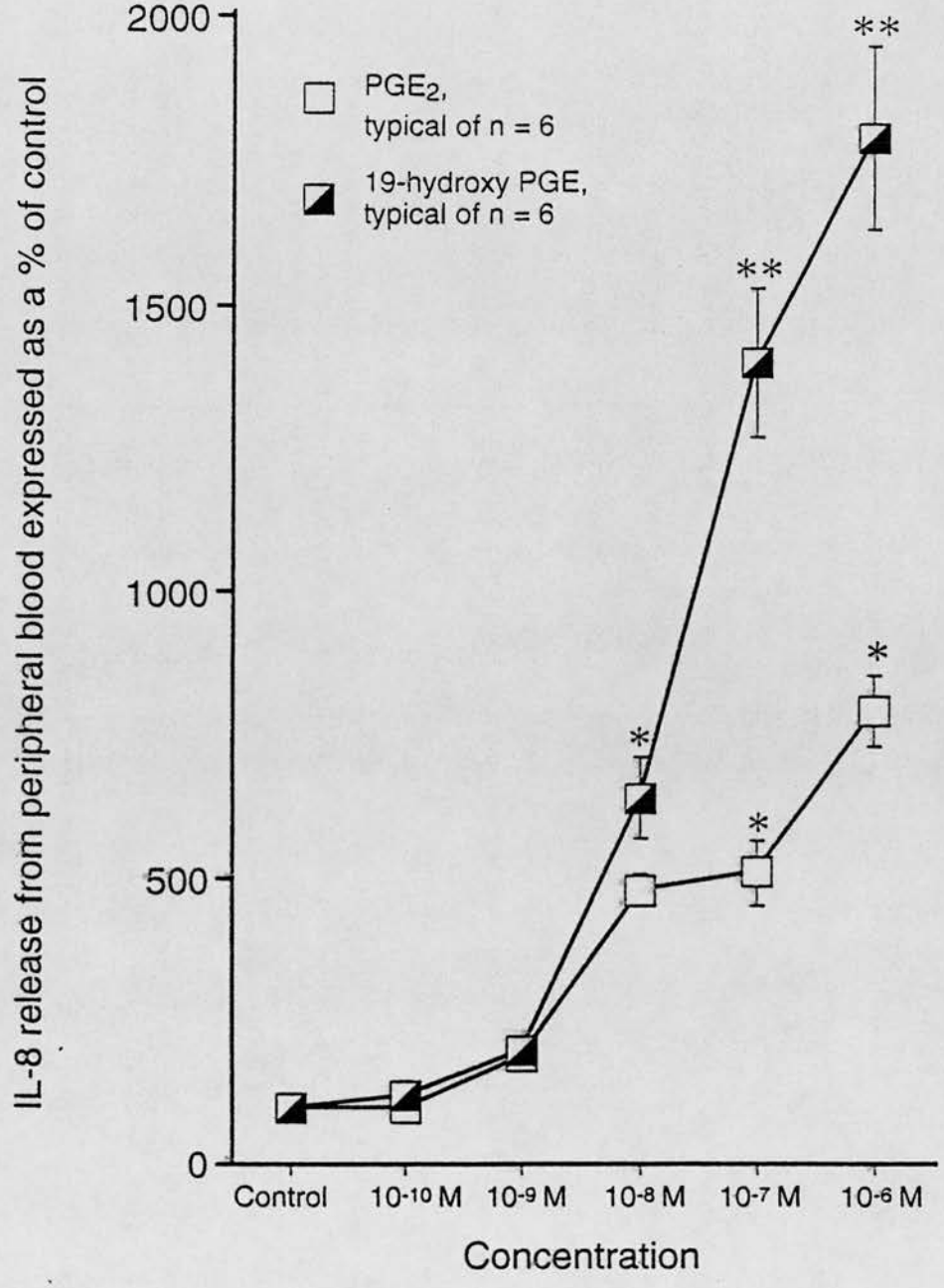


Figure 8

The effect of PGE_2 and 19-hydroxy PGE on IL-8 release from peripheral blood. IL-8 release was significantly stimulated by both PGE_2 and 19-hydroxy PGE. At concentrations of greater than 10^{-8}M, 19-hydroxy PGE was significantly more effective in stimulating IL-8 release than PGE_2. Values are expressed as IL-8 release as % of control; mean ± s.e.m. Typical of n = 6 separate experiments, treatments in quadruplicate. Significance of difference from control; *, $p<0.05$; **, $p<0.005$.

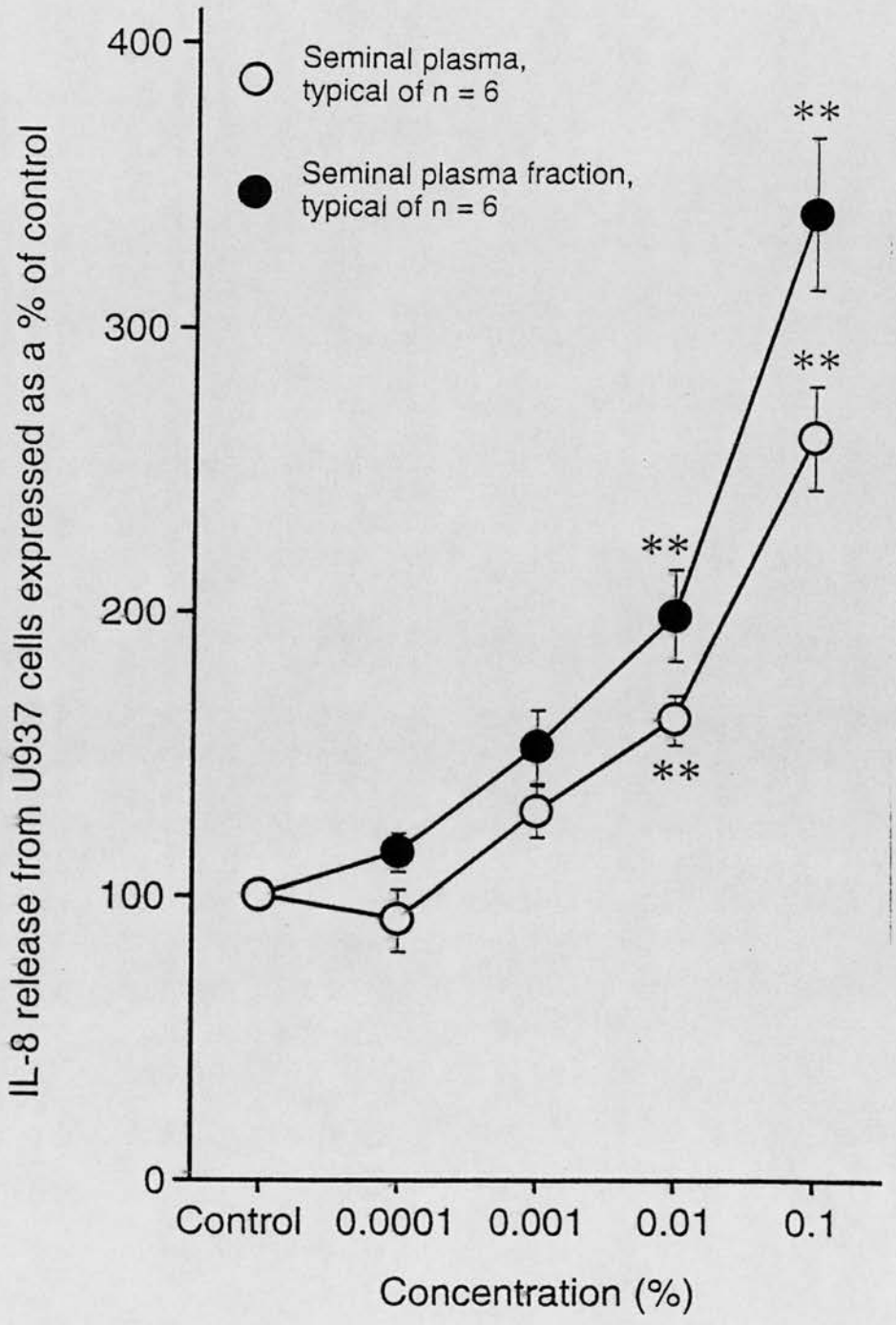


Figure 9

The effect of seminal plasma and seminal plasma fraction on IL-8 release from U937 cells. IL-8 release was significantly stimulated by both seminal plasma and seminal plasma fraction. Values are expressed as IL-8 release as % of control; mean ± s.e.m. Typical of n = 6 separate experiments, treatments in quadruplicate. Significance of difference from control; **, $p<0.005$.

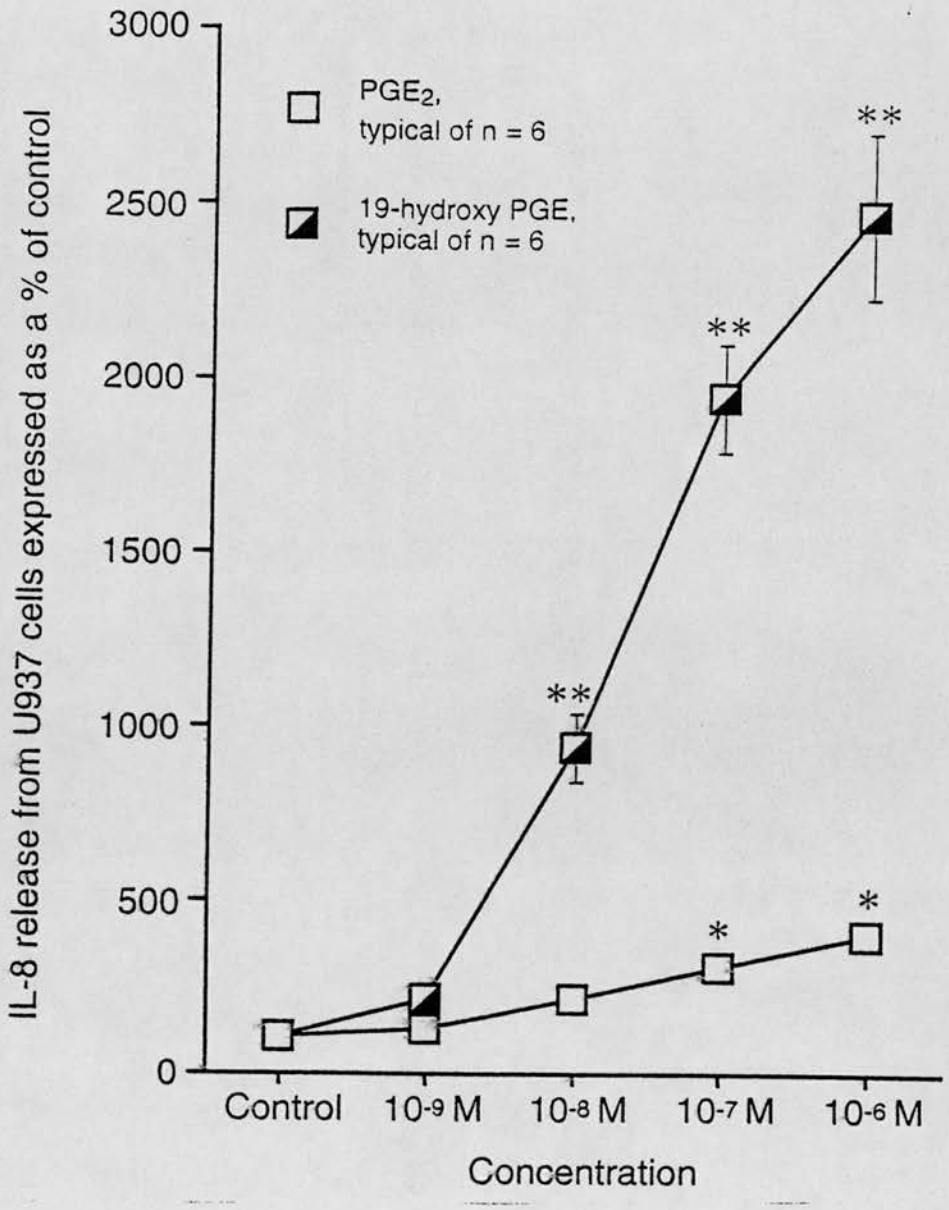


Figure 10

The effect of PGE_2 and 19-hydroxy PGE on IL-8 release from U937 cells. IL-8 release was significantly stimulated by both PGE_2 and 19-hydroxy PGE. 19-hydroxy PGE was significantly more effective in stimulating IL-8 release than PGE_2. Values are expressed as IL-8 release as % of control; mean ± s.e.m. Typical of n = 6 separate experiments, treatments in quadruplicate. Significance of difference from control; *, $p<0.05$; **, $p<0.005$.

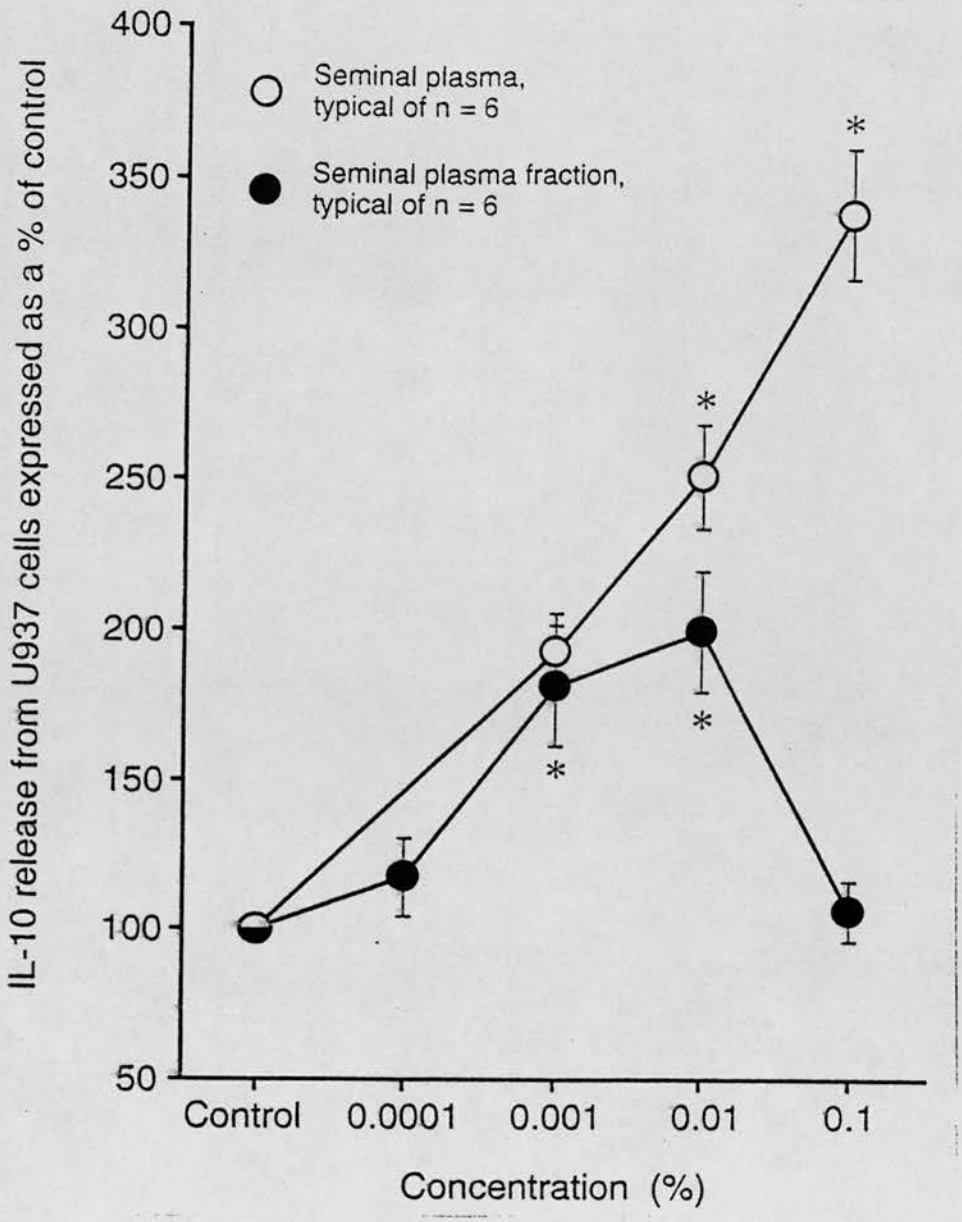


Figure 11

The effect of seminal plasma and seminal plasma fraction on IL-10 release from U937 cells. Seminal plasma significantly stimulated IL-10 release from U937 cells. Seminal plasma fraction significantly stimulated IL-10 release at 0.001 % and 0.01 % but inhibited its release back to control values at 0.1 %. Values are expressed as IL-10 release as % of control; mean ± s.e.m. Typical of n = 6 separate experiments, treatments in quadruplicate. Significance of difference from control; *, $p<0.05$.

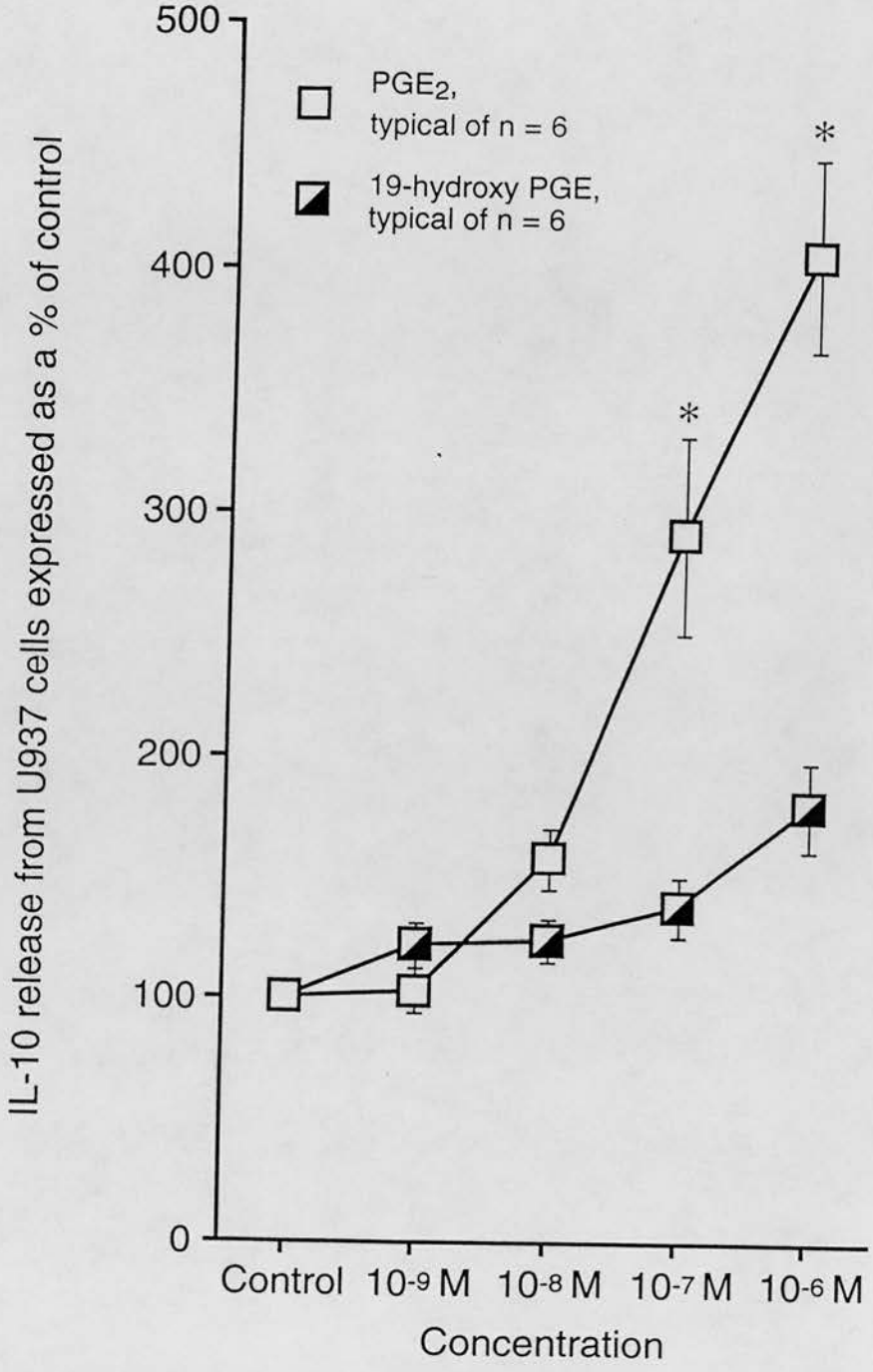


Figure 12

The effect of PGE_2 and 19-hydroxy PGE on IL-10 release from U937 cells. PGE_2 significantly stimulated IL-10 release from U937 cells. 19-hydroxy PGE stimulated IL-10 release but this failed to reach significance. Values are expressed as IL-10 release as % of control; mean ± s.e.m. Typical of n = 6 separate experiments, treatments in quadruplicate. Significance of difference from control; *, $p<0.05$.

3.4 DISCUSSION

Prostaglandin E_2 (Samuelsson, 1963) and 19-hydroxy PGE (Taylor and Kelly, 1974) are thought to be the principal immunosuppressive agents present in semen. In addition to favouring development of a Th-2 type of cytokine release from $CD4^+$ T cells (Kelly, et al., 1997), PGE_2 and IL-10, whose release it stimulates (Kelly, et al., 1997), inhibit the expression of the T-cell co-stimulatory ligands B7-1 and B7-2 (Ding, et al., 1993; Iglesias, et al., 1997). Antigens, which are presented to T-cells in the absence of such molecules are rendered anergic or non-responsive to that specific antigen and do not recognise it when it is represented in a subsequent occasion. IL-10 is also capable of inducing anergy in $CD4^+$ T-cells directly (Groux, et al., 1996).

Less is known about the function of 19-hydroxy PGE, which is synthesised in the microsomal fraction of the seminal vesicles from prostaglandins by PGE 19-hydroxylase. Like PGE_2, it is thought to act principally as an immunosuppressive agent inhibiting NK cell activity (Tarter, et al., 1986), elevating levels of cAMP (Kelly, et al., 1994) and stimulating IL-10 release. Despite the fact that *in vitro* 19-hydroxy PGE is a less potent immunosuppressive agent than PGE_2, its three-fold higher levels within seminal plasma may confer on it greater immunosuppressive activity *in vivo*. In addition, 19-hydroxy PGE is a relatively selective EP-2 receptor agonist (Woodward, et al., 1993), unlike PGE_2 which binds to all of the EP receptors. Given that the primate cervix contains mainly EP-2 receptors (Smith, et al., 1998) then the higher affinity of 19-hydroxy PGE for the EP-2 receptor may make it more active *in vivo*.

This study demonstrates that seminal plasma, a lipid extract, PGE_2 and 19-hydroxy PGE stimulate IL-10 release by U937 cells. IL-10 release by cervical explants was below the detection limit of the assay employed. This data is supported by previous studies, in peripheral blood and peripheral blood mononuclear cells in which semen and its component prostaglandins stimulated expression and release of IL-10 (Jeremias, et al., 1998; Kelly, et al., 1997).

It is hypothesised that stimulation of IL-10 release by seminal plasma prostaglandins would favour development of a pregnancy favourable Th-2 response locally within the upper vaginal tract. In other animals, seminal plasma, its component plasma proteins and lymphocytes are transported from the vagina and uterus to draining lymph nodes

(Carballada and Esponda, 1997; Ibata, et al., 1997). These nodes have been shown to enlarge post-coitus (Alexander and Anderson, 1987) and in mice to alter their cytokine receptor profile in direct response to the presence of seminal plasma within the uterus (Bischof, et al., 1994). Given that some seminal plasma also enters the uterus (Egli and Newton, 1961) after intercourse in women, then it is further suggested that transport of seminal plasma prostaglandins to draining lymph nodes from either the upper vagina or uterus may stimulate IL-10 release and a Th-2 type response in endometrium and the draining lymph nodes. A shift to a pro-pregnancy Th-2 type immune response would potentially favour implantation (Lim, et al., 1998) and the development of a successful pregnancy.

PGE_2 is an enigmatic mediator possessing both anti- and pro-inflammatory (Williams and Peck, 1977) properties. However, recently several novel pro-inflammatory properties have been described. Within human skin it synergises with the neutrophil chemotactic and activating agent IL-8 to promote neutrophil chemotaxis and activation (Colditz, 1990). Prostaglandin E_2 also promotes release of IL-6 and IL-8 from IL-1β stimulated synovial fibroblasts (Agro, et al., 1996), mediates bradykinin induced stimulation of IL-8 release from cultured human airway epithelial cells (Pang and Knox, 1998) and stimulates IL-8 release from human colonic epithelial cells (Yu and Chadee, 1998). The mechanism by which PGE_2 stimulates IL-8 is not certain but may be via post-transcriptional stabilisation of its mRNA. There is no direct evidence that seminal plasma PGE_2 stimulates IL-8 release either within semen or the female genital tract. However, elevated levels of IL-8 within seminal plasma do correlate with leukocytospermia, a condition characterised by abnormally high levels of pro-inflammatory leukocytes in seminal plasma (Shimoya, et al., 1993). A pro-inflammatory action for 19-hydroxy PGE has not been previously described.

This study demonstrates that seminal plasma and its component prostaglandins stimulate IL-8 release. The only exception to this was in peripheral blood where whole semen inhibited IL-8. However, seminal plasma is a potent stimulator of IL-10 release in peripheral blood and given that there is mutual repression between release of IL-10 and IL-8 (Kang, et al., 1998; Lu, et al., 1995) then the high levels of IL-10 generated may have inhibited IL-8 release. In addition, other factors within seminal plasma may also affect IL-8 release in peripheral blood preparations.

It is therefore suggested that PGE_2 and 19-hydroxy PGE, by stimulating release of the neutrophil chemoattractant IL-8 from the female genital tract mucosa, may have a role in inducing the post-coital leukocytic reaction in humans. Spermatozoa not involved in fertilisation could then either be phagocytosed by the invading leukocytes (Moyer, et al., 1970), or endocytosed by vaginal (Phillips and Mahler, 1975) or uterine epithelium (Carballada and Esponda, 1997). The pro-inflammatory action of seminal plasma in stimulating IL-8 release may seem to conflict with its anti-inflammatory effect in stimulating IL-10 release. However, IL-8 is also a potent chemotactic factor for T-lymphocytes (Taub, et al., 1996). It could therefore induce their entry into the upper vaginal tract where they could be presented with spermatozoal antigen by the submucosal dendritic antigen presenting cells. Presentation of antigen in the presence of high concentrations of immunomodulatory agents such as PGE_2, TGF-β and IL-10, either found in or induced by seminal plasma would favour development of an anergic response to spermatozoa. In addition, dendritic cells which mature in the presence of PGE_2 show impaired IL-12 production and thus their naive Th cell development is biased towards a Th-2 type (Kalinski, et al., 1998). In combination, these immune responses would facilitate survival of those sperm which were destined to be involved in fertilisation (Groux, et al., 1996; Tremellen, et al., 1998) and prevent their destruction by a hostile maternal immune response.

It has been suggested that pure seminal plasma stripped of sperm is not capable of leukocytosis and therefore the hypothesis proposed would be invalid. The published data are however conflicting and this may be due to different methods of collecting and purifying the seminal plasma and the age of the semen used for the studies. In addition, pure spermatozoa washed of seminal plasma have also been reported to induce leukocytosis (Thompson, et al., 1992). However, washing spermatozoa may induce alterations in membrane structure including lipid peroxidation generating oxygen free radicles and leukotrienes both of which are chemotactic for neutrophils (Krauss, et al., 1994; Wizemann and Laskin, 1994). This could account for the observed leukocytosis post insemination of pure sperm. It is likely therefore that *in vivo* a combination of seminal plasma constituents such as PGE_2, 19-hydroxy PGE and spermatozoa would act together to induce leukocytosis.

In conclusion, this study demonstrates that seminal plasma prostaglandins can induce release of both pro-and anti-inflammatory cytokines and that PGE_2 and 19-hydroxy PGE differ in their capacity to induce cytokine secretion. The potential immunomodulatory

properties of seminal plasma prostaglandins in women have many parallels with the mouse model. In mice, seminal plasma TGF-β (analogous to PGE_2) stimulates release of GM-CSF (analogous to IL-8), which induces an inflammatory leukocytosis within the uterus (Tremellen, et al., 1998). TGF-β also induces tolerance and a Th-2 type of immune response within the uterus. It is suggested that the anti- and pro-inflammatory effects elicited by seminal plasma prostaglandins in humans are not mutually exclusive. The different cytokines released may act in combination to promote sperm survival, facilitate removal of excess sperm from the female reproductive tract and also to favour development of a pregnancy favourable Th-2 type of immune response within the genital tract and local immune system.

CHAPTER 4

DIFFERENTIAL SECRETION OF CHEMOKINES FROM PERIPHERAL BLOOD IN PREGNANT COMPARED WITH NON-PREGNANT WOMEN

4.1 INTRODUCTION

The initiation and maintenance of pregnancy may necessitate profound changes in the immune system with the development of a Th-2 bias in immune responses (Wegmann, et al., 1993). These immunological alterations have marked effects on the presentation and course of diseases during pregnancy. For example, conditions such as rheumatoid arthritis (Hensch, 1938; Nelson and Ostensen, 1997) and psoriasis (Boyd, et al., 1996), which are mediated by a Th-1 type of immune response, often remit during pregnancy and those associated with a Th-2 response such as systemic lupus erythematosis are frequently exacerbated (Kitridou and Mintz, 1993). These clinical observations suggest that the maternal adaptation of the immune response extends beyond the feto-placental unit to circulating leukocytes and the peripheral immune system.

Early studies, which attempted to identify alterations in the immunological potential of circulating leukocytes, frequently yielded conflicting results. The non-specific immunity afforded by polymorphonuclear leukocytes was demonstrated to be both up- (Crouch, et al., 1995) and down-regulated (Shibuya, et al., 1991) and the proliferative response of leukocytes to noxious stimuli to be increased and decreased (Bermas and Hill, 1997) during pregnancy. More recently, the potential of circulating cells to release cytokines (Elsasser-Beile, et al., 1992), and their concentrations within peripheral plasma have been used to assess the state of the peripheral immune response. The reported findings, again frequently conflict, with results depending on the nature and timing of sampling during pregnancy, assay technique employed and cytokines being investigated. For example, studies have variously demonstrated that serum concentrations of IL-1 and IL-6 correlate positively with gestation (Augustulen, et al., 1994), plasma levels of IL-8 to be unaffected (Laham, et al., 1993) and plasma concentrations of TNF-α to either increase (Beckmann, et al., 1997) or be unaffected by gestation (Laham, et al., 1994).

A further factor, which is frequently ignored in studies examining peripheral immune responses, are the profound haemodynamic alterations that occur during pregnancy. By 15 weeks gestation (Clapp, et al., 1988), a 20 % increase in plasma volume has occurred. Less than 20 weeks later, it has expanded a further 30 % giving a final plasma volume of approximately 3800 ml (de Swiet and Chamberlain, 1997). The bone marrow responds by hyperplasia and increased erythropoietin output, thus stimulating erythrocyte production and effecting a gradual increase in red cell mass. This is accompanied by a

gradual haemodilution, due to preferential expansion of plasma volume over red cell volume, reducing the haemoglobin and haematocrit to about 11 g/dl and 37 %, respectively (Whitfield, 1995). Of direct relevance to studies on immune potential of circulating leukocytes, is the tendency towards leukocytosis during pregnancy. This elevation, which occurs relatively early in gestation and is attributable principally to doubling of polymorphonuclear leukocyte counts (Pitkin and Witte, 1979), is frequently not corrected for in peripheral blood studies. Whether monocyte, basophil, eosinophil or platelet counts increase, decrease or remain unaltered during pregnancy is less certain (Damm, et al., 1991; Pitkin and Witte, 1979; Sejeny, et al., 1975; Tsakonas, et al., 1995).

The aim of this study was therefore to examine plasma concentrations and potential release of immunomodulatory chemotactic cytokines by peripheral blood during pregnancy. The cytokines studied were MCP-1, IL-8 and RANTES, chosen as markers of monocyte, neutrophil and T-cell function, respectively.

4.2 MATERIALS AND METHODS

Sample Collection

Peripheral blood was obtained in the morning by sterile venepuncture in Li-Heparin tubes from pregnant (n = 18) and non-pregnant (n = 36) women (Section 2.211). The patient characteristics are demonstrated in Table 5. Blood was analysed for haemoglobin, leukocyte count, including differential count, and platelet count using an automated NE Sysmex counter.

Characteristic	Patient Group	
	Non-pregnant, n = 18	Pregnant, n = 36
Age	22 - 47 (μ = 30)	16 - 37 (μ = 26)
Parity		
P 0	12	24
P 1	2	9
P 2	1	3
P 3	2	
P 4	1	
Smoker	2	7
Non Smoker	16	29

Table 5

Patient characteristics for peripheral blood samples

Tissue culture

Whole blood was centrifuged at 1500 rpm for 5 minutes at 4°C, the upper plasma layer removed and stored at -20°C prior to immunoassay.

Whole blood was cultured and neutrophils, PBMC preparations and $CD14^+$ and $CD14^-$ cells were separated and cultured (Section 2.221). To assess the effect of steroid antagonism, PBMC preparations were cultured at $1x10^5$ cells/ml in complete media

supplemented with 10^{-6}M progesterone for 48 hours. The media were then removed and replaced with media supplemented with 10^{-6}M mifepristone for 24 hours prior to removing and storing the media at -20°C until analysis.

Assays

Media and plasma were analysed for MCP-1, IL-8 and RANTES by ELISA (Section 2.23).

Statistics

Statistical analysis was performed using ANOVA multiple variate analysis in StatView. Assignment of difference to individual groups was achieved using Fisher's probability of least squared differences (PLSD) with significance taken to be $p<0.05$. Results are expressed as mean +/- standard error of the mean (s.e.m).

Ethical Approval

Ethical approval for the collection of all samples was obtained from the Lothian Trust Ethical Committee with the informed and written consent of patients.

4.3 RESULTS

Haematological Parameters

The haemoglobin levels were significantly lower during labour ($p<0.01$) compared with non-pregnant samples. There was a tendency towards leukocytosis during pregnancy but this failed to reach significance. The neutrophil count was ($p<0.01$) significantly greater at term than during the first trimester or in non-pregnant samples. There was no difference in any of the other haematological parameters measured between the different patient groups (Table 6).

Plasma chemokine levels

MCP-1 and RANTES were detectable in non-pregnant (187 ± 7 pg/ml; 23 ± 4 ng/ml) and pregnant (155 ± 8 pg/ml; 20 ± 2 ng/ml) plasma, respectively. There was no significant difference in plasma MCP-1 and RANTES concentrations between non-pregnant and pregnant samples. IL-8 was not detectable in peripheral plasma.

Peripheral blood cultures

Significantly ($p<0.001$) higher concentrations of MCP-1 were secreted by peripheral blood cultures from pregnant (term not in labour, TNIL; n = 10; 4325 ± 857 pg/ml) compared with non-pregnant (n = 12; 1739 ± 391 pg/ml) women (Figure 13). In a separate study, significantly ($p<0.001$) greater amounts of MCP-1 were released at term compared with first trimester (3890 ± 481 pg/ml) or non-pregnant (2531 ± 319 pg/ml) cultures. More MCP-1 was released by samples taken at term from women not in labour (n = 14; 7635 ± 612 pg/ml) compared with those in labour (term in labour, TIL; n = 12; 6125 ± 521 pg/ml) although this was not statistically significant (Figure 14).

Both IL-8 and RANTES were detectable in non-pregnant (456 ± 96 pg/ml; 736 ± 251 pg/ml) and pregnant (540 ± 182 pg/ml; 1222 ± 247 pg/ml) culture supernatants. There were no significant differences in IL-8 and RANTES release by non-pregnant and pregnant cultures (Figure 13).

Neutrophil separation

Given that observed differences in neutrophil count between patient groups, it was possible that the increased release of MCP-1 from pregnant whole blood cultures was due to its release by neutrophils present in the cultures. Neutrophils were therefore extracted

from non-pregnant (n = 6) and pregnant (TNIL, n = 6) blood samples, counted and cultured at $1x10^5$ cells/ml. There were no significant differences in release of MCP-1 or IL-8 from non-pregnant (59.6 ± 12.3 pg/ml; 91.3 ± 14.5 pg/ml) or pregnant (81.9 ± 13.6 pg/ml; 94.1 ± 17.2 pg/ml) preparations. RANTES release was not detectable from neutrophil preparations (Table 7).

PBMC preparations

PBMC preparations were prepared to investigate whether mononuclear cells in peripheral blood could be responsible for the differences in MCP-1 release. Significantly ($p<0.05$) more MCP-1 was secreted by pregnant (TNIL, n = 6) compared with non-pregnant (n = 6) preparations with this differential being maintained over 72 hours culture (Figure 15). There was no difference in the capacity of non-pregnant and pregnant PBMC preparations to release IL-8 and RANTES (data not shown).

Steroid antagonism studies were performed to assess the effect on chemokine release of removing pregnant peripheral blood from the progesterone rich environment of pregnancy. Progesterone antagonism had no effect on MCP-1, IL-8 or RANTES release.

$CD14^+/CD14^-$ separated cells

To further characterise the cells responsible for MCP-1, IL-8 and RANTES release, PBMC preparations were separated into $CD14^+$ (monocyte enriched) and $CD14^-$ (monocyte depleted) cell fractions. MCP-1 and IL-8 were secreted predominately by $CD14^+$ cells with there being a tendency towards more MCP-1 and IL-8 being released by $CD14^+$ cells isolated from pregnant (TNIL, n = 3; 726 ± 219 pg/ml; 2573 ± 791 pg/ml) compared with non-pregnant (n = 3; 210 ± 184 pg/ml; 1279 ± 942 pg/ml) women. RANTES was released mainly by $CD14^-$ cells with more being released by $CD14^-$ from non-pregnant (1378 ± 535 pg/ml) compared with pregnant (976 ± 230 pg/ml) women (Table 8). There was no correlation in release of MCP-1, IL-8 or RANTES from separated cells with gestation (data not shown).

Parameter	Non-pregnant	First trimester	TNIL	TIL
Haemoglobin (g/l)	132.1 ± 3.4*	120.3 ± 3.2	114.9 ± 3.5	101.2 ± 2.8*
Leukocyte count ($x10^9$/l)	6.73 ± 0.51	7.72 ± 1.10	8.75 ± 0.47	9.2 ± 0.91
Neutrophil ($x10^9$/l)	3.92 ± 0.32	5.45 ± 0.96	6.4 ± 0.45 *	6.9 ± 0.41*
Lymphocyte ($x10^9$/l)	1.77 ± 0.13	1.77 ± 0.15	1.75 ± 0.17	1.79 ± 0.20
Monocyte ($x10^9$/l)	0.30 ± 0.04	0.3 ± 0.03	0.32 ± 0.06	0.3 ± 0.04
Eosinophil ($x10^9$/l)	0.14 ± 0.02	0.16 ± 0.02	0.16 ± 0.02	0.15 ± 0.02
Basophil ($x10^9$/l)	0.08 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Platelet count ($x10^9$/l)	250.6 ± 14.2	286.3 ± 19.9	311.6 ± 22.9	251 ± 21.7

Table 6

Haematological parameters. The haemoglobin levels were significantly lower during labour compared with non-pregnant samples. There was a tendency towards leukocytosis during pregnancy but this failed to reach significance. The neutrophil count was significantly greater at term than during the first trimester or in non-pregnant samples. TNIL, term not in labour; TIL, term in labour; Significance; *, $p<0.01$.

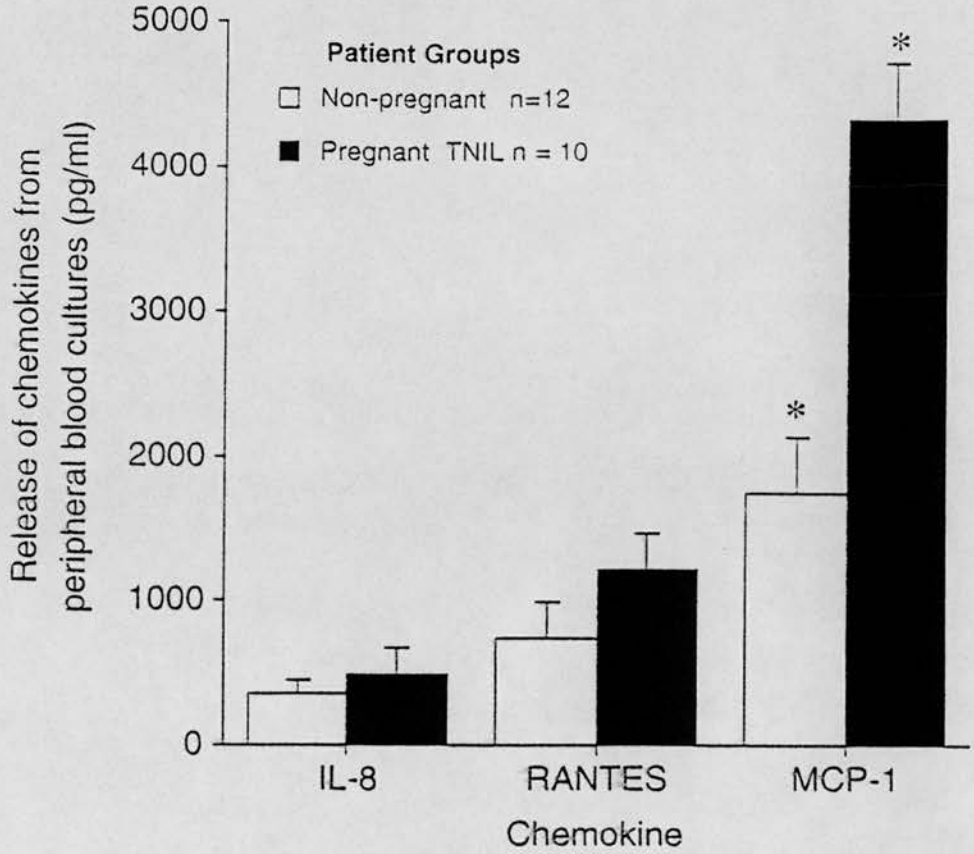


Figure 13

The difference in release of the chemokines IL-8, MCP-1 and RANTES from cultured peripheral blood. Significantly more MCP-1 was released from pregnant (n = 10) compared with non-pregnant samples (n = 12). There was no significant difference in release of either IL-8 or RANTES between pregnant and non-pregnant samples. TNIL, term not in labour. Values are expressed as pg/ml; mean ± s.e.m. Significance; *, $p<0.001$.

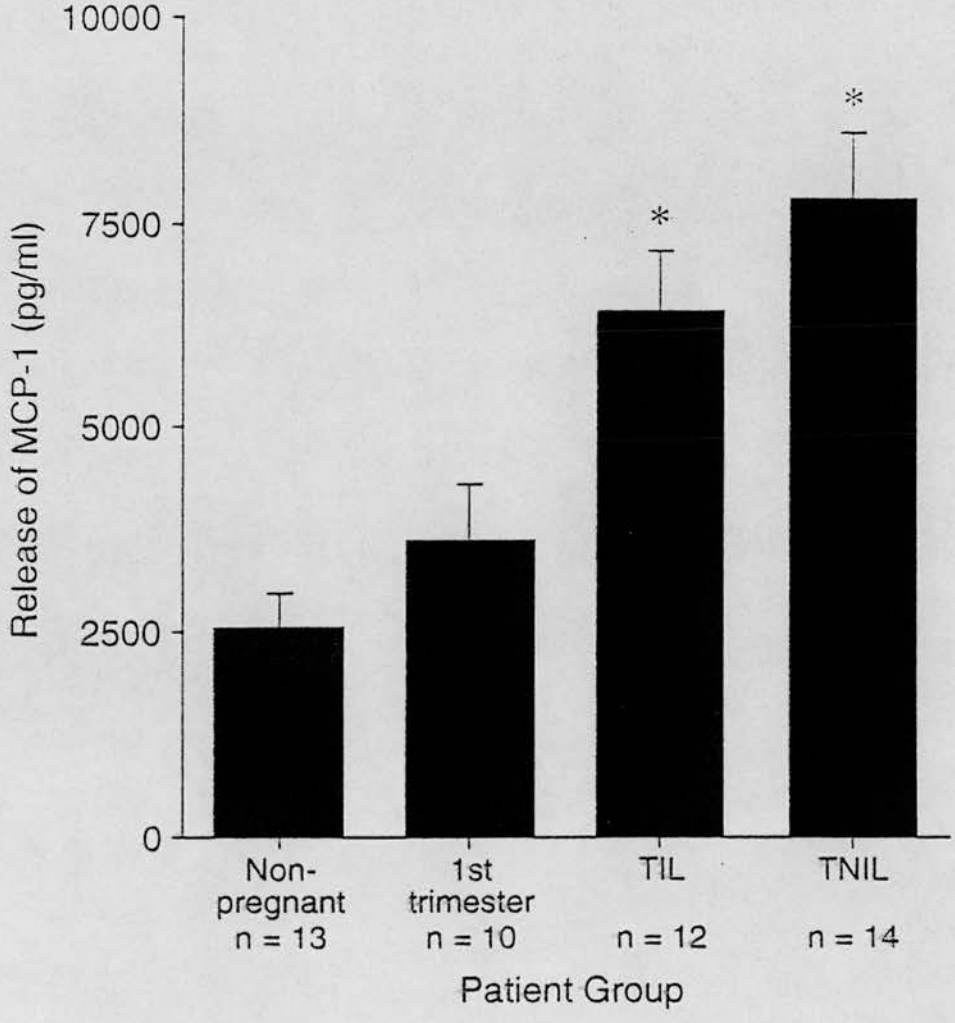


Figure 14

Differential release of MCP-1 through gestation from peripheral blood in a separate study. Significantly higher levels of MCP-1 were released from peripheral blood cultures taken at the end of pregnancy (TNIL, n = 14; TNIL, n = 12) compared with those from first trimester (n = 10) or from women who were not pregnant (n = 13). TNIL, term not in labour; TIL, term in labour. Values expressed as pg/ml; mean ± s.e.m. Significance; *, $p<0.05$.

Sample type	Chemokine release, pg/ml (s.e.m)					
	MCP-1		IL-8		RANTES	
	NP (n = 6)	TNIL (n = 6)	NP (n = 6)	TNIL (n = 6)	NP (n = 6)	TNIL (n = 6)
Peripheral blood	2652 (278)**	8697 (896)**	209 (32)	185 (69)	1472 (121)	1820 (390)
Neutrophil	60 (12)	82 (14)	91 (14)	94 (17)	n.d	n.d
PBMC	1292 (211)*	2665 (214)*	2105 (401)	4319 (362)	3161 (389)	5048 (1171)

Table 7

The release of MCP-1, IL-8 and RANTES from peripheral blood cultures compared with that from peripheral blood mononuclear cell preparations prepared from the same blood sample. Significantly more MCP-1 was produced by both the cultured ($p<0.001$) and the PBMC preparations ($p<0.005$) from pregnant compared with non-pregnant women. PBMC, peripheral blood mononuclear cells; NP, non-pregnant; TNIL, term not in labour; n.d, not detectable; Significance; * $p<0.05$; ** $p<0.001$.

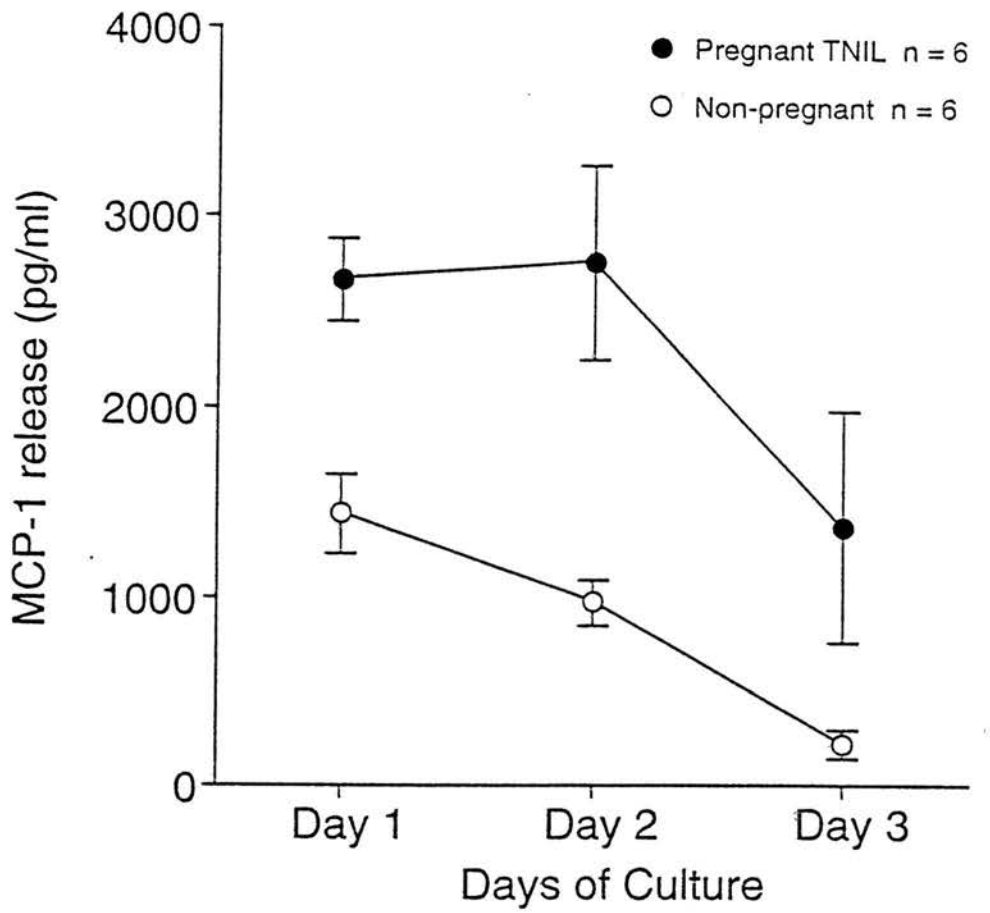


Figure 15

Release of MCP-1 from peripheral blood mononuclear cell preparations over three days culture with media being replaced and assayed every 24 hours. Even after three days culture, more MCP-1 was released from peripheral blood mononuclear cell preparations from pregnant (n = 6) compared with non-pregnant women (n = 6). PBMC, peripheral blood mononuclear cell; TNIL, term not in labour. Values are expressed as pg/ml; mean ± s.e.m.

Cell population	Chemokine release, pg/ml/10^5 cells (s.e.m)					
	MCP-1		IL-8		RANTES	
	NP (n = 3)	TNIL (n = 3)	NP (n = 3)	TNIL (n = 3)	NP (n = 3)	TNIL (n = 3)
$CD14^+$	210 (184)	726 (219)	1279 (942)	2573 (791)	225 (55)	293 (86)
$CD14^-$	17 (2)	70 (26)	197 (51)	108 (20)	1378 (535)	976 (230)

Table 8

The production of MCP-1, IL-8 and RANTES from $CD14^+$ and $CD14^-$ cells. MCP-1 and IL-8 were released predominately by the $CD14^+$ cells whereas RANTES was released predominately by the $CD14^-$ cells. There was no statistical difference in release of any of the chemokines measured between pregnant and non-pregnant blood although this may be due to the small sample size. NP, non-pregnant; TNIL, term not in labour.

4.4 DISCUSSION

This study demonstrates that significantly more MCP-1 is released by peripheral blood cultures from pregnant compared with non-pregnant women with secretion increasing during gestation. The mononuclear cell fraction of peripheral blood, specifically the $CD14^+$ monocyte cells are the likely cellular source of MCP-1. There was no significant difference in secretion of IL-8 or RANTES by peripheral blood preparations or separated cells between pregnant and non-pregnant women.

High levels of circulating progesterone are essential for the initiation and maintenance of pregnancy. In addition, progesterone is an important immunomodulator, inhibiting release of many inflammatory mediators including MCP-1 (Kelly, et al., 1997) and IL-8 (Arici, et al., 1996; Ito, et al., 1994) possibly via the transcription factor NFκB. It could therefore be argued that, on removing blood from the inhibitory, progesterone rich environment of pregnancy, there would be a rebound stimulation of MCP-1 secretion. The experimental data presented discounts this proposal on several counts. Increased release of MCP-1 by pregnant compared with non-pregnant mononuclear cells was still observed after 72 hours culture, long after any inhibitory effect of progesterone would be expected to have dissipated. In addition, since IL-8 is also inhibited by progesterone and if progesterone were an important regulatory factor, then one would also expect a rebound stimulation of IL-8 release, analogous to that observed for MCP-1. This was not observed and was further supported by the failure of steroid antagonism studies to demonstrate any effect on MCP-1 or IL-8 release. Finally, whether pregnant leukocytes do (Paldi, et al., 1994; Szekeres-Bartho, 1992) or do not (Mansour, et al., 1994; Schust, et al., 1996) possess a functioning progesterone receptor, and could therefore respond to progesterone, is controversial.

These studies demonstrated the development of a peripheral leukocytosis and an increase in neutrophil count during pregnancy. Unlike other studies, the former failed to reach significance (Pitkin and Witte, 1979). This may have been due to the relatively small sample size in this study. One explanation, which could be proposed to account for the increased release of MCP-1 by peripheral blood cultures, is that it was purely a function of there being more cells, for example neutrophils, in the pregnant cultures. However, when neutrophils were separated from peripheral blood, counted and cultured, there was no difference in cytokine release between pregnant and non-pregnant cultures. In contrast,

significantly more MCP-1 was released from mononuclear cells from pregnant compared with non-pregnant women suggesting that there might be an intrinsic difference between pregnant and non-pregnant monocytes.

Due to their capacity to present antigens and synthesise a wide range of different cytokines, monocytes and their counterparts, the resident tissue macrophages, play pivotal roles in the induction and regulation of local immune responses. At the feto-maternal interface, resident macrophages are thought to preferentially secrete Th-2 type cytokines (IL-4, IL-5 and IL-10), thus preparing the uterus for implantation and maintenance of the early conceptus (Lin, et al., 1993). Similarly, peripheral blood mononuclear cells from women with normal reproductive histories, which are exposed to trophoblast antigen preferentially secrete Th-2 type cytokines (IL-4 and IL-10) (Marzi, et al., 1996). In contrast, in women with endometrial dysfunction, which results in the inability to permit implantation, endometrial leukocyte populations tend to express more Th-1 (IFN-γ) than Th-2 (IL-4 and IL-10) type cytokines (StewartAkers, et al., 1998). A similar pattern is observed peripherally in women with a history of recurrent spontaneous miscarriage with exposure of peripheral blood mononuclear cells to trophoblast antigen inducing a Th-1 (IL-2, TNF-α, IFN-γ) or embryotoxic response (Hill, 1995). Similarly, mononuclear cells from women who subsequently miscarry or who have small for gestational age fetuses also preferentially secrete Th-1 type cytokines (IL-2 and IFN-γ; Marzi, et al., 1996).

The C-C chemokine MCP-1 was initially described as a monocyte chemoattractant and activating agent. Recently, it has been suggested that it may act as an immunomodulator, favouring development of a Th-2 type of immune response. Studies in mice have demonstrated that MCP-1 expression was increased in the secondary, Th-2 granuloma mediated response to schistosomal infection (Chensue, et al., 1995; Chensue, et al., 1996). In addition, exposure of naïve, murine T-cells to MCP-1 induced them to differentiate into the Th-2 phenotype, capable of releasing IL-4 (Karpus, et al., 1997). It is hypothesised that the increased release of MCP-1 by pregnant compared with non-pregnant monocytes may therefore reflect the Th-2 bias that exists during pregnancy. MCP-1 may act to direct monocyte cytokine synthesis away from a Th-1 type of response and towards a Th-2 type or pregnancy favourable response.

In summary, this study demonstrates that peripheral blood, specifically the monocyte cell fraction releases more MCP-1 than non-pregnant preparations. This suggests that

pregnant monocytes are intrinsically different from non-pregnant monocytes in their cytokine synthetic capacity. This is supported by previous data that demonstrated that monocytes from pregnant women released more IL-1 and TNF-α (Fukuoka, et al., 1993) and expressed higher concentrations of the surface β2-integrins CD18, CD11a and CD11c (Thilaganathan, et al., 1995) than those from non-pregnant women. Finally, it is proposed that the increased secretion of MCP-1 may bias the immune response towards a pregnancy favourable Th-2 type of immune response and facilitate the initiation and maintenance of a normal pregnancy.

CHAPTER 5

DIFFERENTIAL CONCENTRATIONS OF MCP-1 AND IL-8 WITHIN THE FLUID COMPARTMENTS PRESENT DURING THE FIRST TRIMESTER OF PREGNANCY

5.1 INTRODUCTION

During the first trimester, the human embryo is surrounded by two fluid-filled compartments, the amniotic fluid cavity and the extra-embryonic coelom fluid cavity, which also contains the yolk sac (Figure 16).

The amniotic cavity first appears as a slit-like space between the cytotrophoblast and the adjacent embryonic ectoderm. It enlarges to form a small cavity by the 12th post-ovulatory day whose walls and roof are formed by cytotrophoblast and floor by embryonic ectoderm. The amniotic cavity expands at the expense of the extra-embryonic coelom with the developing embryo bulging into the expanded amniotic cavity. Further enlargement results in complete obliteration of the extra-embryonic coelomic cavity with ultimate fusion of the amnion with the chorion to form the amniochorionic membrane (Fox, 1989).

