Mechanisms of Glucocorticoid-mediated Inhibition of Angiogenesis

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Abstract

Angiogenesis, the formation of new blood vessels from pre-existing vascular beds, is a requirement of embryonic development but only occurs in a limited number of discrete processes in the healthy adult. In contrast, abnormal angiogenesis is central to many pathological processes including: tumour growth, diabetic retinopathy and arthritis. Consequently, pharmacological manipulation of angiogenesis has great clinical potential.

Angiogenesis is inhibited by glucocorticoids and this is exploited clinically for the treatment of proliferating capillary haemangiomas in children. Despite this, the exact mechanism(s) through which glucocorticoids inhibit angiogenesis is (are) unknown. Whilst glucocorticoids can act directly on the vessel wall their effects on individual cell types and on molecular signalling remain unclear. The work in this thesis explores the hypothesis that glucocorticoid-mediated inhibition of angiogenesis is the result of direct modulation of growth factor signalling within the vascular endothelial cells.

A well-characterised 2-dimensional *in vitro* model of human endothelial tube formation was introduced. Glucocorticoids were shown to inhibit tube formation in this model via stimulation of glucocorticoid receptors and this process was not influenced by intra-cellular glucocorticoid metabolism by 11β-hydroxysteroid dehydrogenases. This demonstration that glucocorticoids inhibit angiogenesis by acting directly on the endothelium is consistent with, and extends, observations of glucocorticoid-mediated angiostasis in rodent aortic rings and during cutaneous wound healing. Molecular and biochemical assays suggested that glucocorticoids inhibit tube formation by altering the balance of pro- and anti-angiogenic factor activity. Time-lapse imaging of tube formation, combined with assays of endothelial cell migration and proliferation, indicated that glucocorticoids reduce tube formation, rather than accelerating degradation of existing tubes, by preventing morphological changes in the cells but do not inhibit cell division or migration.

In conclusion, these studies demonstrate that glucocorticoids can inhibit angiogenesis by directly inhibiting morphological changes required for tube formation by endothelial cells but without altering migration or proliferation.

Declaration

I declare that this thesis was written by me and that the data presented within it are a result of my own work with the exception listed below:

Miss Sadaf Ali, a BSc honours pharmacology student, cultured human aortic endothelial cells and performed a series of experiments investigating the effects of glucocorticoids on tube-like structure formation under my supervision.

I declare that this work has not been submitted for any other degree.

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AA	Anecortave acetate
ABC	Avidin:biotinylated enzyme complex
ACTH	Adrenocorticotrophic hormone
ALS	Amyotrophic lateral sclerosis
AM	Acetoxymethyl
AMD	Age-related macular degeneration
AMV-RT	Avian myeloblastosis virus reverse transcriptase
AP-1	Activating protein-1
Ang	Angiopoietin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
bp	Base pairs
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CEC	Choroidal endothelial cells
CHO	Chinese hamster ovary
COX-2	Cyclooxygenase-2
Cp	Crossing point
CRH	Corticotrophin releasing hormone
CTP	Cytosine triphosphate

DAB	3,3'-diaminobenzidine
DAPI	4',6-diamino-2-phenylindole
DiI-Ac-LDL	Fluorescent labelled acetylated low density lipoprotein
11-DHC	11-dehydrocorticosterone
Dll4	Delta-like ligand 4
DMEM	Dublbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo-nucleic acid
dNTP	Deoxyribonucleotide
DTT	Dithiothreitol
E	Cortisone
EC_{50}	Half maximal effective concentration
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EHS	Engelbreth-Holm-Swarm (mouse sarcoma)
EGM-2	Endothelial cell growth medium-2
EG-VEGF	Endocrine gland-derived VEGF
ELAM-1	Endothelial-leukocyte adhesion molecule-1
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelium-derived nitric oxide synthase
EPC	Endothelial progenitor cell
ERK	Extracellular signal-regulated kinase
F	Cortisol
F-actin	Filamentous-actin
FAK	Focal adhesion kinase

FAM	Carboxyfluorescein
FBS	Foetal bovine serum
FCS	Foetal calf serum
FW	Formula weight
G	Gravitational constant
GA-1000	Gentamycin/amphotericin B
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFR	Growth factor reduced
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTP(ase)	Guanosine triphosphat(as)e
GC	Glucocorticoid
^{3}H-	Tritiated
H+L	Heavy and light chains
HAoEC	Human aortic endothelial cell
HCl	Hydrochloric acid
hEGF	Human recombinant epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hFGF-B	Human fibroblast growth factor-B
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HMEC-1	Human microvascular endothelial cell
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase

11β-HSD1	11β-Hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-Hydroxysteroid dehydrogenase type 2
Hsp	Heat shock protein
HuDMEC	Human dermal microvascular endothelial cell
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
R^3-IGF-1	Human recombinant insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
JNK	c-jun N-terminal kinase
KCl	Potassium chloride
kDa	KiloDaltons
KDR	Kinase insert domain receptor (VEGF-R2)
KGF	Keratinocyte growth factor
LPS	Lipopolysaccharide
2-ME	2-methoxyestradiol
MAPK	Mitogen-activated protein kinase
MCID	Micro-computer imaging device
MCP	Monocyte chemotactic protein
MEK	MAPK/ERK kinase
MES	2-(*N*-morpholino) ethanesulfonic acid
MGB	Minor groove binder
ml	Millilitre

mM	Millimolar
MMP	Matrix metalloproteinase
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
n	Number of replicate experiments
NaCl	Sodium chloride
Na_3VO_4	Sodium orthovanadate
NF-κB	Nuclear factor-κB
nGRE	Negative GRE
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
p	Passage
p	Probability
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule-1/CD31
PEDF	Pigment epithelial-derived growth factor
pERK	Phosphorylated ERK
PET	Polyethylene terephthalate
PI3K	Phosphotidylinositol 3-kinase
PLA2	Phospholipase A2
PLC	Phospholipase C

PlGF	Placental growth factor
PKC	Protein kinase C
PF4	Platelet factor 4
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PPIA	Peptidylprolyl isomerase A (cylophilin A)
PVDF	Polyvinylidene difluoride
Qrt-PCR	Quantitative real-time PCR
RFU	Relative fluorescence units
RNA	Ribo-nucleic acid
RNase	Ribonuclease
ROP	Retinopathy of prematurity
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription-polymerase chain reaction
RTK	Receptor tyrosine kinase
SAID	Steroidal anti-inflammatory drug
sc	Subcutaneous
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	Small interfering RNA
α-SMA	Smooth muscle α-actin
SMC	Smooth muscle cell
TA	Triamcinolone acetonide
TBE	Tris/borate/EDTA

TBS	Tris buffered saline
TE	Tris/EDTA
tERK	Total ERK
TGF-β	Tissue growth factor-β
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol
TIMP	Tissue inhibitor of matrix metalloproteinase
TM	Trabecular meshwork
TMB	Tetra-methylbenzidine
TNF-α	Tissue necrosis factor-α
TLS	Tube-like structure
TM	Trabecular meshwork
TNS	Trypsin neutralising solution
TIMP	Tissue inhibitor of metalloprotease
TNS	6-p-toluidino-2-naphthalenesulfonic acid
TSP-1	Thrombospondin-1
u-PA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cell
v/v	Volume/volume
VVO	Vesiculo-vacuolar organelle
vWF	Von-Willebrand factor
w/v	Weight/volume

List of publications, presentations and awards

Original articles

Logie JJ, Ali S, Marshall KM, Heck, MMS, Walker BR and Hadoke PWF. Inhibition of angiogenesis by glucocorticoids involves blocking cytoskeletal remodelling and tube-like structure formation by human vascular endothelial cells. (*Submitted*)

Reviews

Hadoke PW, Macdonald L, **Logie JJ**, Small GR, Dover AR and Walker BR (2006). Intra-vascular glucocorticoid metabolism as a modulator of vascular structure and function. *Cell. Mol. Life Sci.* **63(5)**: 565:578.

Abstracts

Logie JJ, Walker BR and Hadoke PWF (2005). 11β-HSD1 does not influence the inhibitory effect of glucocorticoids in a 2D-model of angiogenesis. Oral presentation at the Simpson Symposium Meeting, Edinburgh.

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Logie JJ, Walker BR and Hadoke PWF (2006). Glucocorticoids inhibit tube-like structure formation in human endothelial cells, a model for *in vivo* angiogenesis. Poster presentation at the Scottish Cardiovascular Forum 9th Annual Meeting, Glasgow.

Logie JJ, Walker BR and Hadoke PWF (2006). 11β-HSD1 activity does not regulate glucocorticoid-mediated inhibition of tube formation by endothelial cells. *Microcirculation* **13(6)**: 511:534. Poster presentation at the 56th British Microcirculation Society Meeting, Dundee.

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McDermott JH, Liew A, MacCarthy F, Sheahan J, **Logie JJ**, Hadoke PW, Walker BR, O'Brien T (2007). Deleterious effects of hydrocortisone on endothelial progenitor cell number and function *in vitro*. *Ir.J.Med.Sci.* **176(9)**: S351-83. Oral presentation at the 32nd Annual Meeting of the Irish Endocrine Society, Belfast.

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Scottish Society for Experimental Medicine Autumn Meeting (2005, Glasgow) Poster Prize.

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Chapter 1

Introduction

Angiogenesis, the formation of new blood vessels from the existing vasculature, is crucial to normal development. Since oxygen, which is an absolute requirement for living cells, can only diffuse a short distance (100-200μm) through tissues, it is necessary that almost every cell in the body is situated close to a capillary. Consequently, tissue growth must be accompanied by the concomitant development of a supporting vascular network. Dysregulation of angiogenesis contributes to a large number of clinically-significant conditions characterised by excessive (tumour growth, diabetic retinopathy) or impaired (Alzheimer's disease, diabetes) vessel growth. The mechanisms that regulate angiogenesis have been the subject of much scientific investigation over the past 30 years; greater understanding of these processes opens the possibility that new treatments for conditions such as cancer and ischaemic heart disease can be developed.