The extra-embryonic coelomic fluid cavity is formed during the 4th week of gestation from cavities within the extra-embryonic mesoderm, which is derived from the outer layer of the trophoblast (Jones and Jauniaux, 1995). It is contiguous externally with the mesenchyme of the placental plate and is bounded internally by the mesothelial layer of the amnion membrane. With the expansion of the amniotic cavity, it becomes completely obliterated at the end of the first trimester.

The human secondary yolk sac is an independent organ present within the extra-embryonic coelomic cavity. It develops during the 6th week of gestation as cystic, spherical structure enveloped by numerous small vessels, which merge to form the vitelline duct. It is connected via this duct to the ventral surface of the embryo, its gut and ultimately to the main embryonic blood circulation (Gonzales-Crussi and Roth, 1976; Jones and Jauniaux, 1995). The yolk sac wall is formed by an external mesothelial layer facing the coelomic cavity, a vascular mesenchyme and an internal endodermal layer. It regresses during the 10th week of gestation, rapidly becomes non-functional (Jones and Jauniaux, 1995) and is completely obliterated along with the extra-embryonic coelomic cavity at the end of the first trimester.

The composition of amniotic fluid and extra-embryonic coelom differs considerably in hormonal, biochemical and protein composition (Docherty, et al., 1996; Jauniaux, et al., 1993). Amniotic fluid comprises mainly transcutaneous secretions from the

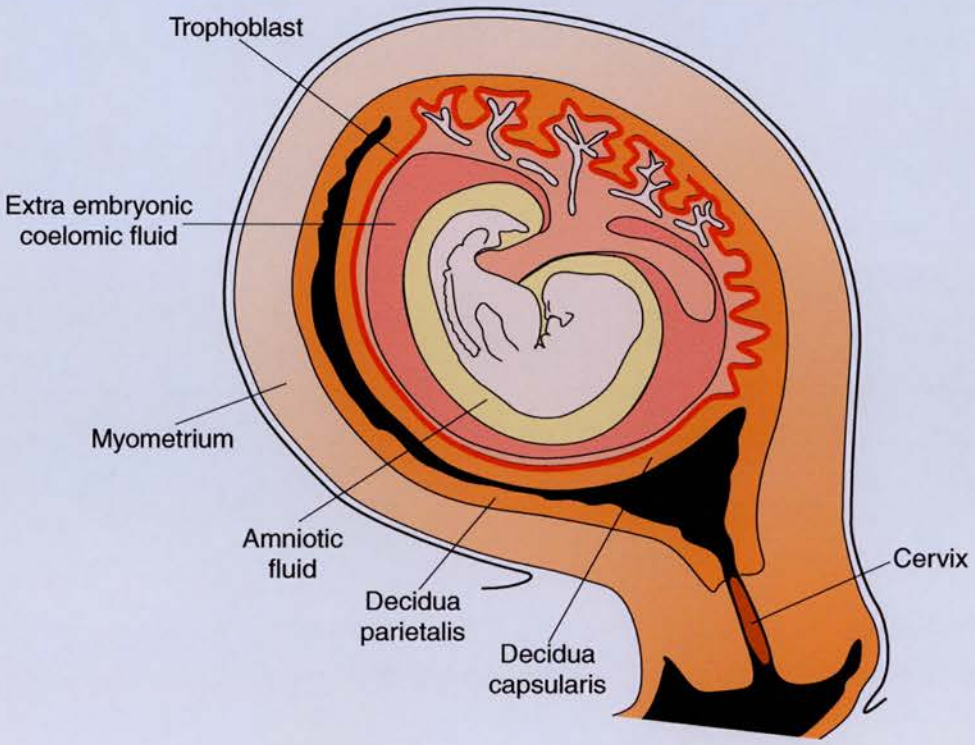


Figure 16
Figure demonstrating the fluid compartments present in the uterus during the first trimester.

fetus and digestive and respiratory tract secretions, after the oropharyngeal membrane has opened (McCarthy and Saunders, 1978). In contrast, extra-embryonic coelom is the site of molecular exchanges between mother and fetus and its contents are the result of an ultra-filtrate of maternal serum with additional contributions from the placenta and secondary yolk sac. Generally, extra-embryonic coelom has higher concentrations of proteins hormones such as hCG and steroids, enzymes and fetal waste products, including urea, creatinine and bilirubin than amniotic fluid. Consequently, extra-embryonic coelom has been proposed as the "physiological liquid extension of the early placenta which may act as a reservoir for nutrients needed by the developing embryo" (Gulbis, et al., 1998). The mechanism for such segregation is not fully understood, however the amnion is thought to play an important role by actively transporting substances between compartments. This concept is supported by the presence of high concentrations of bicarbonate in amniotic fluid and phosphate in extra-embryonic coelom, suggesting the presence of ion anti-ports driving active transport channels (Docherty, et al., 1996). In addition, amnion has rich stores of glycogen, which may represent an energy source for active transport (Jones and Jauniaux, 1995).

The yolk sac has a role in haemopoiesis (Migliaccio, et al., 1986), is the source of primordial germ cells and is involved in protein biosynthesis prior to maturation of the embryonic liver (Gitlin and Perricelli, 1970; Shi, et al., 1985). It is also an important zone of transfer between the extra-embryonic and embryonic compartments (Gulbis, et al., 1998).

Despite this, the role of each compartment in early fetal development remains poorly understood. Cytokines have an essential role in the initiation and maintenance of the early pregnancy by preparing the uterus for implantation and subsequent fetal development. However, whether cytokines are present in the first trimester fluid compartments, and could thus act locally from within the uterus at the feto-maternal interface, is not known. With the advent of modern ultra-sound scans, detailed study of these compartments is now possible with selective aspiration of fluid. Given that studies presented in Chapter 4 had previously demonstrated significant differences in the secretion of MCP-1 but not IL-8 and RANTES by pregnant compared with non-pregnant peripheral blood, then it was of interest to establish whether these chemokines were also present within the first trimester uterus. The aim of this study was therefore to determine the concentrations of MCP-1, IL-8 and RANTES in extra-embryonic coelom, amniotic fluid and maternal serum. Specifically, this study examined whether these cytokines could potentially play a role in

initial development of the fetus and placenta and maintenance of the early pregnancy from within the uterus.

5.2 MATERIALS AND METHODS

Collection of samples

Amniotic and extra-embryonic coelomic fluid were collected by transvaginal ultrasound-guided needle aspiration between 8 - 11 weeks of gestation prior to the termination of pregnancy under general anaesthesia, as described in detail previously (Wathen, et al., 1991). Briefly, women with vaginal bleeding or uncertain menstrual dates were excluded from the study. Peripheral blood (10 mls) was collected prior to the induction of anaesthesia. The perineum and vagina were prepared with Betadine solution. Transvaginal ultrasound scanning was performed to assess fetal viability, gestational age by measurement of the crown-rump length and uterine position. An 18-gauge needle guide was fitted to the probe and a 22 cm 18-gauge needle with trocar was used for the amniocentesis. The needle was advanced under ultrasound guidance through the vaginal vault and myometrium into the fluid. The extra-embryonic coelomic fluid was usually aspirated first due to anatomical reasons. The trocar was then reintroduced and amniotic fluid was aspirated. The extra-embryonic coelomic fluid was always darker than the amniotic fluid. All samples, which were a kind gift from Wathen and Chard and were stored at -20°C prior to immunoassay.

Cytokine ELISAs

MCP-1, IL-8 and RANTES were analysed by ELISA (Section 2.23). Serial dilutions of amniotic fluid, maternal serum and extra-embryonic coelomic fluid gave dose response curves parallel to that of immunopurified standard for MCP-1 and IL-8 (data not shown).

Statistical analysis

The data were normally distributed and statistical analyses of concentrations of MCP-1 and IL-8 between and within compartments with gestation were performed using ANOVA (StatView 4.1, Abacus Inc., Berkeley, CA, USA). The data are expressed as pg/ml mean ± s.e.m with a statistical significant difference considered to be $p<0.05$.

Ethical Approval

These protocols were approved by the Ethics Committee, St Bartholomew's Hospital and informed written consent was obtained from all patients.

5.3 RESULTS

MCP-1 was present in matched samples of amniotic fluid, maternal serum and extra-embryonic coelomic fluid. High levels of MCP-1 were present in all compartments measured with levels of MCP-1 being significantly higher in extra-embryonic coelomic fluid (337.3 ± 40.2 pg/ml) compared with amniotic fluid (151.5 ± 31.5 pg/ml; $p<0.0001$) and maternal serum (142.6 ± 15.9 pg/ml; $p<0.0001$; Figure 17). There was no difference in levels of MCP-1 between amniotic fluid and maternal serum. There was no correlation in levels of MCP-1 in any compartment with gestational age.

IL-8 was present in all compartments measured. The concentration of IL-8 in the extra-embryonic coelomic fluid (339.6 ± 53.8 pg/ml) was significantly higher than those in the maternal serum (33.1 ± 8.2 pg/ml; $p<0.0005$) and amniotic fluid (9.72 ± 6.6 pg/ml; $p<0.0001$). Moreover, significantly greater levels of IL-8 were present in maternal serum compared with amniotic fluid ($p<0.05$; Figure 18). There was no correlation in levels of IL-8 in any compartment with gestation.

The ratios of MCP-1 to IL-8 in amniotic fluid, maternal serum and extra-embryonic coelomic fluid were 15 : 1, 4 : 1 and 1 : 1 respectively.

RANTES was not detectable in extra-embryonic coelomic or amniotic fluid but was present in maternal serum (14.1 ± 5.8 ng/ml).

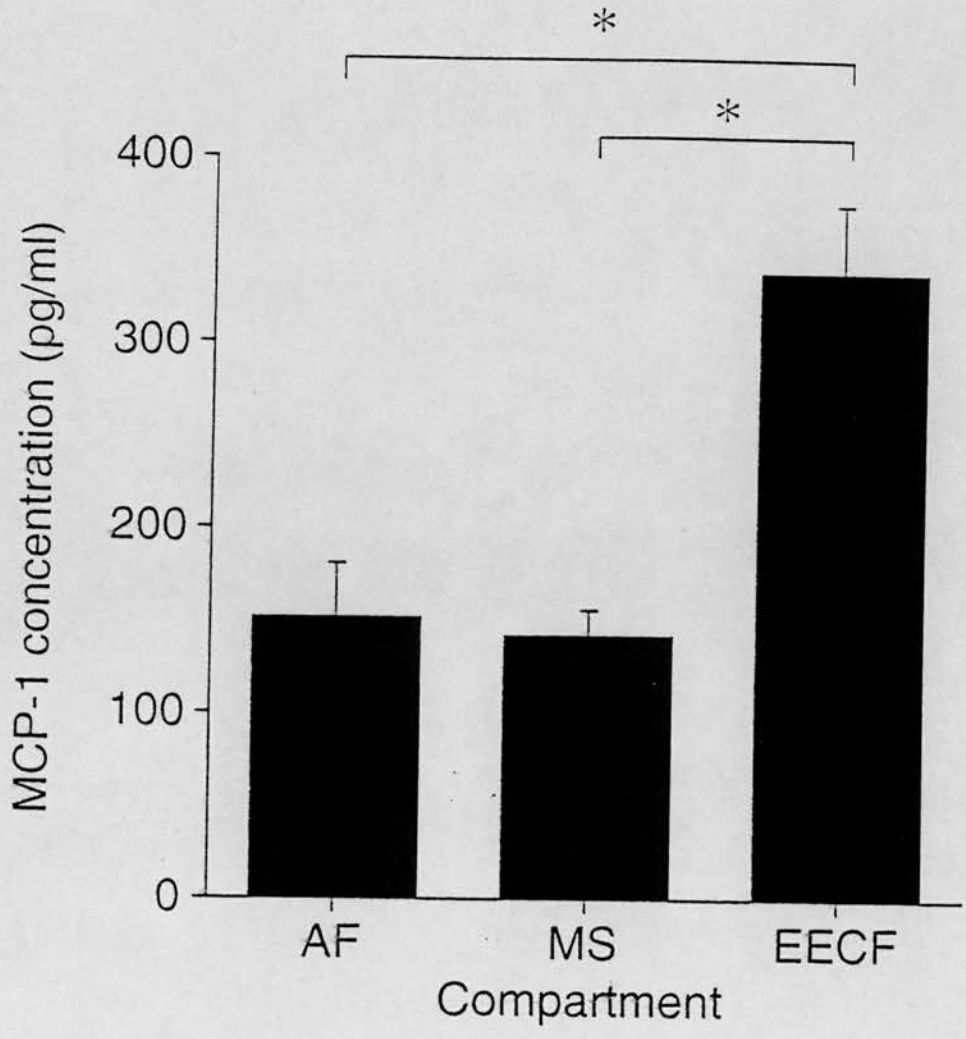


Figure 17

Concentrations of MCP-1 in matched samples of amniotic fluid, maternal serum and extra-embryonic coelom. Significantly higher levels of MCP-1 were present in extra-embryonic coelomic fluid compared with those in amniotic fluid and maternal serum. AF, amniotic fluid; MS, maternal serum and EECF, extra-embryonic coelomic fluid. Values are expressed as pg/ml; mean ± s.e.m. Samples collected from 27 women at 8 - 11 weeks of pregnancy. Significance; *, $p<0.0001$.

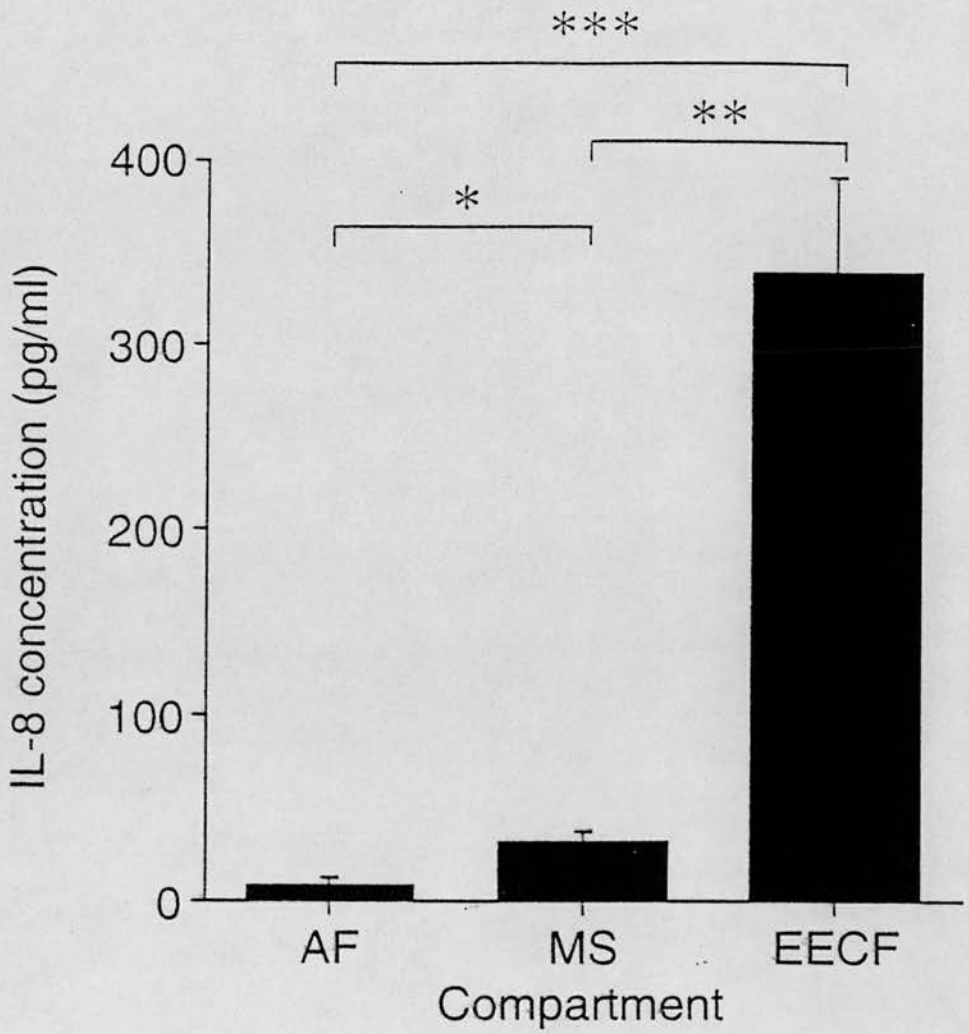


Figure 18

Concentrations of IL-8 in matched samples of amniotic fluid, maternal serum and extra-embryonic coelomic fluid. Significantly higher levels of IL-8 were present in extra-embryonic coelomic fluid compared with those in amniotic fluid and maternal serum. The concentration of IL-8 was significantly higher in maternal serum compared with amniotic fluid. AF, amniotic fluid; MS, maternal serum and EECF, extra-embryonic coelomic fluid. Values are expressed as pg/ml; mean ± s.e.m. Samples collected from 27 women at 8 - 11 weeks of pregnancy. Significance; *, $p<0.027$; **, $p<0.0001$, ***, $p<0.0005$.

5.4 DISCUSSION

This study demonstrates the presence of MCP-1 and IL-8 in amniotic fluid, maternal serum and extra-embryonic coelom during the first trimester, with the highest concentrations of both chemokines being in the latter compartment. RANTES was detectable in peripheral serum but in neither amniotic fluid nor extra-embryonic coelom.

During normal pregnancy, it is postulated that a Th-1 to Th-2 shift occurs in the maternal immune response (Wegmann, et al., 1993) with Th-2 type cytokines being preferentially expressed and secreted at the materno-fetal interface (Lin, et al., 1993). In addition to being a potent monocyte chemotactic and activating factor, MCP-1 facilitates development of a Th-2 type of immune response (Chensue, et al., 1996). MCP-1 is secreted by and has been immunolocalised to perivascular cells present in first trimester decidua (Jones, et al., 1997) and chorio-decidual cells (Kelly, et al., 1997). Although extra-embryonic coelomic fluid mainly comprises an ultra-filtrate of maternal serum, it receives additional contributions from decidua, secondary yolk sac, chorion, chorionic plate and the developing trophoblast. It is therefore hypothesised that the high levels of MCP-1 in the extra-embryonic coelom, which may be due to contributions from maternal serum, decidua and chorion, would facilitate the occurrence of a Th-2 response at the materno-fetal interface.

Amniotic fluid also contains relatively high concentrations of MCP-1. This is unusual in that amniotic fluid is devoid of most proteins during the first trimester (Docherty, et al., 1996). If however, MCP-1 is promoting a pregnancy favourable Th-2 response, then comparable levels would not be unexpected between compartments as demonstrated in this study. MCP-1 could be actively transported from the extra-embryonic coelom across the amnion to the amniotic cavity, gain access to amniotic fluid via yolk sac secretions and the vitelline circulation or be secreted by the embryo as has been demonstrated for alpha feto-protein (Jauniaux, et al., 1993) and cancer antigen 125 (Campbell, et al., 1992), respectively.

IL-8 was located predominately within extra-embryonic coelom with minimal levels in either amniotic fluid or maternal serum. It has been proposed that extra-embryonic coelom may segregate highly active molecules including differentiation factors (insulin-like growth factor-1 and insulin-like growth factor binding protein-1 (Wathen, et al., 1992))

and hormones (progesterone, oestradiol (Jauniaux, et al., 1993)) thus protecting the poorly keratinised fetus when crucial differentiation and organogenesis are occurring (Docherty, et al., 1996). IL-8, due to its potent chemotactic and pro-inflammatory properties (Barclay, et al., 1993; Cherouny, et al., 1992; Sennstrom, et al., 1997) could therefore be confined to extra-embryonic coelom to minimise pro-inflammatory responses within the developing fetus. In contrast, towards the end of the first trimester, the extra-embryonic coelom is obliterated by expansion of the amniotic cavity with subsequent fusion of the amnion with the chorion to form the amniochorionic membrane. Little is known about the mechanism of this fusion process or whether inflammatory cells are recruited but the leucoattractant properties of IL-8 could be beneficial and play a role in this process.

During the first trimester, there is extensive angiogenesis in the chorionic plate and primary villi and haemopoiesis within the secondary yolk sac. The mechanisms and mediators of angiogenesis in the first trimester are poorly understood, however factors such as vascular endothelial growth factor have been implicated. In addition to being a highly specific neutrophil chemoattractant, IL-8 is also angiogenic, haemopoietic (Hu, et al., 1993; Yoshida, et al., 1997) and chemotactic for vascular smooth muscle (Yue, et al., 1994), although the mechanism of the former is not fully understood (Petzelbauer, et al., 1995). IL-8 and vascular endothelial growth factor are both released by and have been immunolocalised to first trimester cytotrophoblast, syncytiotrophoblast and syncytial sprouts, decidua and Hofbauer cells (Athanassiades, et al., 1998; Elliott, et al., 1998; Kelly, et al., 1992; Saito, et al., 1994; Shiraishi, et al., 1996). In other sites, IL-8 and vascular endothelial growth factor synergise to effect angiogenesis with the process being induced by TNF-α (Yoshida, et al., 1997) and hypoxia induced NFκB activation and inhibited to a similar extent by neutralising antibodies to IL-8 and vascular endothelial growth factor (Yoshida, et al., 1998). It is therefore hypothesised that the high concentrations of IL-8 within extra-embryonic coelom, which is adjacent to the actively angiogenic chorionic plate and primary villi, makes IL-8 ideally located to act as a potent mediator of angiogenesis and vascular smooth muscle migration. Even less is known about the factors regulating yolk sac haemopoeisis. However, IL-8 could also play a role in extra-embryonic haemopoiesis, in conjunction with erythropoietin, which has the same distribution in the fluid compartments as IL-8 and has also been implicated in this process (Campbell, et al., 1992).

In summary, this study demonstrates the differential secretion of MCP-1 and IL-8 into the various fluid compartments present during the first trimester. The role of these potent chemokines is not established, however they may be involved in mediating a Th-1 to Th-2 cytokine shift in pregnancy and could play a role in early fetal and placental development.

GENERAL CONCLUSION - PART 1

The first part of this thesis provides support for the hypothesis that cytokines are important in the initiation and maintenance of pregnancy.

Adaptation of maternal immune response

The studies with seminal plasma (Chapter 3) demonstrated that adaptation of the maternal immune system may begin at conception with the principal prostaglandins found in semen, PGE_2 and 19-hydroxy PGE, inducing release of IL-10. This supports Wegmann's hypothesis that a Th-1 to Th-2 shift in immune response may occur during pregnancy and provides a possible mechanism by which this could be induced even prior to fertilisation. Further support for Wegmann's hypothesis was obtained from the peripheral blood studies (Chapter 4), which demonstrated that peripheral blood cultures and monocytes from pregnant women released more MCP-1, which induces Th-2 type cytokine release, than those from non-pregnant women. Although this effect was demonstrated only in circulating monocytes, it is likely that monocytes and macrophages, which are resident within the uterus would also secrete a predominant Th-2 type of cytokine profile. This is supported by previous data that demonstrated that resident macrophages within the uterus preferentially secrete the Th-2 type cytokines IL-4, IL-5 and IL-10 (Lin, et al., 1993). Finally, it was shown that cytokines are present in the fluid compartments of the first trimester of pregnancy with high levels of MCP-1 being present in both amniotic and extra-embryonic coelom (Chapter 5). It was speculated that MCP-1 may be facilitating occurrence of a Th-2 response at the materno-fetal interface. Further studies should be undertaken to examine the secretion and distribution of other Th-1 and Th-2 type cytokines by monocytes and their distribution within the fluid compartments of the first trimester uterus.

Chemokines, cellular recruitment and early fetal development

Seminal plasma prostaglandins stimulated IL-8 release and it was suggested that an increase in the pro-inflammatory, neutrophil chemoattractant IL-8 may have a role in mediating post-coital cervical leukocytosis. It was also hypothesised that IL-8, present in high concentrations in extra-embryonic coelom, may be involved in cellular recruitment during obliteration of the extra-embryonic cavity at the end of the first trimester. Although its role in cellular recruitment within the cervix post-coitus and late first trimester uterus

are novel hypotheses, they are consistent with the known roles of IL-8 in mediating leukocyte infiltration in asthma, dermatitis and menstruation (Jones, et al., 1997; Park, et al., 1998; Strickland, et al., 1997). In addition, it is suggested that IL-8 may play a role in early placental and fetal development. Unfortunately, some of the studies which might be undertaken to further investigate these hypotheses, such as taking post-coital cervical biopsies and samples of the fetal membranes during the fusion process, would be difficult due to ethical and practical reasons.

In summary, the first part of this thesis provides evidence that cytokines have an important role in modulating the maternal immune response to accommodate pregnancy and support Wegmann's hypothesis that a Th-1 to Th-2 shift in immune response may be central to this process. In addition, preliminary data suggests that cytokines are likely involved in cellular recruitment and early fetal and placental development.

PART 2

INFLAMMATORY MEDIATORS: CERVICAL RIPENING AND PARTURITION

GENERAL INTRODUCTION - PART 2

The processes of cervical ripening and myometrial contractility resulting in the ultimate expulsion of the fetus from the uterine cavity at parturition conclude every successful pregnancy. At the local level within the uterus, these events have many analogies with an acute inflammatory reaction, both being associated with cellular infiltration, a rise in inflammatory mediators and tissue remodelling. However, the mediators and regulatory factors involved in cervical ripening and parturition within the third trimester uterus are poorly understood. In the first part of this thesis, the roles of inflammatory mediators in the initiation and maintenance of pregnancy were considered. The second part of this thesis will consider the roles that these factors may play in cervical ripening, parturition and the conclusion of pregnancy. The specific questions which will be addressed are as follows.

1. **Cervical ripening**

   What inflammatory mediators, MMPs and TIMPs are released by the cervix and how are they regulated *in vitro*? What effect does *in vivo* treatment with an antigestogen have on MMP, TIMP and leukocyte immunolocalisation?

2. **Inflammatory mediators and intra-uterine tissues**

   Are MCP-1, IL-8, RANTES and IL-10 released by third trimester amnion, chorion, decidua and placenta and is their release affected by PGE_2 *in vitro*? What are the likely roles of these mediators in parturition?

3. **Anti-inflammatory agents and parturition**

   Is the anti-inflammatory agent secretory leukocyte protease inhibitor released by third trimester intra-uterine structures and present within amniotic fluid? What putative roles may this anti-inflammatory mediator play in the pro-inflammatory process of parturition?

CHAPTER 6

INFLAMMATORY MEDIATOR RELEASE BY THE CERVIX

6.1 INTRODUCTION

Cervical ripening, which has been compared to an inflammatory reaction, is an essential pre-requisite to parturition. Cytokines, in particular the chemokines are thought to play a pivotal role in co-ordinating cellular recruitment, degranulation and activation. However, their paracrine interactions with other inflammatory mediators, metalloproteinases, and hormones are not well understood. The aim of these studies was therefore to investigate these interactions in non-pregnant and pregnant cervical explants using an *in vitro* culture system and *in vivo* treatment, respectively.

Specifically, the following questions were addressed:

6.2 *In vitro* studies on cervix

6.21 Release and regulation of cytokines

The aim was to investigate the release and regulation of IL-8, secretory leukocyte protease inhibitor and PGE_2 by non-pregnant cervical explants and to identify their cellular source of secretion. The regulatory factors studied included progesterone, dexamethasone, PGE_2 and nitric oxide.

6.22 Release and regulation of MMP-2, MMP-9 and their inhibitors by non-pregnant cervical explants.

The aim was to examine whether the gelatinases MMP-2 and MMP-9 and their endogenous inhibitors TIMPs-1, -2, -3 and -4 were released by the non-pregnant cervix, to identify their source of secretion and to study whether their release was affected by administration of PGE_2 and nitric oxide to explants *in vitro*.

6.3 *In vivo* studies on cervix

6.31 Effect of mifepristone administration on the immunolocalisation of MMPs, TIMPs and leukocyte populations during the first trimester

The aim was to investigate whether mifepristone given 6, 12, 24 or 36 hours prior to first trimester termination of pregnancy altered expression of MMP-1, -2, -8 and -9, TIMP-1, -2 and -4, CD45 (leukocyte common antigen), neutrophil elastase and CD68 (monocyte/macrophage marker) as detected by immunohistochemistry.

6.2 *IN VITRO* STUDIES

6.21 RELEASE AND REGULATION OF INFLAMMATORY MEDIATORS BY NON-PREGNANT CERVICAL EXPLANTS

6.211 INTRODUCTION

A variety of mediators have been implicated in cervical remodelling including IL-8 and PGE_2. Interleukin-8 is released *in vitro* (Barclay, et al., 1993) and *in vivo* (Sennstrom, et al., 1997) by the human cervix. In animal models, topical application of IL-8 has been shown to effect cervical ripening, which is morphologically identical to the physiological process (Chwalisz, et al., 1994; El Maradny, et al., 1994). In humans, the concentration of IL-8 within the term cervix increases during ripening and is associated with the release of MMP-8 and MMP-9 (Osmers, et al., 1995) and neutrophil invasion of the lower uterine segment (Winkler, et al., 1997). IL-8 is thought to initiate cervical ripening by promoting neutrophil chemotaxis and activation within cervical stroma.

Prostaglandin E_2 is the most effective and widely utilised cervical ripening agent in clinical practice (Calder, 1990) and has been shown to rise in amniotic fluid prior to the establishment of labour (Challis and Lye, 1994; Gravett, et al., 1994). The mechanism of action of PGE_2 is not well understood although it may involve vasodilation and inducing alterations in stromal and fibroblast proteoglycan metabolism and composition (Carbonne, et al., 1996; Norman, et al., 1993). Whether PGE_2 affects release of other inflammatory mediators, such as IL-8 is not known.

It is likely however, that the highly complex process of cervical ripening will involve a wide range of inflammatory mediators in addition to PGE_2 and IL-8. One such mediator is secretory leukocyte protease inhibitor, which is present in cervical mucus (Helmig, et al., 1995). Secretory leukocyte protease inhibitor is a potent inhibitor of neutrophil function (Sallenave, et al., 1997) and thus might be expected to oppose the action of IL-8 during inflammatory responses such as cervical ripening.

In order to effect controlled, co-ordinated cervical ripening, the release of inflammatory mediators must be tightly regulated. Two factors, which may be involved are nitric oxide and progesterone. Nitric oxide has very recently been shown in humans (Thomson, et al., 1997) and animal models (Chwalisz, et al., 1997) to effect cervical ripening. Its mechanism of action is not fully defined but is thought to be complex involving vasodilation, initiation of apoptosis and induction of synthesis of GAGs and collagen degrading MMPs (Chwalisz and Garfield, 1998). Progesterone appears to exert some control over cervical ripening since progesterone withdrawal initiates cervical softening in

humans throughout gestation despite there being no fall in peripheral serum progesterone prior to cervical remodelling (Chwalisz, 1994). However, the role of nitric oxide and progesterone in regulating release of inflammatory mediators, such as IL-8, secretory leukocyte protease inhibitor and PGE_2, from the cervix has not been well characterised.

The purpose of this study was therefore to investigate the release and regulation of inflammatory modulators within the cervix with the aim of better understanding their role in cervical ripening.

6.212 MATERIALS AND METHODS

Sample collection

Biopsies from the anterior lip of the cervix, approximately 20 - 35 mg in weight, 15 - 20 mm in length and 2 - 3 mm in diameter, were taken from healthy, non-pregnant women (ages 29 - 45) with regular menstrual cycles undergoing a hysterectomy for a non-malignant condition. All women were Caucasian, had no underlying hormonal dysfunction and had not used hormonal contraception, hormone replacement therapy or received GNRH antagonists in the 6 months prior to surgery. The women were at various stages of the menstrual cycle. A different set of biopsies was used for each of the studies.

Tissue culture

Biopsies (n = 39 different women) and cervical fibroblasts (n = 6 different women) were collected, dissected and isolated (Section 2.222). Explants (1 - 2 mg) and fibroblasts (1 x 10^5 cells/ml) were cultured (treatments in quadruplicate) in complete media supplemented with progesterone 10^{-7}M, dexamethasone 10^{-7}M, PGE_2 10^{-7}M and spermine NONOate 50 μg/ml as a nitric oxide donor for 24 hours at 37°C in 95 % air and 5 % carbon dioxide under humid conditions. Spermine NONOate releases nitric oxide at predictable rates and in predictable amounts, with decomposition of the complex generating two molecules of nitric oxide and one molecule of free spermine (Maragos, et al., 1991). To assess the effect of steroid antagonism, explants were cultured in media supplemented with progesterone 10^{-7}M or dexamethasone 10^{-7}M for 24 hours prior to changing and supplementing the media in addition with the anti-gestogen and anti-glucocorticoid mifepristone 10^{-7}M for a further 24 hours (n = 6 different women). The harvested media were then split into two portions, one frozen at -20°C for subsequent ELISA and the other treated 1 : 1 with methyloximating solution overnight at 4°C for prostaglandin assay.

Cervical epithelial cells (n = 6 different women) were isolated by incubating washed, steeped cervical biopsies in Dispase I (1 U/ml in PBS) for 2 - 3 hours at 37°C with gentle agitation. The ecto-cervical epithelium was then sheared off in sheets using a scalpel and cultured to confluence in complete media as previously described. Media was removed from epithelial cell cultures and stored at -20°C until analysis. Subculturing and treatment of cervical epithelial cells was unfortunately not possible.

ELISA assays

IL-8, secretory leukocyte protease inhibitor and PGE_2 release into culture media were analysed by ELISA (Section 2.23).

Statistical analysis

Statistical analysis of concentrations of IL-8, secretory leukocyte protease inhibitor and PGE_2 in culture media was performed using ANOVA StatView 4.1 (Abacus Inc.; Berkley, CA, USA). Fisher's protected least significant difference was used as a post-hoc test to determine significance. The data are expressed as pg/mg mean ± s.e.m with a statistical significant difference being $p<0.05$.

Ethical Approval

Ethical approval for the collection of all samples was obtained from the Lothian Trust Ethical Committee with the informed and written consent of patients.

6.213 RESULTS

IL-8 release

Cervical explants released IL-8. IL-8 release was significantly stimulated by PGE_2 in a dose dependant manner with maximal stimulation at 10^{-6}M ($p<0.001$; Figures 19 and 20a, n = 15 and n = 3 different women with treatments in quadruplicate, respectively). IL-8 release correlated with PGE_2 release by cervical explants (correlation coefficient = 0.82; n = 19 different women, Figure 20b). Nitric oxide ($p<0.05$) stimulated IL-8 release. IL-8 release was inhibited by dexamethasone ($p<0.005$) and progesterone ($p<0.05$). In the studies, which assessed the effect of steroid antagonism on IL-8 release, the inhibition of IL-8 release from explants cultured with progesterone and dexamethasone was antagonised by the addition of mifepristone. There was no significant change in IL-8 release from control biopsies or those cultured with mifepristone alone for 48 hours (Table 9).

IL-8 was released by cervical fibroblasts (n = 6 different women) and epithelial cells (n = 6 different women). Dexamethasone and progesterone significantly inhibited (both $p<0.05$) IL-8 release by cervical fibroblasts (Figure 21).

Secretory leukocyte protease inhibitor release

Cervical explants release secretory leukocyte protease inhibitor (n = 15 different women with treatments in quadruplicate). Secretory leukocyte protease inhibitor release was stimulated by progesterone ($p<0.005$) and inhibited by PGE_2 ($p<0.05$). Nitric oxide, dexamethasone and IL-8 had no effect on secretory leukocyte protease inhibitor release (Figure 22).

Secretory leukocyte protease inhibitor was released by cervical fibroblasts (n = 6 different women) and epithelial cells (n = 6 different women). Release of secretory leukocyte protease inhibitor by cervical fibroblasts was not affected by any of the culture conditions employed (Figure 23).

PGE_2 release

Cervical explants release PGE_2 (n = 15 different women with treatments in quadruplicate). PGE_2 release was stimulated by nitric oxide ($p<0.05$). Progesterone, dexamethasone and IL-8 had no effect on PGE_2 release (Figure 24).

PGE_2 was released by cervical fibroblasts (n = 6 different women) and epithelial cells (n = 6 different women). Release of PGE_2 from cervical fibroblasts was significantly stimulated by nitric oxide and inhibited by dexamethasone (Figure 25; both $p<0.05$).

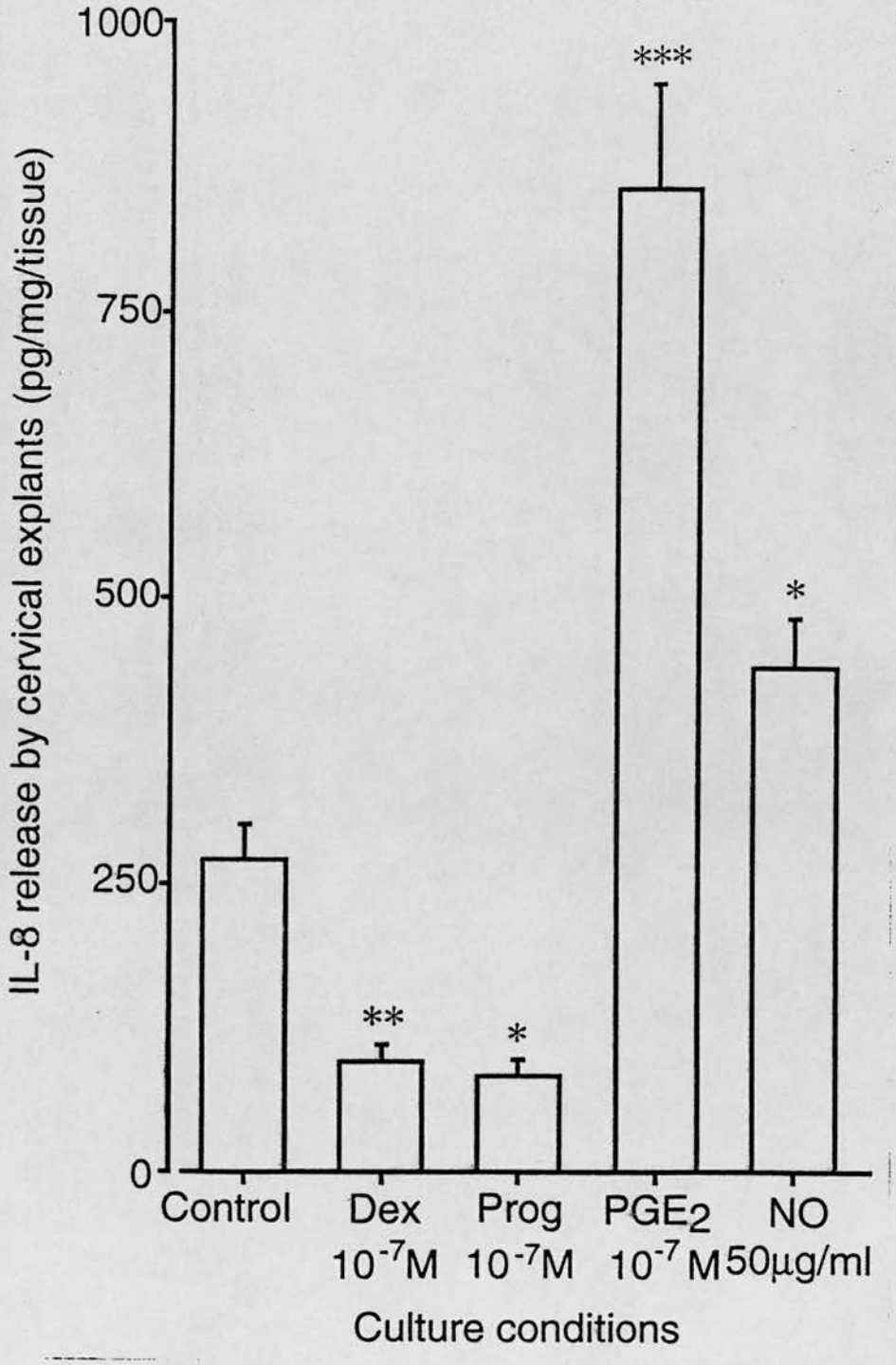


Figure 19

Modulation of IL-8 release by cervical explants by different culture conditions. IL-8 was significantly inhibited by progesterone and dexamethasone and stimulated by PGE_2 and nitric oxide donor. Dex, dexamethasone; Prog, progesterone; NO, nitric oxide donor. Values are expressed as pg/mg wet weight tissue; mean ± s.e.m. n = 15 different women, treatments in quadruplicate. Significance of treatment versus control; *, $p<0.05$, **, $p<0.005$, ***, $p<0.001$.

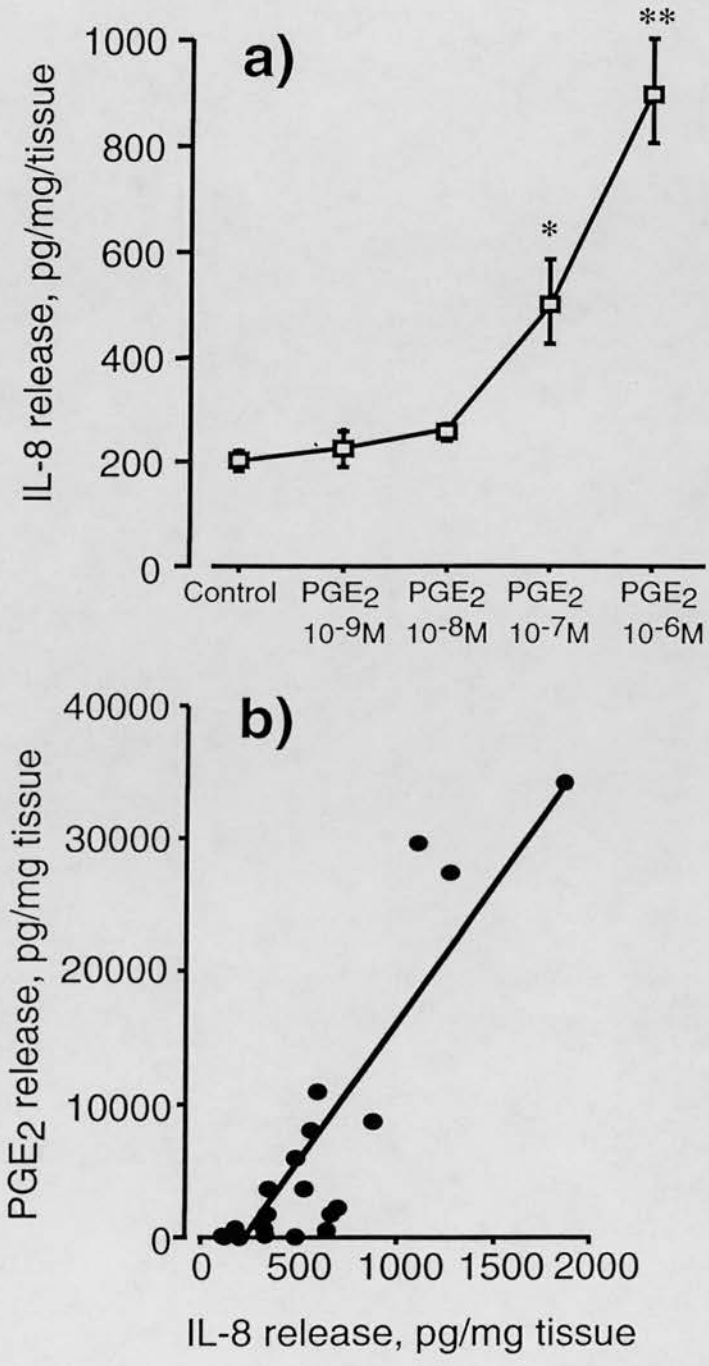


Figure 20

A) Dose response curve of PGE_2 stimulation of IL-8 release with maximal stimulation at 10^{-6}M. Values are expressed as pg/mg wet weight tissue; mean ± s.e.m. Typical of n = 3. Significance; *, $p<0.05$, **, $p<0.005$.

B) IL-8 release correlates positively with PGE_2. Values are expressed as pg/mg wet weight. n = 19 different women, r = 0.82.

Figure 20

A) Dose response curve of PGE_2 stimulation of IL-8 release with maximal stimulation at 10^{-6}M. Values are expressed as pg/mg wet weight tissue; mean ± s.e.m. Typical of n = 3. Significance; *, $p<0.05$, **, $p<0.005$.

B) IL-8 release correlates positively with PGE_2. Values are expressed as pg/mg wet weight. n = 19 different women, r = 0.82.

	Culture condition (IL-8 release expressed as % of control ± s.e.m)			
	Control n = 6	Progesterone 10^{-7}M n = 6	Dexamethason e 10^{-7}M n = 6	Mifepristone 10^{-7}M n = 6
First 24 hours	100	61 (9)*	53 (3)*	103 (8)
Second 24 hours (mifepristone 10^{-7}M added)	100	187 (31)*	156 (13)*	140 (27)

Table 9

The effect of steroid antagonism on IL-8 release by cervical explants. Explants were cultured with steroids for 24 hours prior to changing media and supplementing it with mifepristone 10^{-7}M for a further 24 hours. Treatment with progesterone 10^{-7}M and dexamethasone 10^{-7}M for the first 24 hours inhibited IL-8 release. This inhibition was antagonised by addition of mifepristone to cultures during the second 24 hours. Treatment with mifepristone alone for 48 hours did not affect IL-8 release. Significance of difference from control; *, $p<0.05$.

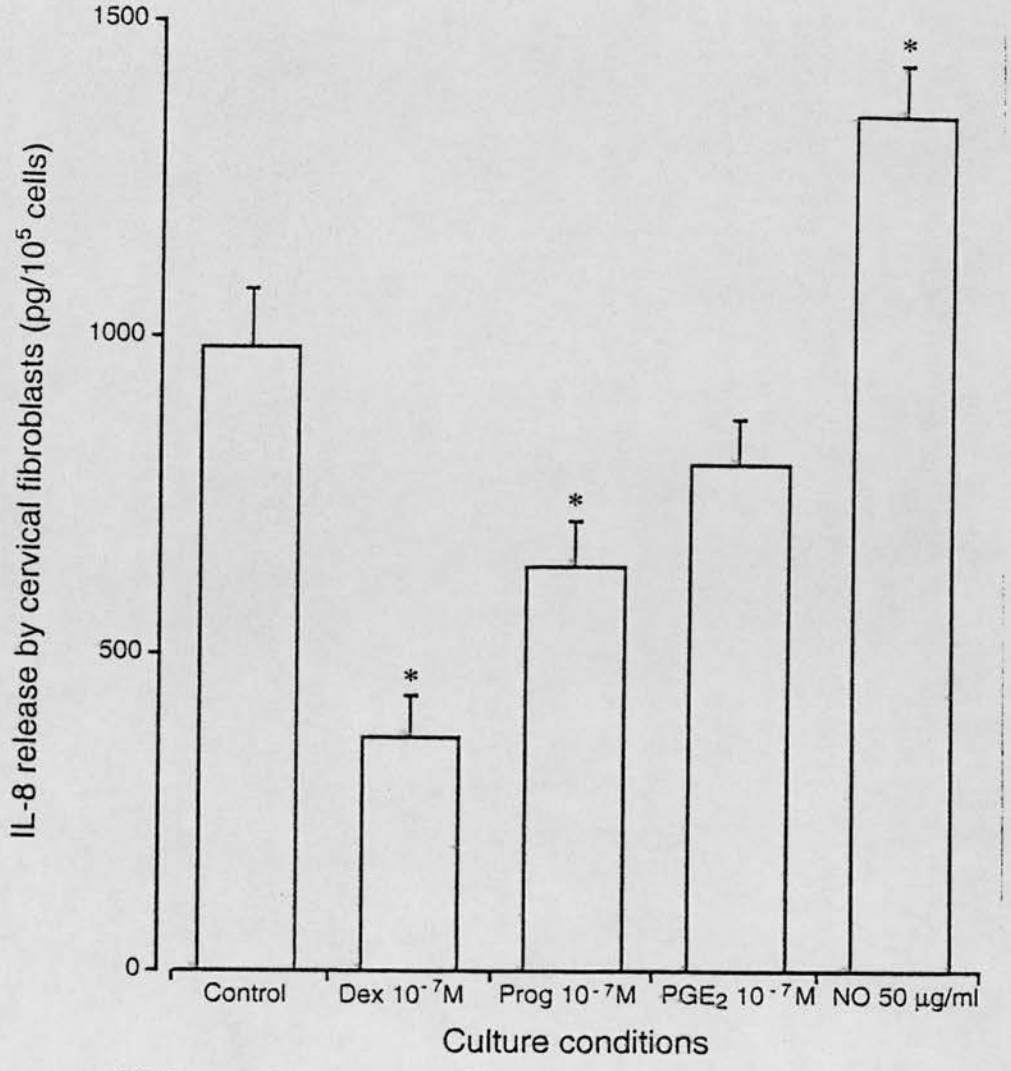


Figure 21

IL-8 release by cervical fibroblasts. Dexamethasone and progesterone significantly inhibited IL-8 release by cervical fibroblasts. Dex, dexamethasone; Prog, progesterone; NO, nitric oxide donor. Values are expressed as pg/10^5 cells; mean ± s.e.m. n = 6 different women, treatments in quadruplicate. Significance of difference from control; *, $p<0.05$.

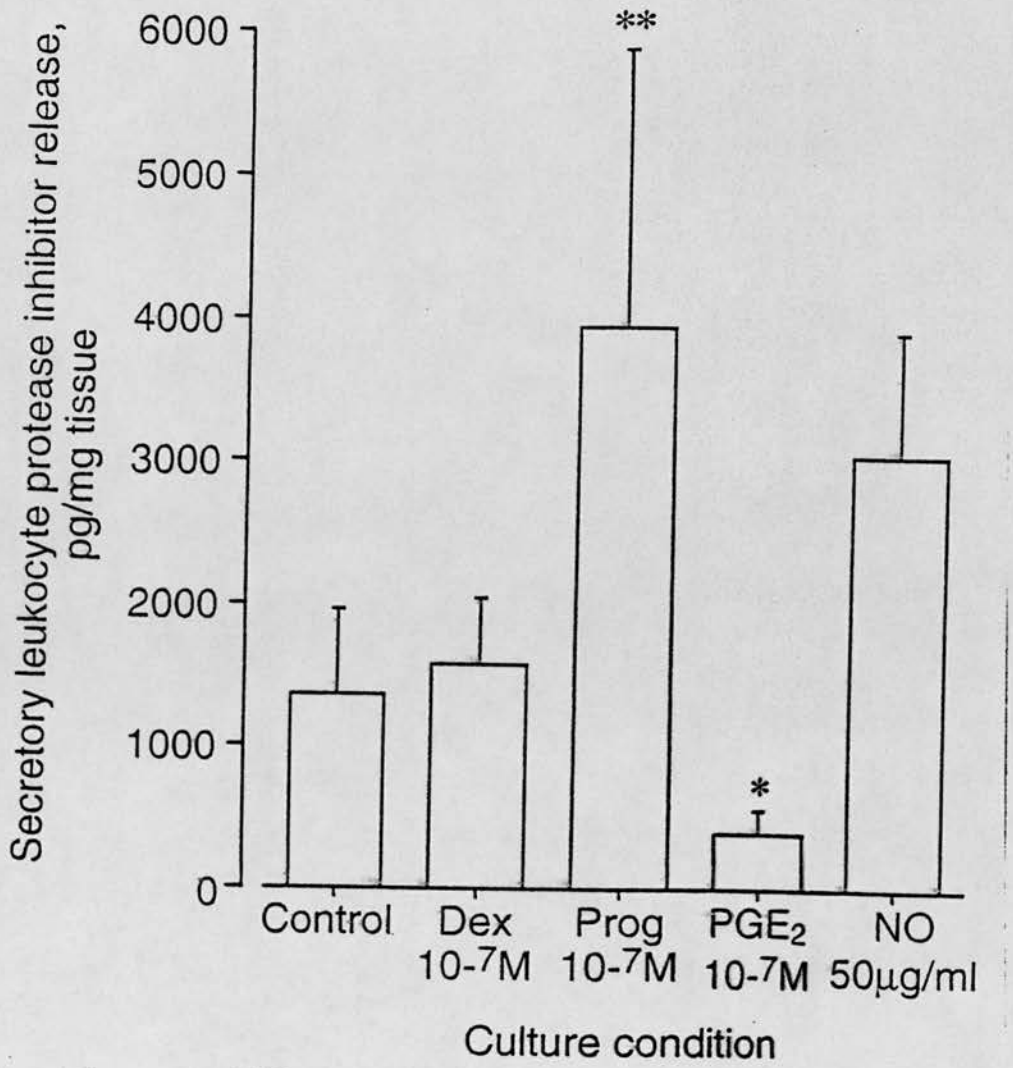


Figure 22

Modulation of secretory leukocyte protease inhibitor release by cervical explants by different culture conditions. Secretory leukocyte protease inhibitor was significantly stimulated by progesterone and inhibited by PGE_2. Dex, dexamethasone; Prog, progesterone; NO, nitric oxide donor. Values are expressed as pg/mg wet weight; mean ± s.e.m. n = 15 different women, treatments in quadruplicate. Significance of treatment versus control; *, $p<0.05$ and **, $p<0.005$.

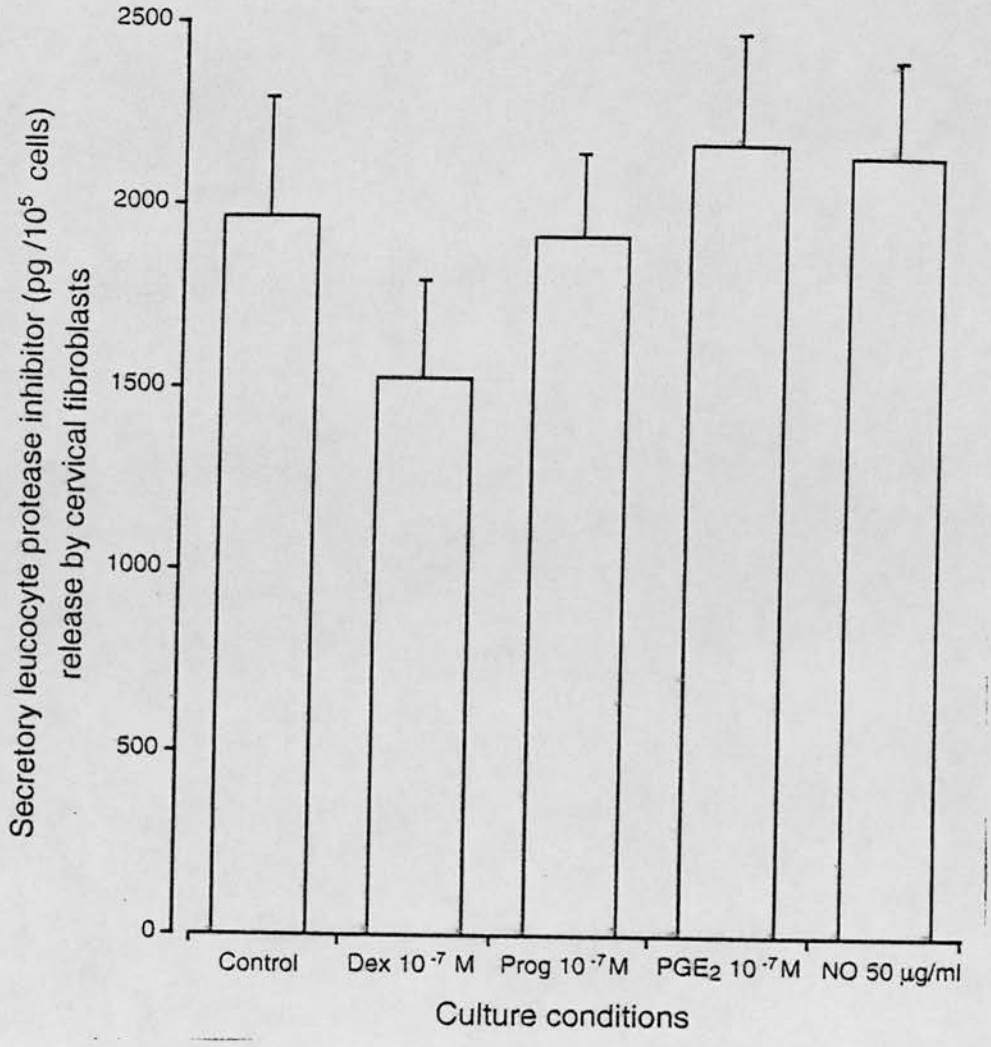


Figure 23

Secretory leukocyte protease release by cervical fibroblasts. Release of secretory leukocyte protease inhibitor was unaffected by the culture conditions employed. Dex, dexamethasone; Prog, progesterone; NO, nitric oxide donor. n = 6 different women; treatments in quadruplicate. Values are expressed as pg/10^5 cells; mean ± s.e.m.

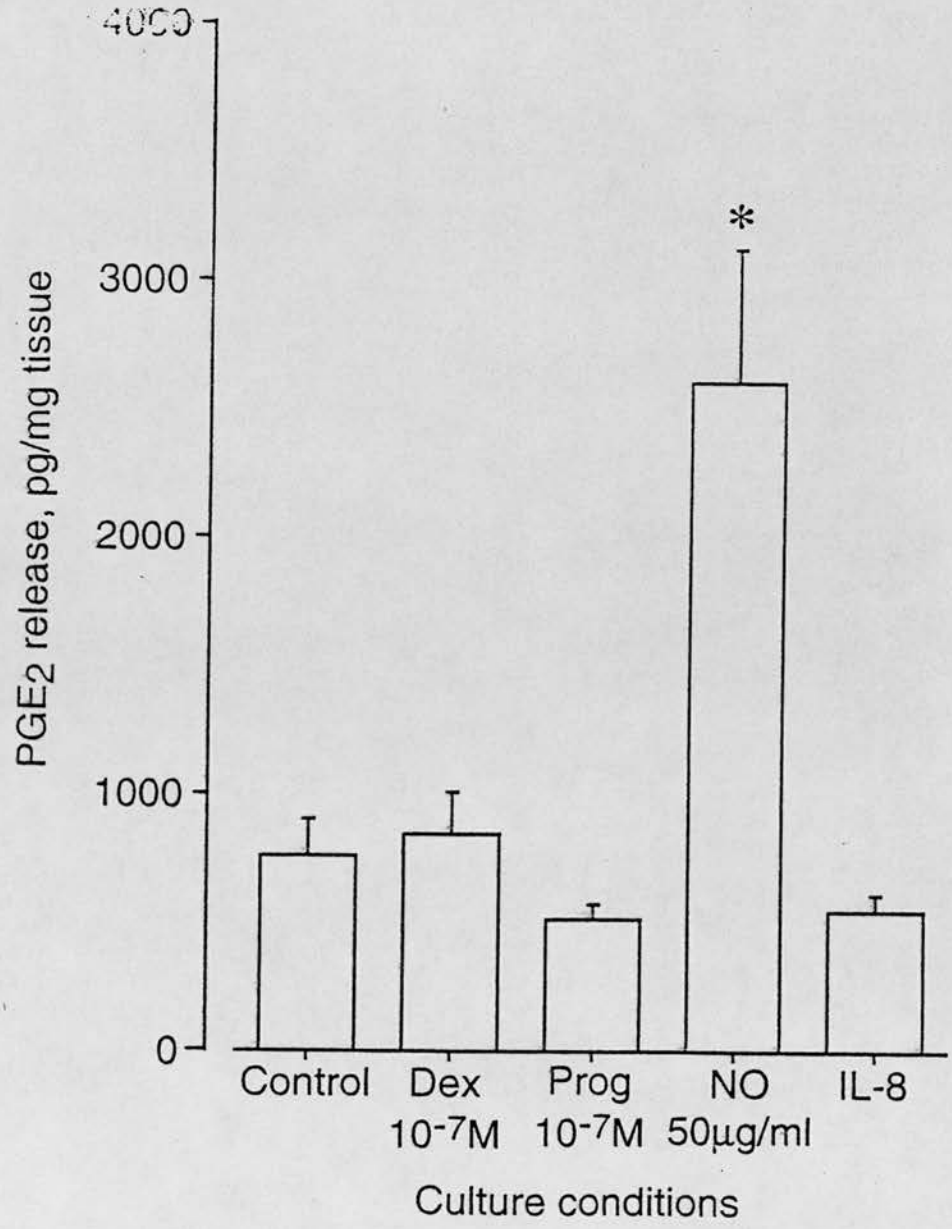


Figure 24

Modulation of PGE_2 release by cervical explants by different culture conditions. PGE_2 was significantly stimulated by nitric oxide (NO). Progesterone (Prog), dexamethasone (Dex) and IL-8 had no effect. Values are expressed as pg/mg wet weight tissue; mean ± s.e.m. n = 15 different women, treatments in quadruplicate. Significance of treatment versus control; * $p<0.05$.

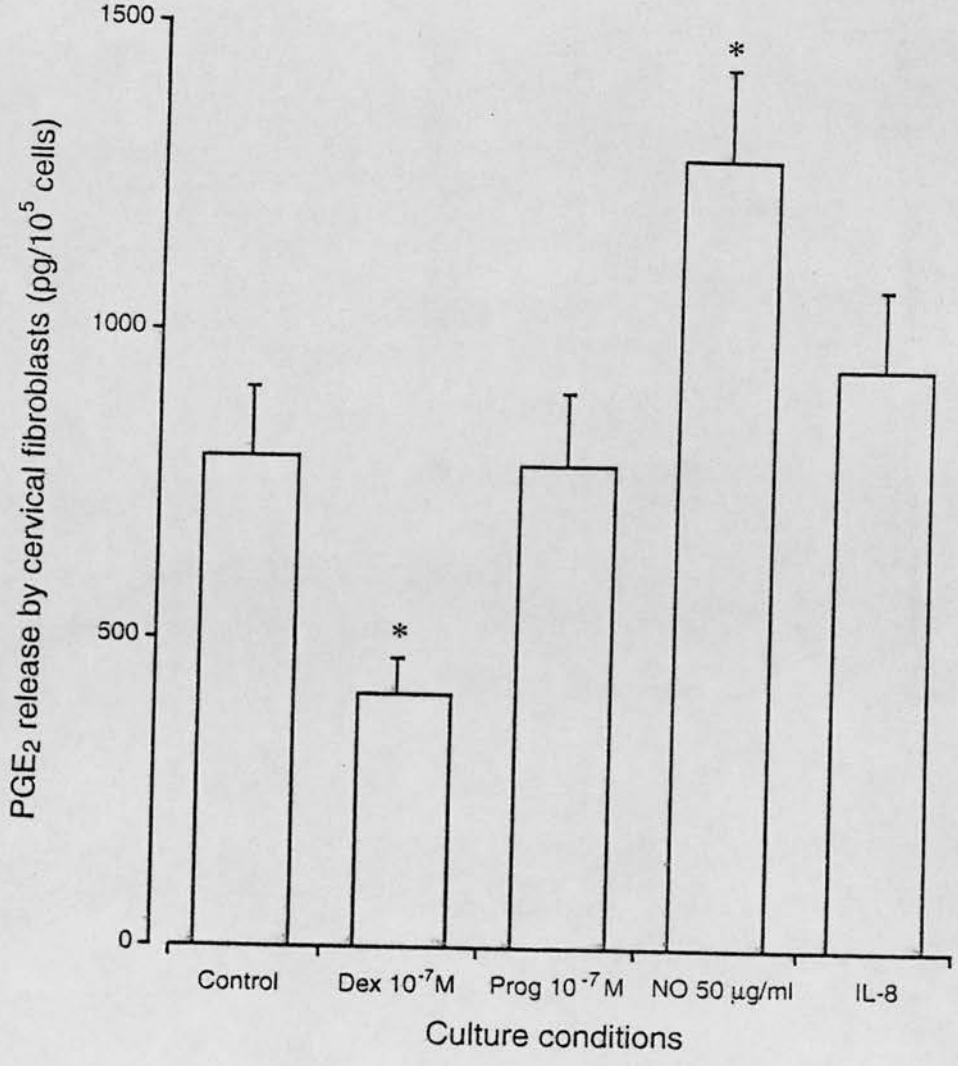


Figure 25

PGE_2 release by cervical fibroblasts. Release of PGE_2 from cervical fibroblasts was significantly stimulated by nitric oxide and inhibited by dexamethasone. Dex, dexamethasone; Prog, progesterone; NO, nitric oxide donor. Values are expressed as $pg/10^5$ cells; mean ± s.e.m. n = 6 different women, treatments in quadruplicate. Significance of treatment versus control; *, $p<0.05$.

6.214 DISCUSSION

These results further our understanding of the mechanism of cervical ripening by demonstrating that complex interactions occur between inflammatory mediators within the cervix. Release of IL-8 by cervical explants was significantly stimulated by nitric oxide and PGE_2 in a dose dependant manner and inhibited by progesterone and dexamethasone. IL-8 was secreted by cervical epithelium and fibroblasts, with release from the latter cell type being stimulated by nitric oxide and inhibited by progesterone and dexamethasone. Inhibition of IL-8 release from cervical fibroblasts by progesterone has been previously demonstrated in the rabbit (Ito, et al., 1994). In contrast to previous studies (Agro, et al., 1996), IL-8 release by cervical fibroblasts was not affected by PGE_2. In the latter studies, PGE_2 mediated stimulation of IL-8 was observed in stimulated human fibroblasts, whereas the cultures employed in these studies were unstimulated. However, during an inflammatory process such as cervical ripening, cervical fibroblasts may be stimulated by a rise in pro-inflammatory cytokines, such as IL-1, and may therefore be primed to increase their secretion of IL-8 in response to PGE_2. Alternatively, PGE_2 may stimulate IL-8 release from cervical epithelial cells, as has been demonstrated in human colonic epithelium (Yu and Chadee, 1998).

Secretory leukocyte protease inhibitor was released by cervical explants, fibroblasts and epithelial cells. Although secretory leukocyte protease inhibitor release was significantly stimulated by progesterone and inhibited by PGE_2 in cervical explants, culture condition had no effect on its release by cervical fibroblasts. It could be therefore that cervical epithelial cells might be responsible for the effect of culture condition on secretory leukocyte protease inhibitor release from cervical explants.

Finally, PGE_2 was released by cervical explants, fibroblasts and epithelial cells with its secretion being stimulated by nitric oxide in explants and fibroblasts and inhibited by dexamethasone in fibroblast preparations. Nitric oxide has previously been shown to stimulate PGE_2 release, via induction of COX-2 in both fibroblasts (Salvemini, et al., 1993) and epithelial cells (Watkins, et al., 1997). It may be that therefore that cervical epithelial cells might also be partially responsible for the nitric oxide mediated stimulation of PGE_2 observed in the tissue explants.

These studies demonstrate that progesterone and dexamethasone inhibit IL-8 and stimulate secretory leukocyte protease inhibitor release from cervical explants. Progesterone plays a vital role in the maintenance of pregnancy and uterine quiescence. In animals such as sheep, a profound reduction in progesterone at term is thought to trigger parturition (Challis and Lye, 1994). In humans, despite there being no peripheral fall in progesterone during the third trimester, indirect evidence suggests that a local reduction within the feto-placental unit may herald the onset of labour (Baird, 1993). Cervical ripening and labour are thought to be co-ordinated at a local level by inflammatory mediators such as the neutrophil chemoattractant IL-8. Progesterone (Kalkhoven, et al., 1996) and glucocorticoids (Mukaida, et al., 1994) inhibit transcription of these mediators, including IL-8 via mutual repression between the steroid receptors and the RelA (p65) subunit of NFκB. This factor is involved in transcriptional regulation of many inflammatory mediators. Glucocorticoids are also known to stimulate secretory leukocyte protease inhibitor synthesis (Abbinantenissen, et al., 1995), although the mechanism is not fully understood. In addition, secretory leukocyte protease inhibitor inhibits cytokine activation by NFκB probably by interfering with the NFκB/rel binding site (Jin, et al., 1997).

It is hypothesised that a local fall in progesterone or interference with its action would stimulate IL-8 and inhibit secretory leukocyte protease inhibitor release from cervical tissue, possibly via NFκB. A rise in IL-8, which has been shown to occur during cervical ripening (Sennstrom, et al., 1997), would initiate neutrophil chemotaxis, emigration and activation within the cervical stroma. Secretory leukocyte protease inhibitor is a potent inhibitor of neutrophil function and elastase release; thus, a fall in its levels would facilitate neutrophil recruitment and activation within the cervical stroma. The overall result would be to favour inflammation and cervical ripening (Figure 26).

It is widely believed that PGE_2 plays an important role in initiating cervical ripening and labour but its mechanisms are not fully understood. In other inflammatory processes, PGE_2 is thought to act principally as a vasoactive agent thus facilitating inflammatory cell infiltration. However, in extra-uterine sites it also upregulates release of many inflammatory cytokines such as IL-8 (Agro, et al., 1996), synergises with IL-8 to augment neutrophil chemotaxis (Colditz, 1990) and stimulates MMP release by monocytes. This latter effect can be inhibited by secretory leukocyte protease inhibitor via inhibition of COX-2, the inducible form of the enzyme, and is independent of secretory leukocyte protease inhibitor's antiprotease activity and regulatory effects on other

cytokines (Zhang, et al., 1997). Finally, PGE_2 is potentially inhibited by progesterone that acts via NFκB to inhibit COX-2 expression.

The data presented demonstrates that *in vitro* treatment of cervical explants with PGE_2 has the dual effect of stimulating IL-8 and inhibiting secretory leukocyte protease inhibitor release. At term, progesterone withdrawal and possible upregulation of NFκB would simulate PGE_2, which would act with IL-8 to promote neutrophil emigration and activation within cervical stroma. In addition to facilitating neutrophil and monocyte chemotaxis and activation, a reduction in secretory leukocyte protease inhibitor would further increase PGE_2 synthesis via upregulation of NFκB mediated COX-2 transcription (Figure 26).

Nitric oxide is a pleiotrophic short-lived free radical gas which mediates a wide range of physiological processes including inhibition of platelet aggregation, smooth muscle relaxation, host immunity and inflammation (Kroncke, et al., 1995). Experimental and clinical evidence also suggests that that nitric oxide plays an important role during pregnancy by maintaining uterine quiescence and placental perfusion. In addition, it may be involved in initiating parturition (Chwalisz and Garfield, 1998). In the rat uterus and placenta, nitric oxide is up-regulated in a gestationally dependant manner during pregnancy and is significantly downregulated prior to parturition (Buhimschi, et al., 1996; Purcell, et al., 1997). Paradoxically, in the rat cervix nitric oxide is regulated in the opposite direction with levels being low during pregnancy but increasing significantly prior to both preterm and term parturition. This may be explained by nitric oxide being synthesised by invading neutrophils (Takeichi, et al., 1998) and monocytes. It is not known if a similar pattern of nitric oxide release exists in humans but recent data suggest that uterine and placental nitric oxide levels do not fall prior to parturition (Thomson, et al., 1997). However, in that study nitric oxide was measured in uteri after 37 weeks by which time any reduction in nitric oxide may have already occurred. Regulation of nitric oxide synthesis is controversial but iNOS appears to be inhibited by progesterone (Miller, et al., 1996) and glucocorticoids possibly via NFκB (De Vera, et al., 1997) and to be stimulated by a variety of inflammatory mediators including IL-1 and TNF-α. Moreover, nitric oxide, like PGE_2 plays a complex immunomodulatory role and can induce both anti- and pro-inflammatory immune responses with high levels, such as might be expected during cervical ripening, favouring inflammation. Nitric oxide induces COX-2 synthesis (Swaisgood, et al., 1997), stimulates MMP-2 secretion by rat mesangial cells (Trachtman,

et al., 1996) and elastase and IL-8 release from peripheral blood (Cuthbertson, et al., 1998), the latter by a cGMP-independent pathway (Corriveau, et al., 1998).

The data presented here demonstrate that nitric oxide stimulates both IL-8 and PGE_2 release from cervical explants. Thus, any fall in progesterone at term and subsequent neutrophil and monocyte invasion of the cervical stroma may produce a rise in nitric oxide via activation of NFκB and local synthesis by invading cells. Nitric oxide would stimulate IL-8 and elastase release and promote PGE_2 synthesis via COX-2 induction leading to a pro-inflammatory state and cervical remodelling (Figure 26).

In summary, the data presented demonstrate that complex interactions occur between inflammatory mediators treated *in vitro*. It is suggested that cervical ripening is not due to a single inflammatory mediator but is the consequence of a cascade of several such agents that interact with each other (Figure 26). Whilst a change in progesterone or its cognate receptor may be the initiating event, studies on progesterone receptor subtype status, local levels and bioavailability of progesterone at term within the cervix need to be performed to confirm this. In addition, these studies have been performed on non-pregnant cervical explants, which have obvious limitations as a model for term cervical ripening, and should be repeated on pregnant tissues to assess whether prior steroid exposure during pregnancy affects interactions between mediators.

Figure 26

Hypothesis of cervical ripening at term. PGE_2 stimulates the pro-inflammatory IL-8 and inhibits the anti-inflammatory secretory leukocyte protease inhibitor. The pro-inflammatory cascade may be further enhanced by stimulation of PGE_2 and IL-8 by rising nitric oxide levels and the effect of falling progesterone on inflammatory mediators.

6.22 RELEASE AND REGULATION OF MATRIX METALLOPROTEINASES -2 AND -9 AND TISSUE INHIBITORS OF METALLOPROTEINASES -1, -2 AND -4 BY NON-PREGNANT CERVICAL EXPLANTS

6.221 INTRODUCTION

During the process of cervical ripening, the cervix undergoes profound tissue remodelling resulting in disorganisation and dissolution of the collagen fibrils, which comprise the bulk of the ECM. Release and activation of MMPs, which include gelatinases (MMP-2 and MMP-9) and collagenases (MMP-1 and MMP-8), are thought to be pivotal in effecting these alterations in animal and human cervices at term (Osmers, et al., 1991; Rajabi, et al., 1991; Uldbjerg, et al., 1983). In rabbits, increased levels of proMMP-2 and pro-MMP-9 are released by term cervical fibroblasts and are associated with cervical remodelling (Imada, et al., 1997). In women, collagenolytic activity increases within the cervix during remodelling (Uldbjerg, et al., 1983) and concentrations of MMP-8 and MMP-9 increase within the lower uterine segment at parturition, correlating with the degree of cervical ripening and dilatation (Osmers, et al., 1995). However, the exact MMPs and their endogenous TIMPs, which are present in the human cervix, their cellular site of secretion and the mechanisms by which their activities are controlled during cervical ripening are not fully understood.

Topical administration of prostaglandins (Calder and Embrey, 1973; Calder, 1990) and more recently nitric oxide (Thomson, et al., 1997) have been used to soften the cervix *in vivo*. However, the mechanisms by which they effect this and how they interact with other mediators of cervical ripening remains unclear, although both have been demonstrated to increase the activity of gelatinases (MMP-2 and MMP-9) in other tissues (Murrell, et al., 1995; Zahner, et al., 1997; Zeng, et al., 1996). It is hypothesised that PGE_2 and nitric oxide may mediate cervical ripening by activating MMP-2 and MMP-9 thus degrading type IV collagen within the basement membrane and endothelium. This would facilitate emigration of neutrophils and macrophages into the cervical stroma, which by degranulation and release of collagenolytic enzymes, could effect remodelling of cervical ECM.

The aims of this study were therefore to examine whether MMPs -2 and -9 and TIMPs -1, -2 and -4 were released by the cervix, to identify their cellular sources and to investigate whether administration of PGE_2 and nitric oxide to explants *in vitro* affected their release.

6.222 MATERIALS AND METHODS

Sample Collection

Cervical biopsies were obtained from healthy, non-pregnant, premenopausal women (n = 15) with regular menstrual cycles undergoing a hysterectomy for non-malignant conditions (Table 10). Tissue was either transported to the laboratory in RPMI 1640 at 4°C or placed immediately in 10 % NBF and fixed overnight at 4°C and stored in 70 % ethanol prior to routine paraffin embedding.

Characteristic	Number patients
Total number	15
Age	28 - 40 (μ = 36)
Parity	
P 0	3
P 1	6
P 2	6
Smoker	7
Non Smoker	8
Indication for hysterectomy	
Menorrhagia	6
Pelvic pain	7
Miscellaneous	2

Table 10

Patient details for cervical biopsies.

Tissue Culture

Cervical biopsies were dissected and cultured in RPMI 1640 supplemented with antibiotics (Section 2.222). Explants were either treated with media alone or media supplemented with PGE_2 (10^{-6}M - 10^{-9}M) and the nitric oxide donor spermine NONOate (50 μg/ml) with treatments in quadruplicate. Cervical fibroblasts were isolated and

cultured in complete media (Section 2.222). Viability was > 95 % by trypan blue exclusion and > 95 % of cells were positive for vimentin immunoreactivity. Prior to experimentation, fibroblasts were cultured in RPMI 1640 supplemented with antibiotics for 24 hours. Media were removed and stored at -20°C prior to freeze drying and reconstituting in 0.1 % SDS for zymography and reverse zymography or sample buffer for western blotting.

Zymography, reverse zymography and western blotting

Gelatinase zymography, reverse zymography and western blotting were performed for MMP-2, MMP-9, TIMP-1 and TIMP-4 (Sections 2.24, 2.25 and 2.26).

Immunohistochemistry

Paraffin embedded cervical sections were immunostained for MMP-2, MMP-9, TIMP-1 and TIMP-4 (Section 2.27).

Ethical Approval

Ethical approval for the collection of all samples was obtained from the Lothian Trust Ethical Committee with the informed and written consent of patients.

6.223 RESULTS

Production of MMP-2 and -9 by cervix

Zymography showed gelatinase activities corresponding to MMP-2 and MMP-9 at 72 and 92 kDa molecular weight, respectively (Figures 27 and 28). Cervical tissue explants released predominantly MMP-2 (72 kDa latent pro-form, 66 kDa active form) with lesser amounts of MMP-9 activity (92 kDa latent pro-form, 86 kDa active form; Figures 27a and 28). Cervical fibroblasts released only MMP-2 (Figure 27b). Western blotting confirmed the presence of MMP-2 protein in non-pregnant cervical explants and in cervical fibroblasts (Figure 29a). MMP-9 protein was not detected in either tissue by western blotting (Figure 29b). Localisation using immunohistochemistry showed that MMP-2 was mainly present in the stromal connective tissue with minimal staining in blood vessels or cervical smooth muscle (Figure 30a). MMP-9 was localised weakly in some perivascular cells and connective tissue stroma (Figure 30b).

Effect of PGE_2 or nitric oxide donor treatment on release of MMP-2 and MMP-9

Treatment with PGE_2 or spermine NONOate *in vitro* had no effect on release of MMP-2 and MMP-9 by explants or cervical fibroblasts, as characterised by zymography (Figure 28 and data not shown; Figures 27a and 27b, respectively), western blot (data not shown; Figures 29a and 29b, respectively) or immunohistochemistry (data not shown for either PGE_2 or spermine NONOate).

Production of TIMPs by cervix

Reverse zymography detected TIMP secretion by non-pregnant cervical tissue and fibroblasts (Figure 31). A band representing the 27 - 30 kDa TIMPs (which may include TIMP-1, -4 and the glycosylated form of TIMP-3) was demonstrated in both explant and fibroblast groups. Western blotting was performed to further delineate the components of the 27 - 30 kDa band obtained by reverse zymography (Figures 29c and 29d). This was unlikely to consist of TIMP-3 since no discrete banding pattern had been obtained on zymography for the unglycosylated 28 kDa form. Analysis for TIMPs -1 and -4 was therefore performed by western blotting and confirmed the presence of both proteins in the cervical tissue explants of cervical biopsies from non-pregnant women (Figures 29c and 29d). Cervical fibroblasts produced TIMP-1 but not TIMP-4 (Figures 29c and 29d). TIMP-1 was localised to blood vessels and connective tissue stroma (Figure 30c). TIMP-

4 was present predominately in the cervical connective tissue stromal cells (Figure 30d).

Effect of PGE_2 and nitric oxide donor treatment on release of TIMPs

In vitro treatment with PGE_2 or spermine NONOate of biopsies from non-pregnant women had no effect on TIMP activity as confirmed by reverse zymography (data not shown; Figures 31a and b, respectively), western blot (data not shown; Figures 29c and 29d, respectively) or immunohistochemistry (data not shown for either PGE_2 or spermine NONOate).

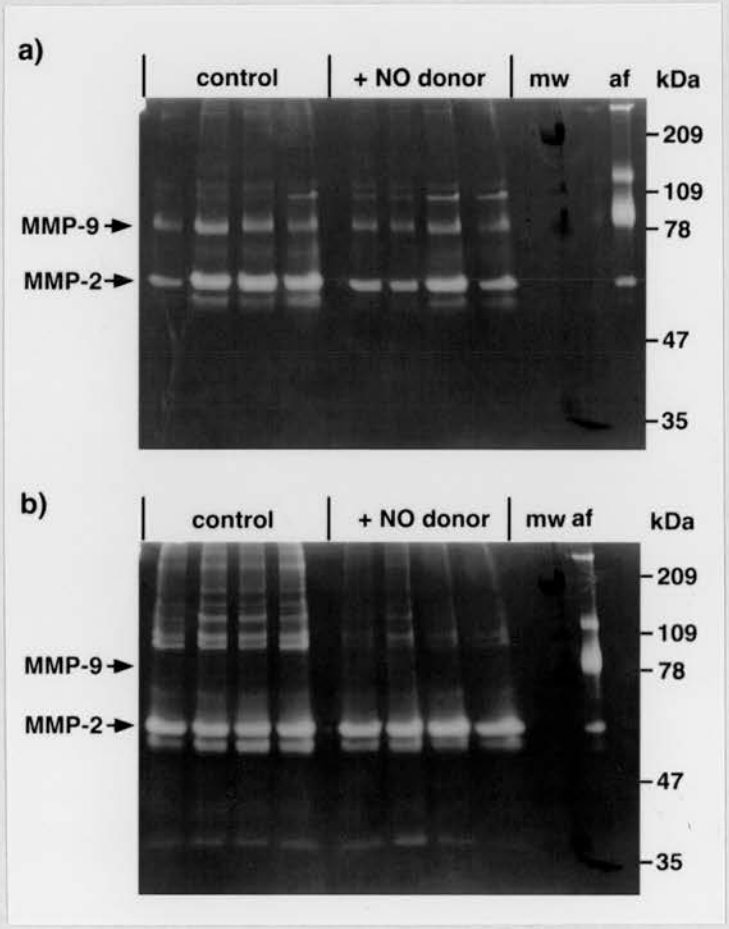


Figure 27

Zymography showing gelatinase activity in culture media of (**a**) non-pregnant cervical tissue explants and (**b**) fibroblasts. Matrix metalloproteinase (MMP)-2 activity is observed predominantly at 72 kDa (latent pro-form) and 66 kDa (active form). MMP-9 activity is shown at 92 kDa (latent pro-form) and 86 kDa (active form). Both explants and fibroblasts were untreated (control) or treated with the NO donor spermine NONOate (+ NO donor). NO, nitric oxide. mw - molecular weight markers as marked kDa. af - amniotic fluid used as positive control.

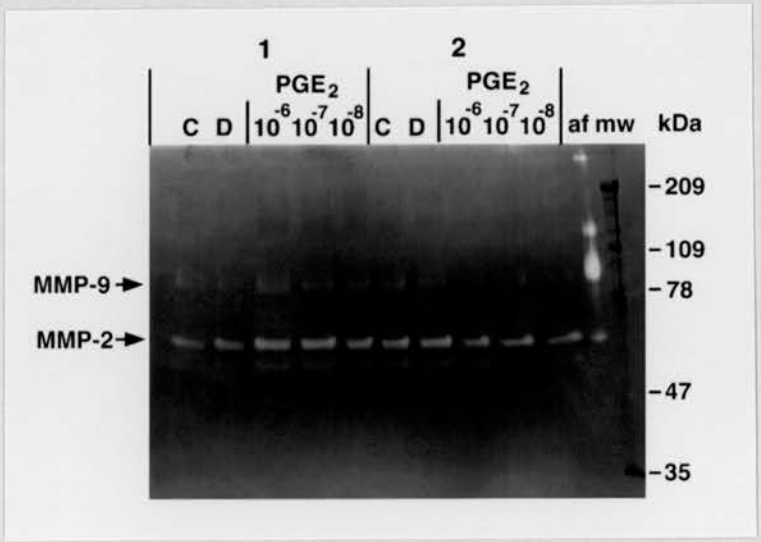


Figure 28

Zymography showing gelatinase activity (visualised as light bands) in culture media of non-pregnant cervical tissue explants. Matrix metalloproteinase (MMP)-2 activity is observed predominantly at 72 kDa (latent pro-form) and 66 kDa (active form). MMP-9 activity is shown at 92 kDa (latent pro-form) and 86 kDa (active form). Explants were untreated (C) or were treated with dexamethasone (D) or PGE_2 (PGE_2; 10^{-6} - 10^{-8} M). mw - molecular weight markers as marked kDa. af - amniotic fluid used as positive control. 1 and 2, samples from different women.

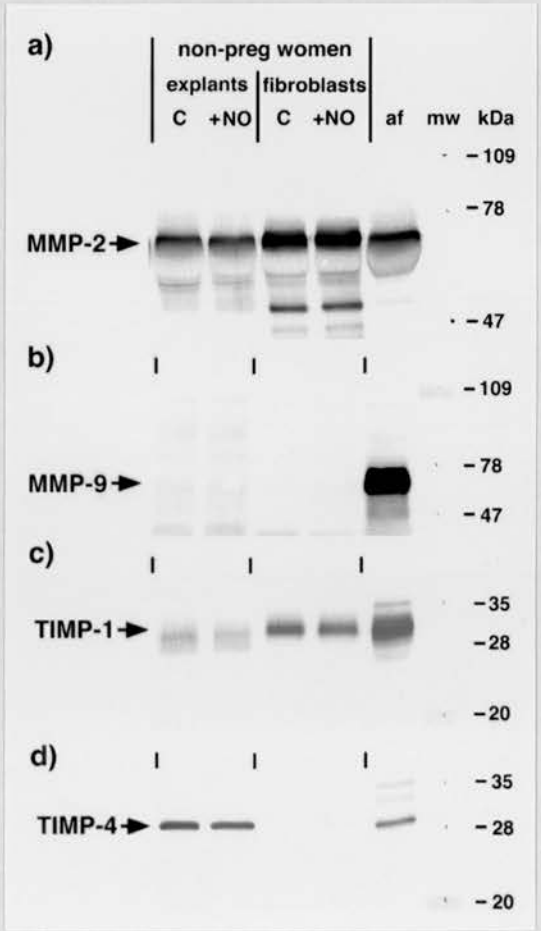


Figure 29

Western blot analysis using antibodies to (**a**) MMP-2, (**b**) MMP-9, (**c**) TIMP-1 and (**d**) TIMP-4. MMP-2 is shown at 72 kDa (latent pro-form) and 66 kDa (active form). MMP-9 is shown at 92 kDa (latent pro-form) and 86 kDa (active form). TIMP-1 and -4 are shown at 27 - 30 kDa. Samples of culture supernatant from explants and fibroblasts from non-pregnant women include controls (C) and those treated *in vitro* with spermine NONOate donor (+NO). mw-molecular weight markers. af - amniotic fluid used as a positive control.

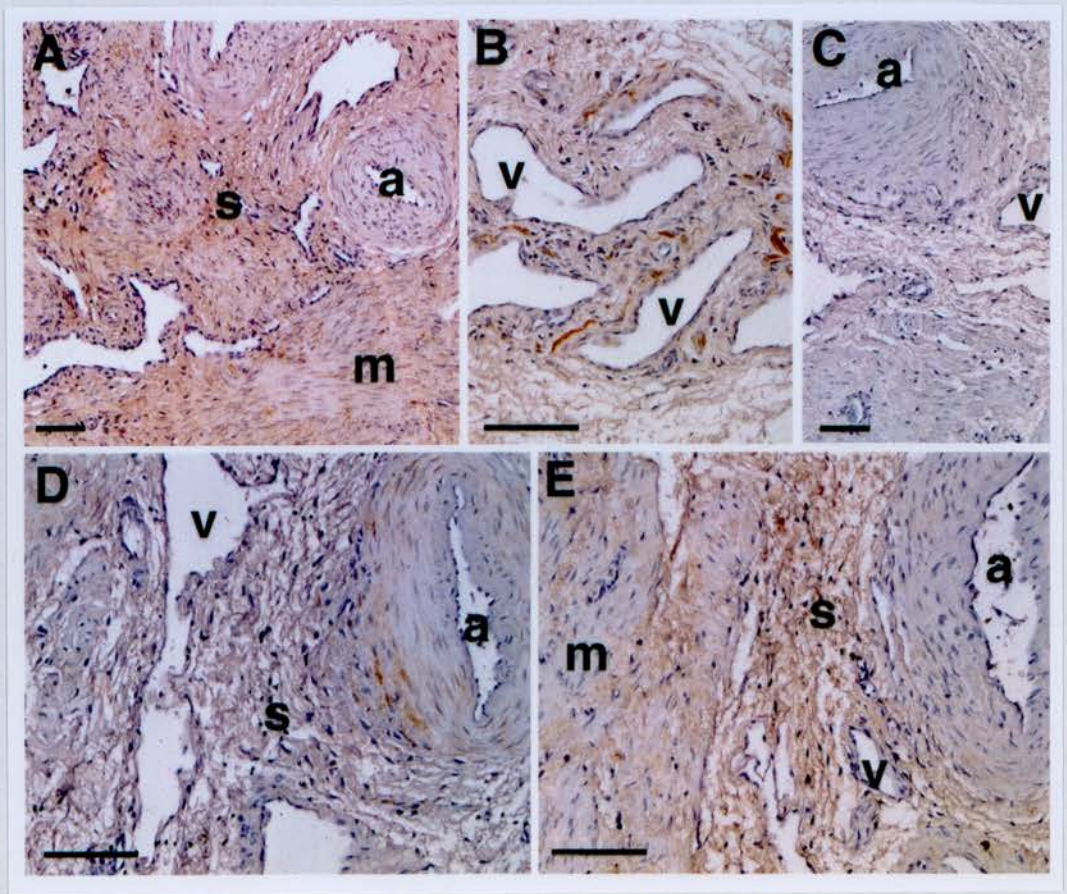


Figure 30

Immunohistochemical localisation in non-pregnant cervical tissue of **A)** MMP-2, **B)** MMP-9, **C)** negative control, **D)** TIMP-1 and **E)** TIMP-4.

Key: a = artery, v = vein, s = stroma, m = muscle. Scale bars = 100 μm.

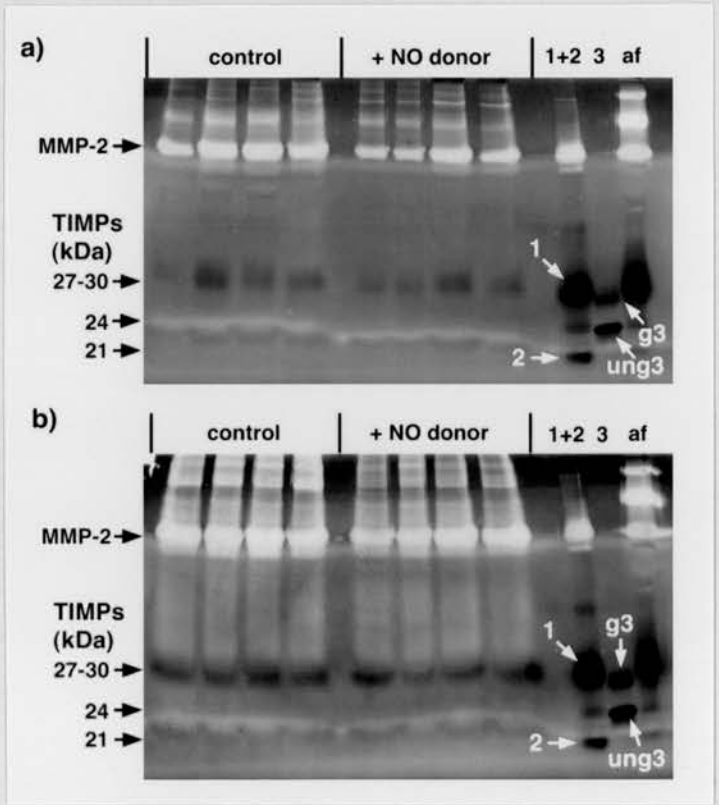


Figure 31

Reverse zymography to detect TIMPs (visualised as dark bands) in culture supernatant from **(a)** cervical explants and **(b)** cervical fibroblasts either untreated (control) or treated with spermine NONOate 50 μg/ml (+NO). Standards of TIMPs 1 and 2 (1+2, separately identified on the gel by arrows) and of TIMP 3 (3; identified on the gel in the 27 - 30 kDa glycosylated form {g3} and 24 kDa unglycosylated form {ung3}). NO, nitric oxide. af - amniotic fluid used as a positive control.

6.224 DISCUSSION

This study demonstrates that MMPs -2 and -9 and TIMPs -1 and -4 are released by and immunolocalised to the non-pregnant human uterine cervix. These results are supported by that of previous studies, which have previously demonstrated the presence of MMPs -2 and -9 in cultured non-pregnant endocervical cells by zymography and western blotting (Agarwal, et al., 1994). These MMPs and their endogenous inhibitors may play a role in the normal turnover of the ECM type IV collagen, elastin and fibronectin in the cervix.

MMP-2 was released by cervical explants and fibroblasts whereas MMP-9 was only released by explants. This was confirmed by detection of MMP-2 but not MMP-9 protein in cervical fibroblast culture supernatants by western blot. These data suggest that cervical fibroblasts may not be the source of MMP-9 in the non-pregnant human cervix. This is supported by previous studies in which MMP-9 was neither released by nor expressed in unstimulated cultured rabbit cervical (Imada, et al., 1997) and human thyroid fibroblast cultures (Hofmann, et al., 1998), respectively. MMP-9 has however been immunolocalised to epithelium, neutrophils, monocyte-macrophages and eosinophils in normal cycling human endometrium (Jeziorska, et al., 1996). It is likely therefore that the source of MMP-9 in non-pregnant explants is either the surface epithelium or bone-marrow derived cells. An alternative explanation for the lack of MMP-9 secretion by cervical fibroblasts is that the amount of protein present in the fibroblast cultures was considerably less than that in the equivalent whole tissue sample and therefore failed to reach the detection limit of either zymography or western blot. In contrast, release of MMP-2 by fibroblasts is supported by studies, which have previously demonstrated its release by such cell types (Hofmann, et al., 1998; Imada, et al., 1997).

Reverse zymography demonstrated that 27 - 30 kDa TIMPs were released by cervical explants. By western blotting it was confirmed that TIMP-1 was released by cervical explants and fibroblasts whereas TIMP-4 was only released by explants. Release of TIMPs have not been previously demonstrated by cervical explants. However, the release and immunolocalisation of TIMP-1 are consistent with studies showing its presence in human epithelium (Mackay, et al., 1992), basement membrane and connective tissue stroma (Ye and Azar, 1998). TIMP-4 has only been identified and cloned recently (Greene, et al., 1996) and little is known about its secretion and localisation in human tissues.

The vasodilators PGE_2 and nitric oxide have been demonstrated to modulate MMP-2 and MMP-9 release in other sites (Murrell, et al., 1995; Zahner, et al., 1997; Zeng, et al., 1996). The mechanisms by which they effect cervical ripening *in vivo* and whether remodelling enzymes are involved are however poorly understood. In this study, *in vitro* administration of PGE_2 and nitric oxide had no effect on release of MMP-2, MMP-9, TIMP-1 or TIMP-4 by cervical explants. A variety of explanations could be proposed to explain these results. During physiological cervical ripening, neutrophils and macrophages invade the cervical stroma and degranulate, releasing their inflammatory mediators. By using an *in vitro* model there is no cellular infiltrate and therefore, if PGE_2 and nitric oxide were to affect MMP release by infiltrating cells, then this could not be examined in the *in vitro* system utilised in these studies. Alternatively, PGE_2 and nitric oxide may affect release of other MMPs such as MMP-1 and MMP-8 (Murrell, et al., 1995), which have also been implicated in cervical ripening (Osmers, et al., 1995). MMP-1 and MMP-8 were not examined in these studies. Finally, these experiments were undertaken on non-pregnant cervical explants which are morphologically different to pregnant cervices and have not been exposed to the high concentrations of circulating progesterone found in pregnancy. Consequently, their response to PGE_2 and nitric oxide may be different from pregnant cervical explants.

In summary, these studies have demonstrated the release and localisation of MMP-2, MMP-9, TIMP-1 and TIMP-4 by non-pregnant cervical explants. Administration of PGE_2 and nitric oxide *in vitro* had no effect on the secretion and immunolocalisation of the MMPs and the endogenous inhibitors studied.

6.3 *IN VIVO* STUDIES ON CERVIX

6.31 EFFECT OF MIFEPRISTONE ON IMMUNOLOCALISATION OF MATRIX METALLOPROTEINASES, TISSUE INHIBITORS OF METALLOPROTEINASES AND LEUKOCYTE POPULATIONS IN THE FIRST TRIMESTER CERVIX

6.311 INTRODUCTION

Progesterone is essential for the initiation and maintenance of pregnancy with its withdrawal initiating cervical ripening and parturition throughout gestation. The anti-gestogen, anti-glucocorticoid mifepristone (RU486), which was first described in 1981 (Philibert, et al., 1981), is highly effective in inducing cervical ripening in women (Bugalho, et al., 1996; Carbonne, et al., 1995; Elliott, et al., 1998). Mifepristone acts as a progesterone antagonist or a partial agonist, depending on whether progesterone is present or absent, respectively (Spitz and Bardin, 1993). Its mechanism of action at the cellular level is highly complex and a variety of hypotheses have been proposed. These include interfering with the dissociation of receptor and heat shock proteins, which is an essential pre-requisite for receptor dimerisation and gene association and inducing a conformational change in the receptor rendering it able to bind to but unable to activate the progesterone response element of responsive genes (Elashry, et al., 1989).

However, the exact mechanisms by which mifepristone increases cervical compliance, reduces cervical resistance and effects cervical ripening *in vivo* are not well understood. Studies have failed to demonstrate any alterations in cervical morphology, collagenolytic activity, collagen content, plasminogen activator levels, muscle contractility, 12-hydroxyeicosatetraenoic acid production or subsequent *in vitro* bioconversion of radiolabelled arachidonic acid to thromboxane, PGE_2 or $PGF_{2\alpha}$ after *in vivo* administration of mifepristone (Bokstrom, et al., 1998; Bokstrom and Norstrom, 1995; Bokstrom, et al., 1994; Heidvall, et al., 1992; Radestad, et al., 1990). A decrease in the ratio of α-2 to β-adrenoceptors (Falkay, 1990), an increase in mast cells and signs of new capillary formation in cervical stroma (Radestad, et al., 1993) have however been found after mifepristone administration. Whether mifepristone affects release of MMPs, their inhibitors or favours cellular infiltration into the cervix, as has been demonstrated in animal models and in human endometrium (Jones, et al., 1997; Marbaix, et al., 1992) is not known.

There is increasing evidence that MMPs and their endogenous inhibitors, which play a pivotal role in effecting cervical remodelling, are differentially regulated by progesterone. However, whether such regulatory mechanisms exist within the cervix and could therefore be implicated in mifepristone induced cervical ripening are not known.

The aims of this study were therefore to investigate the effect of *in vivo* administration of mifepristone on the immunolocalisation of the basement membrane digesting gelatinases MMP-2 and -9, fibrillar collagenases MMP-1 and -8, and their endogenous inhibitors TIMP-1, -2 and -4 within the first trimester human cervix. In addition, given that cervical ripening has been compared to an inflammatory reaction characterised by leukocyte invasion, the effect of mifepristone on leukocyte, neutrophil and monocyte invasion of cervical stroma was also studied.

6.312 MATERIALS AND METHODS

Sample Collection

Cervical biopsies were obtained from women who had received mifepristone at 6, 12, 24 and 36 hours prior to first trimester termination of pregnancy. In addition, there was a control group (untreated) who had not received mifepristone prior to termination (Section 2.211, Table 1). Biopsies were fixed in NBF prior to routine paraffin embedding.

Immunohistochemistry

Immunohistochemistry was performed for MMP-1, -2, -8 and -9, TIMP-1, -2 and -4, CD45 (leukocyte common antigen), neutrophil elastase and CD68 (monocyte marker; Section 2.27). TIMP-3 was not examined because preliminary studies failed to demonstrate its immunolocalisation within cervical stroma.

Ethical Approval

Ethical approval for the collection of all samples was obtained from the Lothian Trust Ethical Committee with the informed and written consent of patients.

6.313 RESULTS

Immunolocalisation

Negative controls

All negative controls were immunonegative for the respective antigen. Representative negative control sections for CD45, CD68, neutrophil elastase and TIMP-2 are demonstrated in Figure 32.

MMP-2

In the untreated biopsies, diffuse positive immunoreactivity for MMP-2 was visualised as dark brown staining in the epithelium, stromal cells and ECM connective tissue with minimal staining in cervical smooth muscle (Figure 33A). *In vivo* treatment with mifepristone had no effect on MMP-2 immunolocalisation (Figure 33B).

MMP-9

In untreated women, MMP-9 was immunolocalised weakly to some cervical epithelial cells (Figure 33C). In biopsies taken more than 24 hours after mifepristone administration, there was a marked increase in positive immunoreactivity for MMP-9 throughout the cervical epithelium, basement membrane and stromal cells and ECM connective tissue (Figure 33D).

MMP-1 (interstitial collagenase)

In women who had not received mifepristone, there was weak, patchy positive immunoreactivity for MMP-1 in cervical epithelium and stromal connective tissue. In addition, there were a few scattered cells of fibroblast morphology, which stained specifically for MMP-1 in cervical stromal (Figure 34A). In biopsies taken more than 6 hours post-mifepristone administration, there was a marked increase in stromal cells staining specifically for MMP-1. The patchy positive immunostaining in the epithelium and stroma was comparable with untreated women (Figure 34B and 34C).

MMP-8 (neutrophil collagenase)

Patchy positive immunostaining was observed for MMP-8 in cervical epithelium and stromal ECM in untreated women (Figure 34D). In addition, there were a few isolated stromal cells which stained specifically for MMP-8. After 24 hours treatment with

mifepristone, there was a marked increase in the population of stromal cells, which stained specifically for MMP-8. The patchy positive immunostaining of epithelium and stromal ECM was comparable with control sections (Figure 34E and 34F).

TIMPs-1, -2 and -4

In untreated women, TIMPs-1, -2 and -4 were immunolocalised to cervical epithelium and connective tissue stroma ECM with relative sparing of blood vessel endothelium and smooth muscle (Figure 35A, 35C and 36A). Mifepristone administration had no effect on the pattern of immunostaining for TIMP-1, -2 or -4 at any timepoint (Figures 35B, 35D and 36B).

Leukocyte markers

CD45 (leukocyte common antigen)

A few isolated cells, which were mainly sub-epithelial, stained immunopositive for CD45 in biopsies from untreated women (Figure 37A). There was a marked increase in the number of discrete, immunopositive cells in biopsies taken more than 12 to 24 hours after mifepristone administration (Figure 37B and 37C). These cells were located predominately in the sup-epithelial layer and diffusely scattered within the cervical stromal ECM.

Neutrophil elastase

A few sub-epithelial cells stained immunopositive for neutrophil elastase in biopsies from untreated women (Figure 38A). There was a marked increase in the number of immunopositive cells in biopsies taken more than 12 hours after mifepristone administration. These cells were located mainly within cervical stroma ECM and within the lumen of stromal blood vessels (Figure 38B and 38C).

CD68 (monocyte/macrophage cell marker)

Biopsies taken from women who had not received mifepristone were immunonegative for CD68 (Figure 39A). After 12 hours treatment with mifepristone, there was an increase in numbers of cells within cervical stromal ECM and in the sub-epithelial layer stained immunopositive for CD68 (Figure 39B and 39C, cells highlighted by arrows).

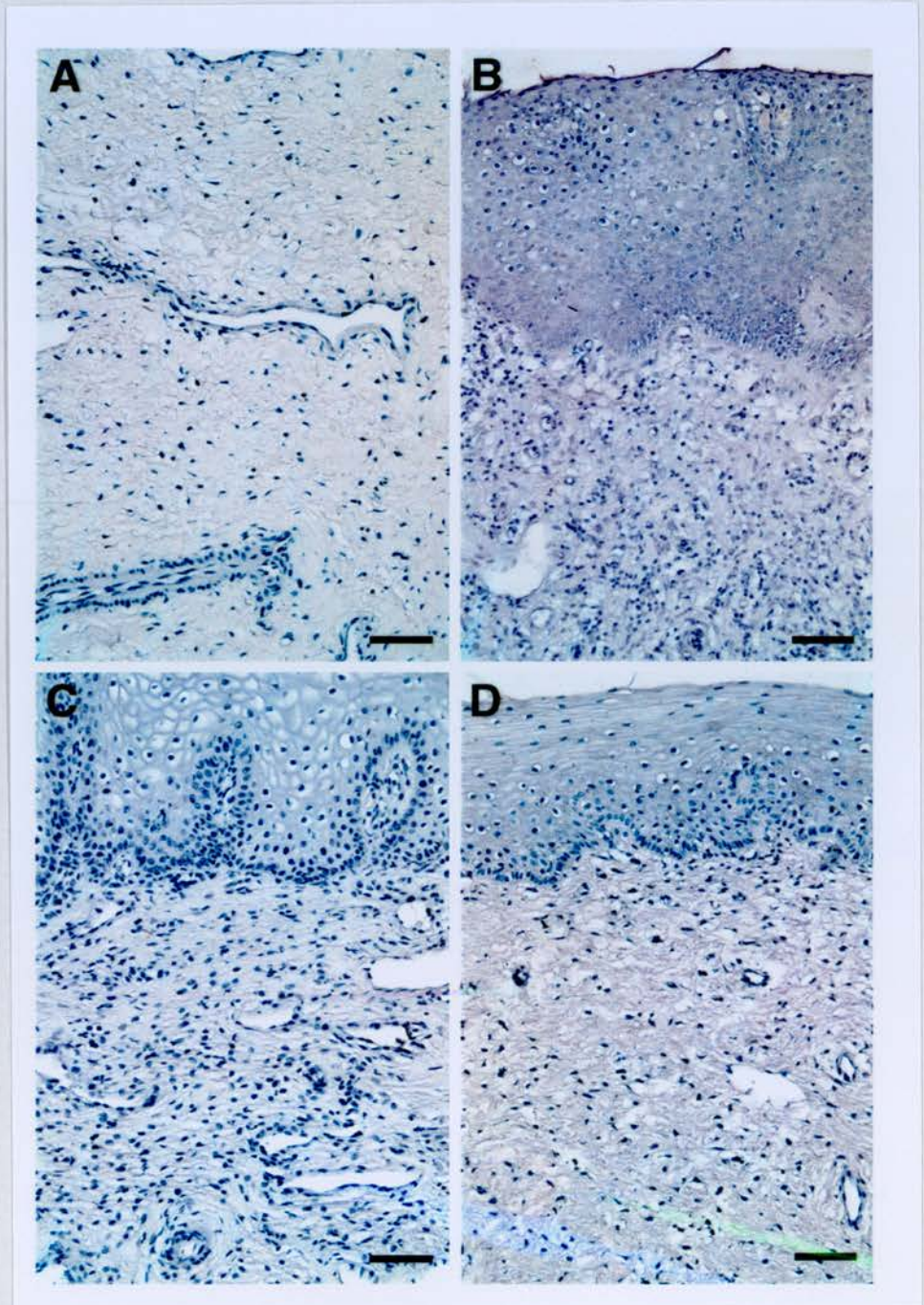


Figure 32

Representative negative control sections of first trimester cervical biopsies stained for **A**) CD45, **B**) CD68, **C**) neutrophil elastase and **D**) TIMP-2. Scale bars = 100 μm.

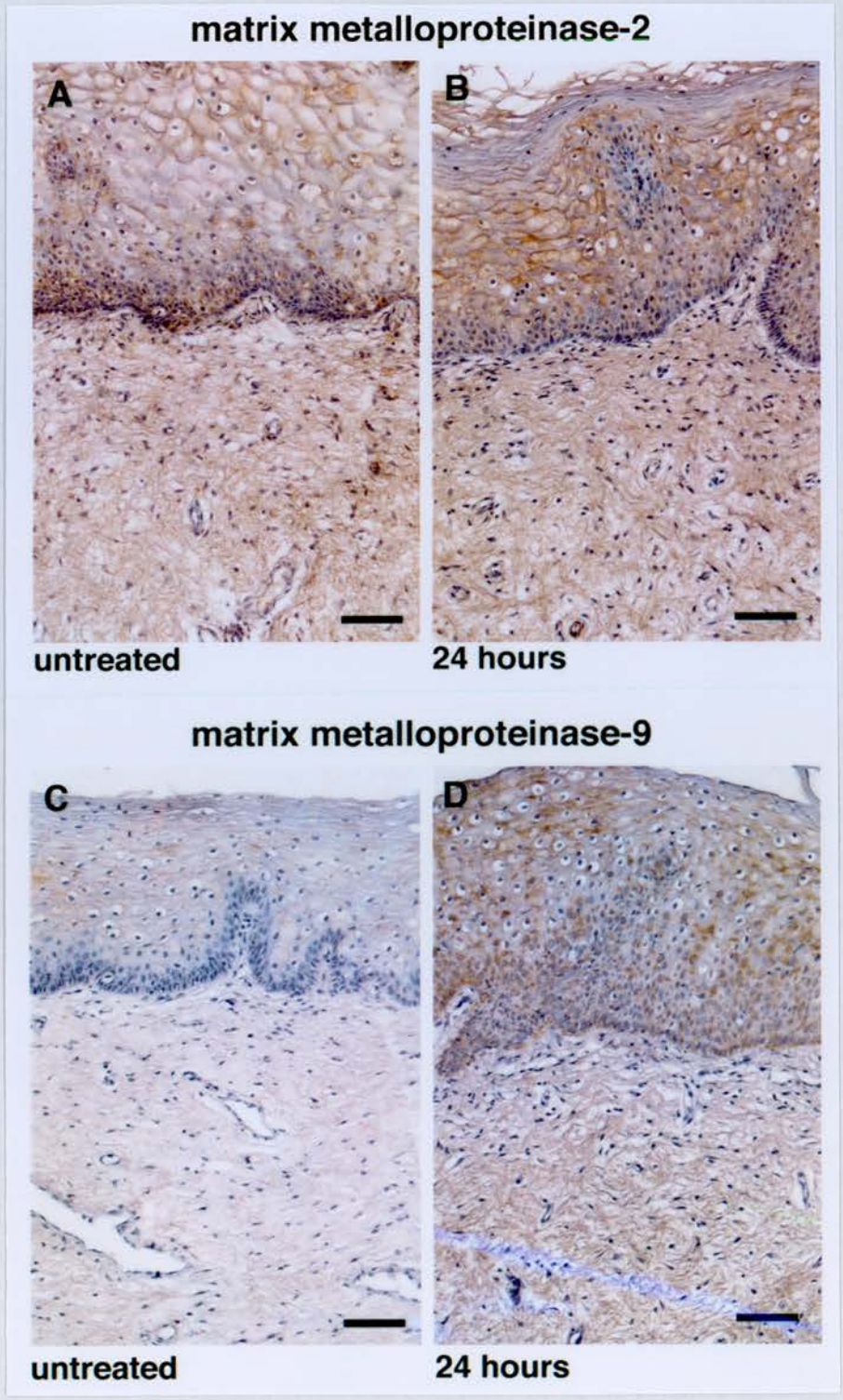


Figure 33

MMP-2 and -9 immunolocalisation in human cervix after mifepristone administration. **A)** and **C)** untreated; **B)** and **D)** 24 hours after treatment. MMP-2 immunolocalisation was unaffected by mifepristone administration. MMP-9 staining increased in epithelial, stromal cells and ECM 24 hours after mifepristone treatment. Scale bars = 100 μm.

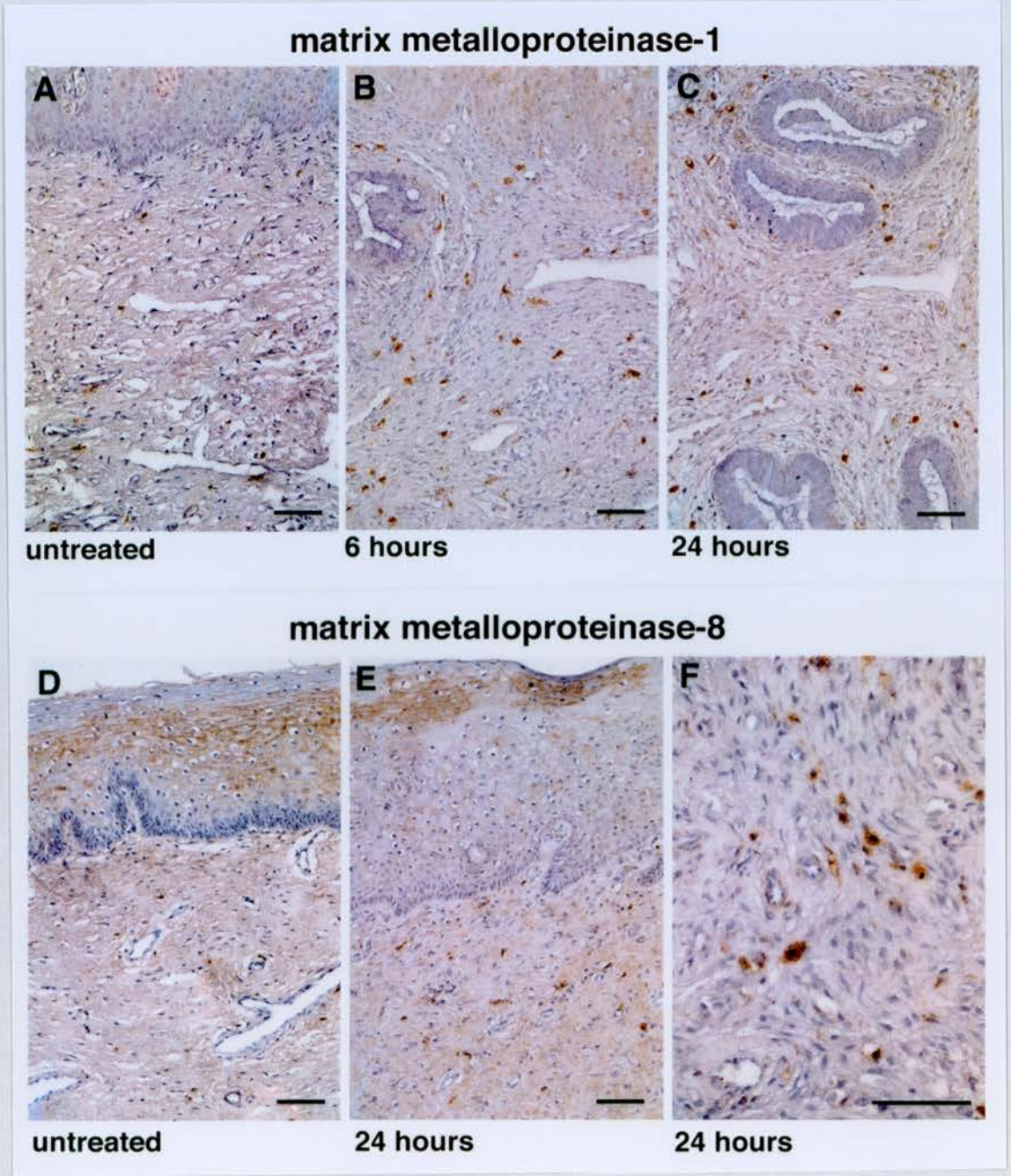


Figure 34

MMP-1 and -8 immunolocalisation in human cervix after administration of mifepristone *in vivo*. **A)** untreated, **B)** 6 hours, **C)** 24 hours, **D)** untreated, **E)** and **F)** 24 hours after treatment. There was increased number of stromal cells staining specifically for MMP-1 and -8 in biopsies taken more than 6 hours and 24 hours, respectively after mifepristone administration. Epithelial staining for MMP-1 and -8 was unaffected by mifepristone treatment. Scale bars = 100 μm.