Glucocorticoids (such as cortisol) are hormones that have many effects in the body but are best renowned for their anti-inflammatory effects; a discovery that led to the award of a Nobel prize to Philip Hench in 1950 (Hench, 1950). As well as limiting inflammation, glucocorticoids are well recognised inhibitors of angiogenesis and this property is exploited in the clinic where they are routinely administered for the treatment of vascular tumours; for example in the treatment of proliferating capillary haemangiomas of infancy (Hasan *et al.*, 2000; Hasan *et al.*, 2003). Despite this practical exploitation of their anti-angiogenic properties the precise mechanism(s) through which glucocorticoids inhibit new blood vessel formation has not been established.

The work described in this thesis explores the mechanisms of glucocorticoid-induced inhibition of angiogenesis. In order to understand these investigations fully, it is necessary to consider the mechanisms that regulate physiological angiogenesis (and their dysregulation in pathophysiological situations), the processes that regulate glucocorticoid activity and the existing literature addressing the influence of glucocorticoids on vascular structure and function.

1.1 Angiogenesis

Angiogenesis, the formation of new blood vessels (neovascularisation) from pre-existing vasculature continues to be a topic of major scientific interest. Angiogenesis is normally tightly regulated in adult physiology, but when uncontrolled, is central to many disease pathologies (Carmeliet, 2003). Consequently, modulation of angiogenesis is regarded as an attractive therapeutic goal in a variety of conditions. Indeed the therapeutic potential of targeting the angiogenic response has been realised with the recent milestone of the first anti-angiogenic treatment (bevacizumab/Avastin) to reach the clinic (Willett *et al.*, 2004) and several more agents are now in late phase clinical trials. In this section the process of angiogenesis, and its cellular and molecular basis, will be considered as a model for regulation by endogenous glucocorticoids.

1.1.1 Types of new blood vessel formation

Normal tissue function depends on an adequate supply of oxygen and nutrients delivered by blood vessels. Nearly every cell in the body is located within 100-200 μm of a capillary since this is the maximum distance over which oxygen can diffuse into cells (Carmeliet & Jain, 2000). Blood vessels are constructed by two distinct processes; vasculogenesis, whereby a primitive vascular network is established during embryogenesis from pluripotent mesenchymal progenitor cells (Carmeliet, 2000; Conway *et al.*, 2001) and angiogenesis, the subsequent growth, expansion and remodelling of this network (Hanahan, 1997; Folkman & D'Amore, 1996; Risau, 1997). Both processes contribute to the development of the embryonic vasculature. It was originally thought that neovascularisation in the adult occurs exclusively by a 'sprouting' angiogenesis but more recent evidence suggests that incorporation of bone marrow-derived endothelial precursor cells (EPCs) may contribute to growing blood vessels, thus complementing sprouting of resident endothelial cells (Asahara *et al.*, 1997; Tepper *et al.*, 2004).

1.1.2 The cellular and molecular basis of angiogenesis

Angiogenesis is a complex process tightly regulated by a balance between counteracting endogenous activators and inhibitors (Carmeliet, 2004). The activators (Table 1.1) comprise a set of pleiotropic growth factors, matrix and adhesion molecules, differentiation/specification signals, chemoattractants, and enzymes that participate in controlling the various steps that go into forming a new capillary (Tomanek & Schatteman, 2000). The inhibitors (Table 1.1) are equally diverse and function by blocking or markedly attenuating vessel formation, stabilisation, branching, vessel remodelling or specialisation (Talks & Harris, 2000). Together these counteracting factors provide the necessary balance required to maintain a normally quiescent yet highly responsive population of vascular cells. In particular, endothelial cells play a central role in angiogenesis. For example, they normally divide approximately every 3 years but can be rapidly induced to proliferate in response to a variety of physiological or pathological stimuli (Polverini, 2002). The onset of angiogenesis, or the 'angiogenic switch', occurs when angiogenic growth factors are produced in excess of angiogenesis inhibitors. The molecular basis of angiogenesis is most easily characterised by viewing the process as a step-wise progression (Figure 1.1) involving: existing vessel dilatation and increase in vascular permeability; degradation of the extra-cellular matrix (ECM); endothelial tube formation (by collective endothelial cell migration, proliferation and morphogenesis); stabilisation (including formation of a new basement membrane); and specialisation (vein or artery) (Conway *et al.*, 2001).

Endogenous activators of angiogenesis	Abbreviation	Endogenous inhibitors of angiogenesis	Abbreviation
Vascular endothelial growth factor	VEGF family	Thrombospondin	TSP-1
Fibroblast growth factor	FGF family	Interleukin	IL-4, -12, -18
Angiopoietins	Ang1, Ang2	Platelet factor	PF-4
Hypoxia inducible factor	HIF-1α	Tissue inhibitor of metalloprotease	TIMP-1 to -4
Platelet-derived growth factor	PDGF-B	Interferon	IFN-α
Matrix metalloprotease	MMP-2, -9	2-methoxyoestradiol	2-ME
Cyclo-oxygenase	COX-2	Retinoic acid	RA
Transforming growth factor	TGF-α, -β	Plasminogen activator inhibitor	PAI-1
Tumour necrosis factor	TNF-α	Pigment epithelial-derived factor	PEDF
Nitric oxide synthase	NOS	Secreted protein, acidic and rich in cysteine	SPARC
Integrins	αvβ3, α5β1	Tetrahydrocortisol-S	
Epidermal growth factor	EGF	Canstatin	
Insulin-like growth factor	IGF-1	Maspin	
Hepatocyte growth factor	HGF	Vasostatin	
Granulocyte colony-stimulating factor	GC-CSF	Plasminogen kringle	
Interleukin	IL-1β, -6, -8	Glucocorticoids	
Monocyte chemotactic protein	MCP-1	Angiostatin	
Platelet-endothelial cell adhesion molecule	PECAM-1 or CD31	Endostatin	
Vascular endothelial cadherin	VE-cadherin	Prolactin	
Urokinase-type plasminogen activator	u-PA		
Angiogenin			
Angiotropin			
Erythropoietin			
Ephrins			
Leptin			
Insulin			
Neuropeptides			

Table 1.1 Endogenous activators and inhibitors of angiogenesis

In normal adult physiology, angiogenesis is tightly regulated by two counteracting groups of endogenous factors; pro-angiogenic factors which drive this process and anti-angiogenic factors which act as the "brakes" to prevent the process. When angiogenic factors are produced in excess of inhibitors, the balance is tipped in favour of blood vessel growth, when the inhibitors are in excess, angiogenesis is stopped. The normal healthy body carefully regulates a perfect spatial and temporal balance of these modulators when and where a new blood supply is required. The key players identified to date are listed above but there are likely to be many more, as yet unidentified, factors which control angiogenesis. Table compiled from (Conway *et al.*, 2001; Folkman, 2001; Polverini, 2002).

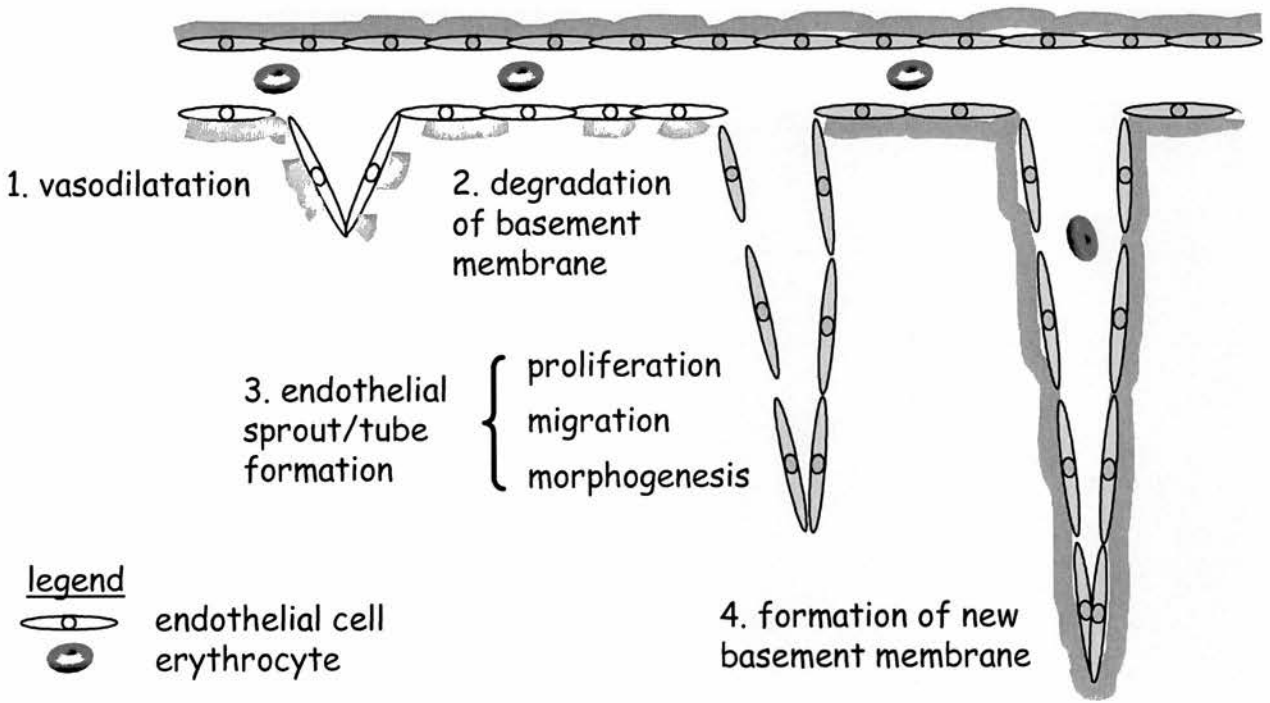


Figure 1.1 Steps in the angiogenesis cascade

Angiogenesis is a step-wise progression of events comprising; (1) dilatation of existing vessels and an increase in vascular permeability, (2) degradation of the extra-cellular matrix (ECM) which allows (3) endothelial tube formation (by a combination of endothelial cell migration, proliferation and morphogenesis) and (4) stabilisation of nascent sprouts (including formation of a new basement membrane) and eventually, vessel specialisation (into vein or artery). Figure adapted from (Tomanek & Schatteman, 2000).

1.1.2.1 Vasodilatation

Hypoxia inducible factor Low tissue oxygen tension generates hypoxia inducible factors (HIFs), specific transcription factors for over 70 genes, whose products stimulate angiogenesis including: endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF) and angiopoietin-2 (Pugh & Ratcliffe, 2003). HIF-1 is a key mediator of the physiological tissue response to hypoxia during embryogenesis (Semenza, 2001) and, in pathology, has been shown to promote neovascularisation in response to myocardial (Martin *et al.*, 1998) and retinal ischaemia (Pierce *et al.*, 1995) by activating transcription of the gene encoding VEGF.

Vascular endothelial growth factor Vascular endothelial cell growth factor (VEGF) is the most critical stimulus of angiogenesis since it is crucial for vascular development both in the embryo and in adult tissues and it is endothelial cell specific. VEGF-A, often referred to simply as VEGF, is the founding member of a family of closely related peptides including VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) (Ferrara *et al.*, 2003). Several different isoforms exist of which $VEGF_{121}$ and $VEGF_{165}$ are the most common freely diffusible forms while $VEGF_{189}$ and $VEGF_{206}$ are usually sequestered in the ECM (Ferrara *et al.*, 2003). VEGF family members have a number of discrete as well as overlapping functions mediated by a set of cell surface receptors that initiate signals through activation of signal transduction cascades (Eriksson & Alitalo, 1999). These receptors comprise a family of three closely related receptor tyrosine kinases (RTKs): VEGF-R1 (formerly Flt-1), VEGF-R2 (formerly Flk-1), and VEGF-R3. VEGF-R2 seems to mediate the major growth and permeability actions of VEGF, whereas VEGF-R1 may have a negative role either by acting as a decoy receptor or by suppressing signalling through VEGF-R2 (Hiratsuka *et al.*, 1998). VEGF-R3 is expressed on lymphatic vessels and is thought to be critical in their development. Disruption of even a single VEGF allele in mice leads to embryonic lethality due to severe vascular abnormalities (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996) and mice lacking VEGF-R1 have excess formation of endothelial cells which coalesce into disorganised tubules (Fong *et al.*, 1995). It is now clear that VEGF signalling must

be delicately regulated in a spatial, temporal and quantitative manner to avoid vascular catastrophe (Yancopoulos *et al.*, 2000).

Regulation of VEGF expression Oxygen tension plays a major role in the regulation of VEGF gene expression and VEGF is transcriptionally upregulated by HIF-1 (Pugh & Ratcliffe, 2003) which is regulated by nitric oxide (NO) (Kimura *et al.*, 2001). Several growth factors including; epidermal growth factor (EGF), transforming growth factor-α (TGF-α), TGF-β, keratinocyte growth factor (KGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) up-regulate VEGF mRNA expression, suggesting that paracrine or autocrine release of these factors co-operate with local hypoxia in regulating VEGF release in the tissue microenvironment (Ferrara *et al.*, 2003). Inflammatory cytokines such as interleukin-1β (IL-1β) and IL-6 induce expression of VEGF whereas IL-10 and IL-13 inhibit the release of VEGF, which is in agreement with the hypothesis that VEGF is a mediator of angiogenesis and permeability in inflammatory disorders (Neufeld *et al.*, 1999). Furthermore, VEGF is produced in response to endurance exercise (Waters *et al.*, 2004), and by the oncogene K-*ras* (Okada *et al.*, 1998), demonstrating that it is a key regulator of physiological and pathological tissue expansion.

Actions of VEGF VEGF is among the most potent permeability-enhancing factors known and was originally called vascular permeability factor (VPF) (Dvorak *et al.*, 1991). An increase in vascular permeability is accomplished by redistribution of intercellular adhesion molecules including platelet-endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)-cadherin, and alterations in cell membrane structure (Gale & Yancopoulos, 1999; Eliceiri *et al.*, 1999). This leads to the formation of vesiculo-vacuolar organelles (VVOs) which allow extravasation of plasma proteins and, eventually, activated endothelial cells. Consistent with a role in the regulation of vascular permeability, VEGF induces endothelial fenestrations (Roberts & Palade, 1995) in vascular beds including the abnormal vasculature in tumours (McDonald & Choyke, 2003). In addition, VEGF acts as a survival factor, mitogen, and it induces endothelial cell migration (Ferrara *et al.*, 2003).

Angiopoietins Although permeability is a prerequisite for angiogenesis, excessive vascular leakage, perhaps not surprisingly, results in pathological outcomes such as intra-cranial hypertension, circulatory collapse, metastasis and blindness (Carmeliet, 2000; Conway *et al.*, 2001). Consequently, permeability changes must be tightly regulated. Angiopoietin 1 (Ang1), a ligand of the endothelial Tie2 receptor, is a natural inhibitor of vascular permeability, tightening pre-existing vessels and preventing excessive plasma leakage (Thurston *et al.*, 2000). Ang1 is though to play a more permissive role by optimising the way in which endothelial cells integrate with supporting cells allowing them to receive critical signals from the local microenvironment (Yancopoulos *et al.*, 2000). Endothelial cell sprouting is further enhanced by Ang2, an inhibitor of Tie2 signalling and a natural antagonist of Ang1. Ang2 is involved in detaching smooth muscle cells (SMCs) and loosening the underlying matrix, and ultimately provides a destabilising signal involved in initiating angiogenic sprouting (Maisonpierre *et al.*, 1997). Whereas Ang1 is expressed widely in adult human tissue, Ang2 is detectable only in the ovary, placenta and uterus which are the three predominant sites of vascular remodelling in the adult. Ang2 together with VEGF act synergistically to promote angiogenesis, a feature of many of the pro-angiogenic factors.

1.1.2.2 Degradation of the extracellular matrix

Degradation of the extracellular matrix involves an array of proteinases which provide space for the migration of endothelial cells, but also results in liberation of growth factors (including VEGF, bFGF and matrikines) which otherwise remain sequestered within the matrix. The most extensively studied proteinases in angiogenesis belong to the group of matrix metalloproteinases (MMPs) (Bellon *et al.*, 2004) of which 28 members have been identified to date. The MMPs can be broadly divided into 2 groups: those that are secreted and those that are membrane-bound (and require initial activation by other members of the family). MMP activity induces changes in the ECM architecture thereby influencing endothelial cell behaviour which is important during all steps of the angiogenic process (Mott & Werb, 2004). Natural inhibitors of MMPs include circulating protease inhibitors such as tissue-localised inhibitors of metalloproteinases (TIMP-1

to TIMP-4) (Brew *et al.*, 2000) as well as thrombospondin-1 (TSP-1) (Conway *et al.*, 2001). The role of these factors is exemplified by the demonstration that Ang1-induced sprouting is, at least partially, mediated through the secretion of MMP-2, MMP-3 and MMP-9, and suppression of TIMP-2 (Kim *et al.*, 2000). Furthermore, MMP-1 and MMP-3 are thought to inhibit tumour angiogenesis by interfering with binding of MMP-2 to integrins (Brooks *et al.*, 1998). MMP-7 and MMP-9 generate angiostatin, a cleavage product of plasminogen and natural angiogenic inhibitor currently in clinical trials, which is thought to act, at least in part, by inhibiting endothelial cell proliferation (Pozzi *et al.*, 2000). Another proteinase implicated in matrix degradation and subsequent endothelial cell migration is urokinase-type plasminogen activator (u-PA) which is essential for revascularisation following myocardial infarction (Heymans *et al.*, 1999).

1.1.2.3 Endothelial cell proliferation and migration

Once destabilised, the ECM is conducive to proliferation and migration of endothelial cells from the vascular bed to distant sites. Interaction between VEGF, FGFs, angiopoietins, and their receptors is intimately involved in endothelial cell proliferation and migration illustrating the high degree of functional interplay between these molecules. VEGF and the angiopoietins appear to be specific mitogens for endothelial cells, whereas FGFs, epidermal growth factor (EGF) and CXC chemokines induce proliferation in a wide variety of cell types (Distler *et al.*, 2003). Since bFGF is a powerful mitogen for SMCs (Lindner & Reidy, 1991), it plays a role in the growth of vessels larger than capillaries. Furthermore, VEGF and bFGF act synergistically *in vitro* to stimulate capillary tube formation which comprises endothelial cell migration and proliferation (Ribatti *et al.*, 2001; Yan *et al.*, 2001). The proliferating endothelial cells migrate along a gradient of chemotactic agents through the disintegrated basement membrane.

Integrins Integrins are cell surface receptors that act as junctional molecules between endothelial cells, and between endothelial cells and the ECM, and play a key role in angiogenic sprouting. They are composed of different α and β sub-units (Humphries, 2000) that bind selectively to their ligands (*e.g.* collagen, laminin,

fibronectin or vitronectin) in the ECM. As well as having a role in cell attachment, they act as important signal transduction mediators that give the cell critical signals about the nature of their surroundings. Consequently, this matrix-integrin-cytoskeletal signalling axis (Davis & Senger, 2005), together with signals arising from receptors for soluble growth factors (*e.g.* VEGF-R, EGF-R), act as important cues for different biological actions including cell migration, proliferation and survival. This is one of the key mechanisms which contributes to vascular morphogenesis, the carefully orchestrated process by which proliferating endothelial cells organise into multi-cellular tubes with functional lumens (Davis & Senger, 2005). It is thought that αvβ3 and α5β1 integrins are important in lumen formation *in vivo* since both are involved in endothelial cell morphogenesis in 3-dimensional (3D) fibrin gels (Bayless *et al.*, 2000).

Thrombospondins TSPs are a family of multifunctional proteins and have roles in angiogenesis, apoptosis, activation of TGF-β and immune regulation. TSPs participate in cell-cell and cell-matrix interactions and 5 members have been identified to date (Lawler, 2000). TSP-1 has multiple receptors, among which CD36, CD47 and integrins are of particular note. TSP-1 acts to inhibit angiogenesis; inhibiting the migration, proliferation (Bagavandoss *et al.*, 1993) and tube formation of endothelial cells by interaction with CD36, expressed on the surface of these cells. Receptor binding also leads to expression of FAS ligand (FasL), activation of Fas and caspases, and ultimately to endothelial cell apoptosis (Jiménez *et al.*, 2000). Moreover, TSP-1 renders endothelial cells refractory to stimulation with VEGF and bFGF (Iruela-Arispe *et al.*, 1999), further augmenting its angiostatic effects. The angiostatic and apoptotic effects of TSP-1 require the sequential activation of CD36, the Src family kinase p59, caspases 3-like proteases and stress-activated p38 mitogen-activated protein kinases (MAPK) (Jiménez *et al.*, 2000).