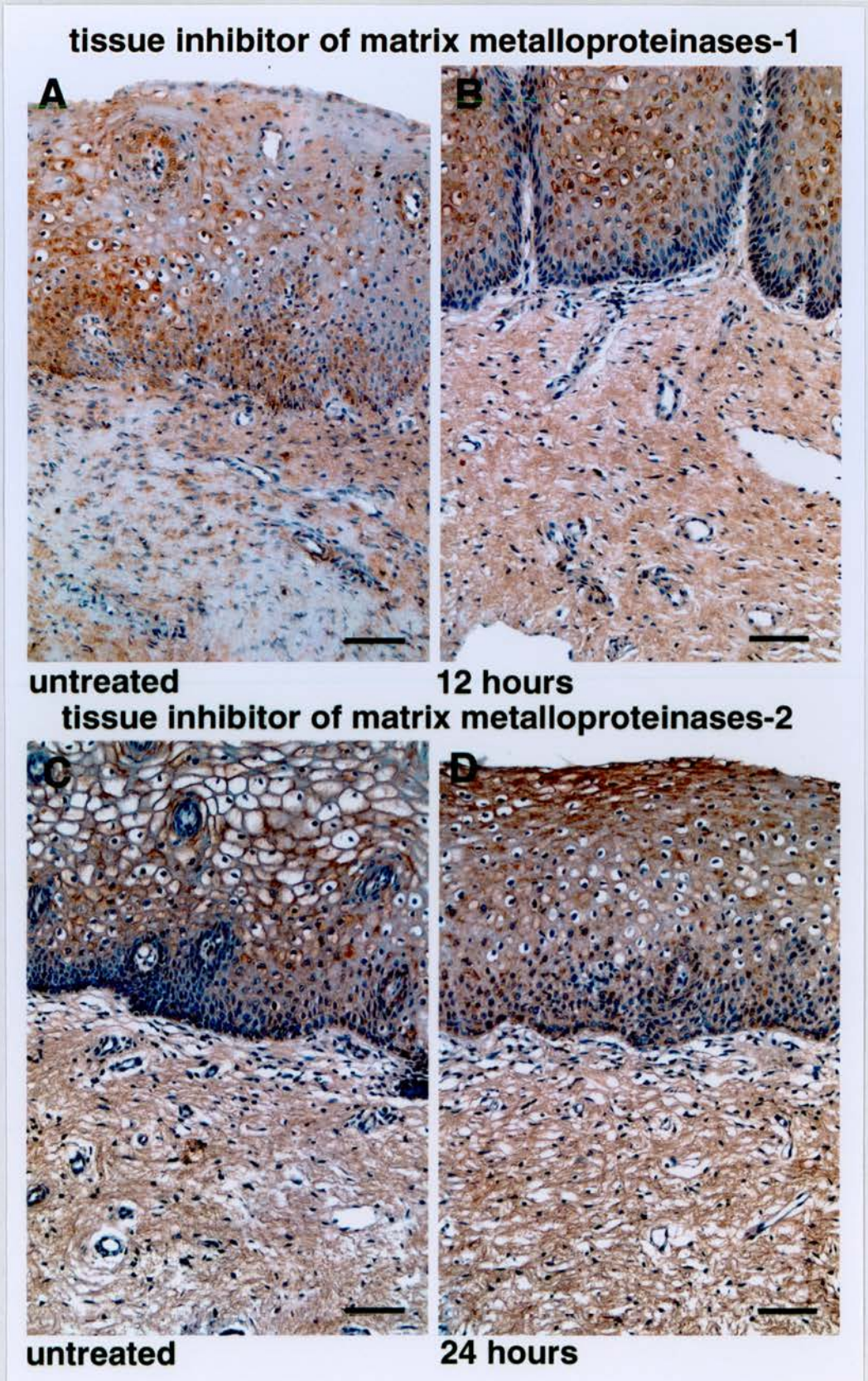


Figure 35

TIMP-1 and -2 immunolocalisation in human cervix after mifepristone treatment. **A)** and **C)** untreated, **B)** 12 hours and **D)** 24 hours post treatment. Mifepristone had no effect on TIMP-1 and -2 immunolocalisation. Scale bars = 100 μm.

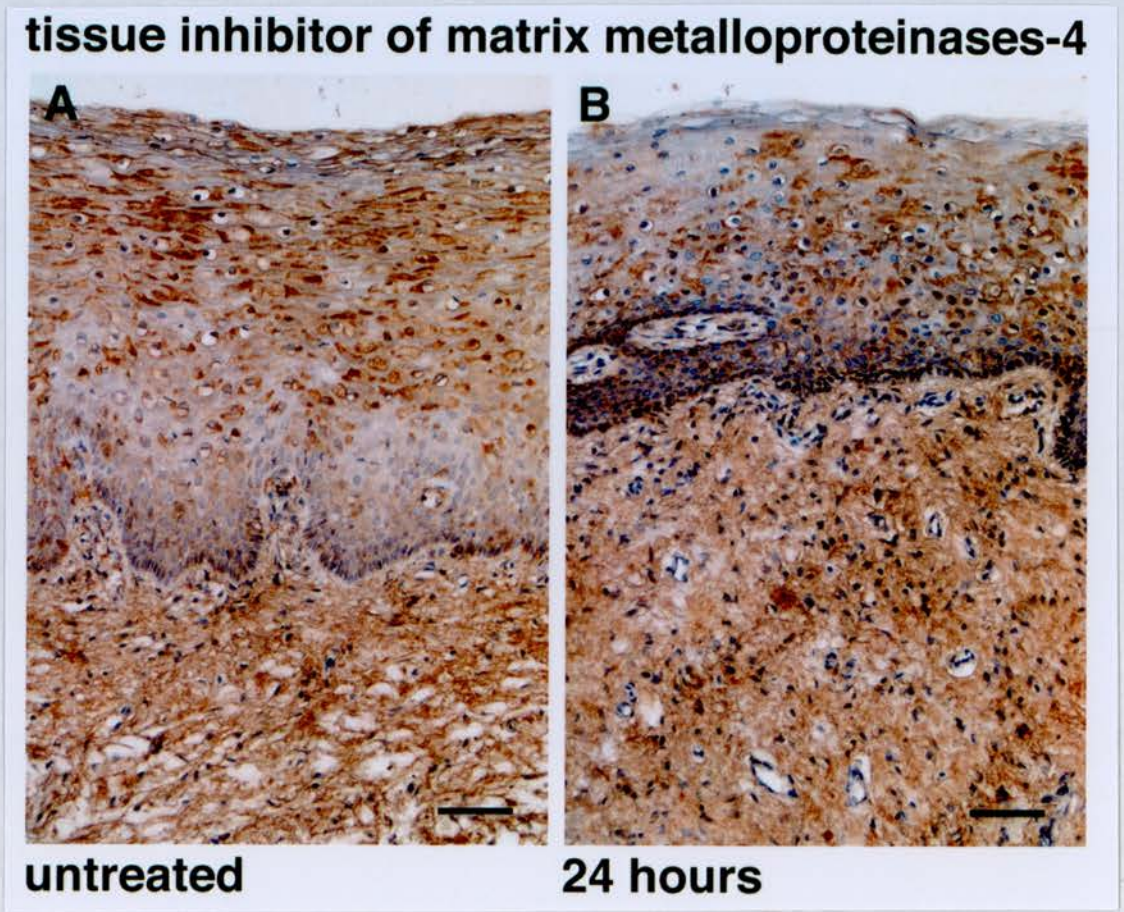


Figure 36

TIMP-4 immunolocalisation in human cervix after administration of mifepristone *in vivo*. **A)** untreated and **B)** 24 hours post treatment. Immunolocalisation of TIMP-4 was unaffected by mifepristone administration. Scale bars = 100 μm.

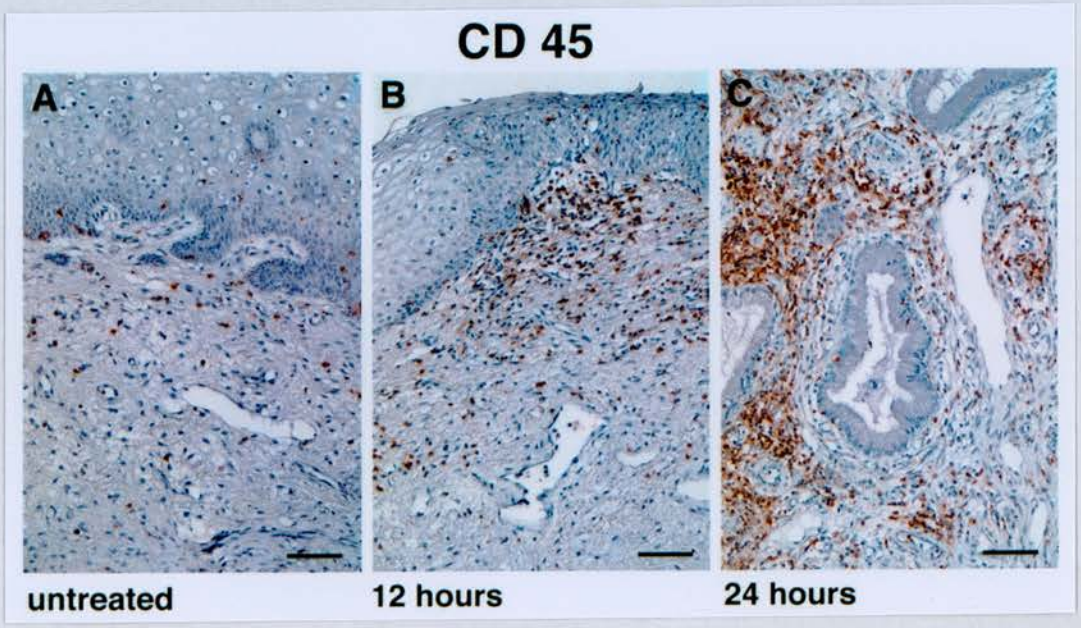


Figure 37

CD45 (leukocyte common antigen) immunolocalisation in human cervix after administration of mifepristone *in vivo*. **A)** untreated, **B)** 12 hours and **C)** 24 hours post treatment. There was an increased number of cells staining specifically for CD45 in the sub-epithelial layer and in cervical stroma in biopsies taken more than 12 hours post mifepristone administration. Scale bars = 100 μm.

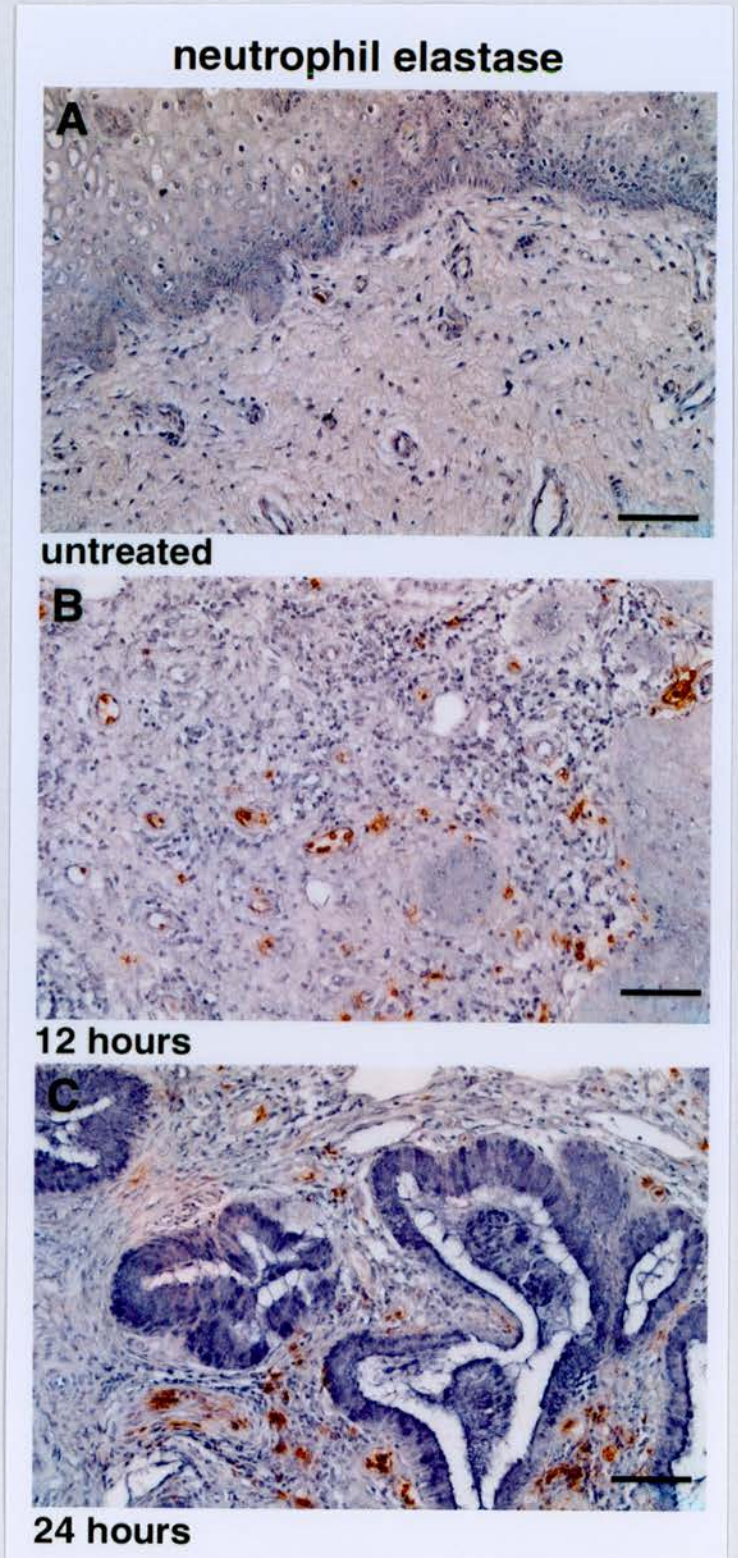
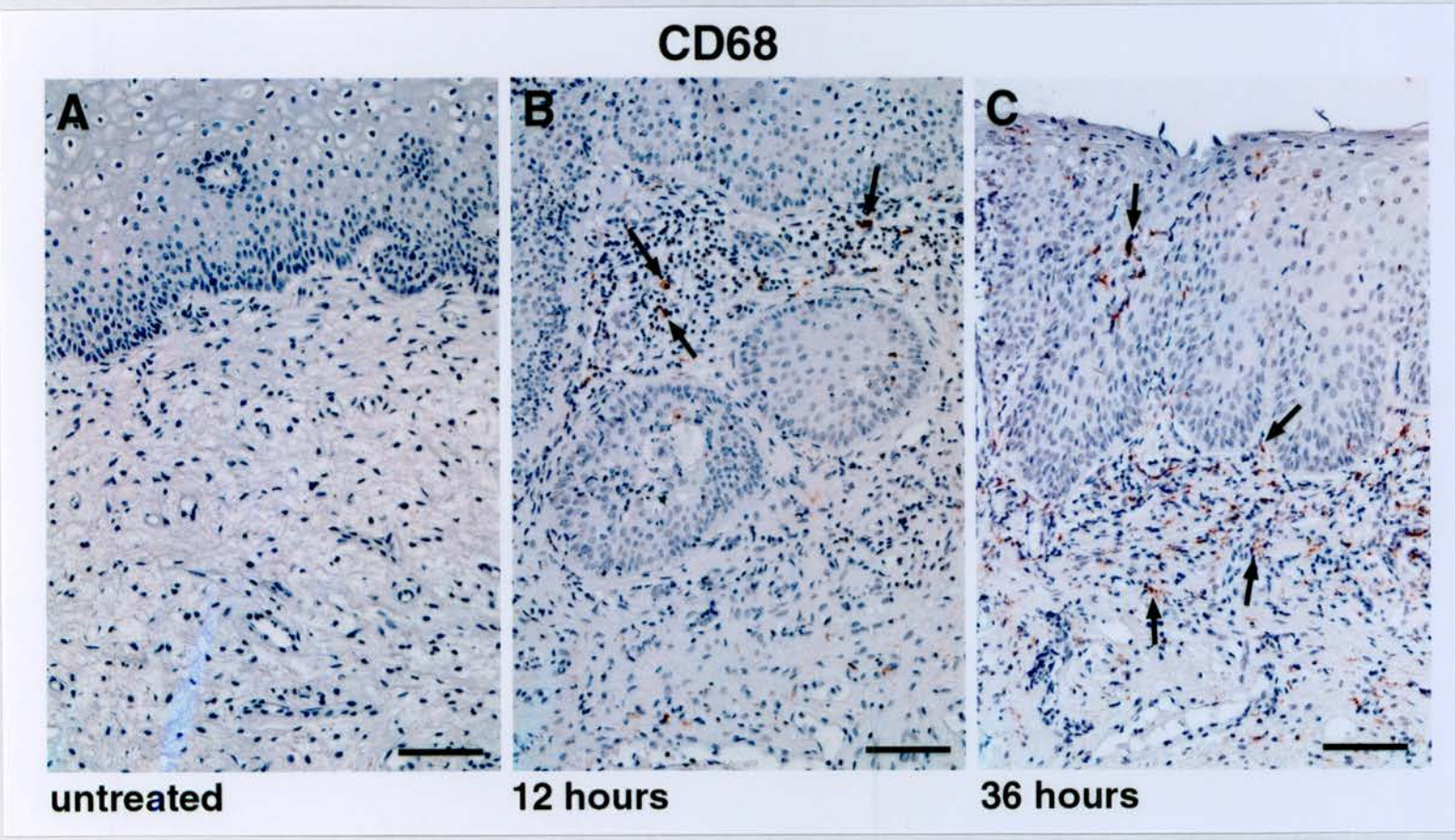


Figure 38

Neutrophil elastase immunolocalisation in human cervix after administration of mifepristone. **A)** untreated, **B)** 6 hours and **C)** 24 hours after mifepristone treatment. There was an increased number of stromal cells staining specifically for neutrophil elastase 12 hours after mifepristone administration. Scale bars = 100 μm.

Figure 39

CD68 (monocyte marker) immunolocalisation in human cervix after administration of mifepristone *in vivo*. **A)** untreated, **B)** 12 hours and **C)** 24 hours after mifepristone treatment. Untreated biopsies were immunonegative for CD68. There was an increased number of cells staining specifically for CD68 in epithelium, sup-epithelium and cervical stroma, more than 12 hours after mifepristone administration. Scale bars = 100 μm.


CD68
A
B
C
untreated
12 hours
36 hours

6.314 DISCUSSION

This study demonstrates that administration of mifepristone increased immunostaining for MMP-1, -8 and -9, CD45, neutrophil elastase and CD68 within cervical biopsies but had no effect on immunostaining of MMP-2, TIMP-1, -2 and -4.

Minimal immunostaining for the gelatinase MMP-9 was observed in control biopsies with a marked increase in immunoreactivity in biopsies taken more than 24 hours after mifepristone administration. Physiological concentrations of progesterone have previously been demonstrated to suppress MMP-9 release by rabbit cervical fibroblasts (Imada, et al., 1997) and human trophoblast cells (Shimonovitz, et al., 1998) with MMP-9 inhibition being antagonised by progesterone withdrawal (Shimonovitz, et al., 1998). The high levels of progesterone in the circulation and within the cervix during normal pregnancy may be a protective mechanism to prevent premature cervical ripening. Up-regulation of MMP-9 post mifepristone administration suggests that MMP-9 may play a role in mifepristone induced cervical ripening.

In contrast, immunolocalisation of the gelatinase MMP-2 was unaffected by mifepristone administration. Whether MMP-2 is under progesterone regulation is controversial with studies variously demonstrating inhibition of release by progesterone (Irwin, et al., 1996; Marbaix, et al., 1992) and no effect (Lockwood, et al., 1998). It may be therefore that cervical MMP-2 is not progesterone regulated or that inhibition of its release was not detectable by immunohistochemistry.

Specific stromal cellular immunostaining for both interstitial (MMP-1) and neutrophil collagenase (MMP-8) was increased in biopsies taken more than 24 hours after mifepristone administration. Upregulation of MMP-1 release by mifepristone has been previously demonstrated in human endometrial stroma and fibroblasts (Lockwood, et al., 1998; Singer, et al., 1997), however stimulation of MMP-8 by progesterone antagonism is a novel finding. MMP-8 is unique in that it is expressed exclusively in inflammatory conditions (Balbin, et al., 1998). Although originally described as being released specifically by neutrophils, it is has recently been shown to be secreted by cytokine activated fibroblasts (Halinen, et al., 1996). The majority of immunopositive stromal cells were of fibroblast morphology. This is therefore consistent with MMP-1 being a

fibroblast product and release of MMP-8 by cytokine activated fibroblasts, such as would be found during the inflammatory process of cervical ripening.

The immunolocalisation of TIMPs -1, -2 and -4 was not affected by mifepristone administration. This conflicts with studies in rabbit cervical fibroblasts in which progesterone stimulated release of TIMP-1 and -2 (Imada, et al., 1994) but is consistent with those in human endometrial stromal cells where progesterone had no effect on TIMP-1 release (Lockwood, et al., 1998).

Progesterone and other steroid hormones, acting via nuclear receptors, such as glucocorticoids and androgens are thought to modulate MMP and TIMP release by a variety of diverse mechanisms (Schroen and Brinckerhoff, 1996). First, steroid receptors and their ligands can act on the promoters of MMP genes to enhance or suppress trans-activation by binding to hormone responsive elements, forming complexes on the DNA with AP-1 proteins and or decreasing mRNA transcription factor expression. Second, nuclear receptors and their ligands can indirectly reduce MMP activity. For example, progesterone both upregulates expression of TIMPs, which complex with MMPs and inhibit enzymatic activity, and stimulate production of TGF-β, which in turn suppresses expression of certain MMPs. Finally, nuclear receptors and their ligands can bind to co-activators, co-repressors and components of the general transcription apparatus, thereby affecting MMP expression and release. Mifepristone, by acting as a progesterone receptor antagonist or partial agonist, could potentially act at any of these regulatory points to modulate MMP or TIMP release.

Mifepristone administration also increased leukocyte invasion into cervical stroma. Further characterisation demonstrated that some of these cells were neutrophils and monocytes, as demonstrated by increased numbers of cells staining immunopositive for neutrophil elastase and CD68 within cervical stroma and blood. It has been previously demonstrated that 24 hours post-mifepristone administration there is a significant increase in mast cells within cervical stroma (Radestad, et al., 1993). Mast cells may therefore account for some of the leukocytes, which did not stain with the neutrophil elastase or monocyte marker. Leukocyte invasion of the connective tissue stroma and their subsequent degranulation releasing collagenolytic enzymes could be a further mechanism by which mifepristone induces cervical softening.

In summary therefore, the following hypothesis can be proposed to explain the mechanisms by which mifepristone could induce cervical ripening. A fall in local progesterone levels, effected by mifepristone administration, would induce new blood capillary formation (Radestad, et al., 1993) and upregulate MMP-9 release, which would degrade vascular basement membrane. Together, these would favour accumulation of leukocytes, specifically neutrophils, monocytes and mast cells, in cervical vasculature and their subsequent emigration and degranulation within the connective tissue stroma. In addition, increased release of the collagenases MMP-1 and MMP-8 by stromal cells would further promote remodelling and loosening of the connective tissue stroma. Finally, the failure of mifepristone to effect changes in TIMP expression would alter homeostatic balance between MMPs and TIMPs towards increased ECM and tissue breakdown

6.4 CONCLUSION

In summary, these studies (Sections 6.21, 6.22 and 6.31) have demonstrated that the non-pregnant and pregnant cervix are capable of releasing a wide range of inflammatory mediators, MMPs and TIMPs. At term, it is likely that these mediators are tightly regulated and act in a co-ordinated manner to effect cervical ripening and tissue remodelling.

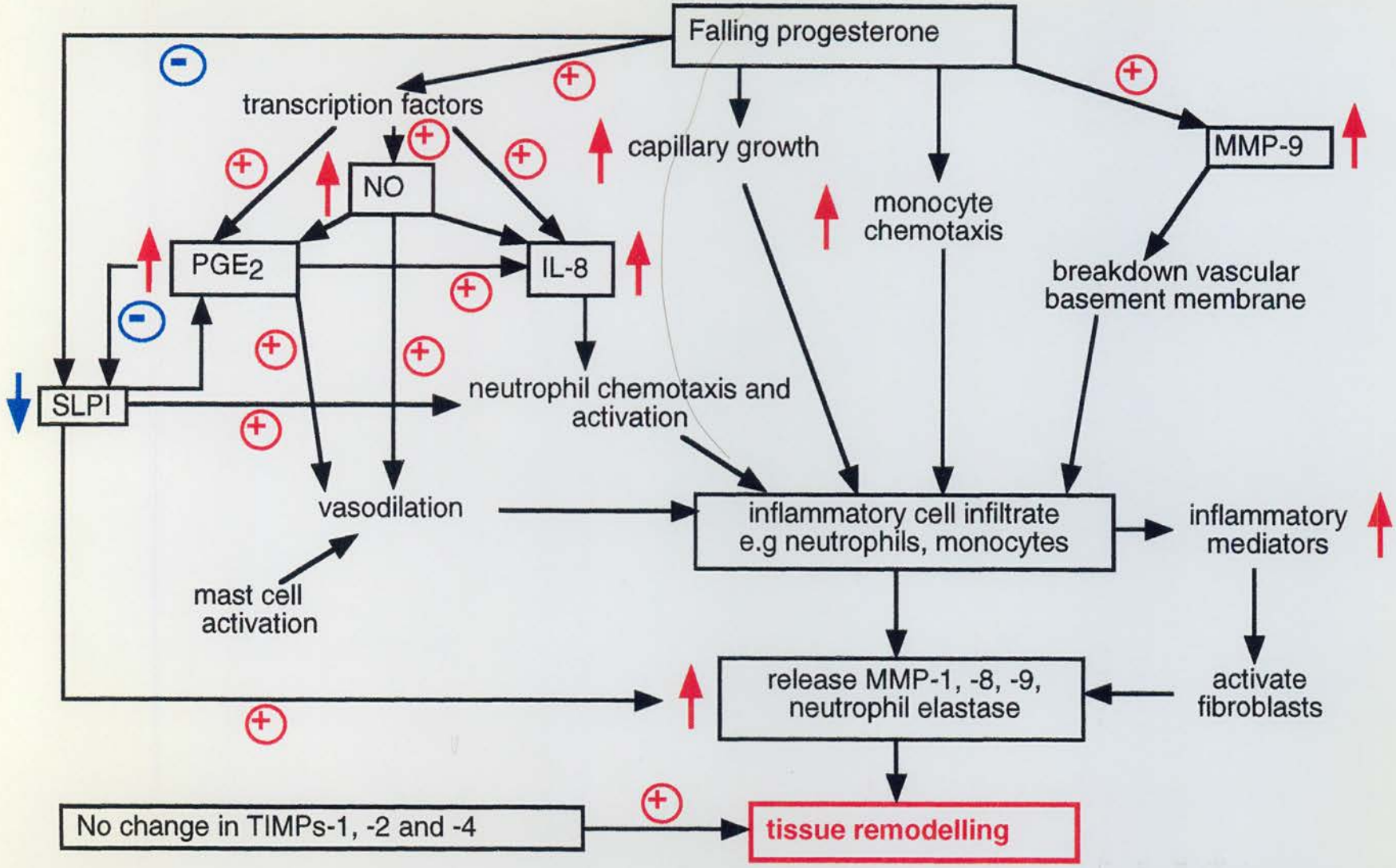
Before attempting to propose a unifying hypothesis to explain cervical ripening, the following caveats are acknowledged. First, it is appreciated that non-pregnant and first trimester cervical tissue are not directly comparable. However, due to difficulty in obtaining cervical biopsies and the small size, particularly of the first trimester samples, it was not possible to perform all the studies on a single sample set. Second, neither sample set are directly comparable to the term pregnant cervix. Ideally, these experiments should be repeated on term samples, however ethically and practically these proved very difficult to collect but should be considered for study in the future. With the preceding caveats, the following hypothesis, illustrated in Figure 40, is therefore proposed to explain cervical ripening at term.

A fall in progesterone or interference with its action at term would stimulate release of the vasodilators PGE_2 and nitric oxide, MMP-9 and promote new capillary growth (Radestad, et al., 1993). The vasodilation would be enhanced by nitric oxide mediated stimulation of PGE_2. Progesterone antagonism would also stimulate release of the neutrophil chemoattractant IL-8 (Kelly, et al., 1994) and inhibit secretion of secretory leukocyte protease inhibitor. The fall in secretory leukocyte protease inhibitor would stimulate PGE_2 release, which could then further up-regulate IL-8 secretion. A rise in the IL-8 and other chemokines, in combination with PGE_2/nitric oxide mediated vasodilation, MMP-9 induced degradation of vascular basement membrane and up-regulation of leukocyte adhesion molecules (Winkler, et al., 1998) would promote cellular infiltration into cervical stroma. The leukocytes, specifically neutrophils (Winkler, et al., 1997), monocytes and mast cells (Radestad, et al., 1993), could then be induced to degranulate by the presence of high concentrations of pro-inflammatory cytokines such as IL-8 and IL-1 (Winkler, et al., 1998) within cervical stroma. Furthermore, cytokines are released by leukocyte degranulation, thus generating a positive feedback by inducing further cellular infiltration, cytokine and MMP release. Digestion of cervical stroma and tissue remodelling would

then ensue mediated by MMPs such as MMP-1, MMP-8 and MMP-9 released by leukocyte degranulation and cytokine activated fibroblasts. This tissue re-organisation would be further promoted by a reduction in secretory leukocyte protease inhibitor, which would normally inhibit neutrophil activation and MMP release. In addition, since progesterone antagonism had no effect on TIMP release, then the balance between MMPs and TIMPs would shift to favour tissue remodelling (Figure 40).

Figure 40

Hypothesis of cervical ripening. It is hypothesised that an increase in pro-inflammatory mediators, inflammatory cell infiltrate and metalloproteinase release, a decrease in anti-inflammatory mediators and no change in levels of tissue inhibitors of metalloproteinases, possibly mediated by falling progesterone, would promote tissue remodelling and cervical ripening.


Falling progesterone
transcription factors
capillary growth
MMP-9
NO
monocyte chemotaxis
PGE2
IL-8
breakdown vascular basement membrane
SLPI
neutrophil chemotaxis and activation
vasodilation
inflammatory cell infiltrate e.g neutrophils, monocytes
inflammatory mediators
mast cell activation
release MMP-1, -8, -9, neutrophil elastase
activate fibroblasts
No change in TIMPs-1, -2 and -4
tissue remodelling

CHAPTER 7

CHEMOKINE SECRETION BY HUMAN FETAL MEMBRANES, DECIDUA AND PLACENTA AT TERM

7.1 INTRODUCTION

Parturition is analogous to an inflammatory reaction. Chemokines, by acting as local effectors and mediators of cell trafficking, are thought to play an important regulatory role at the materno-fetal interface during this process. However, their sites of secretion and regulation within the term uterus have not been fully characterised.

The neutrophil chemoattractant IL-8 has been implicated in the initiation of infection mediated pre-term labour (Cherouny, et al., 1993), cervical ripening (Sennstrom, et al., 1997) and parturition at term. It is found in high concentrations within term amniotic fluid (Laham, et al., 1993) and is released by cervix (Barclay, et al., 1993; Sennstrom, et al., 1997), amnion, chorion, decidua and placenta (Elliott, et al., 1998; Laham, et al., 1997). The monocyte chemotactic factor MCP-1, is released by many reproductive tissues including the ovarian follicles (Arici, et al., 1997), endometrium (Arici, et al., 1995), third trimester chorio-decidua (Kelly, et al., 1997) and is expressed in term placental trophoblasts (Shimoya, et al., 1998). RANTES, which is chemotactic for T-cells, is released by endometrium (Hornung, et al., 1997) and a first trimester trophoblast cell line (Svinarich, et al., 1996). However, whether MCP-1 is released by amnion or placenta or RANTES by amnion, chorion, decidua or placenta during the third trimester is not known.

IL-10 is a potent immunomodulatory cytokine that promotes development of a humoral or Th-2 type of immune response, which is associated with a successful outcome to pregnancy (Wegmann, et al., 1993). In addition, it inhibits release of many pro-inflammatory cytokines including IL-8 (Chen and Manning, 1996), MCP-1 (Kucharzik, et al., 1998) and RANTES (MarfaingKoka, et al., 1996). IL-10 is present in amniotic fluid throughout pregnancy (Dudley, et al., 1997) and is released by most of the intra-uterine structures present during the third trimester (Trautman, et al., 1997). The factors which regulate IL-10 release from these tissues and its function within the uterus at term are however less well defined.

It is now recognised that PGE_2, although initially described as a potent vasodilator, is an important immunomodulator and mediator of inflammation within the uterus (Kelly, et al., 1995). During the third trimester, it is released by amnion (Edwin, et al., 1996), chorion (Dudley, et al., 1996), decidua (Ishihara, et al., 1991) and placenta (Wetzka, et al., 1997)

and a rise in its levels within the uterus is associated with the onset of parturition. The effects of PGE_2 on the release of other cytokines such as IL-8, which may be involved in parturition, is not well understood.

The aim of this study was to investigate the release of MCP-1, IL-8, RANTES and IL-10 from third trimester amnion, chorion, decidua and placenta and to evaluate the possible contribution of these chemokines to parturition. A dynamic dual placental cotyledon perfusion model was then used to study the influence of PGE_2 infusion on placental chemokine release and to assess whether any regulation of chemokine secretion could have implications for the immunological mechanisms involved in the maintenance of pregnancy and parturition.

7.2 MATERIALS AND METHODS

Tissue Collection

Tissue samples for explant cultures were obtained from normal healthy women (n = 7 different women) with uncomplicated pregnancies undergoing elective caesarean section at term as follows:

Amnion	Discs (12 mm diameter; wet weight 10 - 20 mg) were prepared using a cork borer.
Chorion laeve	Discs (9 mm diameter; wet weight 15 - 25 mg) were prepared using a cork borer.
Decidua	Decidual pieces (wet weight 20 - 30 mg) were obtained by curettage off the myometrium.
Placenta	Villous placenta (wet weight 20 - 30 mg) by dissection of placental tissue.

For placental perfusion studies, placentae (n = 8 different women) were collected from normal healthy women with uncomplicated pregnancies at term immediately after spontaneous delivery or elective caesarean section and transported to the laboratory at 4°C.

Explant Culture

Tissue explants (four explants of each tissue from each woman) were placed on absorbent capillary matting and maintained in culture (Section 2.223; (Brennand, et al., 1995)). Media were collected after 24 hours and stored at –20°C until analysis.

Dual perfusion of a placental cotyledon

Term placentae (n = 8), collected after elective caesarean section were perfused with Kreb's solution followed by PGE_2 (Section 2.224). Consecutive 10 minute samples from both the maternal and fetal circulations were collected and stored at -20°C until analysis.

The integrity and viability of each preparation was established throughout the experimental period by ensuring that:

1. The rate of perfusate input in both the fetal and maternal circuits equalled the rate of output.

2. There was adequate exchange of O_2 from the maternal to fetal circuits (step up pressures of <40 to 70 mm Hg in fetal perfusate).
3. Lactate concentrations remained within the normal low range (<2 mM; (Benediktsson, et al., 1997).
4. At the end of the experiment the fetal vasculature responded to a bolus infusion of noradrenaline (20 mg) by a vasoconstriction of > 2 mmHg.
5. Subsequent microscopic histological investigations revealed no significant morphological changes.

Cytokine assays

MCP-1, IL-8, RANTES and IL-10 were analysed by ELISA (Section 2.23).

Statistical analysis

Statistical analysis of the tissue explant data was performed using ANOVA and a Student's paired 't'-test was used for the placental perfusion model (StatView 4.1, Abacus Inc., Berkley, CA, USA). The data were normally distributed and are expressed as pg/mg or pg/min/cotyledon (mean ± s.e.m) with a statistically significant difference defined as $p<0.05$.

Ethical Approval

Ethical approval for the collection of all samples was obtained from the Lothian Trust Ethical Committee with the informed and written consent of patients.

7.3 RESULTS

Tissue explants

Explants of amnion, chorion, decidua and placenta exhibit distinct profiles of cytokine release (Figure 41). Significantly higher levels of MCP-1 were released by chorion (78.2 ± pg/mg; $p<0.001$), decidua (112.4 ± 5.2 pg/mg; $p<0.001$) and placenta (101.8 ± 5.0 pg/mg; $p<0.001$) than from amnion (1.3 ± 0.4 pg/mg). IL-8 was secreted in large amounts by all tissues studied with release from chorion, decidua, placenta and amnion being 52.8 ± 1.9 pg/mg, 42.2 ± 1.5 pg/mg, 45 ± 1.3 pg/mg and 39.9 ± 5 pg/mg, respectively. The placenta (26.9 ± 1.6 pg/mg) and decidua (15.2 ± 1.4 pg/mg) were the principal sources of RANTES with significantly more being released than by chorion (6.0 ± 1.2 pg/mg; $p<0.01$). IL-10 was released mainly by chorion (6.8 ± 0.8 pg/mg) and decidua (9.0 ± 0.9 pg/mg) with levels which were lower, but which failed to reach significance, from placenta (3.3 ± 0.3 pg/mg). Neither RANTES nor IL-10 were released in detectable concentrations by amnion. The ratio of MCP-1 : IL-8 release by amnion was 1 : 31 and the ratios of MCP-1 : IL-8 : RANTES : IL-10 release by chorion, placenta and decidua were 13 : 9 : 1 : 1, 31 : 14 : 8 : 1 and 12 : 5 : 2 : 1, respectively.

Placental perfusion

The basal cytokine output from the perfused placental cotyledon (maternal and fetal combined) prior to commencing the PGE_2 infusion was less than that by placental explants for MCP-1, IL-8 and IL-10 (relative ratios 1 : 4, 1 : 13, 1 : 2) but not for RANTES (relative ratio 1 : 1). The basal output of all cytokines measured was greater from the maternal than the fetal circulation in the perfusion system with the ratios of basal output of MCP-1, IL-8, RANTES and IL-10 between maternal and fetal circuits being 145 : 1, 79 : 1, 7 : 1 and 2 : 1, respectively. The differential release of cytokines between the two circulations was maintained for the duration of the perfusion and study period.

Perfusion of placentae with PGE_2 significantly ($p<0.01$) stimulated release of IL-8, MCP-1 and IL-10 from the maternal and IL-8 and MCP-1 from the fetal circulation. The stimulation became significant approximately 20 minutes after commencing the PGE_2 infusion and was maintained for the duration of the perfusion and study period (Figures 42a, 42b and 43).

RANTES was released into both the maternal and fetal circulation, but secretion was not affected by infusion of PGE_2, unlike the other cytokines measured (Figures 42a and 42b).

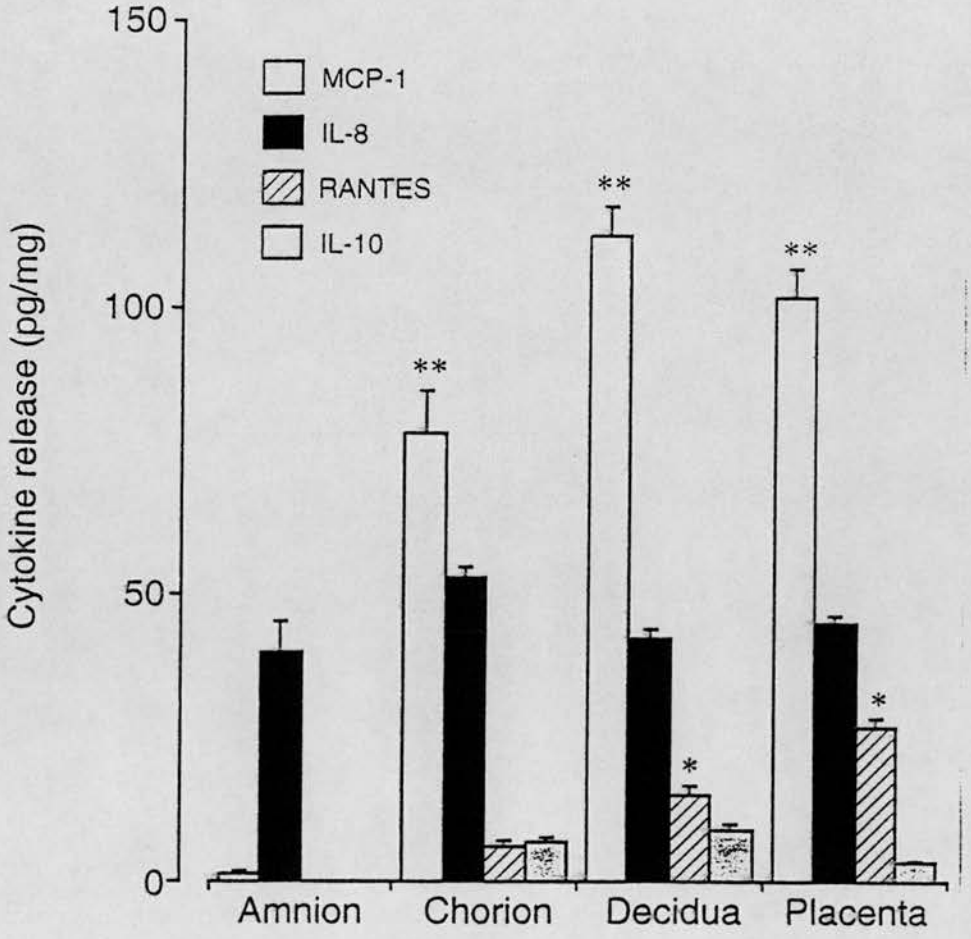


Figure 41

Release of MCP-1, IL-8, RANTES and IL-10 from amnion, chorion, placenta and decidua. Significantly higher levels of MCP-1 were released by chorion, placenta and decidua than from amnion, respectively. RANTES was secreted in significantly greater amounts from decidua and placenta than from chorion. The majority of IL-10 was released by chorion and decidua with lower levels from placenta. Neither RANTES nor IL-10 were released by amnion. Values are expressed as pg/mg; mean ± s.e.m. n = 7 different women. Statistical analysis for treatment versus control; Significance; *, $p<0.01$; **, $p<0.001$.

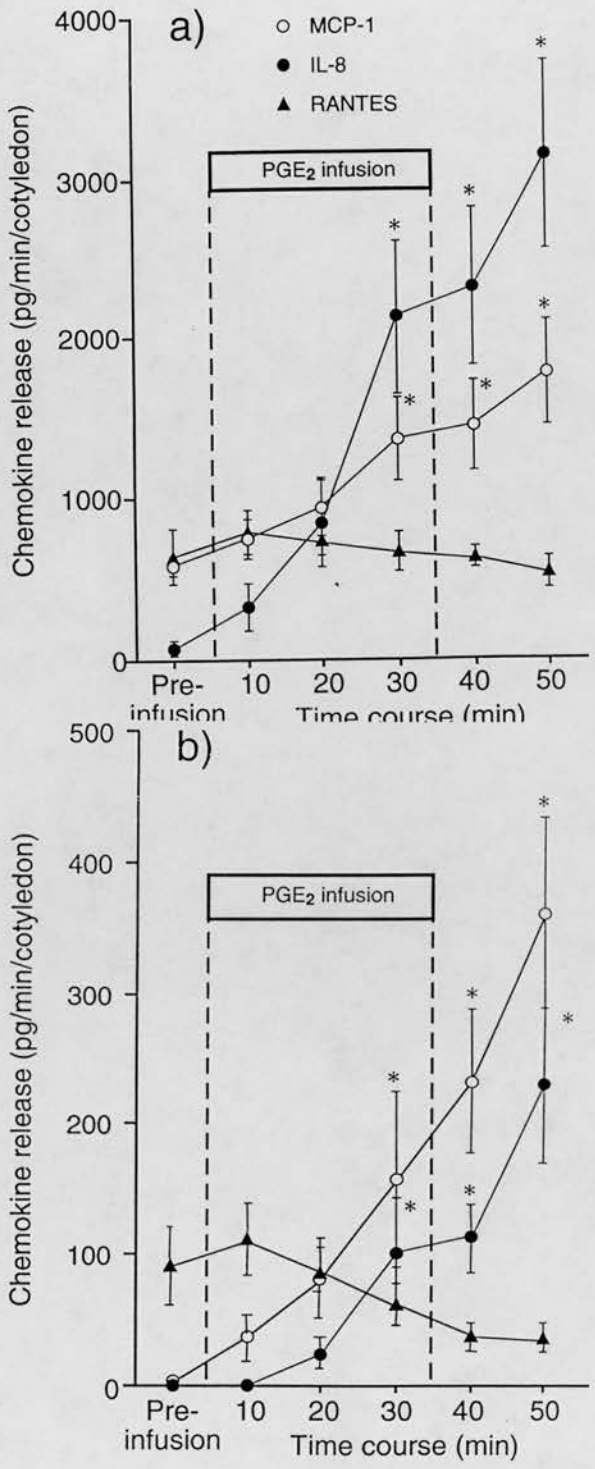


Figure 42

Effect of PGE_2 infusion on the release of MCP-1, IL-8 and RANTES into (a) the maternal and (b) the fetal circulation in a dynamic dually perfused placental cotyledon. The release of MCP-1 and IL-8 release was significantly stimulated into both maternal and fetal circulations approximately 20 minutes after commencing the PGE_2 infusion and levels continued to be elevated until the end of the study period. The PGE_2 infusion had no effect on RANTES release. Values are expressed as pg/min/cotyledon; mean ± s.e.m. n = 8 different placentae. Significance; *, $p<0.01$.

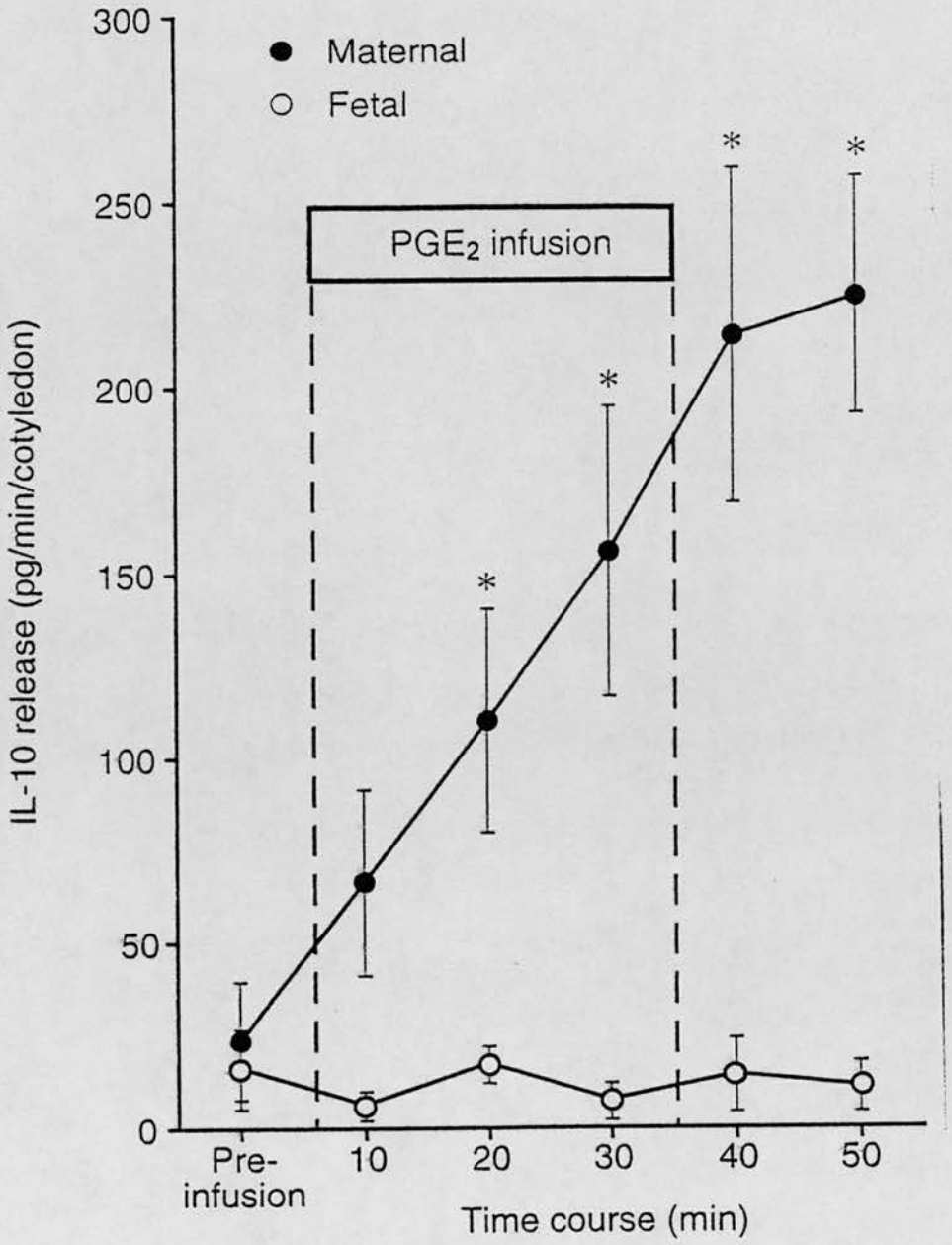


Figure 43

Effect of PGE_2 infusion on the release of IL-10 into the maternal and fetal circulation in dually perfused placental cotyledon. The release of IL-10 was significantly stimulated into the maternal circulation approximately 20 minutes after commencing the PGE_2 infusion and levels continued to be elevated until the end of the study period. There was no rise in levels of IL-10 in the fetal circulation post-infusion. Values are expressed as pg/min/cotyledon; mean ± s.e.m. n = 8 different placentae. Significance; *, $p<0.01$.

7.4 DISCUSSION

The heterogeneous intra-uterine structures present during the third trimester originate from both maternal and fetal tissue and have widely differing functions. This study demonstrates that third trimester amnion, chorion, decidua and placenta explants secrete quite distinct cytokine profiles. MCP-1 was released predominately by chorion, decidua and placenta with low amounts from amnion (relative ratios 62 : 94 : 85 : 1, respectively). High levels of IL-8 were released by amnion, chorion, decidua and placenta (relative ratios 1 : 1 : 1 : 1, respectively). Release of RANTES was not detectable from amnion but was in moderate amounts from chorion, decidua and placenta (relative ratios 1 : 3 : 4). Low levels of IL-10 were secreted by chorion, decidua and placenta with none detectable from amnion (relative ratios 2 : 3 : 1).

The fetal membranes comprise of amnion and chorion. Amnion, which is composed of an epithelial and fibroblast layer, released both IL-8 and MCP-1. Local release of IL-8 from unstimulated (Laham, et al., 1997) and cytokine stimulated amnion explants (Trautman, et al., 1992) has been previously demonstrated and has been postulated to have a role in parturition. The demonstration of MCP-1 release is however novel. IL-8 and MCP-1 could be released from either epithelium or fibroblasts, both of which are capable of their synthesis in extra-uterine sites (Agro, et al., 1996; Arici, et al., 1998; Elner, et al., 1997).

Chorion, whose principal constituent is trophoblast (Chard and Lilford, 1997), released all cytokines measured. MCP-1 and IL-8 release have been previously demonstrated in unstimulated and cytokine stimulated chorion and immunolocalised to chorion trophoblast, stromal fibroblasts and macrophage like cells (Ito, et al., 1994; Kelly, et al., 1997; Laham, et al., 1997). The release of IL-10 by unstimulated chorion explants contrasts with the findings of others. Previous studies have shown IL-10 to be released by stimulated chorion (Dudley, et al., 1997) but have not demonstrated its secretion and only rarely its immunolocalisation in unstimulated explants (Trautman, et al., 1997). In the latter study however, IL-10 release was readily detected from decidua. Decidua, adherent to chorion trophoblast, could therefore be responsible for IL-10 release in both this study and the latter study of Trautman et al., 1997. RANTES could be released from either the chorion trophoblast (Svinarich, et al., 1996), fibroblasts or adherent decidua.

Decidua released high levels of MCP-1 and IL-8 and moderate levels of RANTES and IL-10. The release and immunolocalisation of IL-8 and IL-10 has been previously demonstrated within decidua (Saito, et al., 1994; Trautman, et al., 1997). Although decidua is comprised of a wide range of different cell types, it is likely that decidual macrophages and T-cells would be the principal source of MCP-1 and RANTES, respectively.

Placenta released high levels of MCP-1, IL-8 and RANTES and low levels of IL-10. Secretion of IL-8 and IL-10 by placenta supports previous data demonstrating release and localisation of IL-8 to cytotrophoblast, syncytiotrophoblast, perivascular cells and placental Hofbauer cells (Elliott, et al., 1998; Saito, et al., 1994; Shimoya, et al., 1992) and IL-10 to cytotrophoblast (Roth, et al., 1996). MCP-1 has recently been found to be expressed in increasing amounts by placental trophoblast during pregnancy but its release by placental explants has not previously been demonstrated. Although release of RANTES protein by placenta has not been reported before, a novel chemokine receptor C-C R10 has been described which binds MCP-1 with high and RANTES with much lower affinity. This receptor is uniquely expressed within placenta and fetal liver (Bonini, et al., 1997) and its expression pattern is therefore consistent with placental secretion of both MCP-1 and RANTES. A variety of cells within placenta would be capable of secreting MCP-1 and RANTES including trophoblast, placental macrophages and T-cells. However, some placental explants were contaminated with blood and therefore peripheral leukocytes, in particular monocytes and T-cells could be an additional source of MCP-1 and RANTES, respectively.

Parturition has been likened to an inflammatory reaction with cytokines such as IL-8 playing a key role in cellular recruitment and activation within the utero-placental unit at term (Sennstrom, et al., 1997). Prostaglandin E_2 is also thought to be an important mediator and possible initiator of parturition with concentrations increasing peripartum within amniotic fluid, placenta and cervix. In extra-uterine inflammatory responses, synergy exists between PGE_2 and IL-8 to facilitate neutrophil recruitment (Colditz, 1990). Furthermore, IL-8 release can be upregulated by PGE_2 (Agro, et al., 1996). Whether PGE_2 regulates release of other inflammatory mediators such as MCP-1 and RANTES, which may also be involved in parturition is not known.

This study investigated whether PGE_2 regulated release of MCP-1, IL-8, RANTES and IL-10 within the placenta and studied its effect on cytokine release from a dually infused

placental cotyledon. This demonstrated that PGE_2 stimulated release of MCP-1 and IL-8 into the maternal and fetal circuits of the perfusion system but had no effect on RANTES release. It is hypothesised that a rise in PGE_2 at term would stimulate MCP-1 and IL-8 release thus promoting a local inflammatory reaction, cellular infiltration and favouring parturition. The principal source of cytokines in such a system would be trophoblast although there may be a slight contribution from decidua of the basal plate. Release of cytokines by decidua (Dudley, et al., 1992) and trophoblast (Steinborn, et al., 1995) have previously been implicated in the initiation and propagation of parturition. In addition, cytokines have been shown to stimulate synthesis of PGE_2 in decidua and trophoblast via upregulation of COX-2 (Pollard, et al., 1994). Thus, there is the potential for a positive feedback loop to be initiated between PGE_2 and cytokines to generate pro-inflammatory mediators and therefore propagate the pro-inflammatory parturient response.

The effect of PGE_2 stimulation of cytokine release into the fetal circulation was less than into the maternal circulation and this could be due to inefficient transport of PGE_2 across the trophoblast (Glance, et al., 1986), inability of cells adjacent to the fetal circulation, such as the Hofbauer cells, to respond by stimulation to PGE_2 or metabolism of PGE_2 by the trophoblast (Cheung, et al., 1990). RANTES secretion was not stimulated by PGE_2. This further supports the hypothesis that stimulation of IL-8 and MCP-1 was secondary to PGE_2, was not an artefact of perfusion and is consistent with the literature in which PGE_2 has not been demonstrated to stimulate RANTES release.

Prostaglandin E_2 also stimulated release of IL-10 into the maternal but not the fetal circulation. The maternal immune system maintains tolerance to the fetus throughout pregnancy although the mechanism is poorly understood. Interleukin-10 is present in amniotic fluid in increasing quantities during pregnancy (Greig, et al., 1995), and due to its immunosuppressive properties (Mosmann and Moore, 1991) has been proposed as a factor which may be involved in preventing fetal rejection by promoting a Th-2 type of immune response (Rivera, et al., 1998). It is proposed that at term, stimulation of IL-10 release from the maternal side of the placenta by PGE_2 would enhance local immunosuppression and tolerance towards fetal antigen. The fetus would then be protected from maternal recognition and rejection during the "high risk" inflammatory process of parturition. In addition, IL-10 could inhibit release of IL-8 (Chen and Manning, 1996), MCP-1 (Kucharzik, et al., 1998) and RANTES (MarfaingKoka, et al., 1996) thereby acting as a negative feedback control on the pro-inflammatory parturient response.