1.1.2.4 Endothelial cell survival

Once resident in the vessel wall endothelial cells become quiescent and can survive for several years (Folkman, 2001). Together with the aforementioned functions, VEGF acts as a survival factor for endothelial cells and this has been shown both *in*

vitro (Gerber *et al.*, 1998a; Gerber *et al.*, 1998b) and *in vivo* (Yuan *et al.*, 1996; Benjamin *et al.*, 1999). The pro-survival effects of VEGF are developmentally regulated; VEGF inhibition results in extensive apoptotic changes in the vasculature of neonatal, but not adult, mice (Gerber *et al.*, 1999). VEGF dependence has also been demonstrated in endothelial cells of newly formed vessels, but not of more established, mature vessels within tumours (Yuan *et al.*, 1996; Benjamin *et al.*, 1999). One of the key events resulting in loss of VEGF dependence appears to be recruitment of pericytes to the vessel wall (Benjamin *et al.*, 1999). Gerber and colleagues have shown that this activity leads to up-regulation of the anti-apoptotic bcl-2 gene (Gerber *et al.*, 1998a) mediated by the phosphatidylinositol (PI)-3 kinase-Akt pathway (Gerber *et al.*, 1998b). Endothelial cell survival is also induced by the anti-apoptotic gene *survivin* which interacts with caspases and has been shown to be induced by Ang1-Tie2 signalling (Papapetropoulos *et al.*, 2000). At present, the molecular mechanisms governing its expression *in vivo* remain unclear but it is known to be up-regulated in response to hypoxia independent of VEGF, HIF1α or PlGF (Conway *et al.*, 2003). Furthermore, many of the endogenous angiogenesis inhibitors provide survival advantages to the endothelium (although their exact mechanisms of action are unknown). These include pro-thrombin kringles-1 and -2, antagonists of PECAM-1, IL-4 and IL-12, and cyclo-oxygenase (COX)-2 inhibitors (Conway *et al.*, 2001).

1.1.2.5 Endothelial cell morphogenesis, differentiation and lumen formation

When endothelial cells reach areas of reduced vessel density, they form tube-like structures (TLSs) that contain a functional lumen (Lawley & Kubota, 1989; Taraboletti & Giavazzi, 2004). Compared with the abundance of studies of signals that stimulate endothelial cell migration or proliferation, relatively little is known about the molecular cues that guide endothelial cells into 3D networks. In recent years, however, some novel insights have emerged. As discussed (Section 1.1.2.3), the matrix-integrin-cytoskeletal signalling axis (Davis & Senger, 2005) is likely to play an important role in endothelial cell morphogenesis. Furthermore, in addition to their mitogenic effects, bFGF and VEGF also play key morphogenetic roles in stimulation of endothelial tube formation (Kumar *et al.*, 1998; Saito *et al.*, 2003).

Notch The Notch pathway has recently been implicated as a key player in regulation of endothelial cell differentiation (Gridley, 2007). This pathway regulates sprouting and branching behaviours by influencing formation of vascular 'tip' cells – specialised endothelial cells at the leading edge of vascular sprouts (Hellstrom *et al.*, 2007). These tip cells extend protrusions, called filopodia, that sense the local environment and guide growth of endothelial sprouts along gradients of chemoattractants such as VEGF. Notch signalling is a negative-feedback regulator of VEGF action and occurs downstream of the VEGF signal (Hellstrom *et al.*, 2007; Leslie *et al.*, 2007).

1.1.2.6 Vessel stabilisation, remodelling and specialisation

Although the endothelium has received the most attention in angiogenesis research, the surrounding peri-endothelial cell layers and ECM are critical for ongoing structural and functional support of the vascular network (Kurz, 2000; Conway *et al.*, 2001). This is achieved by interactions between endothelial cells, between endothelial and mural cells, and between endothelials cells and the ECM. Mesenchymal cells in the surrounding tissue migrate to, and proliferate at, the abluminal surface of nascent vessels. These cells then differentiate to become either pericytes, which are located within the basement membrane, or into vascular SMCs (VSMCs), which are situated on the abluminal side of the basement membrane. At least 4 molecular pathways are involved in regulating this process (Jain, 2003). Platelet-derived growth factor (PDGF)-B which is secreted by endothelial cells, and possibly in response to VEGF, is thought to attract pericytes and other mural cells to proliferate and migrate to the vessel wall signalling through PDGFR-β. Furthermore, PDGF-B has autocrine actions on the endothelium, inducing proliferation and tube formation in endothelial cells (Battegay *et al.*, 1994). TGF-β1, a multifunctional cytokine, is responsible for the transformation of fibroblasts into α-smooth muscle actin (SMA)-expressing myofibroblasts (Chambers *et al.*, 2003) via Smad signalling (van den *et al.*, 2003). Finally, sphingosine-1-phosphate-1 (S1P1)-endothelial differentiation sphingolipid G-protein-coupled receptor-1 (EDG1) and Ang1-Tie2 are also involved in vessel stabilisation (Jain, 2003).

Ephrins The ephrin (Eph) system is involved in the demarcation of arteries and veins. EphB2 is a marker for arterial endothelial cells and SMCs and EphB4 is a marker for veinous endothelial cells (Wang *et al.*, 1998). These membrane bound ligands have long been known for their role in axonal guidance in the nervous system (Neufeld *et al.*, 2002), however more recent studies have shown that the ephrins play a similar role in compartmentalising the vascular system (Carmeliet & Tessier-Lavigne, 2005). Analogous to the angiopoietins, interference with ephrin interactions destabilises the developing capillary network (Polverini, 2002).

Organ-specific angiogenesis factors A key unresolved issue in vascular biology at present is/are the mechanism(s) which enable(s) the endothelium of distinct vascular territories to accommodate local physiological requirements with respect to specific tissue compartments. For example, in the blood-brain-barrier, tight cell-cell junctions, comprising occludins, claudins and zona occludens, exist which constitute a barrier that protects nerve cells from potentially toxic molecules in the blood (Jain, 2003). In contrast, fenestrations are present in endothelial cells in endocrine glands to enable secretion of hormones into the bloodstream. This plasticity suggests that endocrine glands produce signals that are essential to maintain the specificity of their endothelial cells and endocrine-gland-derived VEGF (EG-VEGF) has indeed been identified recently (LeCouter *et al.*, 2002). EG-VEGF displays little sequence homology to VEGF-A yet is indistinguishable in its biological activities in select tissues (LeCouter *et al.*, 2002). Another example of a tissue-specific angiogenic factor is fibulin-2, an ECM protein that is a marker for transformed mesenchymal cells in developing cardiac valves, aortic arch vessels, and coronary artery vessels (Tsuda *et al.*, 2001). This tissue-specificity is of therapeutic importance; if tissue-specific blood vessel regulators contribute to the excessive angiogenesis that occurs in cancers and inflammatory disorders then blocking these would be expected to have fewer side effects.

1.1.3 Signal transduction pathways

Intra-cellular signal transduction pathways are an important component of the angiogenic cascade since they convey the necessary signals which lead to gene transcription and a cross-talk of signals that ultimately determine the final cellular behaviour including; proliferation, migration and differentiation (Muñoz-Chápuli *et al.*, 2004). Research in this field has also focussed on the endothelial cell due to its central role in the angiogenesis cascade. Extra-cellular signals that stimulate bio-signalling involved in activation of the endothelium are relatively well known. These signals include the previously mentioned secreted paracrine factors (*e.g.* VEGF and bFGF) which are ligands of surface trans-membrane receptors, and ECM components that usually bind to integrins and to specialised receptors. In contrast, the signal transduction pathways that link the cell surface receptor and the final effectors of the modified cell behaviour are only fragmentarily known. These pathways are important, however, since specific and essential components might provide novel therapeutic targets for pro- and anti-angiogenic therapies (Muñoz-Chápuli *et al.*, 2004).

1.1.3.1 Focal adhesion kinases

The focal adhesion kinases (FAKs) act as a 'switch' for multiple signalling inputs and contribute to integrating the signals from the 2 main sources of angiogenic signals; *i.e.* soluble growth factors and the ECM. FAK activation is best understood in the context of the engagement of integrins and the cell surface (Parsons, 2003) and has an important role in endothelial cell activities associated with angiogenesis including proliferation, migration and differentiation (Ilic *et al.*, 2003; Shen *et al.*, 2005). Members of the FAK family link transmembrane glycoproteins to the actin cytoskeleton and, therefore, have a crucial role in mediating cell shape and motility induced by cell-matrix interactions. Other studies have implicated FAK in the transduction of soluble signals including VEGF (Qi & Claesson-Welsh, 2001). Signals through FAK to Rac and Pak play a role in modulating cell adhesion and migration, actin polymerisation and mitogen-activated protein kinase signalling.

1.1.3.2 Mitogen-activated protein kinases

Of particular importance are the mitogen-activated protein kinase (MAPK) pathways (Figure 1.2), since they carry angiogenic signals necessary for ECM degradation, increases in vascular permeability as well as endothelial cell proliferation, migration, differentiation and morphogenesis (Bates *et al.*, 2001) (Muñoz-Chápuli *et al.*, 2004). The main VEGF-R2 induced signalling proliferative pathway is mediated via the extra-cellular signal-regulated kinase-mitogen activated protein kinase (ERK-MAPK) cascade which can be activated in two ways. Firstly, VEGF-R2 can directly activate phospholipase C-γ (PLCγ) which releases Ca^{2+} from endoplasmic reticulum (Munaron, 2002) and activates protein kinase C (PKC) (Muñoz-Chápuli *et al.*, 2004). Calcium-dependent synthesis of NO (Bauer *et al.*, 2000) and/or arachidonic acid metabolites (via the lipoxygenase cascade) (Antoniotti *et al.*, 2003) are thought to be the links between calcium signalling and endothelial proliferation. Alternatively, phosphorylated (activated) VEGF-R2 can also recruit adaptor proteins (*e.g.* Shc or Skc) which activate the small GTPase, Ras, a main activator of Raf. This serine-threonine kinase, when activated, triggers a phosphorylation cascade and, consequently, activation of MAPK/ERK kinase (MEK)-1/2 and the ERK-MAPKs which translocate to the nucleus and activate transcription factors involved in cell proliferation (Muñoz-Chápuli *et al.*, 2004). Since a significant part of this thesis concerns endothelial tube formation, it is noteworthy that this Ras-ERK-MAPK pathway is also of particular importance in the transduction of morphogenesis signals in endothelial cells (Meadows *et al.*, 2001).