In summary, this study demonstrates that amnion, chorion, placenta and decidua release distinct profiles of both pro- and anti-inflammatory cytokines. Furthermore, *in vitro* perfusion of a placental cotyledon by PGE_2 stimulates release of IL-8, MCP-1 and IL-10 but not RANTES. It is speculated that the pro-inflammatory cytokines MCP-1 and IL-8 may play a role in the inflammatory process of parturition and that the anti-inflammatory IL-10 may be involved in protecting the fetal allograft at parturition and regulating generation of pro-inflammatory cytokines. Moreover, it is suggested that PGE_2 may play an important immunomodulatory role within the placenta at term.

CHAPTER 8

SECRETORY LEUKOCYTE PROTEASE INHIBITOR CONCENTRATION INCREASES IN AMNIOTIC FLUID WITH THE ONSET OF LABOUR: CHARACTERISATION OF ITS SITES OF RELEASE WITHIN THE UTERUS

8.1 INTRODUCTION

Many pro-inflammatory cytokines and chemokines, such as IL-1 (Augustulen, et al., 1994), IL-8 (Laham, et al., 1993), MIP-1α, GRO-α (Cohen, et al., 1996), IL-6 (Arntzen, et al., 1997) and TNF-α (Laham, et al., 1994), have been implicated in the initiation of parturition with levels increasing within peripheral serum and amniotic during gestation fluid with a further increase at the onset of labour. The roles of anti-inflammatory cytokines and cytokine antagonists in the initiation and regulation of labour are however less well defined. It has been proposed that increased production of anti-inflammatory factors, such as IL-10 and TNF receptor antagonist, might prevent premature parturition and their subsequent fall or failure to increase within the utero-placental unit at term, co-incident with increases in pro-inflammatory cytokines, might permit parturition to occur (Arntzen, et al., 1998; Dudley, et al., 1997).

Secretory leukocyte protease inhibitor is a potent anti-inflammatory and immunomodulatory agent. Little is known about its role within the human uterus during pregnancy. In animal uteri, secretory leukocyte protease inhibitor has been found in the endometrium and myometrium of pigs (Reed, et al., 1998), cows and horses (Badinga, et al., 1994) with concentrations of the protein and mRNA tending to increase during pregnancy. In women, secretory leukocyte protease inhibitor protein has not been demonstrated within non-pregnant endometrium or fetal membranes but has been immunolocalised to the crypts of endocervical glands in both the non-pregnant and pregnant cervix (Helmig, et al., 1995). In addition, its concentration increases significantly within amniotic fluid during pregnancy to term (Helmig, et al., 1995). However, any change in concentration with the onset of labour, its site of secretion from uterine tissues and the potential roles of secretory leukocyte protease inhibitor during parturition have not been examined.

The aims of this study were to measure the levels of secretory leukocyte protease inhibitor in amniotic fluid during pregnancy and labour. In addition, the sites of production within the uterus at term have been characterised to determine the putative roles that secretory leukocyte protease inhibitor might play in the paracrine interactions within the uterus for the maintenance of pregnancy and the control of parturition.

8.2 MATERIALS AND METHODS

Tissue Collection

Samples of amniotic fluid (15 - 18 weeks, n = 20; term not in labour, n = 15; term in labour, n = 15), fetal urine (n = 12 neonates; > 37 weeks) and fetal serum (>37 weeks either after spontaneous vaginal delivery, n = 5 or after emergency caesarean section, n = 5) were collected (Sections 2.214, 2.215 and 2.216). All samples were centrifuged at 1000 g for 5 minutes and stored at -20°C prior to immunoassay.

Tissues used for explants were obtained from women undergoing an elective caesarean section at term (n = 6 women; > 37 weeks; not associated with labour; Section 2.213). For placental perfusion, placentae were collected after spontaneous vaginal delivery at term (n = 6; >37 weeks, active labour, not induced). For immunohistochemistry, tissues (n = 4, > 37 weeks, elective caesarean; n = 4, >37 weeks, emergency caesarean section, active labour, not induced) were fixed in 10 % NBF for 24 hours before washing twice in 70 % ethanol prior to mounting in paraffin wax. Ethical approval for the collection of all samples was obtained from the Lothian Trust Ethical Committee with the informed and written consent of patients.

Culture of explants of amnion, chorio-decidua, decidua and placenta

Discs of amnion (12 mm diameter; wet weight 15 - 20 mg), chorio-decidua (9 mm diameter; wet weight 15 - 25 mg), decidua (wet weight 15 - 25 mg) and villous placenta were prepared for and cultured as previously described (Section 2.223; (Brennand, et al., 1995)). The harvested media was then frozen at –20°C until analysis.

Preparation and culture of amnion and chorion cells

Amnion and chorion were collected immediately after delivery from patients undergoing elective caesarean section at term (> 37 weeks, not in labour, Section 2.213). Amnion and chorion cells and chorion fibroblasts were prepared for and maintained in culture (Section 2.223). Media were frozen and stored at -20°C until analysis.

Dual perfusion of placental cotyledons

Term placentae (n = 6) were perfused as previously described (Sections 2.224 and 7.2; (Schneider and Huch, 1985; Schneider, et al., 1972). After a 40 minute equilibration

period, consecutive 10 minute samples were collected from both maternal and fetal circuits. Samples were stored at –20°C until being assayed.

Secretory leukocyte protease inhibitor assay

Secretory leukocyte protease inhibitor was measured by a ELISA (Section 2.23). To validate the assay in amniotic fluid, serial dilutions of amniotic fluids were compared with that of the recombinant standard and assay results were compared with those obtained using a commercial kit which uses the same antibodies (R&D Systems).

Localisation of secretory leukocyte protease inhibitor in tissues by immunohistochemistry

Secretory leukocyte protease inhibitor was immunolocalised by immunohistochemistry (Section 2.27). Sections were counterstained with Harris' haematoxylin, dehydrated, mounted and visualised by light microscopy.

Statistical analysis

Statistical analysis of the data was performed using ANOVA (StatView 4.1, Abacus Inc., Berkley, CA, USA). The data were normally distributed and are expressed as pg/ml, ng/ml or pg/mg/wet weight tissue (mean ± s.e.m) with a statistical significant difference defined as $p<0.05$.

8.3 RESULTS

Validation of secretory leukocyte protease inhibitor assay

Serial dilutions of amniotic fluids gave dose response curves parallel to that of the recombinant standard (Figure 44). The results from this assay and those obtained by a commercial assay (R&D Systems), which uses the same antibodies, were very highly correlated ($r = 0.99$, total degrees of freedom, $n = 5$).

Presence of secretory leukocyte protease inhibitor in amniotic fluid

Secretory leukocyte protease inhibitor was present within amniotic fluid in increasing concentrations from second trimester (24 ± 3 ng/ml; mean ± s.e.m) to term not in labour (751 ± 53 ng/ml) to term in labour (3929 ± 1076 ng/ml) with this rise being significant from second trimester to term ($p<0.05$) to term in labour ($p<0.005$; Figure 45).

Secretory leukocyte protease inhibitor release from amnion, chorion-decidua, decidua and placenta and its concentration in cord serum and fetal urine

Amnion, chorio-decidua, decidua and placenta explants demonstrated a distinct pattern of secretory leukocyte protease inhibitor release with low levels being secreted by placenta (9.2 ± 1.9 pg/mg), more by amnion (55.6 ± 6.0 pg/mg; $p<0.05$) and significantly higher levels from decidua (135.2 ± 12.4 pg/mg; $p<0.01$) and chorio-decidua (325.1 ± 26.4 pg/mg; $p<0.005$; Figure 46). Amnion epithelial cells, chorion trophoblast cells and chorion fibroblasts released 613 ± 88 pg/ml/10^5 cells, 389 ± 62 pg/ml/10^5 cells and 1658 ± 250 pg/ml/10^5 cells secretory leukocyte protease inhibitor, respectively.

Secretory leukocyte protease inhibitor was secreted into both the maternal (7080 ± 840 pg/ml/cotyledon) and fetal (1790 ± 230 pg/ml/cotyledon) circulations in the perfused placental cotyledon system (Figure 47) with significantly higher ($p<0.005$) amounts being released into the maternal than fetal circulation. However, it was undetectable in all cord serum samples ($n = 10$) and below the detection limit of the assay in nine out of the twelve fetal urine samples assayed with levels being 358 pg/ml, 320 pg/ml and 188 pg/ml, respectively in the remaining three samples. There was no evidence of infection or neonatal sepsis in the three neonates in which secretory leukocyte protease inhibitor was detectable.

Immunolocalisation of secretory leukocyte protease inhibitor within fetal membranes, decidua and placenta

Intense specific immunoreactivity for secretory leukocyte protease inhibitor was localised to the layer of decidua parietalis adherent to chorion trophoblast (Figure 48A). Similarly, intense immunostaining was present in decidua parietalis collected from the myometrial aspect (Figure 48B). Only a few isolated cells, which may be fibroblasts, stained immunopositive within the amnion and chorion trophoblast with the majority of the cells constituting these layers being immunonegative (Figure 48A). In placenta, the decidua basalis which underlies the placenta also demonstrated intense specific immunoreactivity (Figure 48C). The extra-villous trophoblast was immunonegative as was the majority of the placenta apart from a small amount of staining around the margins and within fetal blood vessels of the villi. Substitution of the primary antibody with normal donkey (Figure 48D) and normal goat serum (data not shown) resulted in absence of staining in decidua, fetal membranes and placenta.

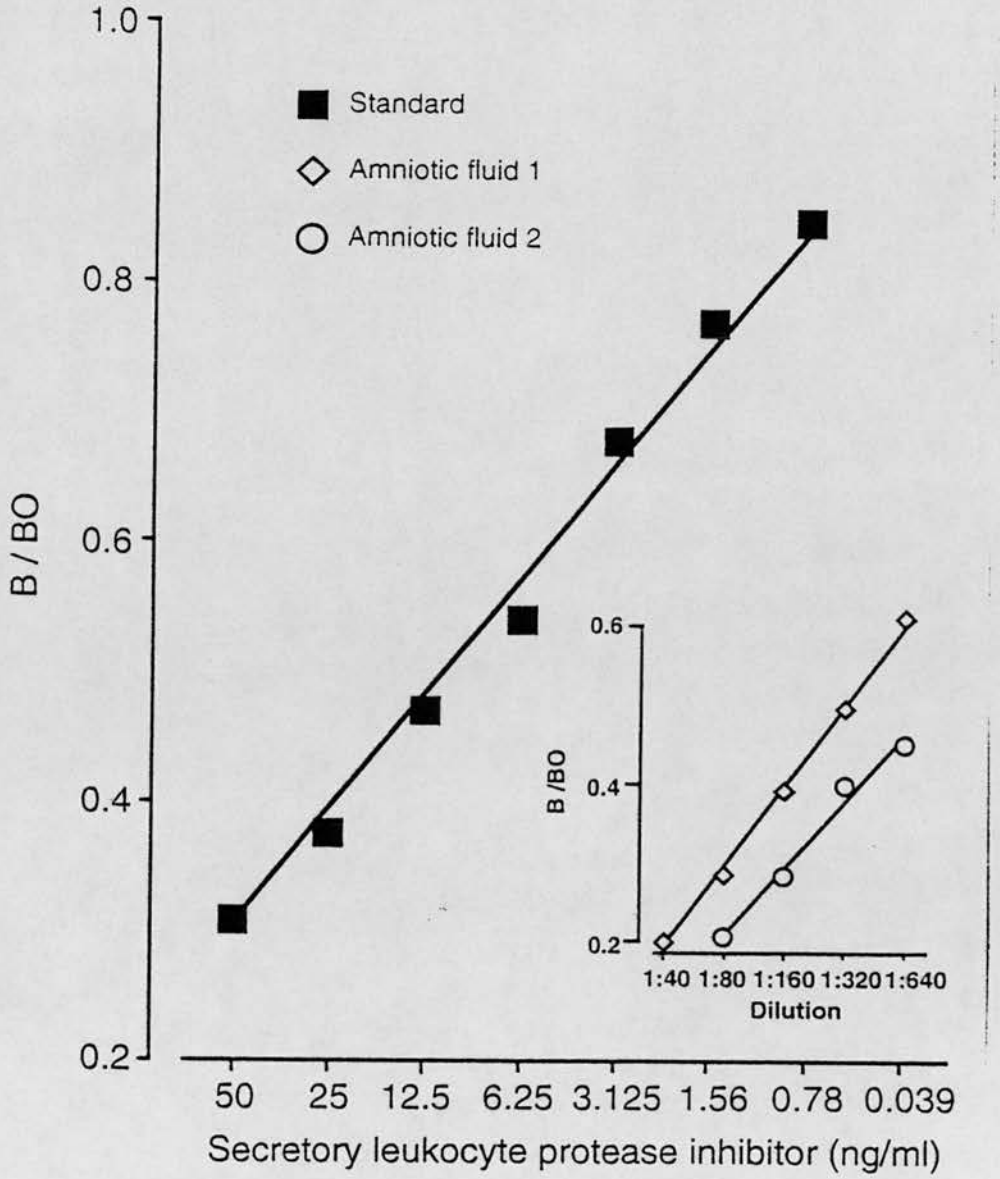


Figure 44

Dose response relationships (B/BO) for recombinant secretory leukocyte protease inhibitor standards and two serially diluted amniotic fluid samples. Serial dilutions of amniotic fluid gave dose responses parallel to that of recombinant standard.

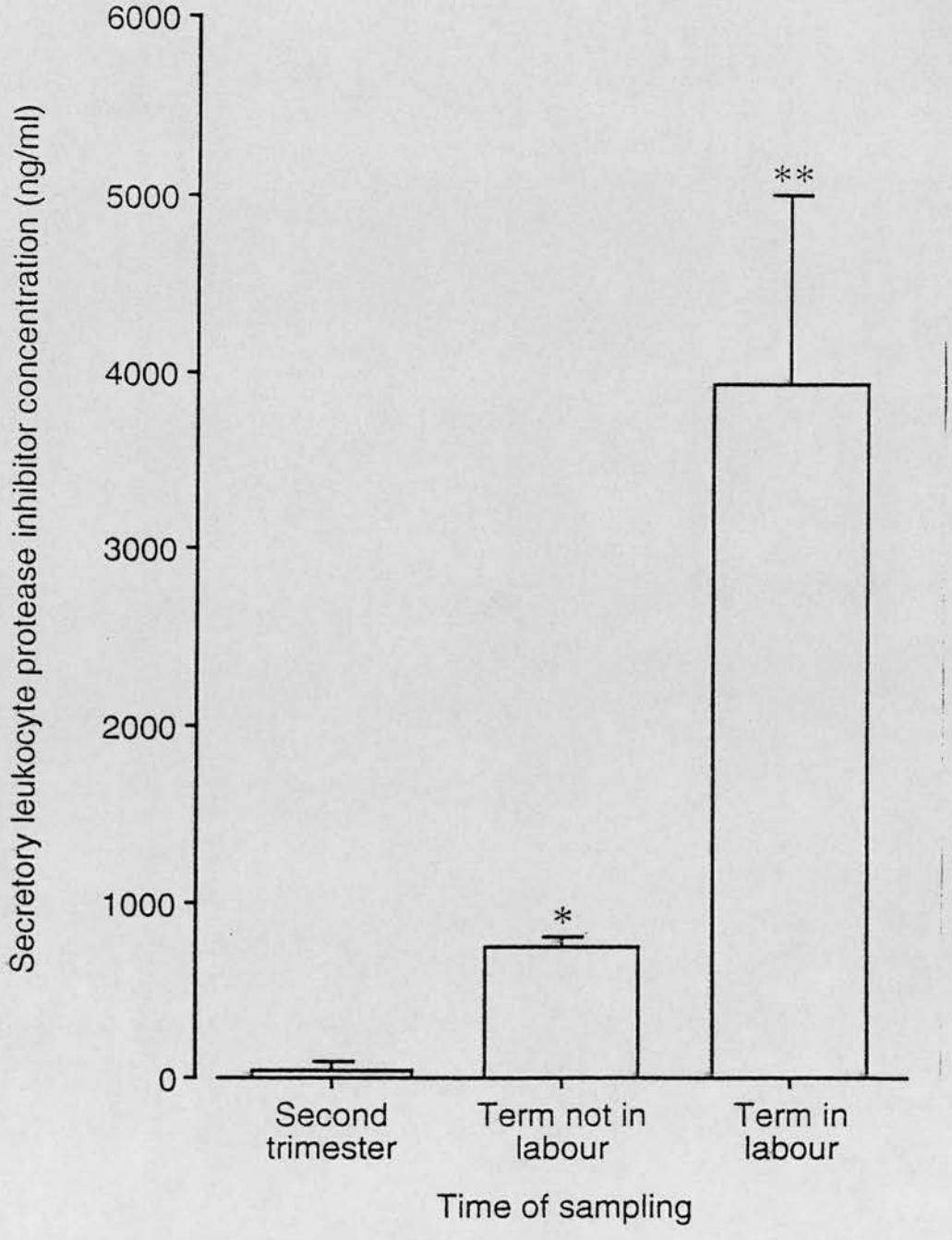


Figure 45

Increase in concentration of secretory leukocyte protease inhibitor in amniotic fluid during pregnancy. The concentration increased significantly during pregnancy from the second trimester (n = 20) to term (n = 15) to term in labour (n = 15). Values are expressed as ng/ml; mean ± s.e.m. Significance; *, $p<0.05$, term not in labour to second trimester; **, $p<0.005$, term not in labour to term in labour.

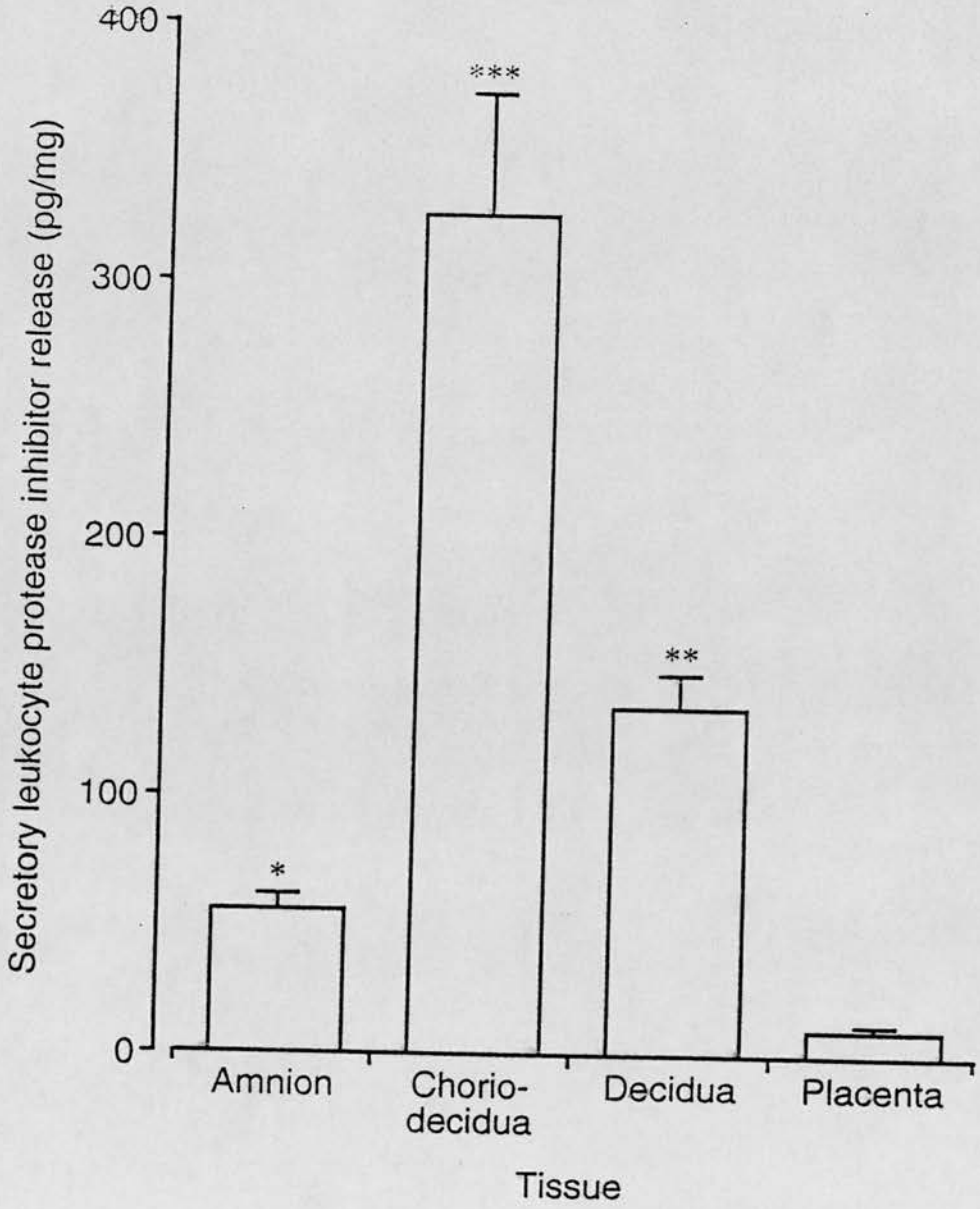


Figure 46

Release of secretory leukocyte protease inhibitor by explants of third trimester amnion, chorio-decidua, decidua and placenta maintained in culture for 24 hours. Significantly more secretory leukocyte protease inhibitor was released by chorio-decidua and decidua than by placenta and amnion. Values are expressed as pg/mg/wet weight; mean ± s.e.m. n = 6 different women. Significance; *, $p<0.05$, amnion compared to placenta; **, $p<0.01$, decidua compared to amnion and placenta; ***, $p<0.005$, chorio-decidua compared to decidua, amnion and placenta.

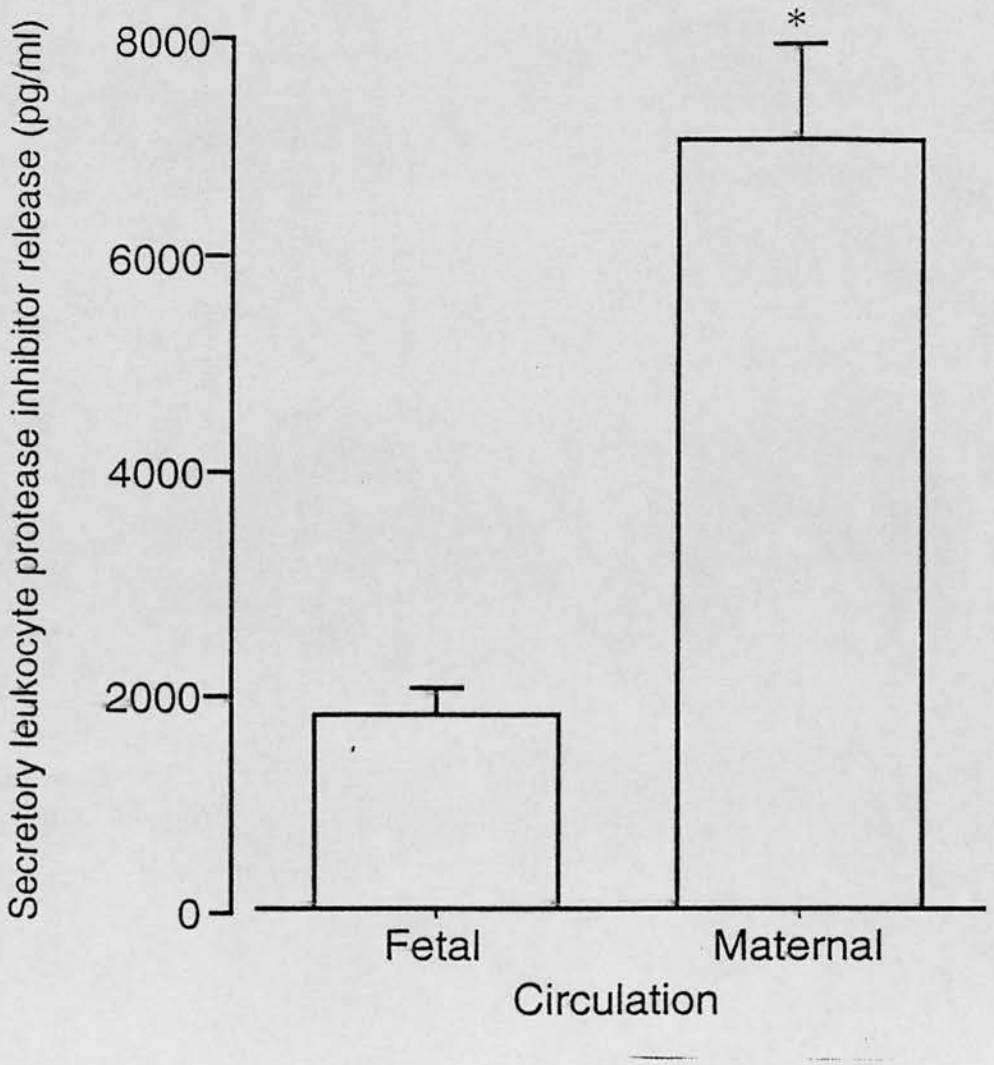


Figure 47

Release of secretory leukocyte protease inhibitor from the dually perfused placental cotyledon. Significantly more secretory leukocyte protease inhibitor was released into the maternal than the fetal circulation. Values are expressed as pg/ml; mean ± s.e.m. n = 6 placentae. Significance; *, $p<0.005$, maternal compared to fetal effluent.

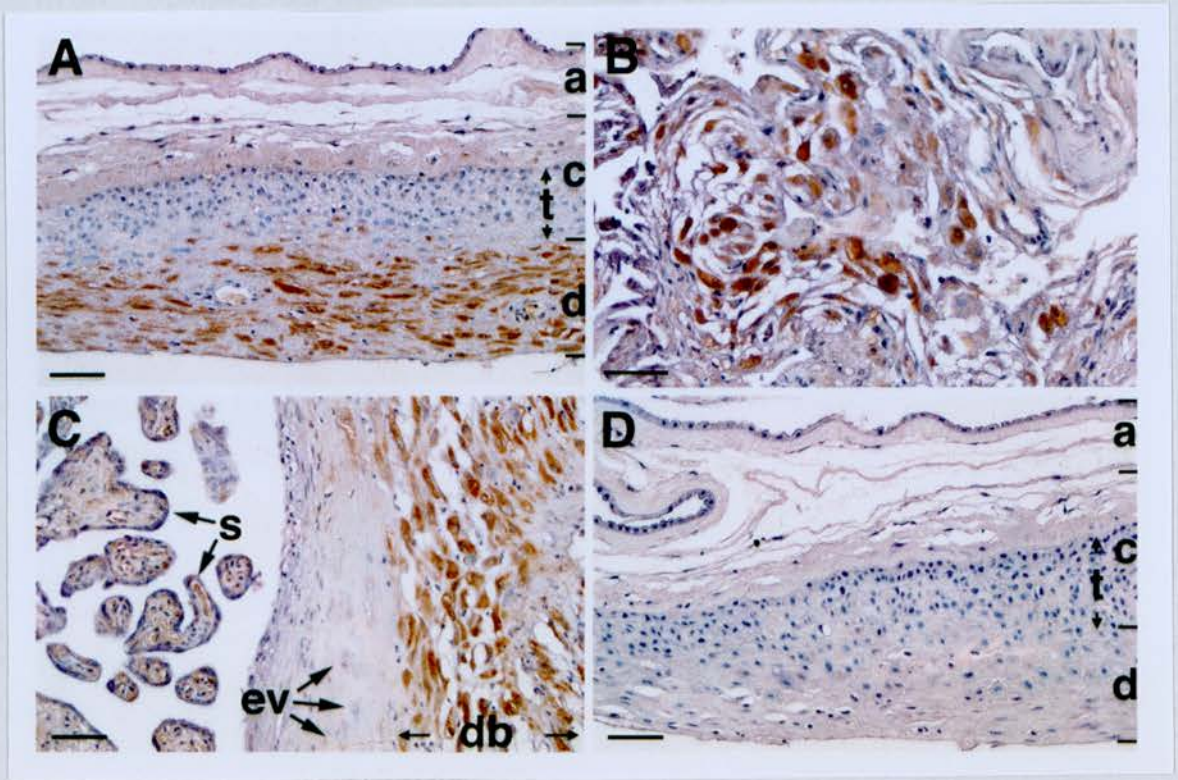


Figure 48

Localisation of secretory leukocyte protease inhibitor by immunohistochemistry in:

A Fetal membranes and adherent decidua. Intense positive immunostaining is localised predominantly in decidual cells and in some cells of fibroblast morphology in chorion and amnion.

B Decidua parietalis with specific positive immunoreactivity in decidual stromal cells.

C Placenta with little or no immunostaining in syncytiotrophoblast cells or extravillous trophoblast in the placental bed. Some positive immunostaining is present in cells of the villous core in the stroma and associated with the vasculature. There is intense immunostaining in cells of the decidua basalis.

D Parallel negative control section of fetal membranes and adherent decidua with no non-specific staining.

Key: a = amnion; c = chorion laeve; d = decidua parietalis adherent to the chorion; t = trophoblast cell layer of chorion; s = syncytiotrophoblast cells; ev = extravillous trophoblast; db = decidua basalis. Scale bars: A & D = 200 μm; B & C 100 μm.

8.4 DISCUSSION

This study demonstrates that secretory leukocyte protease inhibitor is present in increasing concentrations within amniotic fluid during pregnancy and further increases with the onset of labour. Secretory leukocyte protease inhibitor was secreted predominately by explants of decidua and chorio-decidua with lower levels released by amnion and placenta. Immunohistochemistry demonstrated cell-specific localisation of secretory leukocyte protease inhibitor to both decidua parietalis and basalis.

The profound increase in the concentration of secretory leukocyte protease inhibitor within amniotic fluid after the onset of labour is a novel finding. The presence of secretory leukocyte protease inhibitor within amniotic fluid during the second trimester and at term has been previously demonstrated (Helmig, et al., 1995) with the reported values being comparable with our data. Term amniotic fluid comprises mainly of secretions from the surrounding fetal membranes, decidua, fetal lung and from fetal urine (Gilbert and Brace, 1993). Release of secretory leukocyte protease inhibitor from amnion explants *in vitro* was comparatively low. However, the potential release of secretory leukocyte protease inhibitor by amnion *in vivo* may be considerable due to the large surface area of the amniotic membrane. In addition, it has been previously demonstrated that secretory leukocyte protease inhibitor is present in high concentrations within tracheal aspirates from neonates (Ohlsson, et al., 1992) with levels correlating positively with gestational age (Sluis, et al., 1994). Although this study demonstrates that fetal urine contains secretory leukocyte protease inhibitor, it was only detectable in one quarter of all samples tested and therefore unlikely to account for the consistently high levels of secretory leukocyte protease inhibitor demonstrated in all labouring amniotic fluid samples. It is likely therefore that release and transfer of secretory leukocyte protease inhibitor from decidua, as has been suggested for prolactin (Rosenberg, et al., 1980), amnion epithelial and fetal lung secretions may all contribute to secretory leukocyte protease inhibitor in amniotic fluid.

The predominant source of secretory leukocyte protease inhibitor secretion in the uterus is likely to be the decidua, with explants of decidua parietalis and chorio-decidua secreting high levels. In decidua parietalis, intense immunoreactivity for secretory leukocyte protease inhibitor was localised in the maternal aspect of the decidua and in that adjacent to chorion. This adherent decidua is likely to be the principal site of production in chorio-

decidual explants. Decidua collected from the myometrial aspect by curettage is usually contaminated with some myometrium, which is immunonegative for the protein. This may explain why decidual explants secrete slightly less secretory leukocyte protease inhibitor compared to chorio-decidua, unless there is a gradient of secretion with highest outputs from decidua adjacent to the chorion. Term decidua is heterogeneous, consisting of stromal cells (53 %), macrophages (19 %), granulocytes (16 %) and T-cells (8 %) (Adeleye, et al., 1996). Hence the stromal cells, macrophages and granulocytes, which are capable of secretory leukocyte protease inhibitor secretion in other sites (Ohlsson, et al., 1996) are potential sources. In the fetal membranes, amnion explants, which consist of an epithelium and sub-epithelial layers containing fibroblasts, secrete secretory leukocyte protease inhibitor. The potential paracrine interactions between the different cell types present in the term amnion, chorion, composed of reticular fibroblast and the trophoblast cell layers and decidua, to regulate the release of secretory leukocyte protease inhibitor at the materno-fetal interface require further investigation.

Placental explants of villous core tissue released low levels of secretory leukocyte protease inhibitor which is in agreement with only slight immunostaining within the fetal vasculature. This immunostaining could represent either secretory leukocyte protease inhibitor bound to elastin (Rudolphus, et al., 1994) within the syncytiotrophoblast blood vessel wall or specific staining of residual fetal blood cells in the villi. Within the isolated perfused placental cotyledon, secretory leukocyte protease inhibitor was released mainly into the maternal and not the fetal circuit and was undetectable in cord serum. Decidua basalis adherent to the perfused placental cotyledon stains intensely for secretory leukocyte protease inhibitor reactivity and may be the principal source of the secretory leukocyte protease inhibitor secretion into the maternal circulation effluent.

Parturition has been likened to an inflammatory reaction (Liggins, et al., 1977). Secretory leukocyte protease inhibitor may be involved in the regulation of this response at the materno-fetal interface. At the onset of labour, there is increased expression and release of pro-inflammatory cytokines such as IL-1 and IL-6 within amniotic fluid (Cox, et al., 1997) and decidua (Dudley, et al., 1992) which stimulate pro-inflammatory cascades involved in the initiation and maintenance of parturition. However, IL-1 and IL-6 can also stimulate release of secretory leukocyte protease inhibitor (Jin, et al., 1998; Sallenave, et al., 1994). The significant rise in IL-1 and IL-6 within amniotic fluid and decidua at parturition could therefore enhance release of secretory leukocyte protease inhibitor (Jin, et al., 1998; Sallenave, et al., 1994) from amnion epithelium, fetal respiratory tract mucosa

and decidua. Secretory leukocyte protease inhibitor could then exert a negative feedback on release of inflammatory cytokines via inhibition of NFκB (Jin, et al., 1997) and neutrophil function by inhibition of neutrophil elastase. Secretory leukocyte protease inhibitor could also inhibit release of PGE_2 and matrix metalloproteinases, which are also involved in membrane remodelling and rupture, by inhibiting COX-2 (Zhang, et al., 1997). During parturition, the uterine cavity is at high risk of invasion and infection by microbial organisms. Secretory leukocyte protease inhibitor, by virtue of its anti-bacterial, anti-fungal and anti-viral properties could therefore in addition, act as an endogenous block to infection within the decidua and the amniotic cavity.

In summary, it has been demonstrated that secretory leukocyte protease inhibitor is present in increasing concentrations within amniotic fluid during pregnancy and labour. Secretory leukocyte protease inhibitor is also released by and immunolocalised to term decidua. This tissue, together with fetal membranes and possibly the fetal lung, are the likely sources of secretory leukocyte protease inhibitor within the uterus. It is suggested that secretory leukocyte protease inhibitor might act both to limit the pro-inflammatory cascades ongoing during parturition and to protect against microbial invasion and the response to infection. Further studies are required to investigate the regulation of secretory leukocyte protease inhibitor release by intra-uterine tissues and to examine its potential roles in the maintenance of pregnancy and the initiation and progression of parturition, both preterm and at term.

GENERAL CONCLUSION - PART 2

The second part of this thesis provides support for the hypothesis that inflammatory mediators are important in the initiation and regulation of cervical ripening and parturition with complex interactions occurring within the cervix and at the feto-maternal interface.

Cervical ripening

The cervical studies in Chapter 6 demonstrated that the non-pregnant and pregnant cervix release a wide range of inflammatory mediators and it was postulated that these mediators may be involved in maintaining cervical competence and mediating cervical ripening at term. In addition, a fall in progesterone or interference with its action at the local level was proposed as a possible mechanism for initiating these processes. To investigate this, cervical biopsies should also be taken from women at term after caesarean section or spontaneous vaginal delivery post-administration of mifepristone. Furthermore, it is speculated that inflammatory mediators, such as IL-8 and nitric oxide, may have a clinical application as novel cervical ripening agents at term. Alternatively, antagonism of their action may prevent premature cervical ripening and preterm delivery.

Inflammatory mediators and parturition

Chapters 7 and 8 investigated the sites of production of inflammatory mediators within the uterus at term. Chorio-decidua was the principal source of release of the pro-inflammatory mediators MCP-1, IL-8 and RANTES. Anatomically, this tissue comprises the feto-maternal interface and has been proposed as playing a pivotal role in the initiation of a pro-inflammatory parturient response (Dudley, et al., 1992). The findings from the above studies are consistent with this hypothesis and support the concept that paracrine interactions within chorio-decidua may be involved in the initiation of parturition. Future studies should investigate how release of these mediators is affected by the onset of labour and study the secretion of these and other pro-inflammatory cytokines from intra-uterine tissues peri- and intra-partum.

In addition, chorio-decidua was the principal site of secretion of the anti-inflammatory mediators IL-10 and secretory leukocyte protease inhibitor. Together they could, via different mechanisms (Chen and Manning, 1996; Jin, et al., 1997; Kucharzik, et al., 1998; MarfaingKoka, et al., 1996), inhibit release of pro-inflammatory cytokines. Thus they could act both as general inhibitors of an inflammatory response and specifically as a negative feedback control on the pro-inflammatory parturient response. In addition, IL-10

and secretory leukocyte protease inhibitor could protect the fetus during parturition by generating a local Th-2 type immune response, thereby preventing fetal rejection, and by acting as an endogenous block to infection within the decidua and the amniotic cavity, respectively. Future studies could investigate the release of IL-10 and secretory leukocyte protease inhibitor by intra-uterine tissues in pregnancies complicated by adverse outcomes, such as intra-uterine growth retardation, infection, premature rupture of membranes and preterm labour. In addition, release of other anti-inflammatory mediators such as TGF-β, which has many parallels with IL-10 in its action, or other natural anti-microbial agents, such as defensins, at the materno-fetal interface could be investigated. From the clinical perspective, IL-10 and secretory leukocyte protease inhibitor could have a potential therapeutic application in antagonising unwanted inflammatory responses, such as occur during many preterm labours. This therapeutic potential is already being exploited in the field of respiratory medicine, where recombinant secretory leukocyte protease inhibitor protein has been found to boost mucosal immunity (Stolk, et al., 1995) and reduce lung damage mediated by inflammatory processes such as lipopolysaccharide induced emphysema (Rudolphus, et al., 1993).

Finally, preliminary studies were undertaken into the regulation of inflammatory mediator release by PGE_2. Future studies could investigate the molecular mechanisms behind the PGE_2 mediated stimulation of inflammatory mediator release and assess whether transcription factors such as NFκB are involved. In addition, the roles of other factors such as nitric oxide in modulating inflammatory mediator release at the feto-maternal interface could be investigated.

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APPENDIX 1

Recipes for solutions

All chemicals were from Sigma-Aldrich Co. Ltd., Poole, UK unless otherwise stated.

TISSUE COLLECTION

10 % Neutral buffered formalin (NBF)

50 mM Na_2HPO_4
25 mM $NaH_2PO_4.2H_2O$
10 % formaldehyde
Make up to 1 litre with distilled H_20.

CELL CULTURE/PREPARATION MATERIALS

Complete medium

10 % FCS
10 μg/ml gentamicin
50 IU/ml penicillin
50 μg/ml streptomycin — Gibco, Paisley, UK
2 mM L-glutamine
500 mls RPMI 1640

PBMC/CD14 CELL SEPARATION

Cell Separation Buffer

5 mM EDTA
0.5 % BSA
PBS

CELL SEPARATION

Steeping buffer

80 μg/ml gentamicin
5 μg/ml amphotericin B
500 mls PBS — Gibco, Paisley, UK

Transport solution

10 U/ml heparin	LeoLabs Ltd., Risborough, UK
100 mls PBS	Gibco, Paisley, UK

Digestion medium

5 mg/ml trypsin
20 μg/ml DNAse
500 mls RPMI 1640

ELISA SOLUTIONS

Coating Solution **pH 9.6**

400 mM $NaHCO_3$
40 mM Na_2CO_3

Wash Buffer **pH 7 - 7.5**

150 mM NaCl
100 mM Tris - HCl
0.05 % Tween - 20

ELISA Assay Buffer **pH 7.2**

150 mM NaCl
100 mM Tris - HCl
50 mM phenol red solution
2 mM EDTA

1 mM 2-methylisothiazolone	Boehringer Mannheim, Lewes, UK
1 mM bromonitrodioxane	Boehringer Mannheim, Lewes, UK

2 mg/ml BSA
0.05 % Tween - 20

Substrate **pH 6.0**

10 mls 100 mM sodium acetate
1 ml 0.1 g/l tetramethyl benzidine in DMF
1 ml 0.3 g/l urea-hydrogen peroxide in 50 mM sodium acetate

Amplex red substrate **pH 6.0**

9 mls 100 mM sodium acetate

1 ml 0.3 g/l urea-hydrogen peroxide in 50 mM sodium acetate

100 µl 20 µg/ml Amplex Red in DMF

Methyloximating solution **pH 5.6**

0.1 M methoxyamine hydrochloride

10 % ethanol

1M sodium acetate

Blocking solution

50 mM glycine

10 mg/ml BSA (Grade A7888)

ZYMOGRAPHY

Sample application buffer

10 mls 20 % v/v glycerol

1 g 2 % w/v SDS — BioRad, Hemelhempstead, UK

20 mg 0.04 % w/v bromophenol blue — BioRad, Hemelhempstead, UK

Dissolve in 50 mls ElgaStat x1 purified H_20

Store in 1 ml aliquots at 5°C.

Running tank buffer x10 **pH 8.3**

1.9 M glycine

0.25 M Tris — Merck Ltd. (BDH), Lutterworth, UK

1 % SDS — BioRad, Hemelhempstead, UK

Dissolve in 1 litre ElgaStat x1 purified H_20.

Dilute 1 : 10 with ElgaStat x1 purified H_20 for use.

Resolving gel buffer: Stock A — **pH 8.8**

1.5 M Tris — Merck Ltd. (BDH), Lutterworth, UK

Dissolve in 50 mls ElgaStat x1 purified H_20. pH with 5 N HCl.
Make up volume to 100 mls with ElgaStat x1 purified H_20.

Stacking gel buffer: Stock B — **pH 6.8**

0.5 M Tris — Merck Ltd. (BDH), Lutterworth, UK

Dissolve in ~50 mls ElgaStat x1 purified H_20.
Adjust pH with 5 N HCl.
Make up volume to 100 mls with ElgaStat x1 purified H_20.

Stock Wash Buffer x 10 (TBS x 10) — **pH 8.0**

0.5 M Tris — Merck Ltd. (BDH), Lutterworth, UK

1.5 M NaCl — Merck Ltd. (BDH), Lutterworth, UK

Dissolve in ~750 mls ElgaStat x1 purified H_20, adjust pH with 5 N HCl.
Make up volume to 1 litre with ElgaStat x1 purified H_20.
Dilute 1 : 10 with ElgaStat x1 purified H_20 for use (TBS).

Resolving gel (Lower gel)	**7.5 % Acrylamide/Bis**
2.35 mls ElgaStat x1 purified H_20	
1.0 ml gelatin 10 mg/ml ElgaStat x1 purified H_20	Bovine Skin Type III Bloom 225, Sigma, Poole, UK
2.5 mls Stock A Buffer	
100 µl 10 % SDS	BioRad, Hemelhempstead, UK
50 µl 10 % ammonium persulphate	
4.0 mls acrylamide/bis	acrylamide/bis-acrylamide 37.5 : 1 bought in as a 30 % solution from BioRad, Hemelhempstead, UK
Mix by gentle swirling. Degas via water pressure for 5 - 10 minutes.	
5 µl TEMED	
Add TEMED, swirl to mix gently.	

Stacking Gel (Upper gel)	**4 % Acrylamide/Bis**
3.05 mls ElgaStat x1 purified H_20	
1.25 mls stock B. buffer	
50 µl 10 % SDS	BioRad, Hemelhempstead, UK
50 µl 10 % ammonium persulphate	
0.65 mls acrylamide/bis	BioRad, Hemelhempstead, UK
Mix by gentle swirling. Degas by vacuum for 5 - 10 minutes.	
5 µl TEMED	
Swirl to mix gently.	

Triton-X-100 Wash	
2.5 % Triton-X-100 in TBS 1 : 10	Merck Ltd. (BDH), Lutterworth, UK

Digestion buffer **pH 7.6**

50 mM Tris — Merck Ltd. (BDH), Lutterworth, UK

0.2 M NaCl

5 mM $CaCl_2$ — Merck Ltd. (BDH), Lutterworth, UK

1 μM $ZnCl_2$ — Merck Ltd. (BDH), Lutterworth, UK

0.02 % Brij-35

Dissolve in ~750 mls ElgaStat x1 purified H_20, adjust pH with 5N HCl.

Make up volume to 1 litre with ElgaStat x1 purified H_20. Store at 5°C.

Destaining solution

30 % methanol — Merck Ltd. (BDH), Lutterworth, UK

10 % glacial acetic acid — Merck Ltd. (BDH), Lutterworth, UK

600 mls ElgaStat x1 purified H_20

Staining Solution

0.5 % w/v Coomassie brilliant blue R250 in destaining solution. — BioRad, Hemelhempstead, UK

REVERSE ZYMOGRAPHY

Wash buffer/Rinse buffer **pH 7.5**

50 mM Tris — Merck Ltd. (BDH), Lutterworth, UK

5 mM $CaCl_2$ — Merck Ltd. (BDH), Lutterworth, UK

2.5 % Triton-X-100 — Merck Ltd. (BDH), Lutterworth, UK

Dissolve in ~750 mls ElgaStat x1 purified H_20, adjust pH with 5N HCl.

Add 25 mls Triton-X-100, allow to dissolve completely ~ 1 - 1.5 hours on magnetic stirrer.

Make up to 1 litre with ElgaStat x1 purified H_20.

Incubation buffer/Digestion buffer

As rinse buffer but without the Triton-X-100.

Resolving gel (Lower gel)	**12 % Acrylamide/Bis**
1.69 mls ElgaStat x1 purified H_20	
1.0 ml gelatin 10 mg/ml ElgaStat x1 purified H_20	Bovine Skin Type III Bloom 225
2.5 mls stock A buffer	
100 µl 10 % SDS	BioRad, Hemelhempstead, UK
50 µl 10 % ammonium persulphate	
4.0 mls acrylamide/bis	BioRad, Hemelhempstead, UK
0.66 mls solution A	
Mix by gentle swirling. Degas via water pressure/desiccator vacuum for 10 minutes.	
5 µl TEMED	
Swirl gently to mix.	

Stacking gel (Upper gel)	**5 % Acrylamide/Bis**
2.89 mls ElgaStat x1 purified H_20	
1.25 mls stock B buffer	
50 µl 10 % SDS	BioRad, Hemelhempstead, UK
50 µl 10 % ammonium persulphate	
0.812 mls acrylamide/bis	BioRad, Hemelhempstead, UK
Mix by gentle swirling. Degas via water pressure/ vacuum for 5 minutes.	
5 µl TEMED. Swirl gently to mix.	

WESTERN BLOT METHOD

Homogenisation/Extraction buffer	**pH 7.4**
20 mM TrisHCl	Merck Ltd. (BDH), Lutterworth, UK
150 mM NaCl	
1 tablet protease inhibitor	Boehringer Mannheim, Lewes, UK
Dissolve in 40 mls ElgaStat x1 purified H_20, adjust pH with 5 N HCl.	
Make up volume to 50 mls with ElgaStat H_20	
Use straight away or store at -20°C.	

Sample application buffer — **pH 6.75**

1.5 % w/v Tris — Merck Ltd. (BDH), Lutterworth, UK

4.0 % w/v SDS — BioRad, Hemelhempstead, UK

2.0 % w/v dithiothreitol (DTT)

0.05 % w/v bromophenol blue — BioRad, Hemelhempstead, UK

Dissolve Tris in ~ 20 mls ElgaStat x1 purified H_20, adjust pH with 5 N HCl.

Add in other chemicals, dissolve, make volume up to 25 mls.

Store in 0.5 ml aliquots at -20°C.

Stock wash buffer x10 (TBS x10) — **pH 7.4**

0.5 M Tris — Merck Ltd. (BDH), Lutterworth, UK

1.5 M NaCl

Dissolve in ~750 mls ElgaStat x1 purified H_20, adjust pH with 5 N HCl.

Make up volume to 1 litre with ElgaStat x1 purified H_20.

Dilute 1 : 10 with ElgaStat x1 purified H_20 for use and add:-

0.05 % v/v Tween 20

Membrane Blocking Buffer

5 w/v BSA — Sigma A3294

Dissolve in 100 mls TTBS

Make up freshly, do not store. Prepare ~ 30 minutes before use to allow time to dissolve properly.

Transfer/Blotting buffer **pH 8.1 - 8.4**

25 mM Tris — Merck Ltd. (BDH), Lutterworth, UK

192 mM glycine

20 % v/v methanol — Merck Ltd. (BDH), Lutterworth, UK

Dissolve salts in 800 mls ElgaStat x1 purified H_20, check pH, add methanol.
Store at 5°C.

Resolving gel (Lower gel)

	% Acrylamide/Bis		
	7.5 %	**12 %**	
ElgaStat x1 purified H_20	9.7 mls	6.7 mls	
Stock A buffer	5.0 mls	5.0 mls	
10 % SDS	200 µl	200 µl	BioRad, Hemelhempstead, UK
10 % ammonium persulphate	100 µl	100 µl	
acrylamide/bis	5.0 mls	8.0 mls	BioRad, Hemelhempstead, UK

Mix by gentle swirling. Degas via vacuum for 5 - 10 minutes.

TEMED	10 µl	10 µl	

Add TEMED, swirl gently to mix.

Stacking gel (Upper gel) **4 % Acrylamide/Bis**

6.1 mls ElgaStat x1 purified H_20

2.5 mls Stock B Buffer

100 µl 10 % SDS — BioRad, Hemelhempstead, UK

100 µl 10 % ammonium persulphate

1.3 mls acrylamide/bis — BioRad, Hemelhempstead, UK

Mix by gentle swirling. Degas via vacuum for 5 minutes.

10 µl TEMED

Add TEMED, swirl gently to mix.

Blocking solution

5 % BSA or 5 % normal serum

TTBS

Primary antibody solution

Blocking solution or TTBS

0.025 % sodium azide

TTBS

APPENDIX II

AMNION/CHORION DISPERSED CELL METHOD

Method

- Collect membranes directly into PBS containing heparin 10 U/ml.
- Separate amnion and chorion then wash three times with PBS and discard any meconium stained, abnormal tissue and blood clots. Weigh tissue.
- Steep tissue in PBS containing 40 mg gentamicin and 2.5 mg amphotericin B/500 mls (30 - 60 minutes, 4°C) then wash four times with PBS.
- Shred tissue into 3 - 4 mm pieces.
- Incubate chorion in digestion medium (10 minutes, 37°C) to remove red blood cells then wash and retrieve tissue.
- Digest amnion and partially digested chorion in digestion medium with gentle agitation (40 minutes, 37°C).
- Mechanically disperse digested cells then harvest with filter (0.171 mm nylon mesh).
- Centrifuge (1500 rpm/~500 g, 10 minutes, 23°C), decant supernatant, combine pelleted cells and resuspend in a lesser volume (~30 mls) of complete medium.
- Repeat 8 twice then resuspend amnion (10 mls) and chorion (20 mls) in complete medium. Count cells to assess number and viability using trypan blue exclusion. Viability for both amnion and chorion usually greater than 90 %.

ZYMOGRAPHY

Sample preparation

Cell/tissue culture incubation medium

- Remove supernatant and freeze to -70°C prior to lyophilising samples by freeze drying.
- Either reconstitute samples with known volume of 0.1 % SDS or store lyophilised at -20°C until use.

Plate preparation

- Clean (70 % IMS) and assemble plates (8 x 10 cm) with spacers (0.75 mm) between them.
- Prepare the resolving gel according to the recipe and pour immediately to 1.2 cm below top of plate (fill line), avoiding bubbles and leaks if possible.
- Overlay gel with water saturated sec-Butanol to burst any bubbles and give an even level to top of gel.
- Leave to set (45 minutes, 23°C) and pour off the butanol. Wash with water followed by several washes with Tris Buffer (Solution B 1 : 4) and leave with Tris buffer overlay to allow polymerisation to occur (minimum of 2 hours, 23°C).
- Pour off buffer, rinse overlay with water and dry with paper. Clean (70 % IMS) combs (0.75 mm, 15 well) and position between the plates.
- Prepare the stacking gel according to the recipe, apply to the resolving gel as before, removing any bubbles and leave to set (at least 90 minutes, 23°C).

Gel electrophoresis (Discontinuous system)

- Remove the combs from the gels, take gels off the holder and attach to electrophoresis apparatus.
- Place the assembled core in the tank and fill chamber with running buffer (500 mls) to dislodge any trapped air bubbles.
- Load the samples (15 µl - 20 µl; 50 % v/v sample buffer) into the wells, remove any air bubbles and top up the core chamber to about 3 mm below the top edges of the two plates.
- Run at Constant Voltage, 100 V for 90 minutes at 23°C.
- After electrophoresis measure molecular weight markers and dye front.
- Remove gel from plate and immerse in Triton X 100 Wash Buffer.

Wash, digestion and stain

- Wash the gels twice (15 minutes/wash, 23°C) in Triton wash buffer then twice (~ 2 minutes/wash, 23°C) in wash buffer.
- Add digestion buffer and incubate at 37°C overnight. After incubation, decant buffer, wash twice in wash buffer (~2 minutes/wash, 23°C) and add staining solution (~3 hours, 23°C).

- Decant staining solution and replace with destaining solution (~30 minutes then 60 minutes, 23°C). Store destained gels in air tight water-filled container until ready to be photographed (Kodak Ratten Filter No. 21 orange) or scanned.

WESTERN BLOT METHOD

Tissue preparation and cell/tissue culture incubation medium

As for zymography.

Dialysis

Some samples from tissue/cell culture may require a dialysis step to 'clean' them up prior to electrophoresis and blotting. This tubing retains proteins of MW 12000 or greater.

- If samples have been freeze dried reconstitute with extraction buffer (no protease inhibitor added) to original volume, otherwise dialyse culture medium directly.
- Dialyse samples with gentle agitation against distilled water overnight at 4°C.
- Store dialysed samples at -70° C prior to lyophilising.

Gel electrophoresis (Discontinuous system)

- Clean and assemble plates (plate size ~ 8 x 10 cm, spacers 1.5 mm, combs 1.5 mm, 10 well). Prepare gels according to recipe and assemble as described for Zymography.
- Remove combs from the gels and attach plates to cooling core (frozen and stored at -20°C) on electrophoresis apparatus.
- Fill up chamber with running buffer (500 mls x1 running buffer) to dislodge air bubbles trapped in the wells and place the assembled core in the tank.

- Sample preparation.
    - Mix samples (maximum volume 20 µl) with an equal volume of sample application buffer.
    - "Boil" samples for 5 minutes.
    - Centrifuge at 13,000 rpm for 3 minutes at 4° C.
- Load the samples (maximum 40 µl) into the wells (keep samples on ice while loading gel).

- Remove trapped air bubbles and top up core chamber to about 3 mm below the top edges of the two plates.
- Run at constant 100 V for ~2 hours.
- After electrophoresis, remove gels from apparatus, measure molecular weight markers and dye front and immerse in transfer buffer.

Mini trans-blot assembly and transfer

- Equilibrate gels in transfer buffer (~ 30 minutes).
- Soak nitro-cellulose membrane (30 minutes), fibre pads (10 minutes) and filter paper (10 minutes) in transfer buffer.
- Assemble the gel sandwich in gel cassette holder as follows (bottom to top):
  - Pre-soaked fibre pad.
  - Saturated filter paper.
  - Equilibrated gel, face down, on top of the paper. Flood gel surface with a few mls of transfer buffer.
  - Pre-wetted membrane on top of the gel. Mark the position of the MW markers and flood membrane with transfer buffer.
  - Saturated filter paper.
  - Pre-soaked fibre pad.
- Place the assembled gel holder cassette into tank electrode panel and slot cooling unit behind electrode. Add transfer buffer (~400 mls, 5°C) to the tank.
- Electrophorese at constant voltage 100 V for 1 hour (temperature at end of transfer should be no higher than 30°C).
- Dismantle the gel sandwich and transfer membrane to blocking solution for immunoblot (overnight, 4°C).
- Stain gels (~1 - 2 hours) with 0.5 % Coomassie Blue to check the protein transfer, pour off stain, add destaining solution, destain as necessary. After destaining is completed, transfer to water.

Immunoblot-blot

- Drain membrane, incubate with primary antibody (diluted in blocking solution or TTBS plus 0.025 %, 3 - 4 hours, 23°C) then wash three times (TTBS, 10 minutes/wash).
- Incubate membrane with secondary antibody (1 : 200 in TTBS, 45 minutes, 23°C) and prepare ABC (2 drops solution A : 10 mls TTBS : 2 drops solution B). After incubation, wash membrane three times (TTBS, 10 minutes/wash) as before.
- Prepare substrate solution (6 drops buffer solution : 12 drops DAB solution : 6 drops H_2O_2 : 6 drops nickel solution, mixed in glass dish) and add membrane to solution to develop.
- When desired staining intensity achieved, wash membrane in water, dry and scan (Bio-Rad Model GS-700 Imaging Densitometer).