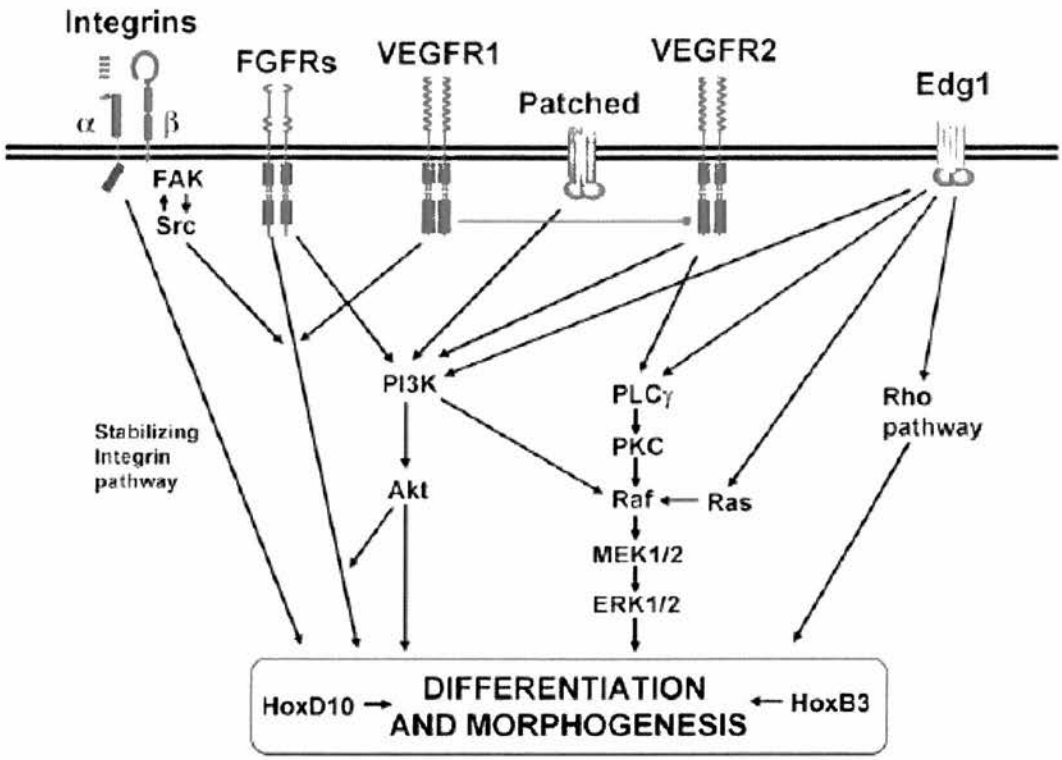


Figure 1.2 Main intra-cellular signalling pathways involved in angiogenesis in endothelial cells

The main signalling pathways involved in the transduction of differentiation and morphogenesis signals in endothelial cells are shown. Other key transmembrane relays including; Tie2, CD44 and VE-cadherin have been omitted from the diagram for the sake of clarity. Signalling factors and their abbreviations are referred to in the main text. Figure taken from (Muñoz-Chápuli *et al.*, 2004).

1.1.4 Physiological and pathological angiogenesis

Physiological angiogenesis is tightly regulated and of limited duration. It is an essential component of reproduction and embryonic development. In postnatal and adult life, angiogenesis is a discrete process (*e.g.* in the reproductive tract, wound healing and exercised muscle) of relatively short duration (days or weeks) (Folkman, 2001). In contrast, pathological angiogenesis is usually persistent and unabated and often continues for months or years (Folkman, 2001). Numerous disorders are characterised by excessive (*e.g.* neoplasia, rheumatoid arthritis and diabetic retinopathy) or insufficient (*e.g.* atherosclerosis and pre-eclampsia) angiogenesis. A wide range of other inflammatory, allergic, infectious, traumatic, metabolic and hormonal disorders which are characterised by aberrant blood vessel growth, and are said to be angiogenesis-dependent, have been described (Carmeliet, 2004).

1.1.4.1 Tumour angiogenesis

In 1971, Judah Folkman proposed that tumours are angiogenesis-dependent and, extending this hypothesis, that targeting the tumour vasculature offered a novel therapeutic target for the prevention of cancerous growth and metastatic spread (Folkman, 1971). Eventual acceptance of the hypothesis came following the emergence of novel cell culture based assays using endothelial cells, the discovery of the first angiogenesis inhibitor (Brem & Folkman, 1975) and, later purification of the first angiogenic protein (basic fibroblast growth factor, bFGF) (Shing *et al.*, 1984). Tumour angiogenesis differs significantly from physiological angiogenesis. Differences include aberrant vascular structure (dilated and tortuous), altered endothelial cell-pericyte interactions, abnormal blood flow (slow and sometimes oscillating), increased permeability (leaky) and delayed maturation (Bergers & Benjamin, 2003). The importance of angiogenesis in the progression and metastasis of cancerous growths has led to the development of novel therapies based on targeting the tumour's vascular supply. Anti-angiogenic therapies to date have essentially been designed to suppress the neovasculature in established tumours. However, identification of the mechanisms that cause a tumour to acquire an angiogenic phenotype is starting to lead to the discovery of new medicines that could

be used to block this 'angiogenic switch' (Harper & Moses, 2006). According to The Angiogenesis Foundation's website (www.angio.org), there are currently seven FDA approved anti-cancer therapies with anti-angiogenic properties. These agents fit into two broad categories; monoclonal antibodies directed against specific pro-angiogenic growth factors *e.g.* bevacizumab (Avastin) (Willett *et al.*, 2004) or their receptors *e.g.* trastuzumab (Herceptin) (Izumi *et al.*, 2002) for patients with colon and breast cancers, respectively, and small molecule inhibitors of tyrosine kinases for receptors of multiple growth factors *e.g.* imatinib (Glivec) (Druker *et al.*, 1996) for patients with chronic myelogenous leukaemia. In addition, other anti-cancer agents may block angiogenesis through mechanisms which are not completely understood *e.g.* thalidomide in combination with dexamethasone for patients with multiple myeloma (Kumar & Rajkumar, 2005).

1.1.4.2 Angiogenesis in cardiovascular diseases

Angiogenesis may be important in both the aetiology and treatment of cardiovascular disease. It has been suggested that neovascularisation plays a key role in atherosclerotic plaque progression (Moulton *et al.*, 2003) and that anti-angiogenic agents may 'normalise' the structure and function of intra-plaque vessels and thus stabilise vulnerable, rupture-prone lesions (Jain *et al.*, 2007). Conversely, stimulation of collateral vessels to areas of ischaemic myocardium may result in improvement of cardiac function by increasing perfusion to the ischaemic region. This notion has given rise to the rapidly growing field of therapeutic angiogenesis and extends to ischaemic limbs and peripheral arterial disease in diabetics (Li *et al.*, 2007). Proof-of-concept of therapeutic angiogenesis for ischaemic heart disease was established by Isner and colleagues (Schumacher *et al.*, 1998) following success with similar strategies (administration of FGF, protein) in patients with critical limb ischaemia (Isner *et al.*, 1996). Thus far, however, the promising preclinical trials in dogs (Banai *et al.*, 1994; Unger *et al.*, 1994; Lazarous *et al.*, 1999) and pigs (Lopez *et al.*, 1998; Crottogini *et al.*, 2003; Ninomiya *et al.*, 2003) have not translated into clinically-proven benefit (Kinnaird *et al.*, 2003) and there are currently no approved pro-angiogenic drugs for the treatment of ischaemic cardiovascular disease. The underlying reasons for these negative findings are likely multi-factorial and may be

related to insufficient dosing, problems with drug delivery, patient selection and duration of follow-up (de Muinck & Simons, 2004). Despite these set-backs, therapeutic angiogenesis has enormous potential for the treatment of cardiovascular disease and remains a promising area of research.

1.1.4.3 Other angiogenesis-dependent diseases

A long and growing list of diseases is caused by or characterised by excessive angiogenesis (Folkman, 2001; Carmeliet, 2003). Historically, the best known are cancer, rheumatoid arthritis and diabetic retinopathy but the link with angiogenesis is emerging in more diseases. A large number of diseases are associated with ocular neovascularisation, including, retinopathy of prematurity (ROP) in neonates, cytomegalovirus (CMV) retinitis and age-related macular degeneration (AMD) (Henry *et al.*, 2004). Indeed, topical injection of anti-angiogenic treatments (*e.g.* ranibizumab/Lucentis) for the exudative (or "wet") form of AMD is now in regular use in the NHS. Many dermatologic diseases are either angiogenesis-associated or angiogenesis-dependent *e.g.* infantile capillary haemangiomas, psoriasis, purpura, telangiectasia and neurofibromatosis (Chang *et al.*, 2002). The reproductive tract has been shown to be a site of pathological angiogenesis and manifests as various gynaecologic diseases such as endometriosis, ovarian cysts and pre-eclampsia (Ahmad & Ahmed, 2004). Adipose tissue is rich in blood vessels and adipose tissue mass may be regulated by angiogenesis (Rupnick *et al.*, 2002). In the lungs, reduced angiogenic signalling is evident in pulmonary fibrosis, pulmonary hypertension and emphysema (Koyama *et al.*, 2002; Tuder *et al.*, 2001; Kasahara *et al.*, 2000). In the brain, amyotrophic lateral sclerosis (ALS; progressive motor neuron degeneration) (Azzouz *et al.*, 2004), Alzheimer's disease (de la Torre, 2004) and stroke (Krupinski *et al.*, 1994) have been linked to insufficient angiogenesis. Consequently, modulation of angiogenesis is regarded as an attractive therapeutic goal in a number of conditions.