APPENDIX III

ORAL PRESENTATIONS

Denison, F.C., Kelly, R.W., Riley, S.C., Critchley, H.O.D. and Calder, A.A. Inflammatory mediators and the control of parturition. Presented at the Diamond Jubilee Scottish Society for Experimental Medicine, Edinburgh, November, 1998.

Denison, F.C., Kelly, R.W., Calder, A.A., and Riley, S.C. Cytokine secretion by human fetal membranes, decidua and placenta at term. Presented at the Munro Kerr Society, Glasgow, June 1998.

Denison, F.C., Calder, A.A., and Kelly, R.W. Human seminal plasma has a pro-inflammatory effect on the human cervix. Presented at the 17th Joint Meeting of the British Endocrine Societies, Heriot Watt University, Edinburgh, March 1998.

Denison, F.C., Kelly, R.W. and Calder, A.A. Inflammatory mediators, progesterone and cervical ripening. Presented at the Munro Kerr Society, Edinburgh, February 1998.

Denison, F.C., Kelly, R.W. and Calder, A.A. Differential secretion of monocyte chemotactic peptide (MCP-1) and interleukin-8 (IL-8) during pregnancy. Presented at the Munro Kerr Society, Edinburgh, March 1997.

APPENDIX IV

POSTER PRESENTATIONS

King, A.E., Critchley, H.O.D., Denison, F.C. and Kelly, R.W. Secretory leukocyte protease inhibitor is present in first trimester decidua. Poster presentation at the 189th Meeting of the Society for Endocrinology, London, November 1998.

Denison, F.C., Riley, S.C., Calder, A.A. and Kelly, R.W. Secretory leukocyte protease inhibitor is secreted by third trimester amnion, chorion, placenta and decidua and its concentration increases within amniotic fluid during labour. Poster presentation at The 2nd Conference on Prematurity for Health Care Professionals, Tommy's Campaign, London, September 1998.

Denison, F.C., Kelly, R.W. and Calder, A.A. Prostaglandin E_2, inflammatory mediators and cervical ripening. Poster presentation at 3rd British Maternal and Fetal Medicine Society, Manchester, April 1998.

Howe, D.C., Leask, R., Denison, F.C., Calder, A.A. and Riley, S.C. Production of tissue inhibitors of metalloproteinases (TIMPs) by the fetal membranes and placenta at parturition. Poster presentation at 3rd British Maternal and Fetal Medicine Society, Manchester, April 1998.

Denison, F.C., Kelly, R.W., Riley, S.C., and Calder, A.A. Prostaglandin E_2: Mechanisms and mediators in cervical ripening. Poster presentation at the Theodore Ogg Silver Jubilee Meeting, London, December 1997.

Denison, F.C., Kelly, R.W. and Calder, A.A. Difference in chemokine release from peripheral blood in pregnant compared with non-pregnant women. Poster presentation at the 13th Annual Meeting of the European Society of Reproduction and Embryology Edinburgh, June 1997. Selected for oral presentation at conference.

Denison, F.C., Kelly, R.W. and Calder, A.A. Differential secretion of chemokines from peripheral blood in pregnant compared with non-pregnant women. Poster presentation at the 1st International Conference of Experimental and Reproductive Immunology, Charlottesville, USA, October 1997.

APPENDIX V

PUBLISHED ABSTRACTS

King, A.E., Critchley, H.O.D., Denison, F.C. and Kelly, R.W. (1998) Secretory leukocyte protease inhibitor is present in first trimester decidua. Journal of Endocrinology, 159, S3, P56

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APPENDIX VI

PRIZES

Sir James Black Young Investigators Award, Scottish Society for Experimental Medicine, Edinburgh, November 1998 for oral presentation:

Denison, F.C., Kelly, R.W., Riley, S.C., Critchley, H.O.D. and Calder, A.A. Inflammatory mediators and the control of parturition. Presented at the Diamond Jubilee Scottish Society for Experimental Medicine, Edinburgh, November, 1998.

Prize for best poster in Labour and Delivery Section at 3rd British Maternal and Fetal Medicine Society, Manchester, April 1998 for poster presentation:

Denison, F.C., Kelly, R.W. and Calder, A.A. Prostaglandin E_2, inflammatory mediators and cervical ripening. Poster presentation at 3rd British Maternal and Fetal Medicine Society, Manchester, April 1998.

William Leslie Prize in Medicine (1997), Faculty of Medicine, Edinburgh for the submitted paper:

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APPENDIX VII

PUBLICATIONS

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Differential secretion of chemokines from peripheral blood in pregnant compared with non-pregnant women

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Abstract

The maintenance of a normal pregnancy is dependent on the delicate interaction between the endocrine and the immune systems. Cytokines are thought to play a key role in pregnancy by way of local modulation of the immune system at the level of peripheral leukocytes. This study examined the potential of peripheral venous blood cultures from pregnant women throughout gestation and from non-pregnant women to produce the chemokines monocyte chemotactic protein-1 (MCP-1), interleukin-8 (IL-8) and RANTES. Significantly ($P = < 0.001$), higher levels of MCP-1 were released from peripheral blood cultures from pregnant women at term than during the first trimester or from women who were not pregnant. This could not be accounted for by differences in differential blood counts. Significantly higher levels ($P = < 0.05$) of MCP-1 were released from PBMC preparations from pregnant compared with non-pregnant women. No 'rebound' increase in MCP-1 was observed on withdrawing progesterone support to the PBMC preparations. MCP-1 was secreted predominately from $CD14^+$ cells with those from pregnant women producing more than those from non-pregnant women. There was no statistical difference in release of IL-8 or RANTES from either peripheral blood or PBMC preparations from pregnant or non-pregnant women. IL-8 and RANTES were secreted from $CD14^+$ and $CD14^-$ cells, respectively. The hypothesis proposed is that the monocytes are fundamentally

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Keywords: Monocyte chemotactic protein-1; Pregnancy; Peripheral marker

1. Introduction

Evidence is accumulating that maintenance of a normal pregnancy is dependent on a delicate interaction between the endocrine and immune systems. Disturbance of this balance can result in a wide range of abnormalities including intra-uterine growth retardation (Stallmach et al., 1995), pre-eclampsia (Vinatier and Monnier, 1995), pre-term labour (Romero et al., 1991b) and in the mouse, implantation failure (Krishnan et al., 1996). Cytokines, the local effectors of the immune system, are increasingly thought to play a key role during pregnancy. Chemokines, a subset of cytokines, specific in their ability to attract and activate immune cells, may be particularly important mediators of pregnancy. For example, interleukin-8, a neutrophil chemoattractant and activator, is produced by many tissues, including the amnion (Trautman et al., 1992), placenta (Shimoya et al., 1992), myometrium (Osmers et al., 1995) and cervix (Barclay et al., 1993) and has been implicated in the processes of cervical ripening (El Maradny et al., 1994), pre-term labour (Romero et al., 1991a) and term labour (Osmers et al., 1995). The roles of other chemokines such as monocyte chemotactic protein-1 (MCP-1) and the T-cell chemokine RANTES (regulated upon activation and normally T-cell expressed and presumably secreted) are not known in pregnancy.

From clinical studies it has been shown that pregnancy also affects the presentation of many clinical diseases either aggravating them, for example in carcinoma of the cervix and melanoma (Gustaffson and Kottymeier, 1962; Shiu et al., 1976) or having the opposite effect by ameliorating them, for example in psoriasis or rheumatoid arthritis (Boyd et al., 1996; Nelson and Ostensen, 1997). However, the immunological basis for these differences is often poorly understood and objective evidence regarding functional changes in leucocyte populations is conflicting. For example, Shibuya et al. (1991) demonstrated that phagocyte mediated non-specific immunity was up-regulated in pregnancy with there being an increased oxidative responsiveness, whereas Crouch et al. (1995) demonstrated a significant reduction in respiratory burst in polymorphonuclear leukocytes during pregnancy. Evidence regarding differences in levels of cytokines in peripheral venous plasma are also conflicting and dependent on the cytokine being measured and possibly assay procedures. Laham et al. (1993, 1994) found

no difference in levels of either TNFα or IL-8 in peripheral plasma between pregnant and non- pregnant samples whereas Austgulen et al. (1994) demonstrated a significant increase in levels of IL-6 and IL-1 in pregnant compared with non-pregnant samples. Another approach, which has been used by Elsasser-Beile et al. (1992), is to measure the production of cytokines in mitogen-stimulated peripheral blood cultures as a measure of lymphocyte function.

Two factors might influence circulating leukocytes during pregnancy. First, they may respond to the prolonged exposure of high levels of circulating hormones and second, given the rapid trafficking of leukocytes through the uteroplacental unit, the potential of such cells to release chemokines could reflect events occurring locally within the uterus. A change in chemokine levels could potentially act as a marker of parturition. We have therefore examined the potential production of IL-8 (an α chemokine), monocyte chemotactic peptide (MCP-1, a β chemokine) and RANTES by peripheral blood cells in culture to examine changes during pregnancy.

2. Materials and methods

2.1. Peripheral blood collection

Ethical approval was obtained from the Local Ethical Committee. Peripheral blood (10 ml) was obtained by venepuncture in sterile Li-heparin blood collecting tubes (Monovette, Sarstedt) from non-pregnant (NP) women (ages 22–47) with regular menstrual cycles who were not using any hormonal forms of contraception and from pregnant women (ages 16–37) experiencing a normal pregnancy either during first trimester (FT), at term in labour (TIL) or at term not in labour (TNIL). Exclusion criteria included clinical evidence of infection. Patient characteristics are shown in Table 1.

2.2. Plasma preparations

The peripheral blood with Li-Heparin anticoagulant was centrifuged at 1500 rpm for 5 min at 4°C and the upper plasma layer removed. The cytokine content was measured by ELISA.

2.3. Whole blood cultures

The blood was stored on ice at 4°C until processing within 20 min of collection. Samples were diluted 1:10 in medium RPMI/1640 (Sigma, Poole)

and cultured overnight at 37°C with 5% CO_2. The culture medium was subsequently analysed for levels of IL-8, MCP-1 and RANTES by ELISA as described below.

2.4. Peripheral blood mononuclear cell preparations

Undiluted whole blood (10 ml) was layered on top of 5 ml of lymphocyte separation medium (Sigma, density 1.077). The tubes were then centrifuged at 2500 rpm for 25 min at 25°C. The lymphocyte layer at the interface was removed and the cells were resuspended in 15 ml PBS. They were then centrifuged at 1500 rpm for 40 min at 25°C. Next, the supernatant was removed and the cells were washed twice in PBS (centrifuged at 1500 rpm for 10 min at 25°C). They were then suspended in 10 ml RPMI/1640 and counted with a haemocytometer. Finally the cell suspension was aliquoted at 10^4 cells per well into culture plates and treated as for the whole blood preparations described above.

2.5. Cellular separation

A PBMC preparation was prepared. The supernatant was removed completely and the pellet resuspended in 2 ml separation buffer (PBS + 5 mM EDTA + 0.5% BSA). The solution was passed through a 30-μm filter then centrifuged at 1500 rpm for 15 min at 20°C. The pellet was resuspended in 100 μl of separation buffer. 25 μl of magnetic activated cell sorter (MACS) anti-CD14 Microbeads were added to the sample which was then

Table 1
Age, parity and smoking status of all the patients studied

Characteristic	Patient group	
	Non-pregnant ($n = 18$)	Pregnant ($n = 6$)
Age	22–47 ($\mu = 30$)	16–37 ($\mu = 26$)
Parity		
P0	12	24
P1	2	9
P2	1	3
P3	2	
P4	1	
Smoker	2	7
Non Smoker	16	29

incubated at 4°C for 15–20 min. The MACS column was pre-washed with 500 μl separation buffer. The cell preparation was then added to the column and washed through with 700 μl buffer ($CD14^-$ fraction). The column was then washed with 1.5 ml of buffer prior to being removed from the magnet. A further 1.2 ml of buffer was added to the column to elute the $CD14^+$ fraction. Both $CD14^+$ and $CD14^-$ fractions were resuspended in 5 ml RPMI/1640 and counted with a haemocytometer. The cells were aliquoted at 10^4 cells per well, cultured and the supernatant analysed for chemokine release.

2.6. ELISA assays for cytokines

The culture supernatant was analysed for levels of IL-8, RANTES and MCP-1 using enzyme-linked immunosorbent assays (ELISA) utilising matched pairs of capture and biotinylated labelled detection antibodies for IL-8 and RANTES (R and D detection system) and a peroxidase labelled Fab fragment of antibody for MCP-1 by the method of Ida et al. (1994) as previously described.

2.7. RANTES ELISA

ELISA plates were passively coated overnight at 4°C with 2 μg/ml of capture antibody (R and D, Oxford diluted in PBS pH 7.2) added at 100 μl per well. After incubation, the plates were washed once in water and coating solution was added (Polyvinyl pyrollidone 2%, BSA 5 mg/ml, EDTA 5 mM, Tris 50 mM) at 100 μl per well. The plates were left for 30 min at room temperature then the coating solution was flicked out. The plates were air dried and stored at 4°C. Prior to use, the plates were washed once in water. The culture supernatant was diluted 1:10 in ELISA buffer (10 mM Tris pH 7.2, preservatives, BSA 2 mg/ml (Sigma A3803), 300 μl 0.5% Phenol Red solution per l, NaCl 9 g/l, EDTA 2 mM, Tween-20 0.05% to final pH of 7.2). Standards (R and D, Oxford) were diluted in ELISA buffer with 500 pg/ml used as the top standard. Then 100 μl of sample and standard were added per well. The plates were then sealed and incubated at 4°C overnight or for at least 3 h at room temperature on an orbital shaker. They were then washed 4 times in washing buffer (0.05% Tween-20, 9 g/l NaCl, 100 mM Tris, pH 7–7.5) and the detection antibody (50 μg/ml) was added at 100 μl/well. The plates were then incubated on a shaker at room temperature for 45 min and washed as above. Following this, 100 μl of diluted (1:100) streptavidin peroxidase (Boerhringer) was added per well at 0.2 U/ml and the plates were incubated for 20 min at room temperature on an orbital shaker. Plates were washed as above and 200 μl of substrate added per well

(1:1:10 of urea-hydrogen peroxide 0.3 g/50 ml of 50 mM sodium acetate buffer pH 6.0: tetramethyl benzidine 2 mg/ml in DMF: 100 mM sodium acetate, pH 6.0). The plates were left for 20 min to develop before quenching with 50 μl 2 NH_2SO_4. Absorption was read at 450 nm within 30 min of quenching. The intra- and inter-assay precisions were both 11% R.S.D. and the limit of detection was 15 pg/ml.

2.8. IL-8 ELISA

The methodology was as for the RANTES ELISA above, with the following modifications. The capture antibody was diluted to 4 μg/ml (R and D, Oxford diluted in PBS 7.2) with 100 μl added per well. The standard used was supplied by Toray Industries with the top standard being 500 pg/ml. The intra- and inter-assay precisions were 9.1% and 22% R.S.D., respectively, and the detection limit of the assay was 15 pg/ml.

2.9. MCP-1 ELISA

MCP-1 was measured using a previously described method (Ida et al., 1994). Briefly, plates were coated overnight at 4°C with 100 μl of MCP-1 capture antibody (Toray, a generous gift, diluted to 4 mg/ml in PBS). They were then washed once in tap water and 100 μl coating solution as above was added for 20 min. Plates were completely air dried and stored with a desiccant at 4°C. The samples were diluted 1:20 in ELISA buffer and added at 100 μl/well. The standards (Toray) were diluted in ELISA buffer and added at 100 μl/well with the top standard being 500 pg/ml. The plates were incubated at 4°C overnight or for a minimum of 3 h at room temperature on an orbital plate shaker. Plates were then washed and 100 μl per well of peroxidase-coupled detection antibody (diluted 1:200 in ELISA buffer) was added. Plates were incubated for 45 min at room temperature on an orbital shaker before washing as above. Substrate was added as above and left for 20 min prior to quenching with 50 μl 2 NH_2SO_4. The plates were analysed as described earlier. The intra-assay precision was 6.3% R.S.D. and inter-assay precision was 8.6% R.S.D. and the detection limit of the assay was 15 pg/ml.

2.10. Cell cultures under different conditions

PBMC were diluted and cultured for 24 h in RPMI/1640 supplemented with 10^{-6} M of progesterone. The medium was then removed and replaced with identical medium for a further 24 h. After this period the medium was removed and replaced with RPMI/1640 supplemented with 10^{-6} M RU486

(Roussel-Uclaf) for a final 24 h. The culture supernatants from each 24-h period were then analysed for chemokine release by ELISA.

2.11. Statistics

Statistical analysis was performed using ANOVA multiple variate analysis in StatView. Assignment of difference to individual groups was achieved using Fischers PLSD. Results are expressed as mean $\pm$ S.E.M.

3. Results

3.1. Plasma chemokine levels

IL-8 was not detectable in peripheral plasma. MCP-1 was detectable in pregnant (155 ± 6.7 pg/ml) and non-pregnant samples (187 ± 7.17 pg/ml). RANTES was present at high levels in both pregnant (20.1 ± 2.2 ng/ml) and non-pregnant samples (23 ± 4 ng/ml). There was no statistical difference in levels between pregnant and non-pregnant plasma levels of any of the chemokines measured.

3.2. Peripheral blood cultured samples

IL-8 was present in culture supernatants from non-pregnant and pregnant samples at levels of 356 ± 96 and 490 ± 182 pg/ml, respectively. RANTES was detectable in culture supernatants from samples taken from non-pregnant and pregnant women at levels of 736 ± 251 and 1222 ± 247 pg/ml, respectively. There was no statistically significant difference in levels of IL-8 or RANTES released by culture supernatants from pregnant and non-pregnant samples.

Significantly ($P < 0.001$) higher levels of MCP-1 were released from the culture supernatants of peripheral blood samples taken from pregnant (4325 ± 857 pg/ml) compared with non-pregnant (1739 ± 391 pg/ml) women. Moreover, there was statistically more MCP-1 released from women during the third trimester compared with those during first trimester or who were not pregnant ($P < 0.001$). There was more MCP-1 produced in samples taken from women at term not in labour compared with those in labour although this was not statistically significant (Figs. 1 and 2).

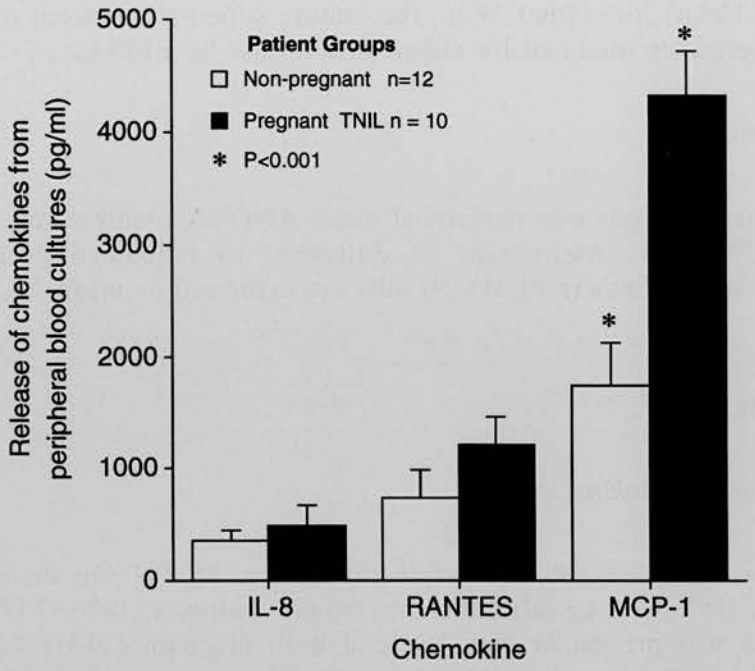

Fig. 1. The difference in release of the chemokines IL-8, MCP-1 and RANTES from cultured peripheral blood. Significantly more MCP-1 ($P < 0.001$) is released from pregnant compared (TNIL, $n = 17$) with non-pregnant samples ($n = 12$). There was no significant difference in release of either IL-8 or RANTES between pregnant and non-pregnant samples.

3.3. PBMC preparations

PBMC preparations released IL-8, MCP-1 and RANTES. In the case of RANTES and IL-8 more chemokine was released from the PBMC preparation than from the whole blood cultures, however, in the case of MCP-1, the converse was true (Table 2). There was no significant difference between pregnant and non-pregnant PBMC preparations in their capacity to release either IL-8 or RANTES. However, PBMC preparations from pregnant compared with non-pregnant released significantly more MCP-1 ($P < 0.05$) and this difference was maintained over 3 days in culture (Fig. 3).

3.4. Separated cells

IL-8 and MCP-1 were released predominantly by the $CD14^+$ cells, whereas RANTES was secreted mainly by the $CD14^-$ cells. There was no statistical difference in production of any of these chemokines between pregnant and non-pregnant samples although there was a tendency towards

Table 2
Release of MCP-1, IL-8 and RANTES from peripheral blood cultures compared with that from peripheral blood mononuclear cell preparations (PBMC) prepared from the same blood sample

Sample type (24 h culture)	Chemokine, pg/ml (S.E.M.)					
	MCP-1		IL-8		RANTES	
	Pregnant ($n=6$)	Non-pregnant ($n=6$)	Pregnant ($n=6$)	Non-pregnant ($n=6$)	Pregnant ($n=6$)	Non-pregnant ($n=6$)
PBMC	2665 (214)*	1292 (211)*	4319 (362)	2105 (401)	5048 (1171)	3161 (389)
Peripheral blood	8697 (896)**	2652 (278)**	185 (69)	209 (32)	1820 (390)	1472 (121)

Significantly more MCP-1 was produced by both the cultured ($P<0.001$) and the PBMC preparations ($P<0.005$) from pregnant women compared with those not pregnant.
* $P<0.05$.
** $P<0.001$.

more IL-8 and MCP-1 being secreted by separated cells from pregnant blood with the converse being the case with RANTES production (Table 3).

3.5. Influence of culture conditions on chemokine release

Culture of PBMC preparations under conditions of progesterone and medroxyprogesterone acetate (MPA) supplementation (10^{-6} M) followed by progesterone withdrawal by addition of the antigestogen RU486 (10^{-6} M) did not affect the release of any of the chemokines being measured.

4. Discussion

This study demonstrates that significantly more MCP-1 is released from peripheral blood cultures from pregnant compared with non-pregnant

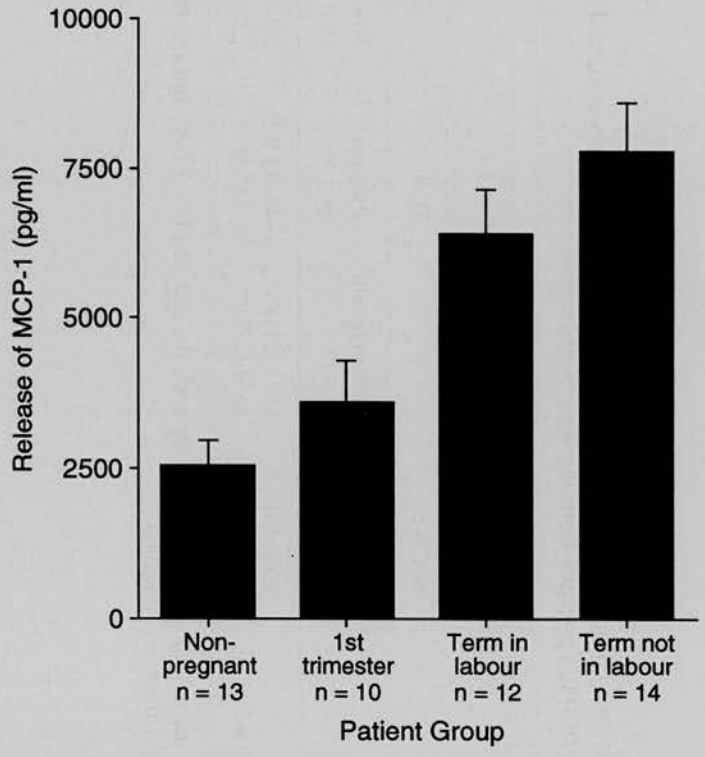

Fig. 2. Differential release of MCP-1 through gestation from peripheral blood in a separate study. Significantly higher ($P < 0.001$) levels of MCP-1 were released from peripheral blood cultures taken at the end of pregnancy (TNIL, $n = 14$; TIL $n = 12$) compared with those from first trimester ($n = 10$) or from women who were not pregnant ($n = 13$).

Table 3
The production of MCP-1, IL-8 and RANTES from $CD14^+$ and $CD14^-$ cells

Cell Population	Chemokine, pg/ml/10^4 cells (S.E.M.)					
	MCP-1		IL-8		RANTES	
	Pregnant ($n = 3$)	Non-pregnant ($n = 3$)	Pregnant (n = 3)	Non-pregnant ($n = 3$)	Pregnant ($n = 3$)	Non-pregnant ($n = 3$)
$CD14^+$	726 (219)	210 (184)	2573 (791)	1279 (942)	293 (86)	225 (55)
$CD14^-$	70 (26)	17 (2)	108 (20)	197 (51)	976 (230)	1378 (535)

MCP-1/ and IL-8 are released predominantly by the $CD14^+$ cells whereas RANTES is released predominantly by the $CD14^-$ cells. There was no statistical difference in release of any of the chemokines measured between pregnant and non-pregnant although this may be due to the small sample size.

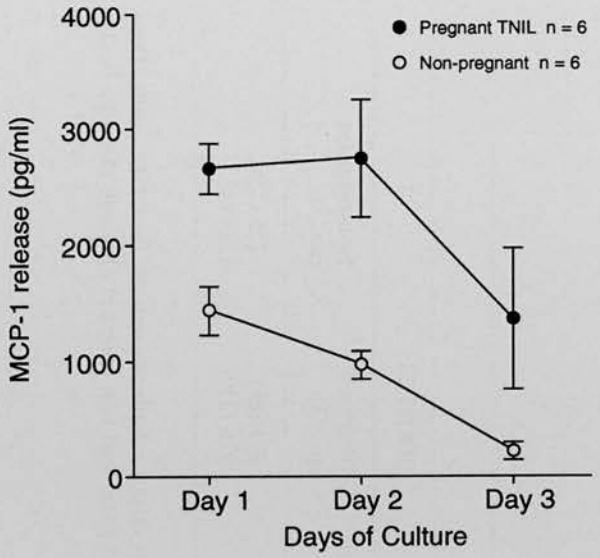

Fig. 3. Release of MCP-1 from PBMC preparations over 3 days culture with media being replaced and assayed every 24 h. Even after three days culture, more MCP-1 is released from PBMC preparations from pregnant (TNIL, $n = 6$) compared with non-pregnant women ($n = 6$).

women and that release of MCP-1 from such cultures is significantly higher at the end of pregnancy compared with first trimester. The differential release of MCP-1 is maintained in the PBMC preparations and from the separated cells it is evident that the majority of the MCP-1 is produced by the $CD14^+$ cells. There was no significant difference in either IL-8 or RANTES production from peripheral blood preparations or from the separated cells between pregnant and non-pregnant samples.

In other tissues, such as the breast cancer cell line T47D and chorio-decidua (Kelly et al., 1997, unpublished results), MCP-1 release is inhibited by progesterone. Given that pregnancy is a progesterone rich environment it could be argued that by removing peripheral blood from this hormonal milieu there could be a rebound secretion of MCP-1. However, the experimental data presented discounts this proposition on several counts. Firstly, if the above hypothesis were valid, one would expect that on culturing the PBMC preparations over 3 days any progesterone withdrawal effect would disappear and the production of MCP-1 from cultures between pregnant and non-pregnant women would become the same. This did not occur and the differential secretion was maintained for the duration of culture. Secondly, the progesterone withdrawal experiments did not stimulate a rise in MCP-1 secretion. Thirdly, IL-8 production is also inhibited by progesterone

(Kelly et al., 1994) and as such were this a factor in secretion, one would expect there to be a rise in IL-8 production analogous to that observed with MCP-1. This was not observed. Fourthly, tissues such as the endometrium and chorio-decidua have definite evidence of a progesterone receptor, whereas there is no conclusive evidence as to whether peripheral lymphocytes do (Szekeres-Bartho, 1995) or do not (Mansour et al., 1994; Schust et al., 1996) possess such a receptor.

The maternal adaptation to pregnancy involves wide ranging changes in peripheral blood. An increase in leucocyte count occurs which is due mainly to an increase in neutrophil count (Pitkin and Witte, 1979). There is conflicting evidence whether normal pregnancy influences the distribution of mononuclear cells with Damm et al. (1991) finding no difference and Tsakonas et al. (1995) finding that there was a significant increase in circulating monocytes with gestation. A second argument which could therefore be used to explain the above findings is that, owing to the leukocytosis of pregnancy, the increased production of MCP-1 is simply a function of there being more cells in the pregnant blood cultures. However, when the peripheral blood was separated into either the PBMC preparations or the $CD14^+$ (a monocyte specific cell surface marker) or $CD14^-$ cells, the number of cells was counted prior to being aliquoted and cultured overnight. When chemokine production was subsequently measured, the level could be corrected for cell number. Under these circumstances, consistently more MCP-1 was produced by pregnant compared with non-pregnant samples.

MCP-1 is a 76 amino acid β chemokine which is secreted by a wide range of cell types, including monocytes (Yoshimura et al., 1989a), macrophages (Brieland et al., 1993), lymphocytes (Yoshimura et al., 1989b) and endothelial cells (Sica et al., 1990). It was initially so named due to its function as a monocyte chemoattractant and activator. Recently, a number of other functions have been ascribed to this chemokine including that of an angiogenic factor and it is increasingly viewed as a key chemokine in orchestrating inflammatory responses. A role has been proposed for MCP-1 in ovulation (Arici et al., 1997) but whether it has a function in pregnancy is not known.

This study demonstrates that there is an increased production of MCP-1 from pregnant monocytes compared with non-pregnant monocytes. This suggests that the monocytes in pregnancy are intrinsically different from those in non-pregnant women. That the higher levels of MCP-1 are a consequence of de novo synthesis is corroborated by there being only low levels of MCP-1 present in peripheral plasma and no difference between pregnant and non-pregnant samples. Moreover RT-PCR data (unpublished results) show MCP-1 message rising only after 3–4 h culture. The statisti-

cally significant rise in MCP-1 production during pregnancy and the further increase in production during labour are intriguing findings suggesting that MCP-1 may play a role which is more important as pregnancy advances. Given the rapid trafficking of leukocytes through the uteroplacental unit, particularly during the third trimester, it is tempting to speculate that the potential of peripheral blood leukocytes to release chemokines could reflect events occurring locally within the uterus with a change in chemokine levels potentially acting as a marker of uterine function.

In conclusion, this study demonstrates that significantly higher levels of MCP-1 are released from peripheral blood cultures from pregnant compared with non-pregnant women. Separation of the peripheral blood demonstrates that the MCP-1 is secreted predominantly by the monocytes.

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Seminal plasma components stimulate interleukin-8 and interleukin-10 release

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Human seminal plasma has potent anti-inflammatory properties which are thought to confer a survival advantage to the spermatozoa within the hostile female genital tract. In contrast, a profound pro-inflammatory leukocytosis has been observed post-coitus in animals and humans. Whether components of seminal plasma are involved in initiating this leukocytic reaction is not known. This study investigated the effect of human seminal plasma, a seminal plasma fraction and its principal constituent prostaglandins, prostaglandin E_2 (PGE_2) and 19-hydroxy PGE, on the release of the pro-inflammatory neutrophil chemotactic factor interleukin-8 (IL-8) and the anti-inflammatory cytokines interleukin-10 (IL-10) and secretory leukocyte protease inhibitor (SLPI). The tissues studied were non-pregnant cervical explants, peripheral blood and the monocyte cell line U937. Seminal plasma fraction (SPF) significantly ($P < 0.05$) stimulated release of IL-8 and inhibited release of SLPI from non-pregnant cervical explants. SPF, PGE_2 and 19-hydroxy PGE significantly ($P < 0.005$) stimulated IL-8 release from peripheral blood and U937 cells. 19-hydroxy PGE was significantly ($P < 0.005$) more effective than PGE_2 in stimulating IL-8 release. Seminal plasma, SPF and PGE_2 significantly ($P < 0.05$) stimulated IL-10 release from U937 cells. 19-hydroxy PGE stimulated IL-10 release from U937 cells but this failed to reach significance. Release of IL-10 by cervical explants and SLPI by peripheral blood and U937 cells were below the detection limit of the assays employed. We suggest that the anti- and pro-inflammatory immune responses which seminal plasma induces might act in combination initially to promote sperm survival and then to facilitate their removal from the female genital tract.

Key words: human seminal plasma/19-hydroxprostaglandin/interleukins/prostaglandin E_2/secretory leukocyte protease inhibitor

Introduction

Human seminal plasma has powerful immunosuppressive properties containing high concentrations of the soluble p55 tumour necrosis factor-α (TNF-α) receptor (Liabakk *et al.*, 1993), receptors for the Fc portion of γ-globulin, transforming growth factorβ (TGFβ) (Nocera and Chu, 1993), spermine (Evans *et al.*, 1995) and complement inhibitors (Kelly, 1995). However, it is the prostaglandins prostaglandin E_2 (PGE_2) and 19-hydroxyprostaglandin E (19-hydroxy PGE), present in mM concentrations within semen (Taylor and Kelly, 1974; Templeton *et al.*, 1978) which are thought to be the principal effectors of the immunosuppression. They are potent stimulators of cAMP, thus inhibiting lymphocyte proliferation and natural killer cell activity, and are likely to modify cytokine release from antigen presenting cells (Kelly, 1995). In addition, human seminal plasma and its component prostaglandins stimulate release of the immunosuppressive T-helper-2 (Th-2) cytokine interleukin-10 (IL-10) and inhibit the release of the Th-1 cytokine interleukin-12 (IL-12) from lipopolysaccharide-stimulated whole blood (Kelly *et al.*, 1997). The effect of PGE_2 and the subsequent cytokine shift would be to induce an anergic response in T-cells (Mannie *et al.*, 1995; Groux *et al.*, 1996) and may therefore have implications for non-recognition of both sperm antigen and viral pathogen.

In contrast, coitus and ejaculation of semen deposits millions of potentially pro-inflammatory immunogenic spermatozoa into the vagina in vaginal inseminators, such as humans and into the uterus in intra-uterine inseminators, such as the horse. Since in most animals only a handful of spermatozoa are required for fertilization, then the majority of spermatozoa are rendered redundant and need to be removed from the reproductive tract. How this is achieved is not well understood but there is good evidence in horses (Kotilainen *et al.*, 1994), rabbits (Tyler, 1977), goats (Mattner, 1968), cattle (Howe and Black, 1963) and mouse (De *et al.*, 1991; Parr and Parr, 1991) that a profound pro-inflammatory leukocytosis develops within the uterus and cervix post-coitus. The invading cells, which are predominately neutrophils, are then thought to phagocytose those spermatozoa not destined to be involved in fertilization. A similar leukocytic reaction is thought to occur post-coitally in humans (Thompson *et al.*, 1992) but whether this performs the same physiological function remains to be established. In addition, the factor or factors which initiate the leukocytosis are unknown and pure spermatozoa, seminal plasma stripped of spermatozoa and a combination of both have all been implicated (Howe and Black, 1963; Mattner, 1968). Finally, heat stable enhancers of neutrophil chemotaxis identified in human semen have not been fully characterized and their role,

if any, in initiating leukocytosis remains uncertain (Clarke and Klebanoff, 1976).

The host responses elicited by seminal plasma are therefore highly complex and potentially conflicting. This study initially investigated the effect of seminal plasma extracts on cytokine release by human cervical explants. The pro-inflammatory cytokine studied was the neutrophil chemotactic factor interleukin-8 (IL-8) and the anti-inflammatory cytokines were secretory leukocyte protease inhibitor (SLPI), an inhibitor of neutrophil function and IL-10. These responses were further characterized by examining the effect of whole seminal plasma, a seminal plasma extract, PGE_2 and 19-hydroxy PGE on release of these mediators by peripheral blood and the monocyte cell line U937.

Materials and methods

Tissue collection

Pooled human seminal plasma was obtained from healthy young men involved in the ongoing semen donor programme (more than 20 men). Cervical biopsies, ~20–35 mg in weight, 15–20 mm in length and 2–3 mm in diameter, were obtained from healthy, non-pregnant women (n = 15, ages 29–45) with regular menstrual cycles undergoing a hysterectomy for a non-malignant condition. These were taken from the anterior lip of the cervix immediately post-hysterectomy. Peripheral blood was taken using a Li-heparin tube (Monovette, Sarstedt, Amsterdam, Holland) from healthy non-pregnant women (n = 6, aged 20–35 years) with normal menstrual cycles not using any hormonal form of contraception. The women were at various stages of the menstrual cycle. Ethical approval was obtained for these studies from the Local Ethics Committee.

Preparation of seminal plasma fraction (SPF)

All reagents were from Sigma (Poole, UK), unless otherwise stated. Human seminal plasma (700 ml) was treated with ethanol (1000 ml) and centrifuged at 4°C for 20 min at 1500 *g*. The supernatant was evaporated at 23°C to a final volume of 450 ml then passed through ENV + extraction columns (International Sorbent Technology Ltd., YstradMynach, Hengoed, UK) which had previously been washed with 1:1 ethanol/hexfluoropropanol (HFP; Aldrich, Poole, UK). Lipid was eluted with 1:1 ethanol/HFP and the liquid evaporated at 23°C to 10 ml. Chloroform (15 ml; Merck, Poole, UK) was added to leach the residue and the chloroform layer evaporated and redissolved in ethanol (4 ml) to give the seminal plasma fraction. PGE_2 and 19-hydroxy PGE were assayed as described below. The seminal plasma fraction contained 165 µg/ml PGE_2 and 705 µg/ml 19-hydroxy PGE. The lipid extraction of the seminal plasma removes the majority of the polyamines such as spermine and spermidine which may have cytotoxic effects (Allen and Roberts, 1986).

Tissue culture

Cervical biopsies

Cervical biopsies were placed immediately in Roswell Park Memorial Institute (RPMI) 1640 medium at 4°C for transport. Explants were washed in phosphate-buffered saline (PBS), dissected into small pieces 1–2 mm^3 then placed in 1 ml complete medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS); 2 mM L-glutamine; 50 µg/ml streptomycin, Gibco, Paisley, UK; 20 µg/ml gentamicin and 50 IU/ml penicillin) in a 24-well plate (Costar, High Wycombe, UK). Explants were treated in quadruplicate with seminal plasma fraction diluted 1:2000 then cultured for 24 h at 37°C in 95% air and 5% carbon dioxide under humid conditions. The harvested media were frozen at –20°C until analysed.

Peripheral blood

Peripheral blood was diluted immediately 1:10 in RPMI 1640 prior to plating out at 1 ml/well and culturing for 24 h at 37°C in 5% CO_2. Media were stored at –20°C until analysis.

U937 cells

U937 cells, a human pro-monocytic cell line, were plated out at 2×10^5 cells/ml in complete media, treated with whole seminal plasma (1–0.0001%), seminal plasma fraction (1–0.0001%), PGE_2 (10^{-6} to 10^{-10} M) and 19-hydroxy PGE (Cayman Chemicals, Ann. Arbor, Michigan, USA; 10^{-6} to 10^{-10} M) at the time of addition. In addition, cells were treated with 10^{-7} M phorbol ester (PMA) for the IL-10 experiments because no IL-10 was released from cells without PMA treatment. U937 cells were cultured for 24 h at 37°C in 5% CO_2 under humid conditions and the media were stored at –20°C until analysis.

Cytokine assays

IL-8 assay

IL-8 was measured by enzyme-linked immunosorbent assay (ELISA) using matched pairs of capture and biotinylated-labelled detection antibodies for IL-8 (R&D Detection Systems, Oxford, UK) as described in detail previously (Denison *et al.*, 1997). The highest concentration of standard was 500 pg/ml, the intra- and inter-assay precision was 9.1% relative standard deviations (r.s.d) and 11% r.s.d. respectively; the detection limit of the assay was 15 pg/ml.

Secretory leukocyte protease inhibitor (SLPI) assay

SLPI was measured by a competitive ELISA. Plates (96-well; Nunc Maxi Sorp; Gibco) were coated with recombinant SLPI (R&D Detection Systems; 0.025 µg/ml in PBS/1% carbonate buffer; 400 mM $NaHCO_3$, 40 mM Na_2CO_3 in 500 ml distilled H_2O, pH 9.6) at 300 µl/well for 60 min at 23°C, blocked with milk powder (0.1 mg/ml in distilled water) at 400 µl/well for 30 min at 23°C then washed (150 mM NaCl, 100 mM Tris, 0.05% Tween-20 to final pH of 7–7.5). ELISA assay buffer (150 mM NaCl; 100 mM Tris; 50 mM Phenol Red solution; 2 mM EDTA; 1 mM 2-methylisothiazolone, Boehringer Mannheim, Lewes, UK; 1 mM bromonitrodioxane, Boehringer Mannheim; 2 mg/ml bovine serum albumin; 0.05% Tween-20 to final pH of 7.2) was added for the non-specific binding (NSB) in singleton at 250 µl/well. Recombinant standards (R&D Detection Systems; highest concentration 50 ng/ml) and samples were added at 200 µl/well and anti-SLPI (polyclonal anti-SLPI, R&D Detection Systems; 2 µg/ml in ELISA buffer) added at 50 µl/well to all wells except the NSB. Plates were incubated for 120 min at 23°C on an orbital shaker then washed and anti-sheep/goat immunoglobulin (Ig)G-peroxidase *Fab* fragments (Boehringer Mannheim; diluted 1:500 in ELISA buffer) added at 100 µl/well. Finally, plates were incubated for 120 min at 23°C on an orbital shaker, washed and substrate (0.3 g/l urea-hydrogen peroxide; 0.1 g/l tetramethyl benzidine in 100 mM sodium acetate, pH 6.0) added at 200 µl/well for 10 min prior to quenching with 2 N H_2SO_4 at 50 µl/well. Absorption was read at 450 nm within 30 min of quenching. The correlation between the results from this assay and those obtained by a commercial assay (n = 10; R&D Detection Systems) was 0.85. The intra- and inter-assay precision was 9.2% r.s.d and 10.1% r.s.d respectively, and the detection limit of the assay was 9.8 pg/ml.

IL-10 assay

All dilutions were in 10% FCS in PBS unless otherwise stated. Plates were coated with capture antibody (Pharmingen, San Diego, USA; 200 ng/ml in PBS) added at 100 µl/well for 60 min at 23°C then blocked (10% FCS in PBS) at 300 µl/well for a further 60 min at

23°C. The plates were then washed and both samples and recombinant standards (Pharmingen) added at 100 µl/well with the top standard being 500 pg/ml. Plates were then incubated on an orbital shaker for 60 min, washed, detection antibody (Pharmingen; 125 ng/ml) added at 100 µl/well and incubated for 60 min at 23°C. After a further wash, polyperoxidase (CLB laboratories, Amsterdam, Holland; 1 ng/ml in ELISA buffer without Tween) was added at 100 µl/well for 30 min. Finally, plates were washed and read as for SLPI. The intra- and inter-assay precision was 6.4% r.s.d and 10.1% r.s.d respectively, and the detection limit of the assay was 15 pg/ml.

PGE_2 ELISA

Samples were treated 1:1 with methyloximating solution (0.1 M methoxyamine hydrochloride in 10% ethanol diluted in 1 M sodium acetate, pH 5.6) overnight at 4°C. Plates (Costar Amine-binding plates, Paisley, UK) were coated with donkey anti-rabbit (DAR serum; Scottish Antibody Production Unit, Carluke, UK) using the direct γ-globulin binding procedure. Briefly, they were coated with rabbit IgG (1 mg/ml diluted in PBS/1% carbonate buffer, pH 9.6) at 200 µl/well for 16 h at 4°C, the solution flicked out and blocking solution (50 mM glycine; bovine serum albumin 10 mg/ml Sigma-A7888 in distilled H_2O) added at 250 µl/well for 120 min at 23°C. They were then washed, DAR serum added at 150 µl/well, incubated for 16 h at 4°C then washed, air-dried and stored with a desiccant at 4°C.

The assay used a PGE–biotin link as a pro-label. To prepare the link, 0.06 M synthetic PGE_2 (kind gift of Applied Therapeutics, Paisley, UK) was added to 320 µl dry dimethylformamide (DMF; Aldrich, Poole, UK), the solution cooled to 4°C and 6 µl tributylamine (Aldrich) and 3 µl butylchloroformate (Aldrich) were added with stirring for 30 min at 4°C. Next 0.05 M biocytin, in 300 µl 1:1 DMF/distilled H_2O was added and the vial left at 4°C for 30 min then at 23°C for 30 min. Finally, the solution was ether extracted and purified by reverse phase chromatography. Rabbit anti-sera were raised against PGE_2 complexed to keyhole limpet haemocyanin and have been previously characterized (Kelly *et al.*, 1989). Samples and synthetic standards (Applied Therapeutics, Paisley, UK; highest concentration 5120 pg/ml) were added in duplicate at 100 µl/well, link (1 in 1.5×10^6) at 50 µl/well and anti-sera (1 in 50 000) at 50 µl/well to all wells except the NSB. The final concentration of methyloximating solution in standards and samples was 12.5%. Plates were incubated at 4°C for 16 h, washed and 100 µl/well of 0.2 IU/ml streptavidin peroxidase (Boerhringer Mannheim) added. The plates were then incubated for 20 min at 23°C on an orbital shaker, washed, substrate added and plates read as for SLPI. The intra- and inter-assay coefficients of variation were 7.8% and 15.0% respectively and the ED_{50} was 195 pg/ml.

19-hydroxy PGE assay

The plates and anti-sera were prepared as described above. 19-hydroxy PGE standards containing equal amounts of 19-hydroxy PGE_1 and 19-hydroxy PGE_2 were prepared as follows. Human seminal plasma (65 ml) was centrifuged at 4°C for 15 min at 1000 *g*, mixed with acetonitrile (Merck; 65 ml), centrifuged at 4°C for 15 min at 2000 *g* prior to freeze-drying the supernatant overnight. The residue was leached with methanol, evaporated, added to a silica column and the prostaglandins (PGE_2 and 19-hydroxy PGE) were eluted with increasing concentrations of methanol (Merck) in methyl formate (Merck). The fractions were analysed by thin layer chromatography and that corresponding to the 19-hydroxy PGE fraction was quantified, using crystalline synthetic PGE_2 as a standard, by treatment with 0.05 M NaOH and measurement of UV absorption at 280 nm in ethanol. To prepare the link, ~1 mg 19-hydroxy PGE was added to 200 µl dry DMF, the solution cooled to 4°C and 3 µl tributylamine and 1.5 µl butylchloroformate were added with stirring for 30 min at 4°C. Finally, 12 mM Pro-Gly-Tyr-Biotin (synthesized in house) in 300 µl 1:1 DMF/distilled H_2O was added and the solution extracted as described above. Standards (5120–78 pg/ml), link (1:20 000) and anti-sera (1:20 000) were diluted in ELISA buffer without Tween and the assay set up as for PGE_2. The intra-assay coefficient of variation was 8.46% and the ED_{50} was 196 pg/ml.

Statistical analysis

Statistical analysis of the data was performed using Statview 4.1 (Abacus Inc, Berkeley, CA, USA). The data were normally distributed and are expressed as pg/ml (mean ± SEM) with a statistically significant difference defined as $P < 0.05$.

Results

Cervical explants

Seminal plasma fraction significantly stimulated ($P < 0.005$) IL-8 and inhibited ($P < 0.005$) SLPI release (Figure 1; n = 15 different women, treatments in quadruplicate). IL-10 release was below the detection limit of the assay employed. Pure seminal plasma was not used to treat cervical explants due to possible immunological interactions between tissues and cytotoxic effects. Cervical biopsies are scarce and difficult to obtain. Hence the experiments performed were limited and insufficient explants were obtained to treat with PGE_2 and 19-hydroxy PGE.

Peripheral blood data

Whole seminal plasma significantly ($P < 0.05$) inhibited IL-8 release at concentrations of >0.1%. Maximum inhibition of IL-8 release down to 8% of control values ($P < 0.005$) was achieved when seminal plasma was added at 1%. In contrast,

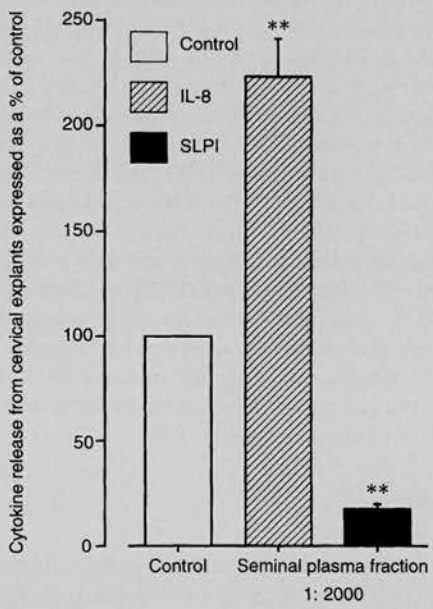

Figure 1. Effect of seminal plasma fraction on interleukin (IL)-8 and secretory leukocyte protease inhibitor (SLPI) release from non-pregnant cervical explants (n = 15 different women, treatments in quadruplicate). IL-8 release was significantly stimulated and SLPI release significantly inhibited by seminal plasma fraction at 1:2000. Values are expressed as cytokine release as percentage of control ± SEM. **Results are significantly different ($P < 0.005$).

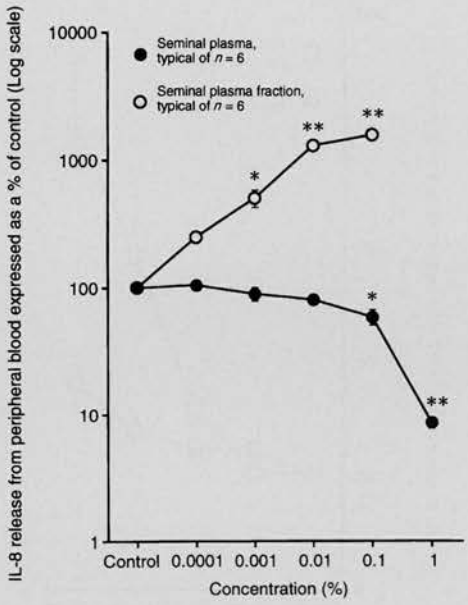

Figure 2. The effect of seminal plasma and seminal plasma fraction on interleukin (IL)-8 release from peripheral blood (typical of six separate experiments, treatments in quadruplicate). IL-8 release was significantly stimulated by the seminal plasma fraction whereas seminal plasma significantly inhibited IL-8 release. Values are expressed as the log of IL-8 release as percentage of control ± SEM. Significantly different from control; $*P < 0.05$; $**P < 0.005$.

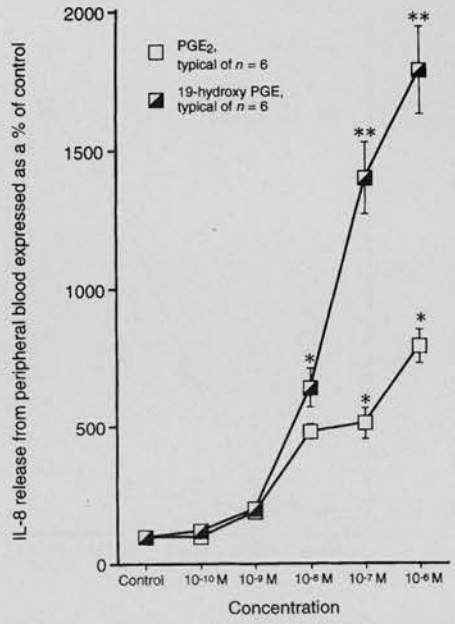

Figure 3. The effect of prostaglandin E_2 (PGE_2) and 19-hydroxy PGE on interleukin (IL)-8 release from peripheral blood (typical of six separate experiments, treatments in quadruplicate). IL-8 release was significantly stimulated by both PGE_2 and 19-hydroxy PGE. At concentrations of $>10^{-8}$ M, 19-hydroxy PGE was significantly ($P < 0.005$) more effective in stimulating IL-8 release than PGE_2. Values are expressed as IL-8 release as percentage of control ± SEM. Significance of difference from control; $*P < 0.05$; $**P < 0.005$.

seminal plasma fraction significantly ($P < 0.05$) stimulated IL-8 release when used at 0.001% with a 10-fold stimulation achieved when peripheral blood was cultured with 0.1% seminal plasma fraction A ($P < 0.005$; Figure 2; $n = 6$ separate experiments, treatments in quadruplicate). Both PGE_2 and 19-hydroxy PGE significantly stimulated ($P < 0.05$; $P < 0.005$ respectively) IL-8 release with maximal stimulation achieved at 10^{-6} M. 19-hydroxy PGE was significantly ($P < 0.05$) more effective in stimulating IL-8 release than PGE_2 at concentrations of $>10^{-8}$ M (Figure 3; $n = 6$ separate experiments, treatments in quadruplicate). SLPI release was below the detection limit of the assay used.

U937 monocyte cell line

Seminal plasma and seminal plasma fraction significantly (both $P < 0.005$) stimulated IL-8 release from U937 cells cultured without PMA by comparable amounts with maximum stimulation at 0.1% (Figure 4; $n = 6$ separate experiments, treatments in quadruplicate). PGE_2 significantly ($P < 0.05$) stimulated IL-8 release from U937 cells with maximum stimulation at 10^{-6} M. 19-hydroxy PGE also stimulated ($P < 0.005$) IL-8 release and as demonstrated in peripheral blood it was significantly ($P < 0.05$) more effective in inducing IL-8 release than PGE_2 (Figure 5; $n = 6$ separate experiments, treatments in quadruplicate). SLPI release was not detectable from U937 cells.

Seminal plasma and seminal plasma fraction significantly stimulated ($P < 0.05$) IL-10 release from U937 cells with maximum stimulation at 0.1 and 0.01% respectively, for seminal plasma and fraction (Figure 6; $n = 6$ separate experiments, treatments in quadruplicate). Seminal plasma

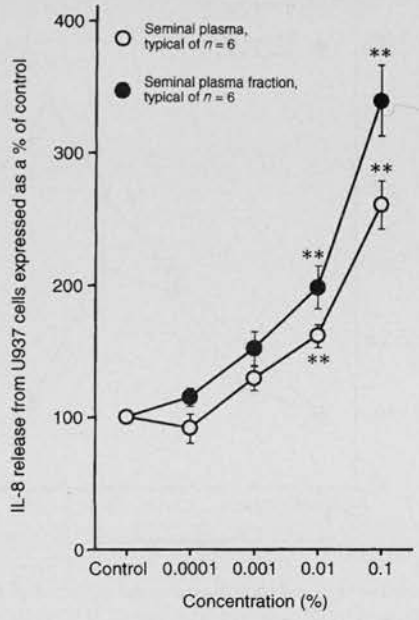

Figure 4. The effect of seminal plasma and seminal plasma fraction on interleukin (IL)-8 release from U937 cells (typical of six separate experiments, treatments in quadruplicate). IL-8 release was significantly stimulated by both seminal plasma and seminal plasma fraction. Values are expressed as IL-8 release as a percentage of control ± SEM. Significance of difference from control; $**P < 0.005$.

fraction inhibited secretion of IL-10 back to control values when added at 0.1%. PGE_2 significantly ($P < 0.05$) stimulated IL-10 release from U937 cells with maximum stimulation at

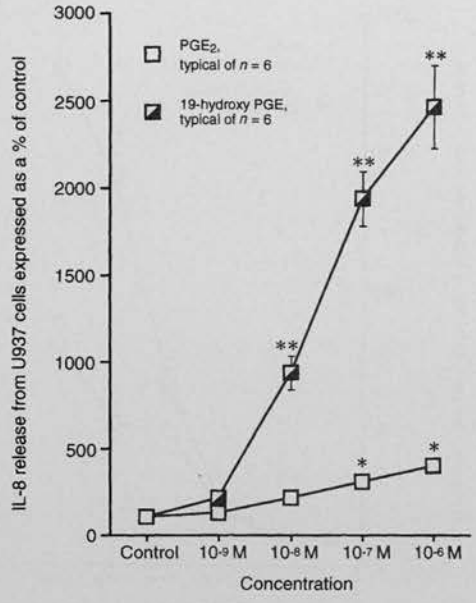

Figure 5. The effect of prostaglandin E_2 (PGE_2) and 19-hydroxy PGE on interleukin (IL)-8 release from U937 cells (typical of six separate experiments, treatments in quadruplicate). IL-8 release was significantly stimulated by both PGE_2 and 19-hydroxy PGE. 19-hydroxy PGE was significantly ($P < 0.005$) more effective in stimulating IL-8 release than PGE_2. Values are expressed as IL-8 release as a percentage of control ± SEM. Significance of difference from control; $*P < 0.05$; $**P < 0.005$.