Glucocorticoids are well recognised inhibitors of angiogenesis and this is exploited in the clinic where they are routinely administered for the treatment of vascular tumours; for example in the treatment of proliferating capillary haemangiomas of infancy (Hasan *et al.*, 2000; Hasan *et al.*, 2003). Further, this property is exploited as a positive control in basic science experiments. The precise mechanism(s) of glucocorticoid-induced angiostasis, however, is (are) unclear. Preliminary data from the Endocrinology Unit, University of Edinburgh, along with suggestions from the literature indicate several possible mechanisms of action but consideration of these requires a comprehensive understanding of glucocorticoid synthesis and action.

1.2 Glucocorticoids

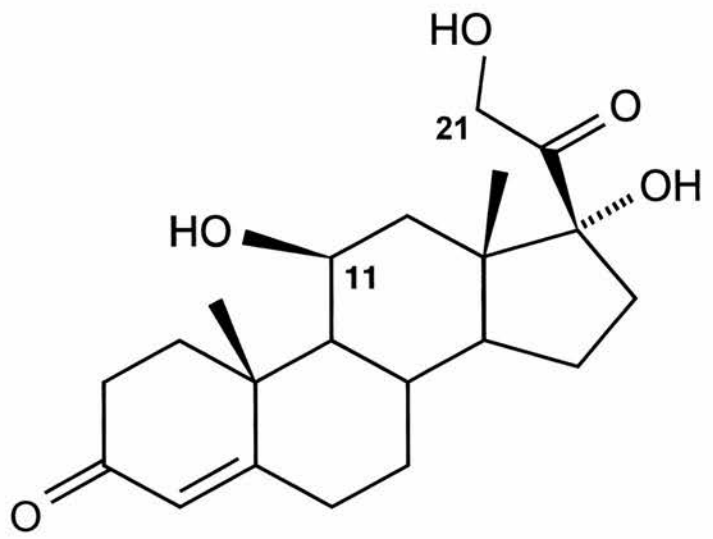
1.2.1 Steroid structure and secretion

Glucocorticoids get their name from their effect of raising the level of blood sugar (glucose). They are a class of steroid hormones alongside mineralocorticoids (*e.g.* aldosterone), androgens (*e.g.* testosterone), oestrogens and progestagens all of which are synthesized from the common four carbon ring precursor molecule, cholesterol. Individually, each steroid gains its unique properties as a result of substitution of chemical groups at various positions on this precursor molecule (Figure 1.3) and by definition, glucocorticoids are steroids which activate the glucocorticoid receptor (GR).

Biosynthesis involves a complex cascade of enzymatic cleavages and side-chain additions, which is subtly different between humans and rodents, ultimately resulting in production of cortisol (in humans) and corticosterone (in rodents). Glucocorticoid synthesis and release from the adrenal gland is controlled by hormonal interactions between the hypothalamus, pituitary gland and adrenal gland (known as the HPA axis), and can be influenced by both neural and chemical stimuli. Briefly, stimulation of the hypothalamus in response to circadian variation entrained to sleep, and in response to psychological and physical stress results in the release of corticotrophin releasing hormone (CRH) which travels via the portal circulation to stimulate CRH receptors present in the anterior pituitary. This results in rapid release of adrenocorticotrophic hormone (ACTH) into the systemic circulation which acts via cell surface receptors (melanocortin-2-receptors) on adrenocortical cells to ultimately stimulate steroidogenesis. Akin to many other biological systems, glucocorticoids themselves provide a negative feedback loop to regulate their own secretion. This occurs at the level of the hypothalamus and pituitary and acts to maintain physiological plasma glucocorticoid levels.

A series of synthetic steroids are potent glucocorticoids, including commonly prescribed drugs such as prednisolone, dexamethasone and beclomethasone. These exploit the anti-inflammatory effects of glucocorticoids for their therapeutic efficacy.

A) Cortisol



B) Cortisone

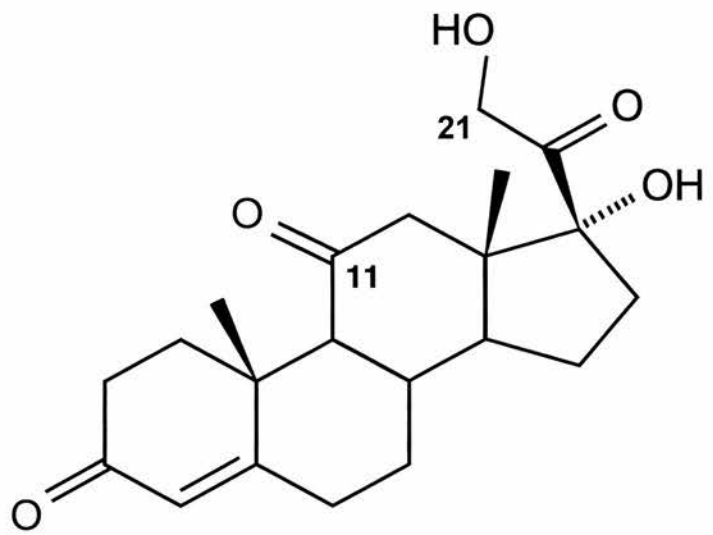


Figure 1.3 Chemical structures of cortisol and cortisone

Glucocorticoids, in common with other steroids, have a molecular backbone comprising three cyclohexane rings and a single cyclopentane ring. The unique properties of each individual steroid are determined by the presence of different chemical groups on the molecule. Functional groups essential for glucocorticoid activity are the C21 hydroxyl and the C11 hydroxyl groups as indicated.

1.2.2 Actions of glucocorticoids

In the blood, glucocorticoids partly circulate freely, but to a greater extent, are bound to albumin and cortisol-binding globulin (CBG, transcortin) (Hammond *et al.*, 1990). As steroids, glucocorticoids are lipophilic molecules that are able to pass through the cell membrane by diffusion and subsequently bind to either mineralocorticoid or glucocorticoid receptors (MR, GR). Pre-receptor metabolism of glucocorticoids by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 prevents illicit occupation of MR in mineralocorticoid-sensitive tissues such as the kidney and colon (Seckl & Walker, 2001). Thus, physiological glucocorticoid effects are predominantly mediated by binding to GR rather than MR in sites where 11β-HSD2 is expressed. GR are a member of the nuclear hormone receptor superfamily that includes receptors for steroids, thyroid hormones, retinoids and vitamin D. The remainder of this discussion will concentrate on the action of glucocorticoids at GR.

1.2.2.1 Transcriptional effects

The important anti-inflammatory and immunomodulatory effects of glucocorticoids are mediated predominantly by effects on gene transcription. Their main characteristics are as follows: First, they are physiologically relevant and therapeutically effective at very small doses, such as maintenance therapy for many rheumatic diseases (Buttgereit *et al.*, 2002). Second, the transcriptional action is slow; significant changes in protein expression are not seen until at least 30 minutes following receptor-ligand binding (Buttgereit *et al.*, 2004). In the absence of ligand, GR resides in the cytoplasm as part of a heat shock protein (HSP) complex (Pratt, 1993). Corticosterone binding activates GR where it dissociates from the complex, interacts with the importin system, and translocates into the nucleus via nuclear pores (Freedman & Yamamoto, 2004). Here, the steroid-bound receptor binds directly to specific DNA recognition motifs known as glucocorticoid response elements (GREs) or negative GREs (nGREs) (Dostert & Heinzel, 2004). These tend to be closely associated with the promoter region of target genes (Yamamoto, 1985). Therefore, upon interaction with the cell's transcriptional machinery, activated GRs are able to either enhance or repress gene transcription (Yamamoto, 1985; Drouin *et al.*, 1993;

Webster & Cidlowski, 1999; Pelaia *et al.*, 2003), a process termed 'transactivation'. In keeping with the diverse array of actions of cortisol, several hundred glucocorticoid-responsive genes have been identified. Putting this in perspective, it is estimated that glucocorticoids influence the transcription of approximately 1% of the entire genome (Goulding NJ & Flower, 2001).

Recent research has uncovered more levels of complexity to this model. Pivotal to this was the demonstration that glucocorticoids remained effective at suppressing inflammatory responses in mice in which a point mutation was inserted into the GR D-loop which prevented dimerisation and hence all transcriptional activation through the classical pathway and did not rely upon DNA binding (Reichardt *et al.*, 2001). Consequently, focus shifted towards examination of direct interactions between the ligand-GR complex and other transcriptional regulators such as nuclear factor-κB (NF-κB), activating protein 1 (AP-1) and Smad3, which play a central role in the induction of pro-inflammatory genes (Refojo *et al.*, 2001; De Bosscher *et al.*, 2003). This 'transrepression' has now emerged as the dominant anti-inflammatory mechanism of glucocorticoids (Goulding, 2004). Pharmacologically, this is a critical area of research, as novel anti-inflammatory compounds that dissociate the transactivating properties of GR from those which repress transcription are reported to have a better side effect profile. Theoretically at least, these 'dissociated steroids' possess strong transrepressional activities via inhibition of AP-1 or NF-κB, but weak transactivation via GREs.

1.2.2.2 Non-transcriptional effects

Some regulatory effects of glucocorticoids arise within a few seconds or minutes and cannot be explained by the above mentioned transcriptional actions because of the time required for their occurrence (Cato *et al.*, 2002; Falkenstein *et al.*, 2000; Croxtall *et al.*, 2000; Hafezi-Moghadam *et al.*, 2002). Much of the initial research focused on neurophysiological and behavioural systems however, new evidence suggest that these rapid effects may also impact upon inflammatory responses (Buttgereit & Scheffold, 2002). One can postulate, therefore, that angiogenesis may also be regulated in this way although evidence to support this is only just emerging.

Three different rapid non-transcriptional actions of glucocorticoids are considered below.

Non-specific actions in the form of physicochemical interactions with cell membranes suggest that high concentrations of glucocorticoids are able to alter cell function by influencing cation transport through the plasma membrane. The resulting inhibition of calcium and sodium cycling across the plasma membrane is thought to contribute to a 'dampening' of the inflammatory response (Schmid *et al.*, 2000).

Alternatively, glucocorticoids may also cause specific non-transcriptional actions that are mediated through membrane-bound GR (mGR) (Bartholome *et al.*, 2004). The search for mGR has been hindered by a lack of sensitivity of conventional tools for detecting membrane receptors expressed at low density. The advancement of more sensitive immunofluorescence techniques and availability of more sensitive antibodies that distinguish between cytosolic (cGR) and mGR has introduced a primitive understanding of these receptors. Although there is a growing body of evidence for their existence, no such data is available pertaining to specific signalling pathways associated with mGR.

Finally, non-transcriptional effects could be mediated by cytosolic GR (cGR) with no requirement for either nuclear translocation or effects on transcription. In this case, chaperones (*e.g.* heat shock proteins, hsps) or co-chaperones (*e.g.* Src) act as signalling components, and therefore, mediators of glucocorticoid effects. Examples exist of rapid glucocorticoid effects on phospholipase A2 (PLA2) and phosphoinositide-3-kinase (PI3K)-mediated eNOS release that are blocked by antagonising ligand binding to GR, but not by transcriptional inhibition (Limbourg *et al.*, 2002; Hafezi-Moghadam *et al.*, 2002).

Thus there are undeniably rapid effects of glucocorticoids that cannot be explained by classical transcriptional mechanisms but their relevance to inflammation and angiogenesis remains speculative.

1.2.2.3 11β-Hydroxysteroid dehydrogenases

The 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes catalyse the inter-conversion of active and inactive glucocorticoids allowing local regulation of corticosteroid receptor activation, a phenomenon described as pre-receptor metabolism (Seckl & Walker, 2001). Two isozymes have been identified; 11β-HSD2 is an exclusive dehydrogenase and converts cortisol (corticosterone in rodents) into its inactive metabolite, cortisone (11-dehydrocorticosterone, 11-DHC, in rodents) (Agarwal *et al.*, 1994), whereas 11β-HSD1 is predominantly a reductase in intact cells and whole organs and regenerates cortisol from cortisone (Agarwal *et al.*, 1989).

1.2.2.4 Physiological roles of the 11β-hydroxysteroid dehydrogenases

11β-HSD1 is widely distributed in glucocorticoid target tissues including liver, adipose tissue, skeletal muscle, vascular smooth muscle, lung, ovary and brain (Monder & White, 1993; Stewart & Krozowski, 1999), where its role is to amplify local glucocorticoid concentrations (Seckl & Walker, 2001) (Figure 1.4).

11β-HSD2 is expressed in the distal nephron of the kidney, colon and sweat glands where it protects MR from inappropriate activation by glucocorticoids, thereby allowing aldosterone selective access to otherwise non-selective MR (Edwards *et al.*, 1988). Congenital absence of this isozyme, as in the case of the syndrome of apparent mineralocorticoid excess (SAME) (Ulick *et al.*, 1979), or by inhibition with liquorice or its derivatives (Stewart *et al.*, 1987), causes glucocorticoids to occupy MR illicitly, resulting in sodium retention, hypokalemia, and hypertension. 11β-HSD2 is also expressed in tissues which are not classic MR targets, including the lung, lymph nodes, heart, blood vessel wall and placenta. In the placenta, 11β-HSD2 protects the foetus from excessive exposure to maternal glucocorticoids (Brown *et al.*, 1996), whereas cardiac 11β-HSD2 activity might have a role in preventing fibrosis resulting from stimulation of MR by glucocorticoids (Konishi *et al.*, 2003).

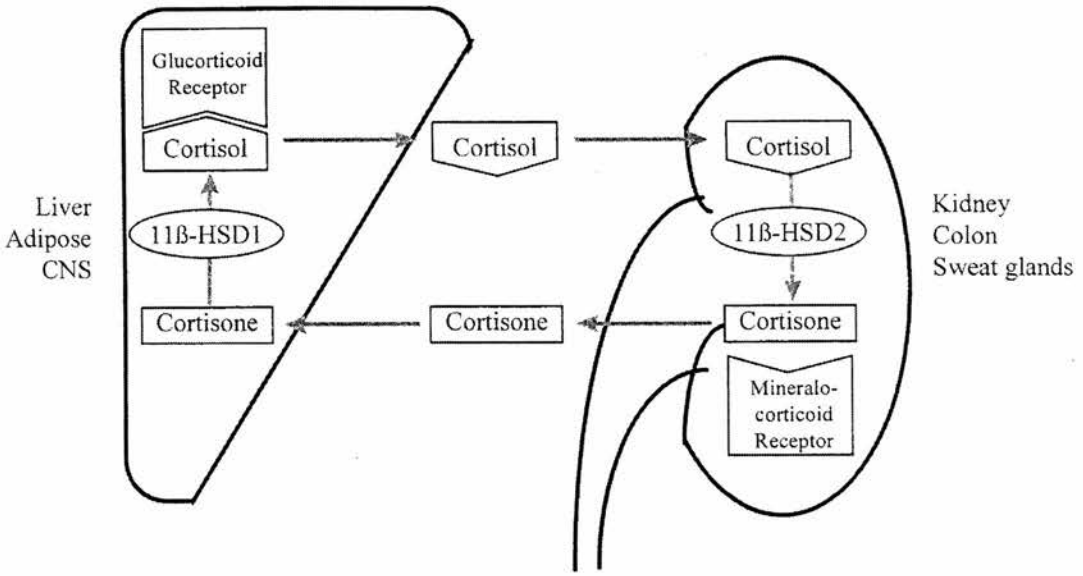


Figure 1.4 Physiological functions of the 11β-hydroxysteroid dehydrogenase isozymes

Diagram of the roles of the 11β-hyrdroxysteroid dehydrogenase (11β-HSD) enzymes. 11β-HSD2 is an exclusive 11β-dehydrogenase that acts in classical aldosterone target tissues to exclude cortisol from otherwise nonselective mineralocorticoid receptors. Inactivation of cortisol also occurs in placenta. 11β-HSD1 is a predominant 11β-reductase *in vivo* that acts in many tissues to increase local intracellular glucocorticoid concentrations and thereby maintain adequate exposure of relatively low affinity glucocorticoid receptors to their ligand. Figure taken from (Seckl & Walker, 2001).

1.2.3 Physiological effects of glucocorticoids

Glucocorticoids influence many physiological systems including; metabolism, the immune system, the cardiovascular system and growth and development. The abundant physiological effects of glucocorticoids were first identified from the clinical observations of the consequences of adrenal disease. Addison's disease, or hypocortisolism, is a manifestation of glucocorticoid insufficiency and is characterised by weight loss, decreased appetite, hypoglycaemia and hypotension (Addison, 1855). Causes include inadequate production of ACTH by the pituitary, or alternatively, the destruction of the adrenal cortex by the body's own immune system (auto-immune disease). In contrast, Cushing's syndrome is caused by glucocorticoid excess and is characterised by increased central fat deposition, impaired glucose tolerance, hypertension, immunosuppression and impaired wound healing (Cushing, 1912; Gordon *et al.*, 1994). It may be the result of an underlying disease (*e.g.* tumour of the adrenal gland), or iatrogenic, *e.g.* in the case of patients given long-term corticosteroid treatment for asthma. Since the subject of this thesis concerns the influence of glucocorticoids on angiogenesis, some of the established effects of glucocorticoids on the vasculature will be discussed.

1.2.3.1 Glucocorticoid effects on the vasculature

Corticosteroid receptors are present in the cells of the vessel wall supporting the idea that glucocorticoids interact directly with the vasculature, reviewed in (Hadoke *et al.*, 2006). GR and MR have been identified in freshly isolated vessels (Christy *et al.*, 2003) and in cultured vascular cells (VSMCs (Meyer & Nicholls, 1981; Scott *et al.*, 1987) and endothelial cells (Inoue *et al.*, 1999; Oberleithner *et al.*, 2003)) from a variety of species, however the distribution of these receptors may vary with vascular territory. For example, MR were detected in rabbit aortic and pulmonary VSMCs but not in small arteries (Lombes *et al.*, 1992). Corticosteroid receptors are active in the vasculature: GR antagonism (with RU38486) blocks dexamethasone-mediated induction of angiotensin-converting enzyme (ACE) activity in rat aortic endothelial cells (Sugiyama *et al.*, 2005), and angiotensin II and aldosterone (an MR agonist)

causes hypertrophy in VSMCs (Hatakeyama *et al.*, 1994), and swelling in endothelial cells (Oberleithner *et al.*, 2003), respectively .

The downstream effects of GR activation in the vessel wall are imperfectly understood. Glucocorticoids are essential for maintenance of blood pressure in healthy individuals (Ullian, 1999) whilst their ability to increase peripheral resistance in animals devoid of renal mass indicates that a non-renal mechanism contributes to glucocorticoid-induced hypertension (Langford & Snavely, 1959). A considerable body of evidence suggests that this non-renal mechanism may involve direct glucocorticoid-mediated alteration of endothelial cell and VSMC function. Consequently, regulation of glucocorticoid availability within the vessel wall is thought to be an important influence on cardiovascular physiology and pathology (Hadoke *et al.*, 2006).

Glucocorticoids have been shown to influence many aspects of vascular function including; vascular tone, haemostasis, inflammation, remodelling and angiogenesis (Hadoke *et al.*, 2006). Some of these properties are exploited clinically. For example, large doses of glucocorticoids have been used to inhibit angiogenesis in vascular tumours *e.g.* in the treatment of proliferating capillary haemangiomas of infancy (Hasan *et al.*, 2000; Hasan *et al.*, 2003). The precise mechanism of this glucocorticoid-induced angiostasis is unclear. Furthermore, the adverse effects of systemic glucocorticoid therapy have restricted their use in this regard.