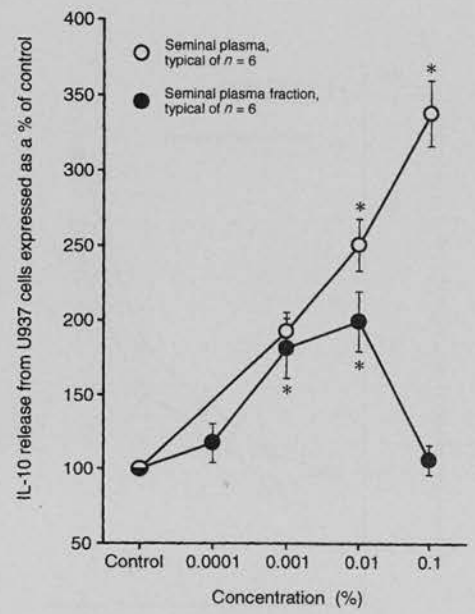

Figure 6. The effect of seminal plasma and seminal plasma fraction on interleukin (IL)-10 release from U937 cells (typical of $n = 6$ separate experiments, treatments in quadruplicate). Seminal plasma significantly ($P < 0.05$) stimulated IL-10 release from U937 cells. Seminal plasma fraction significantly ($P < 0.05$) stimulated IL-10 release at 0.001 and 0.01%, but inhibited its release back to control values at 0.1%. Values are expressed as IL-10 release as a percentage of control ± SEM. Significance of difference from control; $*P < 0.05$.

10^{-6} M. Although 19-hydroxy PGE also stimulated IL-10 release this failed to reach significance (Figure 7; $n = 6$ separate experiments, treatments in quadruplicate).

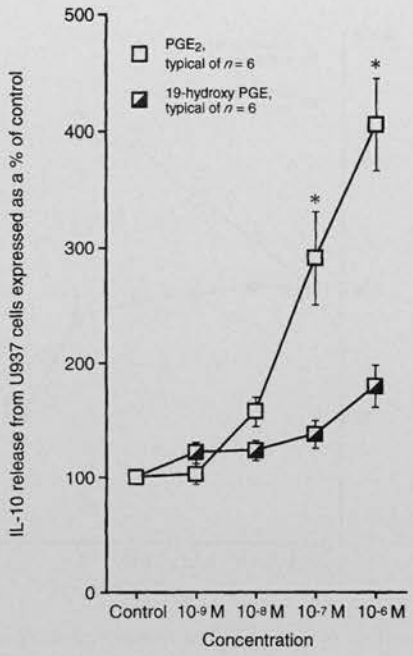

Figure 7. The effect of prostaglandin E_2 (PGE_2) and 19-hydroxy PGE on interleukin (IL)-10 release from U937 cells (typical of six separate experiments, treatments in quadruplicate). PGE_2 significantly stimulated IL-10 release from U937 cells. 19-hydroxy PGE stimulated IL-10 release but this failed to reach significance. Values are expressed as IL-10 release as a percentage of control ± SEM. Significance of difference from control; $*P < 0.05$.

Discussion

In this study, it is reported for the first time that seminal plasma and its principal constituent prostaglandins, PGE_2 and 19-hydroxy PGE stimulated IL-8 release from peripheral blood and the monocyte cell line U937. Moreover, 19-hydroxy PGE was significantly more potent than PGE_2 in inducing IL-8 release. In addition, seminal plasma extracts were effective in stimulating IL-8 release from cultured cervical explants. It was also demonstrated that IL-10 release was stimulated in U937 cells by seminal plasma, seminal plasma fractions, PGE and 19-hydroxy PGE, although the latter was not significant. This confirms previous data (Kelly *et al.*, 1997) which showed stimulation of IL-10 release from lipopolysaccharide-stimulated whole blood under similar conditions.

Human seminal plasma contains very high concentrations of PGE_2 (Samuelsson, 1963) and 19-hydroxy PGE (Taylor and Kelly, 1974) which are mainly synthesized *de novo* within the seminal vesicles (Pourian *et al.*, 1995). Seminal plasma PGE_2 acts primarily to suppress the female genital tract immune system thus conferring a survival advantage to spermatozoa within the hostile female reproductive tract (Kelly and Critchley, 1997). In addition to altering the ratio of IL-10 to IL-12 release, it also reduces expression of the T-cell co-stimulator ligands B7-1 and B7-2 required for normal T-cells activation and antigen recognition (Iglesias *et al.*, 1997). In contrast, in other situations, PGE_2 can act as a potent pro-inflammatory agent by virtue of its vasodilatory properties. Within human skin it synergizes with the neutrophil chemotactic and activating agent IL-8 to promote neutrophil chemotaxis and activation (Colditz, 1990) and in IL-1α stimulated synovial

fibroblasts PGE_2 stimulates IL-8 release (Agro *et al.*, 1996). Whether seminal plasma PGE_2 is capable of inducing a pro-inflammatory response either within semen or the female genital tract is not known. Elevated concentrations of IL-8 within seminal plasma are, however, associated with leukocytospermia, a condition characterized by abnormally high concentrations of pro-inflammatory leukocytes in seminal plasma (Shimoya *et al.*, 1993).

Less is known about the function of 19-hydroxy PGE_2 within seminal plasma although, like PGE_2 it is thought to act principally as an immunosuppressive agent inhibiting natural killer cell activity (Tarter *et al.*, 1986) and elevating concentrations of cAMP (Kelly *et al.*, 1994). Despite the fact that 19-hydroxy PGE is a less potent immunosuppressive agent than PGE_2 *in vitro*, its three-fold higher concentrations within seminal plasma may confer on it greater immunosuppressive activity *in vivo*. In addition, 19-hydroxy PGE is a relatively selective cAMP prostaglandine (EP)-2 receptor agonist (Woodward *et al.*, 1993) unlike PGE_2 which binds to all of the EP receptors. Given that the primate cervix contains mainly EP-2 receptors (Smith *et al.*, 1998) then the higher affinity of 19-hydroxy PGE for the EP-2 receptor may make it even more active *in vivo*. A pro-inflammatory effect for 19-hydroxy PGE has not been demonstrated previously.

These results demonstrate that seminal plasma and its component prostaglandins stimulate IL-8 release. The only exception to this was in peripheral blood where whole semen inhibited IL-8. However, seminal plasma is a potent stimulator of IL-10 release in peripheral blood and given that there is mutual repression between release of IL-10 and IL-8 (Lu *et al.*, 1995; Kang *et al.*, 1998) then the high concentrations of IL-10 generated may have inhibited IL-8 release. In addition, other factors within seminal plasma may also affect IL-8 release in peripheral blood preparations. We therefore suggest that PGE_2 and 19-hydroxy PGE, by stimulating IL-8 release from the female genital tract mucosa, may act as potent neutrophil leukoattractants within seminal plasma. Neutrophils invading into the upper vaginal tract, cervical mucus and stroma would then be ideally placed to phagocytose any spermatozoon not involved in fertilization. In addition, 19-hydroxy PGE may be useful in inducing cervical ripening at term, given that IL-8 induces tissue remodelling (Chwalisz *et al.*, 1994).

It has been suggested that pure seminal plasma stripped of spermatozoa is not capable of leukocytosis and therefore the hypothesis proposed would be invalid. The published data are however conflicting and this may be due to different methods of collecting and purifying the seminal plasma and the age of the semen used for the studies. In addition, pure spermatozoa washed of seminal plasma have also been reported to induce leukocytosis (Thompson *et al.*, 1992). However, washing spermatazoa may induce alterations in membrane structure including lipid peroxidation generating oxygen free radicles and leukotrienes both of which are chemotactic for neutrophils (Krauss *et al.*, 1994; Wizemann and Laskin, 1994). This could account for the observed leukocytosis post insemination of pure spermatozoa. It is likely therefore that *in vivo* a combination of seminal plasma constituents such as PGE_2, 19-hydroxy PGE and spermatozoa would act together to induce leukocytosis.

In this study, we have also demonstrated that seminal plasma and its constituent prostaglandins stimulate release of IL-10 from U937 cells, thus supporting earlier data (Kelly *et al.*, 1997) with PGE_2 being more effective than 19-hydroxy PGE in stimulating IL-10 release. This immunosuppressive action of seminal plasma may seem to conflict with the pro-inflammatory effect of stimulating IL-8 release. However, IL-8 is also a potent chemotactic factor for T-lymphocytes (Taub *et al.*, 1996). It could therefore induce their entry into the upper vaginal tract where they could be presented with antigen in the presence of high concentrations of immunomodulatory agents such as IL-10, PGE_2 and TGF-β which would all favour development of anergy (Groux *et al.*, 1996; Tremellen *et al.*, 1998). In addition, seminal plasma prostaglandins could be transported to draining lymph nodes together with foreign cells (Ibata *et al.*, 1997). These nodes enlarge post-coitus (Alexander and Anderson, 1987) and could be a further site of IL-10 mediated immunosuppression.

In conclusion, it has been demonstrated that seminal plasma prostaglandins can induce release of both pro-and anti-inflammatory cytokines. Moreover, PGE_2 and 19-hydroxy PGE have differing capacities to induce anti- and pro-inflammatory cytokine release respectively. We suggest that these observations are not mutually exclusive and that the different cytokines released may act in combination initially to promote sperm survival, and then to facilitate their removal from the female reproductive tract.

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Differential concentrations of monocyte chemotactic protein-1 and interleukin-8 within the fluid compartments present during the first trimester of pregnancy

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Monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) are important chemokines which effect the chemotaxis of monocytes and neutrophils, respectively. There is increasing evidence that such chemokines play an integral role in the control and maintenance of a normal pregnancy from implantation to parturition. However, little is known about the sites of secretion and function of MCP-1 and IL-8 in particular with respect to establishment of the placenta and membranes during first trimester. The aim of this study was therefore to investigate the concentrations and localization of MCP-1 and IL-8 in amniotic fluid and extra-embryonic coelomic fluid (EECF) collected by ultrasound-guided needle aspiration and maternal serum during the first trimester of pregnancy. Using specific enzyme-linked immunosorbent assays, MCP-1 was present at high concentrations in the EECF, significantly higher than those in amniotic fluid and maternal serum. IL-8 was also present predominantly in the EECF with concentrations being significantly higher than the low values detected in maternal serum and the very low amounts found in amniotic fluid. This strict compartmentalization of these cytokines in the fluid compartments of early pregnancy may be important for establishment and development of a viable pregnancy.

Key words: amniotic fluid/extra-embryonic coelom/first trimester/interleukin-8/monocyte chemotactic peptide-1

Introduction

Monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) are chemokines whose principal role is in effecting leukocyte chemotaxis. MCP-1, a 76-amino acid protein, is a member of the CC class of chemokines which are monocyte chemoattractants in which the first two conserved cysteine (C) residues in the molecule are adjacent. IL-8 is a 72-amino acid molecule and a member of the CXC class of chemokines which attract neutrophils and in which the first two cysteines are separated by an intervening amino acid residue.

The control and regulation of many reproductive processes is dependent on a delicate balance between immune and endocrine systems. Cytokines, acting as local effectors are thought to play pivotal roles in this regulation. MCP-1 is released by many reproductive tissues including the endometrium (Arici *et al.*, 1995) and ovarian follicles (Arici *et al.*, 1997). Although its presence and role in early fetal development and pregnancy has not been defined, significantly higher levels of MCP-1 are released from peripheral blood monocytes from pregnant compared with non-pregnant women (Denison *et al.*, 1997). During pregnancy, IL-8 is produced by tissues including the placenta (Shimoya *et al.*, 1992), cervix (Barclay *et al.*, 1993) and myometrium (Osmers *et al.*, 1995). In amniotic fluid, IL-8 is not detectable during the second trimester or at term not in labour but is present in significant amounts at preterm and term labour (Romero *et al.*, 1991).

During the first trimester, the human embryo is surrounded by two fluid-filled compartments, the amniotic fluid cavity and the extra-embryonic coelomic fluid (EECF) cavity which also contains the yolk sac. The composition of amniotic fluid and EECF differs considerably in hormonal, biochemical, protein and enzymic composition (Jauniaux *et al.*, 1993; Docherty *et al.*, 1996; Miell *et al.*, 1997), although the specific role of each compartment in early fetal development remains uncertain. In addition, the potential for transfer between maternal serum and fetal compartments is site- and gestational age-dependent (Jauniaux *et al.*, 1996). The yolk sac, which is connected with the midgut of the developing fetus via the vitelline duct, is obliterated along with the extra-embryonic coelom by the expansion of the amniotic cavity at the end of the first trimester. Little is known about the function of the yolk sac.

The purpose of this study was to determine the pattern of secretion and possible sources of MCP-1 and IL-8 during the first trimester. With the advent of modern ultrasound scans, study of these compartments is now possible by selective aspiration of fluid (Wathen *et al.*, 1991). Specifically we wanted to establish whether these chemokines could have potential roles in the establishment and maintenance of pregnancy and in the early development of the fetus and placenta.

Materials and methods

Collection of samples

Amniotic and EECF were collected by transvaginal ultrasound-guided needle aspiration between 8–11 weeks of gestation prior to the elective termination of a normal pregnancy under general anaesthesia, as described in detail previously (Wathen *et al.*, 1991). Women with

vaginal bleeding or uncertain menstrual dates, were excluded from the study. Fetal viability and gestational age were confirmed by ultrasonic identification of the fetal heart and by measurement of crown–rump length. Maternal serum was collected before anaesthesia and all samples were stored at –20°C prior to immunoassay. These protocols were approved by the Ethics Committee, St Bartholomew's Hospital and informed written consent was obtained from patients.

Assay for MCP-1

MCP-1 was measured by enzyme-linked immunosorbent assays (ELISA) using a peroxidase-labelled Fab fragment of an antibody to MCP-1 as previously described (Ida *et al.*, 1994). All reagents were from Sigma, Poole, UK, unless otherwise stated. Briefly, plates were coated overnight at 4°C with 100 µl/well of MCP-1 capture antibody (4 mg/ml in phosphate-buffered saline; Toray Industries, Inc., Tokyo, Japan). Next, they were washed once in tap water and coating solution added (2% polyvinyl pyrrollidone, 5 mg/ml BSA, 5 mM EDTA, 50 mM Tris) at 100 µl/well for 30 min. Plates were air-dried and stored with a desiccant at 4°C. Samples were diluted 1:1 in ELISA buffer [100 mM Tris, 100 mM 2-methylisothizolone (Boehringer Mannheim, Germany), 100 mM bromonitrodioxane (Boehringer Mannheim), 2 mg/ml BSA, 50 mM phenol red solution, 150 mM NaCl, 2 mM EDTA, 0.05% Tween 20, to final pH of 7.2] and added at 100 µl/well. Recombinant standards (Toray Industries, Inc.) were diluted in ELISA buffer and added at 100 µl/well with the top standard being 500 pg/ml. The plates were incubated for a minimum of 3 h at 23°C on an orbital plate shaker. Plates were washed (0.05% Tween-20, 150 mM NaCl, 100 mM Tris to final pH of 7–7.5) and peroxidase-coupled detection antibody (diluted 1:200 in ELISA buffer) was added at 100 µl/well. Plates were incubated for 45 min at 23°C on an orbital shaker, washed and substrate added at 200 µl/well [0.3 g/l urea-hydrogen peroxide, 0.1 g/l tetramethyl benzidine (Aldrich, Gillingham, UK) in 100 mM sodium acetate, pH 6.0] for 20 min prior to quenching with 1 M H_2SO_4 at 50 µl/well. Absorption was read at 450 nm within 30 min of quenching. Serial dilutions of amniotic fluid, maternal serum and EECF gave a dose–response curve parallel to that of immunopurified standard (data not shown). The intra- and interassay precisions were 6.3% and 8.6% respectively and the detection limit of the assay was 15 pg/ml.

Assay for IL-8

IL-8 was measured by ELISA utilizing matched pairs of capture and biotinylated labelled detection antibodies for IL-8 (R&D detection system, Oxford, UK). Briefly, plates were coated overnight at 4°C with capture antibody added at 100 µl/well, washed once in water, and coating solution added at 100 µl/well for 30 min at 23°C. The coating solution was removed and the plates stored as for MCP-1. Before use, the plates were washed once in water. All samples were diluted 1:1 in ELISA buffer. Recombinant standards (Toray Industries, Inc.) were diluted in ELISA buffer (500 pg/ml highest standard) and 100 µl of sample and standard were added per well. Plates were incubated for at least 3 h at 23°C on an orbital shaker, washed as above, detection antibody (30 ng/ml in ELISA buffer) added at 100 µl/well and incubated on a shaker for 45 min at 23°C. After a further wash, streptavidin–peroxidase (Boehringer Mannheim) was added at 0.02 U in 100 µl/well and plates were incubated for 20 min at 23°C on an orbital shaker. They were then washed, substrate was added and were read as for MCP-1. Serial dilution of EECF gave a dose–response curve parallel to that of immunopurified standard (data not shown). The intra- and interassay precisions were 9.1% and 22% respectively and the detection limit of the assay was 15 pg/ml.

Table I. Concentrations of monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) in 27 matched samples of amniotic fluid, maternal serum and extra-embryonic coelomic fluid (EECF) collected at 8–11 weeks of pregnancy. Values are expressed as pg/ml ± SEM

Chemokine	Source sample		
	Amniotic fluid (n = 27)	Maternal serum (n = 27)	EECF (n = 27)
MCP-1	151.5 ± 31.5[a]	142.6 ± 15.9[a]	337.3 ± 40.2
IL-8	9.72 ± 6.6[a,c]	33.1 ± 8.2[b]	339.6 ± 53.8

[a] $P < 0.0001$ compared with EECF.
[b] $P < 0.0005$ compared with EECF.
[c] $P < 0.05$ compared with maternal serum.

Statistical analysis

The data were normally distributed and statistical analyses of concentrations of MCP-1 and IL-8 between and within compartments with gestation were performed using ANOVA (Statview 4.1, Abacus Inc., Berkeley, CA, USA). The data are expressed as mean ± SEM; a P value < 0.05 was considered to be statistically significant.

Results

MCP-1 was present in 27 matched samples of amniotic fluid, maternal serum and EECF (Table I). High concentrations of MCP-1 were present in all compartments measured, and were significantly higher in EECF than in either amniotic fluid ($P < 0.0001$) or maternal serum ($P < 0.0001$). There was no correlation in concentrations of MCP-1 in any compartment with gestational age (data not shown), though this may be due either to the low number of samples collected in each week, or to the short period of gestation (8–11 weeks) studied.

IL-8 was present in all compartments measured (Table I), with concentrations in the EECF significantly higher than those in the maternal serum ($P < 0.0005$) and amniotic fluid ($P < 0.0001$). Moreover, significantly greater concentrations of IL-8 were present in maternal serum compared with amniotic fluid ($P < 0.05$). There was no correlation in concentrations of IL-8 in any compartment with gestational age, as was demonstrated with MCP-1.

Discussion

This is the first report to demonstrate the presence of MCP-1 and IL-8 in the various fluid compartments present during the first trimester of pregnancy. The highest concentrations of both chemokines was found in the EECF, while ratios of MCP-1 to IL-8 in amniotic fluid, maternal serum and EECF were 15:1, 4:1 and 1:1, respectively.

MCP-1 was found predominantly in the EECF which, anatomically, is directly adjacent to the chorion, chorionic plate, developing trophoblast and decidua. During pregnancy it is postulated that a T-helper 1 (TH1) to TH2 shift occurs in the maternal immune response with a TH2 or humoral response favouring a successful outcome to pregnancy and a TH1 or cytotoxic response an adverse outcome (Wegmann *et al.*, 1993). The principal function of MCP-1 is in inducing the chemotaxis and activation of monocytes (Wuyts *et al.*, 1994), although recently several novel properties have been ascribed

to it, including that of an immunological modulator, promoting development of a TH2 type of immune response (Chensue *et al.*, 1995). We hypothesize that the high levels of MCP-1 in the EECF may facilitate either the occurrence of a TH2 immune response at the materno-fetal interface, or mediate chemotaxis of macrophages in decidua or trophoblast. Both amniotic fluid and maternal serum also contain relatively high levels of MCP-1, suggesting that MCP-1 may be playing the same role in these two compartments.

IL-8 was found in this study to be mainly located within the EECF with minimal concentrations in both amniotic fluid or maternal serum. Initially discovered as a highly specific neutrophil chemoattractant and activator (Mukaida *et al.*, 1992), IL-8 is now known to have a number of other functions including that of a T-cell chemotaxin (Baldwin *et al.*, 1990), mediator of vascular smooth muscle migration (Yue *et al.*, 1994) and an angiogenic factor (Hu *et al.*, 1993; Yoshida *et al.*, 1997). How it affects angiogenesis is unknown, but IL-8 receptors were not detected on endothelial cells, suggesting an indirect mechanism (Petzelbauer *et al.*, 1995). Only those CXC chemokines, such as IL-8, which have an ELR (Glu-Leu-Arg) N-terminal motif preceding the first cysteine amino acid are angiogenic (Strieter *et al.*, 1995). The mechanism and mediators of angiogenesis in the first trimester placenta are poorly understood, although factors such as vascular endothelial growth factor (Charnock-Jones *et al.*, 1994) and epidermal growth factor (John *et al.*, 1997) have been implicated. IL-8 is released by chorion trophoblast (Kelly *et al.*, 1992), decidua (Saito *et al.*, 1994) and amnion (Trautman *et al.*, 1992) and is localized to the perivascular cells of the placental villi during the first trimester and at term (Elliott *et al.*, 1998). In this study, IL-8 was found predominantly within the EECF which is adjacent to the chorionic plate and primary villi, both of which are sites undergoing extensive angiogenesis. Thus, IL-8 is ideally located to act as a potent angiogenic factor and mediator of vascular smooth muscle migration. Moreover, hypoxia stimulates release of IL-8 (Karakurum *et al.*, 1994) and the hypoxic environment that exists *in utero* during the first trimester (Genbacev *et al.*, 1996), may therefore stimulate IL-8 production.

As a coordinator of phagocytic cells, IL-8 has a role in many inflammatory processes including infection-mediated preterm labour (Cherouny *et al.*, 1992), menstruation (Kelly *et al.*, 1994) and cervical ripening (Barclay *et al.*, 1993; Sennstrom *et al.*, 1997). The mechanisms by which IL-8 mediates inflammation and cellular infiltration are not fully understood, but recent evidence suggests that polarization of chemokine receptors to the leading edge of migrating leukocytes may be a pivotal event (Nieto *et al.*, 1997). Towards the end of the first trimester the EECF is obliterated by the expansion of the amnion which subsequently fuses with the chorion. Little is known about the mechanism of this resorptive process or whether inflammatory cells are recruited, but the leukoattractant properties of IL-8 could play a role.

It has been suggested that the EECF may sequester some biologically active substances, thus protecting the poorly keratinized fetus when crucial organogenesis and differentiation are occurring (Docherty *et al.*, 1996). This could explain why the concentrations of IL-8, which mediates chemotaxis of phagocytic cells, are negligible in amniotic fluid adjacent to the embryo compared with those in the EECF. In addition, given that fibroblasts secrete IL-8 and that the EECF is surrounded by fibromesenchyme, IL-8 could be released directly into the EECF. If MCP-1 is promoting a pregnancy-favourable cytokine environment via a TH2 response, then higher levels would be expected in all fluid compartments, as was found in this study.

In summary, this study demonstrates differential concentrations of MCP-1 and IL-8 within the various fluid compartments present during the first trimester. The role of these potent cytokines is not established, but they may be involved in the TH1 to TH2 shift in pregnancy and could play a role in early fetal and placental development and cellular trafficking.

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The action of prostaglandin E_2 on the human cervix: Stimulation of interleukin 8 and inhibition of secretory leukocyte protease inhibitor

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OBJECTIVE: The objective of this study was to investigate regulation of inflammatory mediators implicated in cervical ripening and to explore the mechanisms by which the clinically effective agent prostaglandin E_2 may mediate cervical ripening.
STUDY DESIGN: Cervical biopsy specimens were taken from healthy, nonpregnant women undergoing a hysterectomy for a benign nonmalignant condition and were cultured, with treatments in quadruplicate, for 24 hours in media supplemented with progesterone, dexamethasone, nitric oxide, interleukin 8, and prostaglandin E_2. Media were collected and assayed by enzyme-linked immunosorbent assay for interleukin 8, secretory leukocyte protease inhibitor, and prostaglandin E_2. Ethical approval was obtained for this study from the local ethics committee.
RESULTS: Interleukin 8 release from cervical explants was stimulated by prostaglandin E_2 and nitric oxide and inhibited by progesterone and dexamethasone. Secretory leukocyte protease inhibitor release from cervical explants was stimulated by progesterone and inhibited by prostaglandin E_2. Prostaglandin E_2 release from cervical explants was stimulated by nitric oxide.
CONCLUSION: Complex interactions occur between inflammatory cytokines within the cervix; these results further our understanding of the mechanism of cervical ripening. (Am J Obstet Gynecol 1999;180:614-20.)

Key words: Cervical ripening, interleukin 8, nitric oxide, prostaglandin E_2, secretory leukocyte protease inhibitor

Cervical ripening is the process by which the cervix changes from a tightly closed structure designed to maintain an intrauterine pregnancy to a soft, compliant organ capable of dilating to accommodate passage of the fetus. This process necessitates profound tissue remodeling and is accompanied by disruption of collagen fibrils, alteration in glycosaminoglycans, and edema of the cervical stroma. Such changes within the cervix have been likened to an inflammatory reaction[1] and are accompanied by extensive neutrophil invasion of the underlying cervical tissue.[2]

Progesterone appears to exert some control on cervical ripening; progesterone withdrawal initiates cervical softening in women throughout gestation, even though there is no fall in peripheral serum progesterone before cervical remodeling. The roles of local inflammatory mediators in leukocyte migration and connective tissue rearrangement during cervical ripening, however, are less well defined.

A variety of mediators have been implicated in cervical remodeling, including prostaglandin (PG) E_2, interleukin (IL) 8, and more recently nitric oxide. The level of PGE_2, the most effective and widely used cervical ripening agent in clinical practice,[3] has been shown to rise in amniotic fluid before the establishment of labor. The mechanism of action of PGE_2 is not known, although it may involve vasodilation. IL-8, a potent neutrophil chemotactic and activating agent,[4] is released in vitro[5] and in vivo[6] by the human cervix. In animal models progesterone inhibits IL-8 release[7] and topical application of IL-8 has been shown to effect cervical ripening that is morphologically identical to the physiologic process.[8] IL-8 is thought to initiate cervical ripening by promoting neutrophil chemotaxis to and activation within cervical stroma. Nitric oxide has quite recently been shown to effect cervical ripening in women[9] and in animal models.[10] Its mechanism of action is not fully defined but is thought to be complex, involving vasodilation, initiation of apoptosis, and induction of synthesis of glycosaminoglycans and collagen degrading matrix metalloproteinases.

It is likely, however, that the highly complex process of

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cervical ripening will prove to involve orchestration of a wide range of inflammatory mediators in addition to PGE_2, IL-8, and nitric oxide. One such mediator is secretory leukocyte protease inhibitor (SLPI), which is present in cervical mucus.[11] SLPI is a potent inhibitor of neutrophil function[12] and thus might be expected to oppose the action of IL-8 during inflammatory responses, such as cervical ripening. The purpose of this study was therefore to investigate the release and regulation of inflammatory modulators (particularly by PGE_2) within the cervix, with the aim of better understanding their roles in cervical ripening.

Material and methods

Tissue. Biopsy specimens from the anterior lip of the cervix (approximately 20-35 mg in weight, 15-20 mm in length, and 2-3 mm in diameter) were taken from healthy, nonpregnant women (aged 29-45 years, n = 39 different women) with regular menstrual cycles undergoing hysterectomy for a nonmalignant condition. All women were white, had no underlying hormonal dysfunction, and had not used hormonal contraception, received hormone replacement therapy, or received gonadotropin-releasing hormone antagonists in the 6 months before the operation. The women were at various stages of the menstrual cycle. A different set of biopsy specimens was used for each of the studies. The study had the approval of the local research ethics committee.

Tissue culture. Biopsy specimens were placed immediately in Roswell Park Memorial Institute medium 1640 at 4°C for transport. Reagents were from Sigma (Poole, United Kingdom) unless otherwise stated. Explants were washed in phosphate-buffered saline solution, dissected into small pieces (1-2 mm^3), and then placed in 1 ml Roswell Park Memorial Institute medium 1640 supplemented with 10% fetal calf serum, 20 μg/mL gentamicin, 50 IU/mL penicillin, 50 μg/mL streptomycin (Gibco, Paisley, United Kingdom), and 2 mmol/L L-glutamine in a 24-well plate (Costar, High Wycombe, United Kingdom). Explants were treated in quadruplicate with 10^{-7} mol/L progesterone, 10^{-7} mol/L dexamethasone, 10^{-7} mol/L PGE_2, and 50 μg/mL spermine NONOate (Alexis Corp, Birmingham, United Kingdom) as a nitric oxide donor before culture for 24 hours at 37°C in 95% air and 5% carbon dioxide under humid conditions. Medium from each explant was analyzed by enzyme-linked immunosorbent assay (ELISA). Steroid antagonism studies were performed with mifepristone. Each harvested medium sample was then split into 2 portions, 1 of which was frozen at –20°C for subsequent ELISA and 1 of which was treated 1:1 with methyloximating solution (1 mol/L sodium acetate, pH 5.6, containing 0.1 mol/L methoxyamine hydrochloride in 10% ethanol) overnight at 4°C for prostaglandin assay.

ELISA

IL-8 assay. IL-8 was measured by ELISA with matched pairs of capture and biotinylated labeled detection antibodies for IL-8, as described in detail previously.[13] The capture antibody (4 μg/mL) and detection antibody (50 μg/mL) were obtained from R & D Systems, (Oxford, United Kingdom) and the standards (recombinant, top standard 500 pg/ml) were a gift from Toray Industries, Inc (Tokyo, Japan). The intra-assay and interassay precision levels were 9.1% relative SDs and 22% relative SDs, respectively, and the detection limit of the assay was 15 pg/mL.

SLPI assay. SLPI was measured by a competitive ELISA. Plates (96-well Nunc Maxi Sorp Plates; Gibco) were coated with recombinant SLPI (R & D Systems) as a solution of 0.025 μg/mL in phosphate-buffered saline solution with 1% carbonate buffer, 400 mmol/L monosodium carbonate, 40 mmol/L disodium carbonate in 500 mL distilled water, pH 9.6) at 300 μL/well for 60 minutes at 23°C, blocked with milk powder (diluted 1:10 in distilled water) at 400 μL/well for 30 minutes at 23°C, then washed with 150 mmol/L sodium chloride, 100 mmol/L tris(hydroxymethyl)aminomethane, and 0.05% polysorbate 20 to final pH of 7 to 7.5. ELISA assay buffer (150 mmol/L sodium chloride, 100 mmol/L tris[hydroxymethyl]aminomethane, 50 mmol/L phenol red solution, 1 mmol/L 2-methylisothiazolone [Boehringer Mannheim, Lewes, United Kingdom], 1 mmol/L bromonitrodioxane [Boehringer Mannheim], 2 mmol/L ethylenediaminetetraacetic acid, 2 mg/mL bovine serum albumin, and 0.05% polysorbate 20 to final pH of 7.2) was added for the nonspecific binding in singleton at 250 μL/well. Recombinant standards were diluted in ELISA buffer and added in triplicate at 200 μL/well from 50 ng/mL to 0.078 ng/mL and samples were added at 200 μL/well. Anti-SLPI (recombinant, 2 μg/mL in ELISA buffer) was added at 50 μL/well to all wells except that used for nonspecific binding. Plates were incubated for 120 minutes at 23°C on an orbital shaker, then washed, and antisheep goat immunoglobulin G–peroxidase (Boehringer Mannheim) diluted 1:500 in ELISA buffer was added at 100 μL/well. Finally, plates were incubated for 120 minutes at 23°C on an orbital shaker and then washed, and substrate (0.3 g/L urea–hydrogen peroxide and 0.1 g/L tetramethyl benzidine in 100 mmol/L sodium acetate, pH 6.0) was added at 200 μL/well for 10 minutes before quenching with 50 μL/well 2N sulfuric acid. Absorption was read at 450 nm within 30 minutes of quenching. Serial dilution of culture medium gave a dose-response curve parallel to that of immunopurified standard (data not shown). The correlation between the results from this assay and those obtained by a commercial assay (n = 10; R & D Systems) was 0.85. The intra-assay and interassay precision levels were 9.2% relative SDs and 10.1% relative SDs, respectively, and the detection limit of the assay was 9.8 pg/mL.

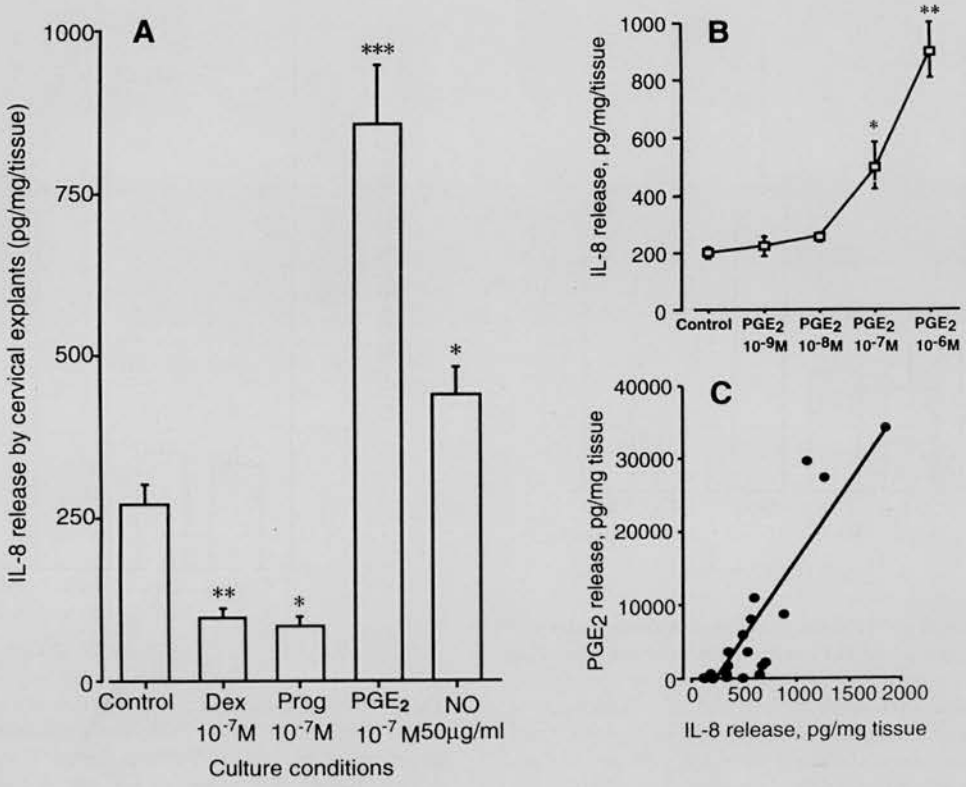

Fig 1. A, Modulation of IL-8 release by cervical explants under different culture conditions. IL-8 was significantly inhibited by 10^{-7} mol/L progesterone *(Prog)* and 10^{-7} mol/L dexamethasone *(Dex)* and was stimulated by 10^{-7} mol/L PGE_2 and 10^{-7} mol/L nitric oxide *(NO)*, n = 15. Statistical analyses were performed for treatment versus control; *asterisk*, $P < .05$; *two asterisks*, $P < .005$; *three asterisks*, $P < .001$. Values are expressed as picograms per milligram wet weight of tissue; *error bars*, SEM. **B,** Dose-response curve for PGE_2 stimulation of IL-8 release. IL-8 is stimulated by PGE_2, with maximal stimulation at 10^{-6} mol/L. Typical of 3 samples. Statistical analyses were performed for treatment versus control; *asterisk*, $P < .05$; *two asterisks*, $P < .005$. Values are expressed as picograms per milligram wet weight of tissue; *error bars*, SEM. **C,** IL-8 release correlated positively with PGE_2 concentration ($r = 0.82$, n = 19). Values are expressed as picograms per milligram wet weight of tissue.

Table I. Effects of steroid antagonism on IL-8 release by cervical explants

	Control	*Progesterone (10–7 mol/L)*	*Dexamethasone (10–7 mol/L)*	*Mifepristone (10–7 mol/L)*
No.	6	6	6	6
First 24 h	100	61 ± 9*	53 ± 3*	103 ± 8
Second 24 h (10–7 mol/L mifepristone added)	100	187 ± 31*	156 ± 13*	140 ± 27

Explants were cultured with corticosteroids for 24 hours before changing media and supplementing with 10^{-7} mol/L mifepristone for a further 24 hours. Treatment with 10^{-7} mol/L progesterone and 10^{-7} mol/L dexamethasone for the first 24 hours inhibited IL-8 release. This inhibition was antagonized by addition of mifepristone to cultures during the second 24 hours. Treatment with mifepristone alone for 48 hours did not affect IL- 8 release. IL-8 release is expressed as a percentage of the control value and SEM.
*$P < .05$.

PGE_2 ELISA. Plates (Amine-binding plates, Costar) were coated with donkey antirabbit antibody by means of the direct γ-globulin binding procedure. Plates were coated with rabbit immunoglobulin G (1 mg/mL diluted in phosphate-buffered saline solution with 1% carbonate buffer, pH 9.6) at 200 µL/well for 16 hours at 4°C, the solution was flicked out, and blocking solution (50 mmol/L glycine, 10 mg/ml bovine serum albumin Sigma-A7888 in distilled water) was added at 250 µL/well for 120 minutes at 23°C. Plates were then washed, donkey antirabbit serum (Scottish Antibody Production Unit, Carluke, United Kingdom) was added at 150 µL/well, and plates were incubated for 16 hours at 4°C before washing, air drying, and storage with a desiccant at 4°C. To prepare the link 20 mg synthetic PGE_2 (a gift from Applied Therapeutics, Paisley, United Kingdom) was added to 320 µL dry dimethylformamide (Aldrich, Poole, United Kingdom), the solution was cooled to 4°C, and 6 µL tributylamine (Aldrich) and 3 µL butylchloroformate (Aldrich) were added with stirring for 30 minutes at 4°C. Finally, 0.05 mol/L biocytin (diluted in 300 µL 1:1 dimethylformamide/distilled water) was added

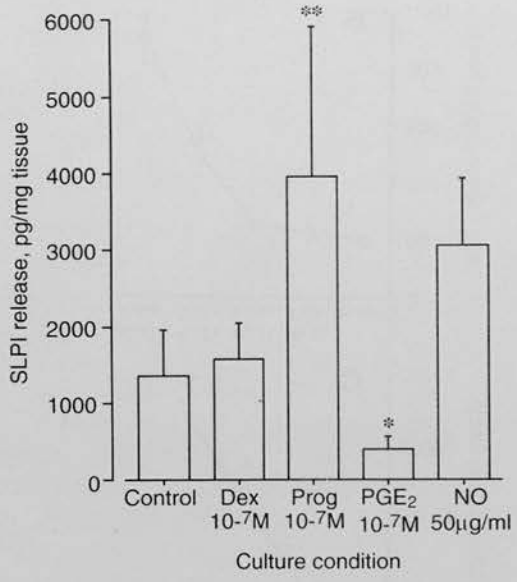

Fig 2. Modulation of SLPI release by cervical explants under different culture conditions. SLPI was significantly stimulated by 10^{-7} mol/L progesterone *(Prog)* and inhibited by 10^{-7} mol/L PGE_2. Nitric oxide *(NO)* at 50 μg/mL and dexamethasone at 10^{-7} mol/L had no effect, n = 15. Statistical analyses were performed for treatment versus control; *asterisk*, $P < .05$; *two asterisks*, $P < .005$. Values are expressed as picograms per milligram wet weight of tissue; *error bars*, SEM.

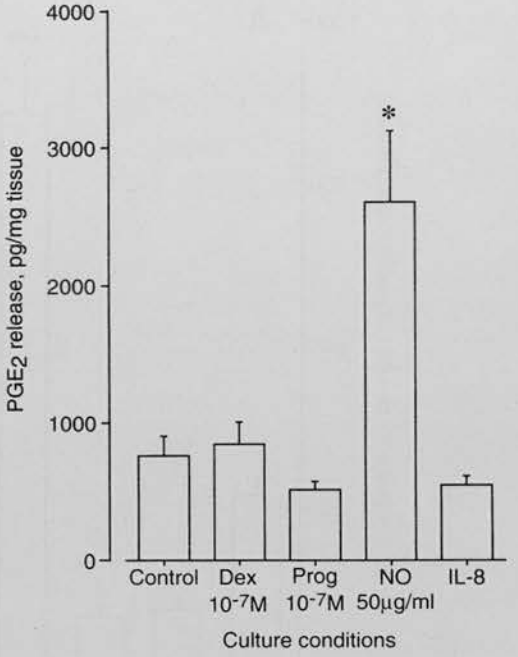

Fig 3. Modulation of PGE_2 release by cervical explants under different culture conditions. PGE_2 was significantly stimulated by 50 μg/mL nitric oxide *(NO)*. Progesterone *(Prog)* at 10^{-7} mol/L, dexamethasone *(Dex)* at 10^{-7} mol/L, and IL-8 at 50 mg/mL had no effect, n = 15. Statistical analyses were performed for treatment versus control; *asterisk*, $P < .05$. Values are expressed as picograms per milligram wet weight of tissue; *error bars*, SEM.

and the solution ether extracted and purified by reverse chromatography. The rabbit antisera that were raised against PGE_2-complexed keyhole limpet hemocyanin have been previously characterized.[14] Samples and synthetic standards (Applied Therapeutics), diluted in ELISA buffer from 5120 to 78 pg/mL, were added in duplicate at 100 μL/well. Link (diluted $1:1.5 \times 10^6$ in ELISA buffer) was added at 50 μL/well to all wells, and antiserum (diluted 1:50,000 in ELISA buffer) was added at 50 μL/well to all wells except that used for nonspecific binding. The final concentration of methyloximating solution in standards and samples was 12.5%. Plates were incubated at 4°C for 16 hours, plates were washed and 100 μL/well of 0.2 unit/mL streptavidin peroxidase (Boehringer Mannheim) was added. The plates were then incubated for 20 minutes at 23°C on an orbital shaker, plates were washed, substrate was added, and plates were read as for SLPI. The intra-assay and interassay coefficients were 7.8% and 15.0%, respectively. The median effective dose was 195 pg/mL.

Statistical analysis. Statistical analysis of concentrations of IL-8, SLPI, and PGE_2 in culture media were performed with analysis of variance (Statview 4.1; Abacus Inc, Berkeley, Calif). The Fisher protected least significant difference was used as a post hoc test to determine significance. The data are expressed as mean and SEM in picograms per milligram. $P < .05$ was considered to represent a statistically significant difference.

Results

IL-8 release. Cervical explants released IL-8. IL-8 release was significantly stimulated by PGE_2 in a dose-dependent manner, with maximal stimulation at 10^{-6} mol/L ($P < .001$; Fig 1, *A* and *B*, n = 15 and n = 3 different women, respectively, with treatments in quadruplicate). IL-8 release correlated with PGE_2 release by cervical explants (correlation coefficient 0.82, n = 19 different women; Fig 1, *C*). Nitric oxide stimulated IL-8 release ($P < .05$). IL-8 release was inhibited by dexamethasone ($P < .005$) and progesterone ($P < .05$). To assess the effect of steroid antagonism, explants were cultured in media supplemented with 10^{-7} mol/L progesterone or 10^{-7} mol/L dexamethasone for 24 hours before the media were changed and supplemented with the antigestogen and antiglucocorticoid mifepristone at 10^{-7} mol/L for a further 24 hours (n = 6 different women). IL-8 inhibition in biopsy specimens cultured with progesterone or dexamethasone was antagonized by mifepristone. There was no significant difference in IL-8 release between control biopsy samples and those cultured with mifepristone for 48 hours (Table I).

SLPI release. Cervical explants release SLPI (n = 15 different women with treatments in quadruplicate). SLPI release was stimulated by progesterone ($P < .005$) and inhibited by PGE_2 ($P < .05$). Nitric oxide, dexamethasone, and IL-8 had no effect on SLPI release (Fig 2).

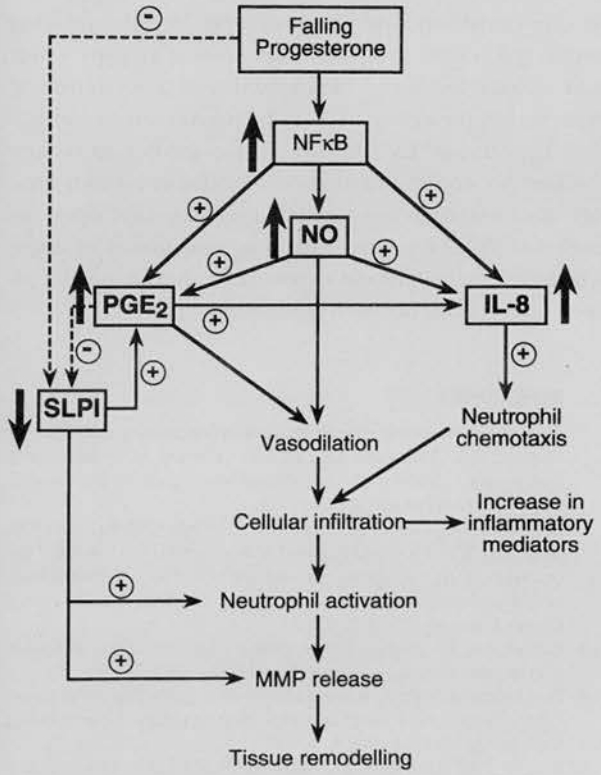

Fig 4. Hypothesis for cervical ripening at term. PGE_2 stimulates proinflammatory IL-8 and inhibits anti-inflammatory SLPI. The proinflammatory cascade may be further enhanced by stimulation of PGE_2 and IL-8 by rising nitric oxide *(NO)* levels and the effect of falling progesterone on inflammatory mediators. *MMP,* Matrix metalloproteinase.

PGE_2 release. Cervical explants released PGE_2 (n = 15 different women with treatments in quadruplicate). PGE_2 release was stimulated by nitric oxide ($P < .05$). Progesterone, dexamethasone, and IL-8 had no effect on PGE_2 release (Fig 3).

Comment

These results further our understanding of the mechanism of cervical ripening by demonstrating that complex interactions occur between inflammatory mediators within the cervix. We demonstrated that release of IL-8 by cervical explants was significantly stimulated by nitric oxide and PGE_2 in a dose-dependent manner and was inhibited by progesterone and dexamethasone. Moreover, SLPI was released by cervical explants and this secretion was significantly stimulated by progesterone and inhibited by PGE_2. Finally, release of PGE_2, the currently preferred clinical cervical ripening agent, was stimulated by nitric oxide, a potential new mediator of cervical ripening.

We demonstrated that progesterone and dexamethasone inhibit IL-8 and stimulate SLPI release from cervical explants. Progesterone plays a vital role in the maintenance of pregnancy and uterine quiescence. In animals such as sheep, a profound reduction in progesterone at term is thought to trigger parturition. In women, although there is no peripheral fall in progesterone during the third trimester, indirect evidence suggests that a local reduction within the fetoplacental unit may herald the onset of labor. Cervical ripening and labor are thought to be coordinated at a local level by inflammatory mediators, such as the neutrophil chemoattractant IL-8. Progesterone[15] and glucocorticoids inhibit transcription of these mediators, including IL-8, through mutual repression between the steroid receptors and the relA (p65) subunit of nuclear factor κ-β (NFκB). This factor is involved in transcriptional regulation of many inflammatory mediators. Glucocorticoids are also known to stimulate SLPI synthesis,[16] although the mechanism is not fully understood and no such effect was demonstrated in this study. In addition, SLPI inhibits cytokine activation by NFκB, probably by interfering with the NFκB-relA binding site.[17]

We hypothesize that a local fall in progesterone or interference with its action would stimulate IL-8 and inhibit SLPI release from cervical tissue, possibly by means of NFκB. A rise in IL-8, which has been shown to occur during cervical ripening,[6] would initiate neutrophil chemotaxis, emigration, and activation within the cervical stroma. SLPI is a potent inhibitor of neutrophil function and elastase release; a fall in its levels thus would facilitate neutrophil recruitment and activation within the cervical stroma. The overall result would be to favor inflammation and cervical ripening (Fig 4).

It is widely believed that PGE_2 plays an important role in initiating cervical ripening and labor, but its mechanisms are not fully understood. In other inflammatory processes PGE_2 is thought to act principally as a vasoactive agent, thus facilitating inflammatory cell infiltration. In extrauterine sites, however, it also upwardly regulates release of many inflammatory cytokines such as IL-8,[18] acts synergistically with IL-8 to augment neutrophil chemotaxis,[19] and stimulates matrix metalloproteinase release by monocytes. This last effect can be inhibited by SLPI through inhibition of cyclooxygenase 2, the inducible form of the enzyme, and is independent of SLPI's antiprotease activity and regulatory effects on other cytokines. Finally, PGE_2 is potentially inhibited by progesterone, which acts through NFκB to inhibit cyclooxygenase 2 expression.

The data presented here demonstrate that in vitro treatment of cervical explants with PGE_2 has the dual effect of stimulating IL-8 and inhibiting SLPI release. At term, progesterone withdrawal and possible upward regulation of NFκB would simulate PGE_2, which would act with IL-8 to promote neutrophil emigration and activation within cervical stroma. In addition to facilitating neutrophil and monocyte chemotaxis and activation, a

reduction in SLPI would further increase PGE_2 synthesis through upward regulation of NFκB-mediated cyclooxygenase 2 transcription (Fig 4).

Nitric oxide is a pleiotropic, short-lived free radical gas that mediates a wide range of physiologic processes, including inhibition of platelet aggregation, smooth muscle relaxation, host immunity, and inflammation. Experimental and clinical evidence also suggests that nitric oxide plays an important role during pregnancy by maintaining uterine quiescence and placental perfusion. In addition, it may be involved in initiating parturition. In the rat uterus and placenta, nitric oxide is upwardly regulated in a gestationally dependent manner during pregnancy and is significantly downwardly regulated before parturition.[20] Paradoxically, in the rat cervix nitric oxide is regulated in the opposite direction, with levels being low during pregnancy but increasing significantly before both preterm and term parturition. This may be explained by nitric oxide being synthesized by invading neutrophils and monocytes. It is not known whether a similar pattern of nitric oxide release exists in women, but recent data suggest that uterine and placental nitric oxide levels do not fall before parturition.[21] In that study, however, nitric oxide was measured in uteri after 37 weeks' gestation, by which time any reduction in nitric oxide could already have occurred. Regulation of nitric oxide synthesis is controversial, but inducible nitric oxide synthase appears to be inhibited by progesterone[22] and glucocorticoids, possibly through NFκB, and to be stimulated by a variety of inflammatory mediators, including IL-1 and tumor necrosis factor α. Moreover, nitric oxide, like PGE_2, plays a complex immunomodulatory role and can induce both anti-inflammatory and proinflammatory immune responses, with high levels such as might be expected during cervical ripening favoring inflammation. Nitric oxide induces cyclooxygenase 2 synthesis,[23] stimulates gelatinase secretion by rat mesangial cells,[24] and stimulates elastase and IL-8 release from peripheral blood,[25] the last by a cyclic guanosine monophosphate–independent pathway.

The data presented here demonstrate that nitric oxide stimulates both IL-8 and PGE_2 release from cervical explants. Thus any fall in progesterone at term and subsequent neutrophil and monocyte invasion of the cervical stroma could produce a rise in nitric oxide through activation of NFκB and local synthesis by invading cells. Nitric oxide would stimulate IL-8 and elastase release and would promote PGE_2 synthesis through cyclooxygenase 2 induction, leading to a proinflammatory state and cervical remodeling (Fig 4).

Complex interactions occur between inflammatory mediators in vitro. We suggest that cervical ripening is not due to a single inflammatory mediator but is the consequence of a cascade of several such agents that interact with each other (Fig 4). Although a change in progesterone or its cognate receptor may be the initiating event, studies on progesterone receptor subtype status and on local levels and bioavailability of progesterone at term within the cervix need to be performed to confirm this hypothesis. In addition, these studies were performed on nonpregnant cervical explants, which have obvious limitations as a model for term cervical ripening, and should be repeated with pregnant tissues to assess whether previous steroid exposure during pregnancy affects interactions between mediators.

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Secretory leukocyte protease inhibitor concentration increases in amniotic fluid with the onset of labour in women: characterization of sites of release within the uterus

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Abstract

Secretory leukocyte protease inhibitor is a potent inhibitor of neutrophil function, a mediator of mucosal immunity and an inhibitor of NFκB regulated inflammatory responses. However, its source, function and regulation within the uterus during pregnancy and at parturition are not well defined. In amniotic fluid, the concentration of secretory leukocyte protease inhibitor increased significantly from 2nd trimester (24 ± 3 ng/ml; mean ± S.E.M.; n=20) to term (751 ± 53 ng/ml; P<0·05; n=15) with a further profound increase (P<0·005) with the onset of labour (3929 ± 1076 ng/ml; n=15). To establish the intrauterine sites of secretion, explants (n=6 different patients per tissue) were collected at term after elective caesarean section. High levels of secretory leukocyte protease inhibitor were released by decidua (135·2 ± 12·4 pg/mg; mean ± S.E.M.) and chorio-decidua (325·1 ± 26·4 pg/mg) with less by amnion (55·6 ± 6·0 pg/mg) and placenta (9·2 ± 1·9 pg/mg). Intense immunoreactivity for secretory leukocyte protease inhibitor was detected predominantly in decidua parietalis cells adherent to the chorion laeve and myometrium, and also in decidua basalis. We propose that, within the pregnant uterus, secretory leukocyte protease inhibitor is released by decidua, fetal membranes and potentially the fetal lung. The increase in secretory leukocyte protease inhibitor may act to modulate pro-inflammatory paracrine interactions for the maintenance of pregnancy and limit those occurring at parturition within the uterus.

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Introduction

Secretory leukocyte protease inhibitor, also known as anti-leukoprotease inhibitor and human mucus proteinase inhibitor, is a 12 kDa member of the chelonianin class of serine protease inhibitors (Francart *et al.* 1997). The protease inhibitory sequence has been identified in the carboxy domain (Meckelein *et al.* 1991). Although initially described as a potent inhibitor of neutrophil elastase and cathepsin-G (Thompson & Ohlsson 1986), secretory leukocyte protease inhibitor has many other functions, including inhibition of mast cell kinases (Pemberton *et al.* 1998), as an anti-bacterial (Hiemstra *et al.* 1996), anti-viral (Shine *et al.* 1997) and anti-fungal (Tomee *et al.* 1997) agent and anti-coagulant (Masuda *et al.* 1995). In addition, it has recently been shown to inhibit the release of matrix metalloproteinases by monocytes via inhibition of cyclo-oxygenase-2 (Zhang *et al.* 1997) and the lipopolysaccharide-induced activation of the pro-inflammatory transcription factor, NFκB (Jin *et al.* 1997). Secretory leukocyte protease inhibitor is found in monocytes and polymorphonuclear leukocytes (Ohlsson *et al.* 1996) and is secreted in large amounts from bronchial epithelium (Marchand *et al.* 1995) and mucus-secreting glands (Bergenfeldt *et al.* 1996) where it acts as an important mediator of innate mucosal immunity.

In the uterus, secretory leukocyte protease inhibitor has been found in the endometrium and myometrium of pigs (Reed *et al.* 1998), cows and horses (Badinga *et al.* 1994), with concentrations of the protein and mRNA tending to increase during pregnancy. In women, secretory leukocyte protease inhibitor protein has not been demonstrated within non-pregnant endometrium or fetal membranes but has been immunolocalised to the crypts of endocervical glands in both the non-pregnant and pregnant cervix (Helmig *et al.* 1995). In addition, its concentration increases significantly within amniotic fluid during pregnancy to term (Helmig *et al.* 1995). However, any change in concentration with the onset of labour, its site of secretion from uterine tissues and the potential roles of secretory leukocyte protease inhibitor during parturition have not been examined.

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The aims of this study were to measure the levels of secretory leukocyte protease inhibitor in amniotic fluid during pregnancy and labour. In addition, the sites of production within the uterus at term have been characterised to determine the putative roles that secretory leukocyte protease inhibitor might play in the paracrine interactions within the uterus for the maintenance of pregnancy and the control of parturition.

Materials and Methods

Tissue collection

Samples of amniotic fluid from the second trimester (n=20 women; 15–18 weeks) were obtained from women undergoing an amniocentesis as part of the karyotype screening programme for Downs' Syndrome. All pregnancies were karyotypically normal. At term, samples of amniotic fluid were obtained from women undergoing elective caesarean section (n=15 women; >37 weeks) and after spontaneous vaginal delivery (n=15 women; >37 weeks, uncomplicated labour, not induced). Fetal urine samples, which were clear and not contaminated with blood, were collected at first micturition immediately after delivery by elective caesarean section from uncomplicated pregnancies (n=12 neonates; >37 weeks). Fetal serum samples were collected at term (>37 weeks) from the placental venous cord vein after spontaneous vaginal delivery (n=5 neonates) and emergency caesarean section (spontaneous active labour, n=5 neonates). All samples were centrifuged at 1000 *g* for 5 min and stored at −20 °C prior to immunoassay. Tissues used for explants were obtained from women undergoing an elective caesarean section at term (n=6 women; >37 weeks; not associated with labour). For placental perfusion, placentae were collected after spontaneous vaginal delivery at term (n=6; >37 weeks, active labour, not induced). For immunohistochemistry, tissues (n=4; >37 weeks, elective caesarean; n=4; >37 weeks, emergency caesarean section, active labour, not induced) were fixed in 10% neutral buffered formalin for 24 h before washing twice in 70% ethanol prior to mounting in paraffin wax. Ethical approval for the collection of all samples was obtained from the Lothian Trust Ethical Committee with the informed and written consent of patients.

Culture of explants of amnion, chorio-decidua, decidua and placenta

Discs of amnion (12 mm diameter; wet weight 15–20 mg) and chorio-decidua (9 mm diameter; wet weight 15–25 mg) were prepared using a cork borer. Decidua (wet weight 15–25 mg) was dissected off the myometrial aspect of the posterior uterine wall from a site away from the placental bed and pieces of villous placental tissue dissected from the middle of a central cotyledon (wet weight 15–30 mg). Tissues explants (four explants of each tissue from each woman) were maintained in culture, as described previously by this laboratory (Brennand *et al.*, 1995). All reagents were obtained from Sigma, Poole, UK unless otherwise stated. Explants were placed on absorbent capillary matting (The Fyba Pot Company Ltd, Knottingley, UK) in a 24-well plate (Costar, High Wycombe, UK) and maintained in complete culture medium (RPMI 1640 with 2 mM L-glutamine, Gibco, Paisley, UK supplemented with 10% fetal calf serum, Gibco; 50 IU/ml penicillin, Gibco; 50 μg/ml streptomycin, and insulin/transferrin/selenium, Gibco) for 24 h at 37 °C in 95% air and 5% CO_2 under humid conditions in 24 well culture plates (Costar). The harvested media was then frozen at −20 °C until analysis.

Preparation and culture of amnion and chorion cells

Amnion and chorion were collected immediately after delivery from patients undergoing elective caesarean section at term (>37 weeks, not in labour), transported to the laboratory in Dulbecco's phosphate buffered saline (DPBS; Gibco) containing heparin (10 U/ml; LeoLabs Ltd, Risborough, UK), then incubated in DPBS containing gentamicin (80 μg/ml) and amphotericin B (5 μg/ml) for 60 min at 23 °C. Amnion and chorion cells were prepared for and maintained in culture adapting methods described previously (Jones *et al.* 1989). Excess decidua was scraped from the chorion laeve, and amnion and chorion were minced, washed and incubated in digestion medium (RPMI 1640 containing 5 mg/ml trypsin and 20 μg/ml DNAse) with mechanical agitation for 40 min at 37 °C. Dispersed cells were collected by passing through a 0·16 mm nylon mesh (Lockertex, Warrington, UK) three times. Cells were washed in complete culture medium and viabilities for all preparations were assessed as >90% by trypan blue exclusion. Cells were cultured in complete culture medium for 2–4 days. Chorion fibroblasts were obtained by dilution of chorion trophoblast cell preparations with subsequent overgrowth of fibroblasts to confluence within 28 days. Chorion fibroblasts were used up to passage five. Amnion cell preparations were >95% positive for the epithelial cell marker cytokeratin and <5% positive to the fibroblast/mesenchyme marker vimentin; chorion trophoblast cells preparations were >90% cytokeratin positive and <5% vimentin positive and chorion fibroblasts were >95% vimentin positive.

Dual perfusion of placental cotyledons

A peripheral cotyledon was selected which was macroscopically intact with parallel chorionic artery and vein, cannulated and mounted in a perfusion chamber as described previously (Schneider *et al.* 1972, Schneider & Huch 1985), with minor modifications (Benediktsson *et al.*

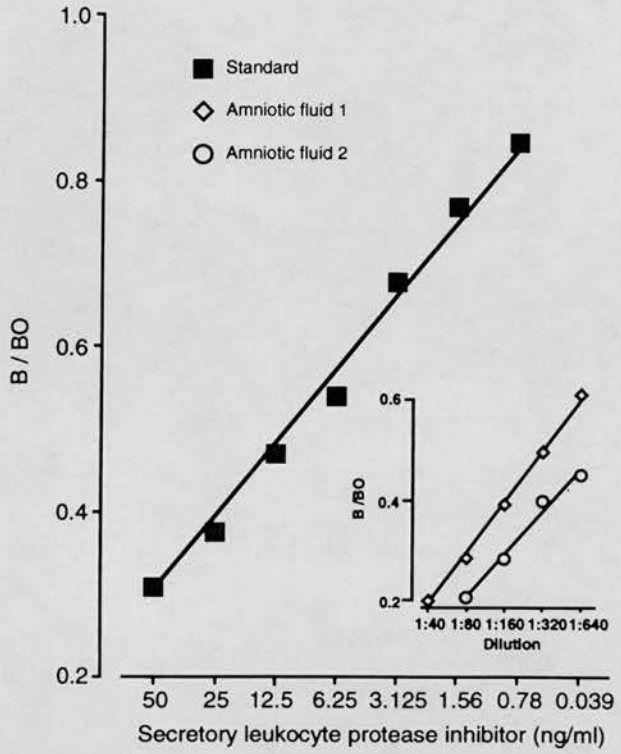

Figure 1 Dose–response relationships (B/BO) for recombinant secretory leukocyte protease inhibitor standards and two serially diluted amniotic fluid samples. Serial dilutions of amniotic fluid gave dose responses parallel to that of recombinant standard.

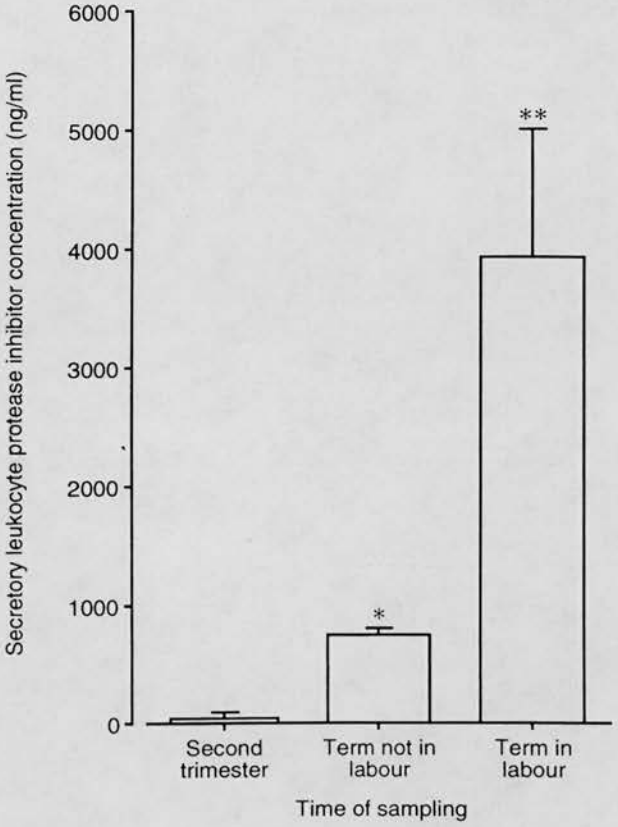

Figure 2 Increase in concentration of secretory leukocyte protease inhibitor in amniotic fluid during pregnancy. The concentration increased significantly during pregnancy from the second trimester (n=20) to term (n=15) to term in labour (n=15). Values are expressed as ng/ml ± S.E.M. Significance: *P<0·05, term not in labour to second trimester; **P<0·005, term not in labour to term in labour.

trimester (24 ± 3 ng/ml; mean ± S.E.M.) to term not in labour (751 ± 53 ng/ml) to term in labour (3929 ± 1076 ng/ml), with this rise being significant from second trimester to term (P<0·05) to term in labour (P<0·005; Fig. 2).

Secretory leukocyte protease inhibitor release from amnion, chorion-decidua, decidua and placenta and its concentration in cord serum and fetal urine

Amnion, chorio-decidua, decidua and placenta explants demonstrated a distinct pattern of secretory leukocyte protease inhibitor release with low levels being secreted by placenta (9·2 ± 1·9 pg/mg), more by amnion (55·6 ± 6·0 pg/mg; P<0·05) and significantly higher levels from decidua (135·2 ± 12·4 pg/mg; P<0·01) and chorio-decidua (325·1 ± 26·4 pg/mg; P<0·005; Fig. 3). Amnion epithelial cells, chorion trophoblast cells and chorion fibroblasts released 613 ± 88 pg/ml/10^5 cells, 389 ± 62 pg/ml/10^5 cells and 1658 ± 250 pg/ml/10^5 cells secretory leukocyte protease inhibitor, respectively.

Secretory leukocyte protease inhibitor was secreted into both the maternal (7080 ± 840 pg/min) and fetal (1790 ± 230 pg/min) circulations in the perfused placental cotyledon system (Fig. 4), with significantly higher (P<0·005) amounts being released into the maternal than fetal circulation. However, it was undetectable in all cord serum samples (n=10) and below the detection limit of the assay in nine out of the twelve fetal urine samples assayed, with levels being 358 pg/ml, 320 pg/ml and 188 pg/ml, respectively in the remaining three samples. There was no evidence of infection or neonatal sepsis in the three neonates in which secretory leukocyte protease inhibitor was detectable.

Immunolocalisation of secretory leukocyte protease inhibitor within fetal membranes, decidua and placenta

Intense specific immunoreactivity for secretory leukocyte protease inhibitor was localised to the layer of decidua parietalis adherent to chorion trophoblast (Fig. 5A). Similarly, intense immunostaining was present in decidua parietalis collected from the myometrial aspect (Fig. 5B). Only a few isolated cells, which may be fibroblasts, stained immunopositive within the amnion and chorion trophoblast, with the majority of the cells constituting these layers

1997). The maternal and fetal compartments were perfused with Kreb's solution at flow rates of 10 and 6 ml/min, and gassed with 95% O_2/5% CO_2 and 95% N_2/5% CO_2, respectively. The perfusate on the fetal side was supplemented with dextran (20 g/l; average molecular weight 74 kDa; Sigma Chemical Co., Poole, UK). Perfusions were started within 10 min of delivery. After a 40 min equilibration period, which permitted the residual blood to be eluted and circulatory perfusion pressures to stabilise, consecutive 10 min samples were collected from both maternal and fetal circuits. Samples were stored at −20 °C before being assayed. The integrity and viability of each preparation were established throughout the experimental period by ensuring that (a) the rate of perfusate input in both the fetal and maternal circuits equalled the rate of output, (b) there was adequate exchange of O_2 from the maternal to fetal circuits (step up pressures of >40 mm Hg in fetal perfusate), (c) lactate concentrations remained within the normal low range (<2 mM; Benediktsson *et al.* 1997), (d) at the end of the experiment a bolus of noradrenaline induced an increase in fetal vascular resistance of >2 mm Hg and (e) subsequent microscopic histological investigations revealed no significant morphological changes.

Secretory leukocyte protease inhibitor assay

Secretory leukocyte protease inhibitor was measured by a competitive enzyme linked immunosorbent assay. Plates (96 well; Nunc Maxi-Sorp, Gibco) were coated with recombinant human secretory leukocyte protease inhibitor (R&D Systems, Oxford, UK; 0·025 μg/ml in PBS/1% carbonate buffer; 400 mM $NaHCO_3$; 40 mM Na_2CO_3; pH 9·6; 300 μl/well) for 60 min at 23 °C then blocked with milk powder (0·1 mg/ml in H_2O; 400 μl/well) for 30 min at 23 °C. Plates were then washed (150 mM NaCl; 100 mM Tris; 0·05% Tween-20; pH 7–7·5) and ELISA assay buffer (150 mM NaCl; 100 mM Tris; 50 mM Phenol Red solution; 2 mM EDTA; 1 mM 2-ethylisothiazolone, Boehringer Mannheim, Lewes, UK; 1 mM bromonitrodioxane, Boehringer Mannheim; 2 mg/ml BSA; 0·05% Tween-20 to final pH of 7·2; 250 μl/well) added for determination of non-specific binding (NSB). Recombinant standards (R&D Systems; highest concentration 50 ng/ml; 200 μl/well) and samples (200 μl/well) were added, followed by the polyclonal antibody raised in goat against recombinant human secretory leukocyte protease inhibitor (R&D Systems; 2 μg/ml in ELISA buffer; 50 μl/well) and incubated for 120 min at 23 °C. Plates were washed and anti sheep/goat IgG Fab fragments raised in donkey conjugated to peroxidase (Boehringer Mannheim; diluted 1:500 in ELISA buffer; 100 μl/well) added and incubated for 120 min at 23 °C. Finally, plates were washed again and substrate (0·3 g/l urea–hydrogen peroxide; 0·1 g/l tetramethyl benzidine in 100 mM sodium acetate, pH 6·0; 200 μl/well) was added prior to quenching with 2N H_2SO_4 (50 μl/well). Absorption was read at 450 nm within 30 min of quenching. The intra- and inter-assay precisions were 9·2% relative standard deviations (r.s.d.) and 10·1% r.s.d., respectively and the detection limit of the assay was 9·8 pg/ml. To validate the assay, serial dilutions of amniotic fluids were compared with that of the recombinant standard and assay results were compared with those obtained using a commercial kit which uses the same antibodies (R&D Systems).

Localisation of secretory leukocyte protease inhibitor in tissues by immunohistochemistry

Sections (5 μm) were dewaxed, rehydrated and endogenous peroxidase quenched with H_2O_2 (1% v/v in methanol) for 30 min at 23 °C. Non-specific binding was eliminated by pre-blocking with 10% normal donkey serum (Scottish Antibody Production Unit, Carluke, UK) for 30 min at 37 °C. The polyclonal secretory leukocyte protease inhibitor primary antibody, previously described (R&D Systems), was then applied (25 μg/ml in 10% normal donkey serum) for 17 h at 4 °C. Sections were washed and specific binding detected using an anti-sheep/goat Fab fragment raised in donkey conjugated to peroxidase (R&D Systems) applied for 90 min at 23 °C. This peroxidase activity was localised using 3,3′-diaminobenzidine (Vector Labs Ltd, Peterborough, UK) as chromagen. Sections were counterstained with Harris' haematoxylin, dehydrated, mounted and visualised by light microscopy.

Statistical analysis

Statistical analysis of the data was performed using ANOVA (Statview 4·1, Abacus Inc., Berkeley, CA, USA). The data were normally distributed and are expressed as pg/ml, ng/ml or pg/mg/wet weight tissue (mean ± S.E.M.) with a statistically significant difference defined as $P<0·05$.

Results

Validation of secretory leukocyte protease inhibitor assay

Serial dilutions of amniotic fluids gave dose–response curves parallel to that of the recombinant standard (Fig. 1). The results from this assay and those obtained by a commercial assay (R&D Systems), which uses the same antibodies, were very highly correlated ($r=0·99$, total degrees of freedom, $n=5$).

Presence of secretory leukocyte protease inhibitor in amniotic fluid

Secretory leukocyte protease inhibitor was present within amniotic fluid in increasing concentrations from second

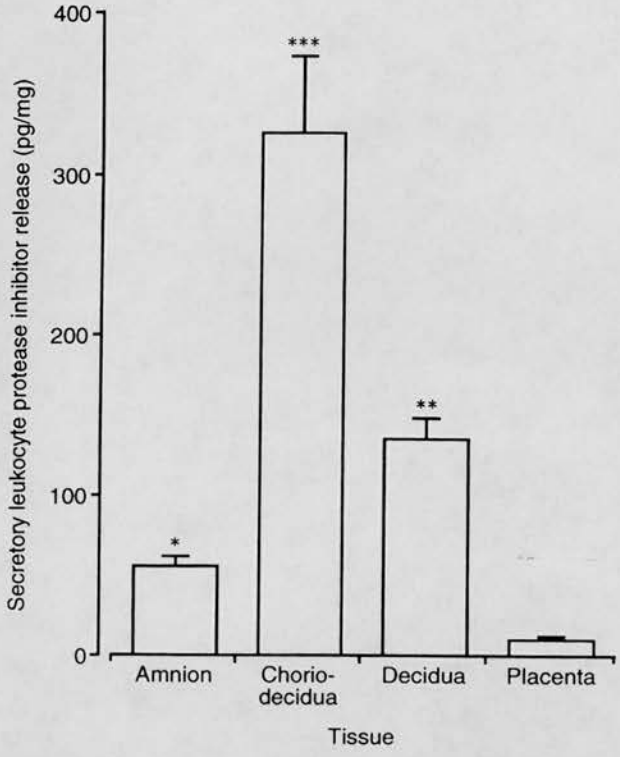

Figure 3 Release of secretory leukocyte protease inhibitor by explants of third trimester amnion, chorio-decidua, decidua and placenta maintained in culture for 24 h. Significantly more secretory leukocyte protease inhibitor was released by chorio-decidua and decidua than by placenta and amnion. Values are expressed as pg/mg/wet weight tissue ± S.E.M.; tissues collected from n=6 different women. Significance: *P<0·05, amnion compared with placenta; **P<0·01, decidua compared with amnion and placenta; ***P<0·005, chorio-decidua compared with decidua, amnion and placenta.

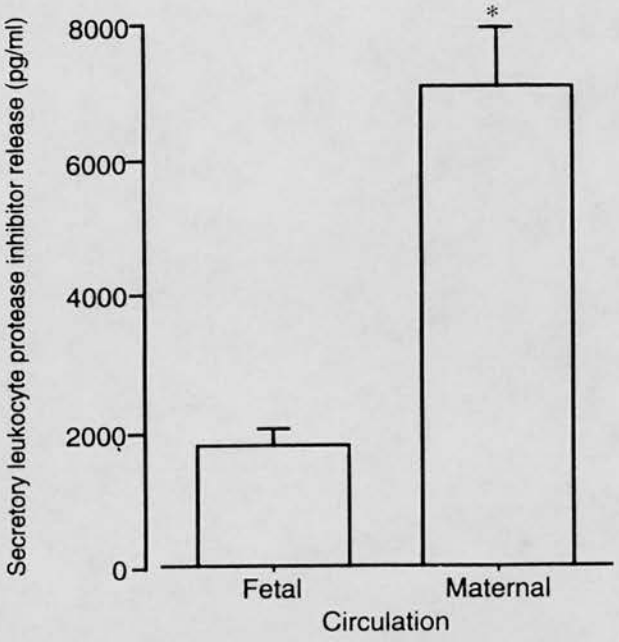

Figure 4 Release of secretory leukocyte protease inhibitor from the dually perfused placental cotyledon. Significantly more secretory leukocyte protease inhibitor was released into the maternal than the fetal circulation. Values are expressed as pg/min ± S.E.M.; n=6 placentae. Significance: *P<0·005, maternal compared with fetal effluent.

being immunonegative (Fig. 5A). In placenta, the decidua basalis which underlies the placenta also demonstrated intense specific immunoreactivity (Fig. 5C). The extra-villous trophoblast was immunonegative, as was the majority of the placenta apart from a small amount of staining around the margins and within fetal blood vessels of the villi. Substitution of the primary antibody with normal donkey (Fig. 5D) and normal goat serum (data not shown) resulted in absence of staining in decidua, fetal membranes and placenta.

Discussion

We demonstrate that secretory leukocyte protease inhibitor is present in increasing concentrations within amniotic fluid during pregnancy and further increases with the onset of labour. Secretory leukocyte protease inhibitor was secreted predominantly by explants of decidua and chorio-decidua, with lower levels released by amnion and placenta. Immunohistochemistry demonstrated cell-specific localisation of secretory leukocyte protease inhibitor to both decidua parietalis and basalis.

The profound increase in the concentration of secretory leukocyte protease inhibitor within amniotic fluid after the onset of labour is a novel finding. The presence of secretory leukocyte protease inhibitor within amniotic fluid during the second trimester and at term has been previously demonstrated (Helmig *et al.* 1995) with the reported values being comparable to our data. Term amniotic fluid comprises mainly of secretions from the surrounding fetal membranes, decidua, fetal lung and from fetal urine (Gilbert & Brace 1993). Release of secretory leukocyte protease inhibitor from amnion explants *in vitro* was comparatively low. However, the potential release of secretory leukocyte protease inhibitor by amnion *in vivo* may be considerable, due to the large surface area of the amniotic membrane. In addition, it has been previously demonstrated that secretory leukocyte protease inhibitor is present in high concentrations within tracheal aspirates from neonates (Ohlsson *et al.* 1992) with levels correlating positively with gestational age (Sluis *et al.* 1994). Although we have demonstrated that fetal urine contains secretory leukocyte protease inhibitor, it was only detectable in one quarter of all samples tested and therefore unlikely to account for the consistently high levels of secretory

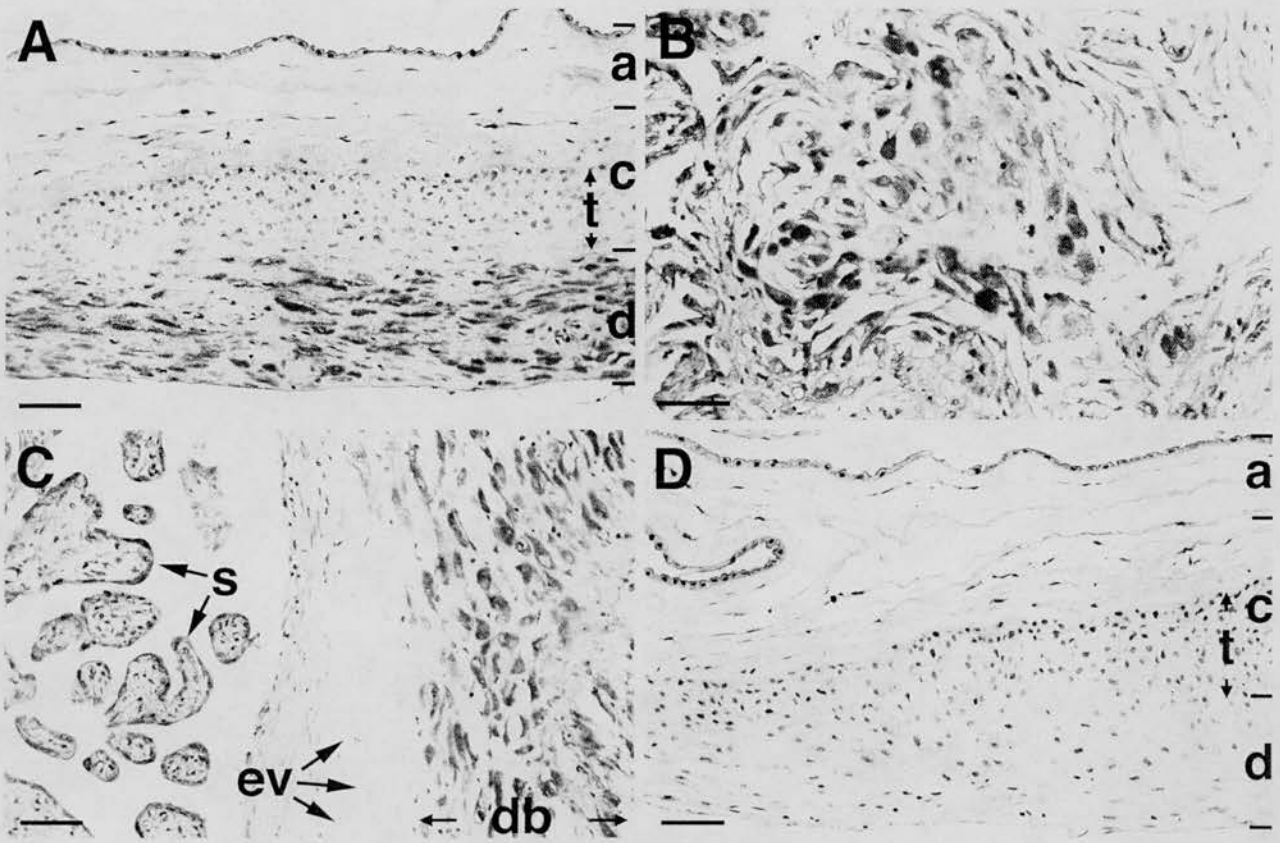

Figure 5 Localisation of secretory leukocyte protease inhibitor by immunohistochemistry in: A, Fetal membranes and adherent decidua collected at emergency caesarean section in active labour. Intense immunostaining for secretory leukocyte protease inhibitor is localised predominantly in decidual cells and in some cells of fibroblast morphology in chorion and amnion. Key: a=amnion; c=chorion laeve; d=decidua parietalis adherent to the chorion; t=trophoblast cell layer of chorion. B, Decidua parietalis collected from the myometrial aspect after emergency caesarean section in active labour with specific positive immunoreactivity in decidual stromal cells. C, Placenta collected at emergency caesarean section in active labour with little or no immunostaining in syncytiotrophoblast cells or extravillous trophoblast in the placental bed. Some positive immunostaining is present in cells of the villous core in the stroma and associated with the vasculature. There is intense immunostaining in cells of the decidua basalis. Key: s=syncytiotrophoblast cells; ev=extravillous trophoblast; db=decidua basalis. D, Parallel negative control section of fetal membranes and adherent decidua with no non-specific staining. Scale bars: A and D, 200 μm; B and C, 100 μm.

leukocyte protease inhibitor demonstrated in all labouring amniotic fluid samples. It is likely therefore that release and transfer of secretory leukocyte protease inhibitor from decidua, as has been suggested for prolactin (Rosenberg *et al.* 1980), amnion epithelial and fetal lung secretions may all contribute to secretory leukocyte protease inhibitor in amniotic fluid.

The predominant source of secretory leukocyte protease inhibitor secretion in the uterus is likely to be the decidua, with explants of decidua parietalis and chorio-decidua secreting high levels. In decidua parietalis, intense immunoreactivity for secretory leukocyte protease inhibitor was localised in the maternal aspect of the decidua and in that adjacent to chorion. This adherent decidua is likely to be the principal site of production in chorio-decidual explants. Decidua collected from the myometrial aspect by curettage is usually contaminated with some myometrium, which is immunonegative for the protein. This may explain why decidua explants secrete slightly less secretory leukocyte protease inhibitor compared with chorio-decidua, unless there is a gradient of secretion with highest outputs from decidua adjacent to the chorion. Term decidua is heterogeneous, consisting of stromal cells (53%), macrophages (19%), granulocytes (16%) and T-cells (8%) (Adeleye *et al.* 1996). Hence the stromal cells, macrophages and granulocytes, which are capable of secretory leukocyte protease inhibitor secretion in other sites (Ohlsson *et al.* 1996), are potential sources. In the fetal membranes, amnion explants, which consist of an epithelium and sub-epithelial layers containing fibroblasts, secrete secretory leukocyte protease inhibitor. The potential for paracrine interactions between the different cell types present in the term amnion, chorion, composed of reticular fibroblast and the trophoblast cell layers and

decidua, to regulate the release of secretory leukocyte protease inhibitor at the materno–fetal interface requires further investigation.

Placental explants of villous core tissue released low levels of secretory leukocyte protease inhibitor, which is in agreement with only slight immunostaining within the fetal vasculature. This immunostaining could represent either secretory leukocyte protease inhibitor bound to elastin (Rudolphus *et al.* 1994) within the blood vessel wall or specific staining of residual fetal blood cells in the villi. Within the isolated perfused placental cotyledon, secretory leukocyte protease inhibitor was released mainly into the maternal and not the fetal circuit and was undetectable in cord serum. Decidua basalis adherent to the perfused placental cotyledon stains intensely for secretory leukocyte protease inhibitor reactivity and may be the principal source of the secretory leukocyte protease inhibitor secretion into the maternal circulation effluent.

Parturition has been likened to an inflammatory reaction (Liggins *et al.* 1977). Secretory leukocyte protease inhibitor may be involved in the regulation of this response at the materno–fetal interface. At the onset of labour, there is increased expression and release of pro-inflammatory cytokines such as IL-1 and IL-6 within amniotic fluid (Cox *et al.* 1997) and decidua (Dudley *et al.* 1992), which stimulate pro-inflammatory cascades involved in the initiation and maintenance of parturition. However, IL-1 and IL-6 can also stimulate release of secretory leukocyte protease inhibitor (Sallenave *et al.* 1994, Jin *et al.* 1998). The significant rise in IL-1 and IL-6 within amniotic fluid and decidua at parturition could therefore enhance release of secretory leukocyte protease inhibitor (Sallenave *et al.* 1994, Jin *et al.* 1998) from amnion epithelium, fetal respiratory tract mucosa and decidua. Secretory leukocyte protease inhibitor could then exert a negative feedback on release of inflammatory cytokines via inhibition of NFκB (Jin *et al.* 1997) and neutrophil function by inhibition of neutrophil elastase. Secretory leukocyte protease inhibitor could also inhibit release of PGE_2 and matrix metalloproteinases, which are also involved in membrane remodelling and rupture, by inhibiting cyclo-oxygenase-2 (Zhang *et al.* 1997). During parturition, the uterine cavity is at high risk of invasion and infection by microbial organisms. Secretory leukocyte protease inhibitor, by virtue of its anti-bacterial, anti-fungal and anti-viral properties, could therefore in addition act as an endogenous block to infection within the decidua and the amniotic cavity.

In summary, we demonstrate that secretory leukocyte protease inhibitor is present in increasing concentrations within amniotic fluid during pregnancy and labour. Secretory leukocyte protease inhibitor is also released by and immunolocalised to term decidua. This tissue, together with fetal membranes and possibly the fetal lung, are the likely sources of secretory leukocyte protease inhibitor within the uterus. We suggest that secretory leukocyte protease inhibitor might act both to limit the pro-inflammatory cascades ongoing during parturition and to protect against microbial invasion and the response to infection. Further studies are required to investigate the regulation of secretory leukocyte protease inhibitor release by intra-uterine tissues and to examine its potential roles in the maintenance of pregnancy and the initiation and progression of parturition, both preterm and at term.

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Cytokine secretion by human fetal membranes, decidua and placenta at term

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Cytokines such as monocyte chemotactic peptide-1 (MCP-1), interleukin-8 (IL-8), RANTES (Regulated on Activation and Normally T-cells Expressed and presumably Secreted) and interleukin-10 (IL-10) are thought to play pivotal roles in immune recognition, acceptance of the fetal allograft, maintenance of pregnancy and parturition. Their secretion and regulation within the third trimester uterus is, however, less well defined. We therefore investigated the release of these cytokines by third trimester amnion, chorion, placenta and decidua, and studied the influence of prostaglandin E_2 (PGE_2) infusion on their release in a dynamic placental cotyledon perfusion system. MCP-1 was released predominately by the chorion (78.2 ± 7.3 pg/mg wet tissue weight; mean ± SEM), decidua (112.4 ± 5.2 pg/mg) and placenta (101.8 ± 5.0 pg/mg) with low amounts from the amnion (1.3 ± 0.4 pg/mg). High concentrations of IL-8 were released by the amnion (39.9 ± 5.3 pg/mg), chorion (52.8 ± 1.9 pg/mg), decidua (42.2 ± 1.5 pg/mg) and placenta (45 ± 1.3 pg/mg). Release of RANTES was not detectable from the amnion but was detected in moderate amounts from the chorion (6.0 ± 1.2 pg/mg), decidua (15.2 ± 1.4 pg/mg) and placenta (26.9 ± 1.6 pg/mg). Low concentrations of IL-10 were secreted by the chorion (6.8 ± 0.8 pg/mg), decidua (9.0 ± 0.9 pg/mg) and placenta (3.3 ± 0.3 pg/mg) with none detectable from the amnion. MCP-1, IL-8, RANTES and IL-10 were all released by perfused placental cotyledons. PGE_2 stimulated release of MCP-1, IL-8 and IL-10 into the maternal and of MCP-1 and IL-8 into the fetal circulation of the placenta but had no effect on RANTES release. It is suggested that MCP-1 and IL-8 may be involved in the inflammatory process of parturition and IL-10 in the protection of the fetal allograft. In addition, PGE_2 may have an important immunomodulatory role within the uterus at term.

Key words: cytokines/placenta/pregnancy/prostaglandin E_2/uterus

Introduction

The control and regulation of intrauterine function during pregnancy and parturition is dependent on a delicate balance between immune and endocrine systems. Chemokines, acting as local effectors and mediators of cell trafficking, are thought to play pivotal roles in the immune recognition and maintenance of pregnancy, acceptance of the fetal allograft and parturition. Monocyte chemotactic peptide-1 (MCP-1), interleukin-8 (IL-8) and RANTES (Regulated on Activation and Normally T-cell Expressed and presumably Secreted) are chemokines whose principal role is in effecting chemotaxis of monocytes, neutrophils and T-cells respectively. IL-8, an α-chemokine, is released by many of the intrauterine tissues present during the third trimester (Elliott *et al.*, 1998), is found in high concentrations within amniotic fluid, and has been implicated in the initiation of infection-mediated pre-term labour (Cherouny *et al.*, 1993) and cervical ripening (Sennstrom *et al.*, 1997). MCP-1, a β-chemokine, is released by many reproductive tissues, including the ovarian follicles (Arici *et al.*, 1997), endometrium (Arici *et al.*, 1995) and third trimester chorio-decidua (Kelly *et al.*, 1997), and RANTES is released by the endometrium (Hornung *et al.*, 1997) and a first trimester trophoblast cell line (Svinarich *et al.*, 1996). However, whether MCP-1 is released by amnion or placenta or RANTES by amnion, chorion, decidua or placenta during the third trimester is not known.

Interleukin-10 (IL-10) is a potent immunomodulatory cytokine that promotes development of a humoral or T-helper 2 (TH-2) type of immune response, which is associated with a successful outcome to pregnancy (Wegmann *et al.*, 1993). IL-10 is present in amniotic fluid throughout pregnancy and is released by most of the intrauterine structures present during the third trimester. The factors which regulate IL-10 release from these tissues and its function within the uterus at term are, however, less well defined.

It is now recognized that prostaglandin E_2 (PGE_2), although initially described as a potent vasodilator, is an important immunomodulator and mediator of inflammation within the uterus (Kelly *et al.*, 1995). During the third trimester, it is released by the amnion (Edwin *et al.*, 1996), chorion (Dudley *et al.*, 1996), decidua (Ishihara *et al.*, 1991) and placenta (Wetzka *et al.*, 1997), and a rise in its concentrations within the uterus is associated with the onset of parturition. The effects of PGE_2 on the release of other cytokines such as IL-8, which may be involved in parturition, are not well understood.

The aim of this study was to investigate the release of MCP-1, IL-8, RANTES and IL-10 from third-trimester amnion, chorion, decidua and placenta and to evaluate the possible contribution of these chemokines to parturition. A dynamic dual placental cotyledon perfusion model was then used to study the influence of PGE_2 infusion on placental chemokine release and to assess whether any regulation of chemokine

secretion could have implications for the immunological mechanisms involved in the maintenance of pregnancy and parturition.

Materials and methods

Tissue collection

Tissue samples for explant cultures were obtained from normal healthy women with uncomplicated pregnancies undergoing elective Caesarean section at term. Discs of amnion (12-mm diameter; wet weight 10–20 mg; $n = 7$ different women) and chorion laeve (9-mm diameter; 15–25 mg; $n = 7$ different women) were prepared using a cork borer, pieces of decidua (20–30 mg; $n = 7$ different women) by curettage off the myometrium and villous placenta (20–30 mg; $n = 7$ different women) by dissection of placental tissue. For placental perfusion studies, placentae ($n = 8$ different placentae) were collected from normal healthy women with uncomplicated pregnancies immediately after elective Caesarean section or spontaneous delivery and were transported to the laboratory on ice. Ethical approval was obtained for this study from the local Ethics Committee.

Explant culture

Discs of amnion, chorion laeve, pieces of decidua and villous placental tissue were placed on absorbent capillary matting and maintained in culture as described previously (Brennand *et al.*, 1995). Four explants of each tissue were cultured from each woman. Culture medium was collected after 24 h and stored at –20°C before being assayed.

Dual perfusion of a placental cotyledon

A peripheral cotyledon was selected which was macroscopically intact with parallel chorionic artery and vein, cannulated and mounted in a perfusion chamber as described previously (Schneider *et al.*, 1972; Schneider and Huch, 1985), with minor modifications (Benediktsson *et al.*, 1997). The maternal and fetal compartments were perfused with Kreb's solution at flow rates of 10 and 6 ml/min, and gassed with 95% O_2/5% CO_2 and 95% N_2/5% CO_2 respectively. The perfusate on the fetal side was supplemented with dextran (20 g/l; average molecular weight 74 kDa; Sigma Chemical Co., Poole, Dorset, UK). Perfusions were started within 10 min of delivery. After a 40-min equilibration period, which permitted the residual blood to be eluted and circulatory perfusion pressures to stabilize, consecutive 10-min samples were collected from both maternal and fetal circuits. A pre-infusion sample was followed by three samples during the infusion of PGE_2 (500 pg/ml for 30 min; Upjohn Co., Kalamazoo, MI, USA) into the maternal circuit, followed by two post-infusion samples. Samples were stored at –20°C before being assayed.

The integrity and viability of each preparation was established throughout the experimental period by ensuring that: (i) the rate of perfusate input in both the fetal and maternal circuits equalled the rate of output; (ii) there was adequate exchange of O_2 from the maternal to fetal circuits (step-up pressures of <40 to 70 mm Hg in fetal perfusate); (iii) lactate concentrations remained within the normal low range (<2 mM; Benediktsson *et al.*, 1997); (iv) at the end of the experiment the fetal vasculature responded to a bolus infusion of noradrenaline (20 mg; Sigma); and (v) subsequent microscopic histological investigations revealed no significant morphological changes.

Cytokine assays

Assay for MCP-1

MCP-1 was measured by an enzyme-linked immunosorbent assay (ELISA) using a peroxidase labelled Fab fragment of an antibody to MCP-1 as described previously (Ida *et al.*, 1994; Kelly *et al.*, 1997). Briefly, plates were coated overnight at 4°C with MCP-1 capture antibody [4 μg/ml in phosphate-buffered saline (PBS); Toray Industries Inc., Tokyo, Japan], then washed once in tap water and coating solution added [50 mM Tris, 5 mg/ml bovine serum albumin (BSA), 5 mM EDTA, 2% polyvinyl pyrrolidone; all reagents were from Sigma, unless otherwise stated] for 30 min. Plates were air dried and stored with a desiccant at 4°C. Samples and standards (Toray Industries, Inc., diluted in ELISA buffer; 150 mM NaCl; 100 mM Tris; 100 mM 2-methylisothizolone, Boehringer Mannheim, Lewes, Sussex, UK; 100 mM bromonitrodioxane, Boehringer Mannheim; 50 mM Phenol Red solution; 2 mM EDTA; 2 mg/ml BSA; 0.05% Tween 20 to final pH of 7.2) were added with the highest concentration of standard being 500 pg/ml. Plates were then incubated for 3 h at 23°C on an orbital plate shaker, washed (150 mM NaCl; 100 mM Tris; 0.05% Tween 20 to final pH of 7–7.5), then peroxidase-coupled detection antibody (diluted 1:200 in ELISA Buffer) was added and incubated for 45 min at 23°C on an orbital shaker. Plates were then washed and substrate (0.3 g/l urea–hydrogen peroxide; 0.1 g/l tetramethyl benzidine in 100 mM sodium acetate, pH 6.0) added prior to quenching with 2 N H_2SO_4. Absorption was read at 450 nm within 30 min of quenching. The intra-assay precision was 6.3% relative standard deviation, inter-assay precision was 8.6% relative standard deviation and the detection limit of the assay was 7.5 pg/ml.

Assay for IL-8

IL-8 was measured by ELISA, utilizing matched pairs of capture and biotinylated-labelled detection antibodies against IL-8 (R&D Systems, Oxford, UK) (Denison *et al.*, 1997). Briefly, plates were coated overnight at 4°C with capture antibody, washed once in water, and coating solution added for 30 min at 23°C. The coating solution was removed and the plates stored as for MCP-1. Prior to use, the plates were washed once in water. Samples and standards (recombinant standards; Toray Industries Inc.; diluted in ELISA buffer) were added, with the top standard being 500 pg/ml. Plates were incubated for 3 h at 23°C on an orbital shaker, washed as above, then detection antibody (30 ng/ml in ELISA buffer) was added and plates were incubated on a shaker for 45 min at 23°C. After a further wash, streptavidin peroxidase (Boehringer Mannheim) was added at 0.02 U/well and plates were incubated for 20 min at 23°C on an orbital shaker. They were then washed, substrate was added and were read as for MCP-1. The intra- and inter-assay precisions were 9.1% relative standard deviation and 22.1% relative standard deviation respectively, and the detection limit of the assay was 7.5 pg/ml.

Assay for RANTES

This assay has been performed previously in this laboratory and has been described in detail (Denison *et al.*, 1997). The capture antibody (coated at 2 μg/ml), standards (recombinant, top standard 500 pg/ml) and detection antibody (added at 50 μg/ml) were all obtained from R&D Systems. The intra- and inter-assay precisions for this assay were 10.4% relative standard deviation and 11.2% relative standard deviation respectively, and the detection limit of the assay was 7.5 pg/ml.

Assay for IL-10

All dilutions were in 10% fetal calf serum in PBS unless otherwise stated. Plates were coated with capture antibody (Pharmingen, San Diego, USA; 200 ng/ml in PBS) added at 100 μl/well for 60 min at 23°C, then blocked (10% fetal calf serum in PBS) at 300 μl/well for a further 60 min at 23°C. The plates were then washed and both samples and standards (recombinant; Pharmingen) added with the top standard being 500 pg/ml. Plates were then incubated on an orbital shaker for 60 min, washed, detection antibody (Pharmingen, San Diego, CA, USA, 100 μl; 125 ng/ml) was added and incubated for 60

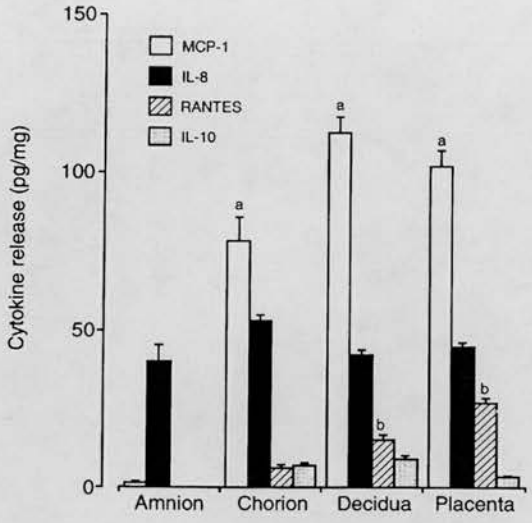

Figure 1. Release of monocyte chemotactic peptide-1 (MCP-1), nterleukin-8 (IL-8), RANTES (Regulated on Activation and Normally T-cells Expressed and presumably Secreted) and nterleukin-10 (IL-10) from the amnion, chorion, placenta and decidua. Significantly higher concentrations of MCP-1 were eleased by the chorion ($P < 0.001$), placenta ($P < 0.001$) and decidua ($P < 0.001$) than from the amnion respectively. RANTES vas secreted in significantly greater amounts from the decidua $P < 0.01$) and placenta ($P < 0.01$) than from the chorion. The najority of IL-10 was released by the chorion and decidua with ower but non-significant concentrations from the placenta. Neither RANTES nor IL-10 were released by the amnion. Significance: a, < 0.001; b, $P < 0.01$; $n = 7$ different women. Values are xpressed as pg/mg wet weight ± SEM.

nin at 23°C. After a further wash, polyperoxidase (CLB laboratories, Amsterdam, The Netherlands; 100 µl; 1 ng/ml in ELISA buffer vithout Tween) was added for 30 min. Finally, plates were washed nd read as for MCP-1. The intra- and inter-assay variations were .4% and 10.1% respectively, and the detection limit of the assay vas 7.5 pg/ml.

tatistical analysis

tatistical analysis of the tissue explant data was performed using nalysis of variance and a Student's paired *t*-test was used for the lacental perfusion model (Statview 4.1, Abacus Inc., Berkeley, CA, JSA). The data were normally distributed and are expressed as g/mg wet weight (mean ± SEM) with a statistically significant ifference defined as $P < 0.05$.

Results

Tissue explants

Explants of amnion, chorion, decidua and placenta exhibit istinct profiles of cytokine release (see Figure 1). Significantly igher concentrations of MCP-1 were released by the chorion 78.2 ± pg/mg wet weight; $P < 0.001$), decidua (112.4 ± 5.2 g/mg; $P < 0.001$) and placenta (101.8 ± 5.0 pg/mg; $P <$ .001) than from the amnion (1.3 ± 0.4 pg/mg). IL-8 was ecreted in large amounts by all tissues studied with release rom the chorion, decidua, placenta and amnion being 52.8 ± .9 pg/mg, 42.2 ± 1.5 pg/mg, 45 ± 1.3 pg/mg and 39.9 ± 5 espectively. The placenta (26.9 ± 1.6 pg/mg) and decidua 15.2 ± 1.4 pg/mg) were the principal sources of RANTES, with significantly more being released than by the chorion (6.0 ± 1.2 pg/mg; $P < 0.01$). IL-10 was released mainly by the chorion (6.8 ± 0.8 pg/mg) and decidua (9.0 ± 0.9 pg/mg) with concentrations which were lower, but which failed to reach significance, from the placenta (3.3 ± 0.3 pg/mg). Neither RANTES nor IL-10 were released in detectable concentrations by the amnion. The ratio of MCP-1:IL-8 release by the amnion was 1:31 and the ratios of MCP-1:IL-8: RANTES: IL-10 release by the chorion, placenta and decidua were 13:9:1:1, 31:14:8:1 and 12:5:2:1 respectively.

Placental perfusion

The basal cytokine output from the perfused placental cotyledon (maternal and fetal combined) prior to commencing the PGE_2 infusion was less than that by placental explants for MCP-1, IL-8 and IL-10 (relative ratios 1:4; 1:13; 1:2) but not for RANTES (relative ratio 1:1). The basal output of all cytokines measured was greater from the maternal than the fetal circulation in the perfusion system, with the ratios of basal output of MCP-1, IL-8, RANTES and IL-10 between maternal and fetal circuits being 145:1, 79:1, 7:1 and 2:1 respectively. The differential release of cytokines between the two circulations was maintained for the duration of the perfusion and study period.

Perfusion of placentae with PGE_2 significantly ($P < 0.01$) stimulated release of IL-8, MCP-1 and IL-10 from the maternal and IL-8 and MCP-1 from the fetal circulation. The stimulation became significant ~20 min after commencing the PGE_2 infusion and was maintained for the duration of the perfusion and study period (Figures 2a, b and 3).

RANTES was released into both the maternal and fetal circulation, but secretion was not affected by infusion of PGE_2, unlike the other cytokines measured (Figures 2a, b).

Discussion

The heterogeneous intrauterine structures present during the third trimester originate from both maternal and fetal tissue and have widely differing functions. In this study it has been demonstrated that third trimester amnion, chorion, decidua and placenta explants secrete quite distinct cytokine profiles. MCP-1 was released predominately by the chorion, decidua and placenta with low amounts from the amnion (relative ratios 62:94:85:1 respectively). High concentrations of IL-8 were released by the amnion, chorion, decidua and placenta (relative ratios 1:1:1:1 respectively). Release of RANTES was not detectable from the amnion but was detected in moderate amounts from the chorion, decidua and placenta (relative ratios 1:3:4). Low concentrations of IL-10 were secreted by the chorion, decidua and placenta with none detectable from the amnion (relative ratios 2:3:1).

The fetal membranes comprise the amnion and chorion. The amnion, which is composed of an epithelial and fibroblast layer, released both IL-8 and MCP-1. Local release of IL-8 from unstimulated (Laham *et al.*, 1997) and cytokine-stimulated amnion explants (Trautman *et al.*, 1992) has been demonstrated previously and has been postulated to have a role in parturition. The demonstration of MCP-1 release is, however, novel and

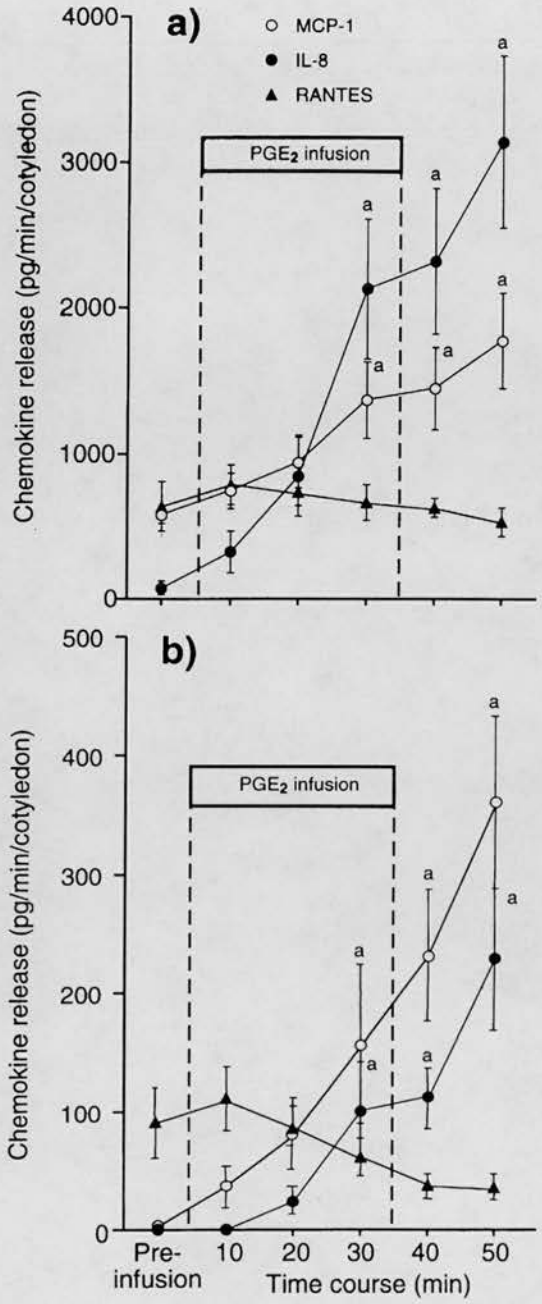

Figure 2. Effect of prostaglandin E_2 (PGE_2) infusion on the release of monocyte chemotactic peptide-1 (MCP-1), interleukin-8 (IL-8), RANTES (Regulated on Activation and Normally T-cells Expressed and presumably Secreted) into (**a**) the maternal and (**b**) the fetal circulation in a dynamic dually perfused placental cotyledon. The release of MCP-1 and IL-8 release was significantly ($P < 0.01$) stimulated into both maternal and fetal circulations ~20 min after commencing the PGE_2 infusion and concentrations continued to be elevated until the end of the study period. The PGE_2 infusion had no effect on RANTES release. Significance: a, $P < 0.01$; $n = 8$ different placentae. Values are expressed as pg/min/cotyledon ± SEM.

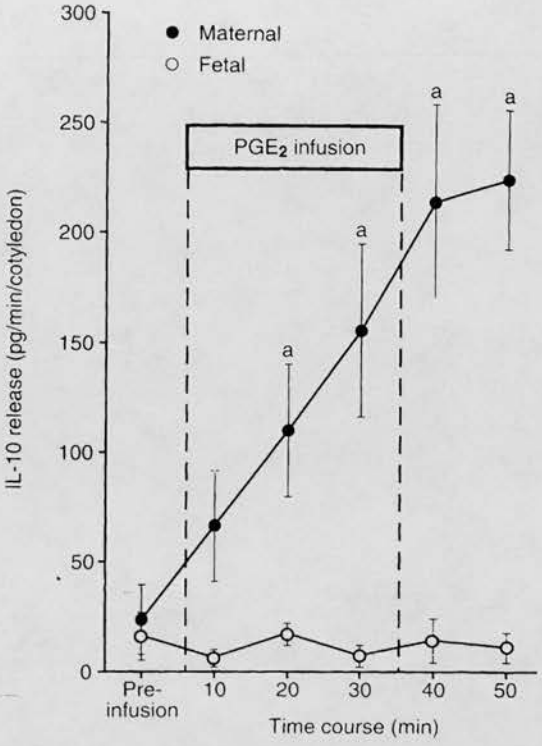

Figure 3. Effect of prostaglandin E_2 (PGE_2) infusion on the release of interleukin-10 (IL-10) into the maternal and fetal circulation in dually perfused placental cotyledon. The release of IL-10 was significantly ($P < 0.01$) stimulated into the maternal circulation ~20 min after commencing the PGE_2 infusion and concentrations continued to be elevated until the end of the study period. There was no rise in concentrations of IL-10 in the fetal circulation post-infusion. Significance: a, $P < 0.01$; $n = 8$ different placentae. Values are expressed as pg/min/cotyledon ± SEM.

could be from either epithelium or fibroblasts, both of which are capable of its synthesis in extra-uterine sites (Elner *et al.*, 1997).

The chorion, whose principal constituent is the trophoblast, released all cytokines measured. IL-8 release has been demonstrated previously and has been immunolocalized in cytokine-stimulated chorion to the trophoblast, stromal fibroblasts and macrophage-like cells (Ito *et al.*, 1994). The release of IL-10 by the chorion contrasts with the findings of others (Trautman *et al.*, 1997) who were not able to demonstrate its release and only rarely its immunolocalization within the chorion. However, they readily detected IL-10 in the decidua, and it may be that the decidua adherent to the chorionic trophoblast is responsible for IL-10 release in these explant preparations.

The decidua released high concentrations of MCP-1 and IL-8 and moderate concentrations of RANTES and IL-10. The release and immunolocalization of IL-8 and IL-10 has been demonstrated previously within the decidua (Saito *et al.*, 1994; Trautman *et al.*, 1997). Although the decidua comprises a wide range of different cell types, it is likely that decidual macrophages and T-cells would be the principal source of MCP-1 and RANTES respectively.

The placenta released high concentrations of MCP-1, IL-8 and RANTES and low concentrations of IL-10. Secretion of IL-8 and IL-10 by the placenta supports previous data demonstrating release and localization of IL-8 to the cytotrophoblast, the syncytiotrophoblast, perivascular cells and placental Hofbauer cells (Saito *et al.*, 1994; Elliott *et al.*, 1998) and of IL-10 to the cytotrophoblast (Roth *et al.*, 1996). Although release of MCP-1 and RANTES protein by the

placenta has not been reported before, a novel chemokine receptor CCR10 has been detected recently, which binds MCP-1 with high affinity and RANTES with much lower affinity. This receptor is uniquely expressed within placenta and fetal liver (Bonini *et al.*, 1997). A variety of cells within the placenta would be capable of secreting MCP-1 and RANTES, including the trophoblast, placental macrophages and T-cells. However, some placental explants were contaminated with blood, and therefore peripheral leukocytes, in particular monocytes and T-cells, could be an additional source of MCP-1 and RANTES respectively.

Parturition has been likened to an inflammatory reaction, with cytokines such as IL-8 playing a key role in cellular recruitment and activation within the utero-placental unit at term (Sennstrom *et al.*, 1997). Prostaglandin E_2 is also thought to be an important mediator and possible initiator of parturition, with concentrations increasing peripartum within amniotic fluid, placenta and cervix. In extra-uterine inflammatory responses, synergy exists between PGE_2 and IL-8 to facilitate neutrophil recruitment (Colditz, 1990). Furthermore, IL-8 release can be up-regulated by PGE_2 (Agro *et al.*, 1996) and, although the mechanism is not well defined, it may be via PGE_2 stimulation of cAMP (Brar *et al.*, 1997; Kaveleers *et al.*, 1997). Whether PGE_2 regulates release of other inflammatory mediators, such as MCP-1 and RANTES, which may also be involved in parturition is not known.

In this study it was investigated whether PGE_2 regulated release of MCP-1, IL-8, RANTES and IL-10 within the placenta and also its effect on cytokine release from a dually infused placental cotyledon. This demonstrated that PGE_2 stimulated release of MCP-1 and IL-8 into the maternal and fetal circuits of the perfusion system but had no effect on RANTES release. It can be postulated that a rise in PGE_2 at term would stimulate MCP-1 and IL-8 release, thus promoting a local inflammatory reaction and favouring parturition. The principal source of cytokines in such a system would be the trophoblast, although there may be a slight contribution from the decidua of the basal plate. The effect of PGE_2 stimulation of cytokine release into the fetal circulation was less than into the maternal circulation, and this could be due to inefficient transport of PGE_2 across the trophoblast, inability of cells adjacent to the fetal circulation, such as the Hofbauer cells, to respond by stimulation to PGE_2, or metabolism of PGE_2 by the trophoblast (Cheung *et al.*, 1990). RANTES secretion was not stimulated by PGE_2. This further supports the hypothesis that stimulation of IL-8 and MCP-1 was secondary to PGE_2 and not an artefact of perfusion.

In addition, PGE_2 stimulated release of IL-10 into the maternal but not the fetal circulation. The maternal immune system maintains tolerance to the fetus throughout pregnancy, although the mechanism is poorly understood. IL-10 is present in amniotic fluid in increasing quantities during pregnancy (Greig *et al.*, 1995) and, because of its immunosuppressive properties (Mosmann and Moore, 1991), has been proposed as a factor which may be involved in preventing fetal rejection (Rivera *et al.*, 1998). In other systems, PGE_2 is a potent stimulator of IL-10 and inhibitor of IL-12, a pro-inflammatory cytokine (Kelly *et al.*, 1997) which favours a TH-1 or cell-mediated immune response (Trinchieri, 1993). A rise in IL-10 and fall in IL-12 concentrations favour immunosuppression and can induce anergy or non-recognition of antigen by T-cells (Groux *et al.*, 1996). We propose that, at term, stimulation of IL-10 release from the maternal side of the placenta by PGE_2 would enhance local immunosuppression and tolerance towards fetal antigen. The fetus would then be protected from maternal recognition and rejection during the 'high risk' inflammatory process of parturition.

In summary, it has been demonstrated that the amnion, chorion, placenta and decidua release distinct profiles of both pro- and anti-inflammatory cytokines. Furthermore, in-vitro perfusion of a placental cotyledon by PGE_2 stimulates release of IL-8, MCP-1 and IL-10, but not RANTES. We speculate that the pro-inflammatory cytokines MCP-1 and IL-8 may play a role in the inflammatory process of parturition and that the anti-inflammatory IL-10 may be involved in protecting the fetal allograft at parturition. Moreover, we suggest that PGE_2 may play an important immunomodulatory role within the placenta at term. Further studies are now underway to assess the effect of PGE_2 and other immunomodulators on cytokine release by the amnion, chorion, decidua and placenta.

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