1.2.3.2 Intra-vascular glucocorticoid metabolism

The influence of 11β-HSD isozyme activity on cardiovascular physiology and pathophysiology is well recognised (Krozowski & Chai, 2003) but details of the role of 11β-HSDs within the vessel wall have emerged only recently and remain somewhat uncertain. Both 11β-HSD isozymes are expressed in the blood vessel wall suggesting that they influence vascular function by regulating local availability of active glucocorticoids (Ullian, 1999). The cellular distribution of vascular 11β-HSD1 and 11β-HSD2 is not completely clear, and may be both species and site-specific. Studies from the Endocrinology Unit, University of Edinburgh, using

rat and mouse aorta suggest that 11β-HSD2 is localised to endothelial cells, whereas 11β-HSD1 is predominantly expressed in VSMCs (Walker *et al.*, 1991; Christy *et al.*, 2003). Others have reported activity of both enzymes in human VSMC and endothelial cells (Hatakeyama *et al.*, 1999; Cai *et al.*, 2001) and in rat aortic endothelial cells (Brem *et al.*, 1998). Furthermore, the literature suggests that 11β-HSD activity differs in vessels from distinct anatomical beds; in the rat 11β-HSD activity was higher in resistance arteries than in conduit arteries (Walker *et al.*, 1991), whereas in contrast, in the mouse, 11β-reductase activity was higher in the aorta than in femoral arteries (Dover *et al.*, 2007). These variations in cellular distribution and activity suggest that the role of intra-vascular glucocorticoid metabolism is not the same in all blood vessels. Whilst these differences may be attributable to species and site-specific differences, comparison between studies is further complicated by the variety of experimental techniques employed (*e.g.* intact vessels versus cultured cells, *in situ* hybridisation versus polymerase chain reaction). Consequently, there is a need to resolve the contributions of these isozymes to glucocorticoid metabolism in each experimental model when investigating the influence of glucocorticoids on vascular function.

Recent studies in the Endocrinology Unit, University of Edinburgh, have demonstrated that mice with genetic inactivation of 11β-HSD1 have enhanced angiogenesis *in vitro*, in aortic rings, and *in vivo* (within implanted sponges, cutaneous wounds and infarcted myocardium) (Small *et al.*, 2005). These intriguing data suggest that endogenous glucocorticoids, including those generated locally by 11β-HSD1, exert tonic inhibition of angiogenesis in physiology and pathophysiology. The mechanisms underlying this inhibition, however, remain unclear. Initial attempts to define this mechanism identified steroid-induced histological changes in inflammation, cell migration, cell proliferation and ECM integrity (Folkman & Ingber, 1987). This thesis focuses on the mechanisms of glucocorticoid-mediated inhibition of angiogenesis, and therefore, a comprehensive review of the proposed mechanisms is required.

1.3 Regulation of angiogenesis by glucocorticoids

1.3.1 Discovery of the angiostatic steroids

Using complementary *in vitro* (chick chorioallantoic membrane assay, CAM) and *in vivo* (rabbit cornea) assays, Folkman and colleagues were the first to demonstrate that cortisone, in the presence of heparin, inhibits angiogenesis (Folkman *et al.*, 1983). Extending these findings, complete tumour regression was observed in mice bearing subcutaneous tumours when both compounds were administered. This activity was retained in nude mice, excluding the possibility that the heparin-cortisone combination acts by promoting an immune response. Furthermore, a role for the anti-coagulant function of heparin was ruled out as similar results were obtained with the non-anti-coagulant hexasaccharide fragment of the heparin molecule. Using a range of glucocorticoids and mineralocorticoids that lack classical GR- and MR-mediated effects (deposition of glycogen in the liver, and sodium excretion in urine, respectively) the same group showed that inhibition of angiogenesis in the rabbit cornea was independent of these properties (Crum *et al.*, 1985). For example, 17α-hydroxyprogesterone has no glucocorticoid or mineralocorticoid activity but has an anti-angiogenic activity equivalent to that of hydrocortisone. Similarly, otherwise inert tetrahydro-S has twice the angiogenic activity of hydrocortisone. Taken together, these studies demonstrated a class of steroids for which inhibition of angiogenesis appears to be the principal function which were thus named the 'angiostatic steroids' (Crum *et al.*, 1985).

In the rat air-pouch model, tetrahydro-derivatives of cortisone and cortisol (THE and THF, respectively), which were amongst the first angiostatic agents to be identified using the CAM assay (Crum *et al.*, 1985), were unexpectedly shown to increase the vascularity index (and hence angiogenesis) when administered in combination with hyaluronan (Alam *et al.*, 1998). Hyaluronan is an essential component of the ECM that is important during wound healing and is commonly used as a topical drug delivery system. Hyaluronan on its own does not modulate granulomatous tissue angiogenesis, in contrast to THF which has an inhibitory effect. Therefore, a possible explanation for this phenomenon is that tetrahydrosteroids and hyaluronan,

when in combination, may modulate different cellular processes which converge to promote angiogenesis. For example, it has been suggested that hyaluronan might enable access of the steroid to a different cellular compartment than would normally occur in isolated cell preparations (Alam *et al.*, 1998). Consequently, it is important to consider the *in vivo* tissue micro-environment, wherever possible, when recapitulating angiogenesis in experimental models.

In the subsequent literature conventional glucocorticoids (*i.e.* GR agonists) and other angiostatic steroids have often been used somewhat interchangeably. The potential mechanisms identified are therefore reviewed together in the following section. As is demonstrated below, however, a wide range of effects of steroids has been observed, and there is a need to delineate which effects (if any) are mediated by conventional GR signalling. Moreover, as discussed above, inflammation and oxidative metabolisms play central roles in the initiation of angiogenesis, so that there is a need to delineate which effects of steroids are mediated directly in the vessel wall and which rely on systemic anti-inflammatory and/or metabolic effects.

1.3.2 Mechanisms of action: Experimental evidence

1.3.2.1 Actions on the basement membrane

The definitive mechanism(s) responsible for these angiostatic actions of steroids at the cellular and molecular level remain imperfectly understood. At the time of their discovery, Folkman hypothesised that physiologically, angiostatic steroids may act synergistically with the heparin-like molecules on the surface of the endothelial cell and within its basement membrane, to prevent capillary growth (Folkman & Ingber, 1987). This proposal was supported by subsequent experimental evidence. First, the distribution of two major basement membrane components, laminin and fibronectin, were studied using immunofluorescence microscopy in avascular regions induced by angiostatic steroids in CAMs (Folkman & Ingber, 1987). As the avascular regions developed over 48 hours, there was progressive loss of laminin and fibronectin. Maragoudakis and colleagues showed that the effects of angiostatic steroids were associated with decreased collagenous protein synthesis *in vivo* (Maragoudakis *et al.*,

1989). Furthermore, regression of growing capillaries was induced pharmacologically by proline analogues (such as L-azetidine carboxylic acid, LACA) which interfere with triple helix formation and prevent collagen type IV deposition (Ingber & Folkman, 1988). Combination of suboptimal doses of proline analogues with either angiostatic steroids or heparin resulted in potentiation of their angiostatic effects (avascular zones in 100% of CAMs and larger and more extensive avascular zones than previously observed with steroid-heparin combinations). In addition, protection of endogenous tissue heparin from degradation using a synthetic arylsulfatase inhibitor appears to synergise with angiostatic steroids to induce capillary regression even in the absence of exogenous heparin (Chen *et al.*, 1988). Thus, an imbalance in ECM turnover and loss of ECM structural integrity are thought to play an important role in steroid-induced capillary regression. Morphologically, degradation of basement membrane leads to gradual rounding, and eventual detachment, of endothelial cells from the substratum and, ultimately, death after only a few hours (Folkman & Moscona, 1978). This model also goes some way towards explaining the specificity of angiostatic steroids for growing capillaries and not for larger, more mature and quiescent vessels (since capillaries have a much higher rate of basement turnover). Soon after the discovery of angiostatic steroids in the CAM, this phenomenon was also shown for murine cerebral endothelial cells *in vivo* (Beck *et al.*, 1986) supporting a potential clinical application of these drugs.

Several mechanistic studies have further enhanced our understanding of the angiostatic effects of steroids through interaction with the basement membrane. The importance of the basement membrane protein laminin during the organisation of isolated endothelial cells into tube-like structures (TLSs) is well established (Kubota *et al.*, 1988; Grant *et al.*, 1989; Grant *et al.*, 1992). Levels of laminin produced by endothelial cells on an ECM substrate have been shown to be regulated by an angiostatic steroid (medroxyprogesterone) (Tokida *et al.*, 1990) which in turn, caused inhibition of TLS formation.

Interactions between cells and the ECM during endothelial tube formation are important because the matrix provides guidance cues for the establishment of

endothelial tubes in the absence of cell-cell contacts. It is now clear that this process is mediated, at the molecular level, via the matrix-integrin-cytoskeletal signalling axis (Davis & Senger, 2005). Glucocorticoids can influence certain aspects of this axis, such as regulation of integrin expression in inflammatory situations which are driven by angiogenesis. In a rat arthritis model, RGD (Arg-Gly-Asp) peptides which are linked to a carrier (liposome) containing dexamethasone, and targeted to αvβ3 integrins on endothelial cells, have proved to be efficacious in reducing inflammation (Koning *et al.*, 2006). Glucocorticoids have also been shown to alter expression levels of various integrins in isolated cell cultures *e.g.* down-regulation of the α2 subunit in cytotrophoblasts (Ryu *et al.*, 1999) and up-regulation of αvβ3 and αvβ5 in osteoblasts (Cheng *et al.*, 2000). However, the significance of this is not yet clear since the pro- versus anti-angiogenic properties of the different integrin sub-units is still under debate and it remains unclear whether altered integrin expression alone is central to the angiostatic effects of glucocorticoids. Furthermore, expression of TSP-1 (which is known to influence endothelial cell function by modulating cell-matrix interactions (Bornstein, 2001)) may be stimulated by glucocorticoids (Flugel-Koch *et al.*, 2004) (Rae *et al.*, manuscript submitted).

1.3.2.2 Protease production and activity

Considering that proteases are now accepted to play an essential role in an early, rate limiting step in angiogenesis (Bellon *et al.*, 2004; Mott & Werb, 2004), the angiostatic effect of steroids may be mediated by the regulation of endothelial cell protease production and activity. Steroid-induced inhibition of plasminogen activator (PA) activity may occur as a result of 2 separate mechanisms: (i) PA activity is increased in isolated endothelial cell monolayers when stimulated with bFGF, and this effect is abrogated by the addition of an angiostatic steroid or glucocorticoids (Blei *et al.*, 1993; Yamamoto *et al.*, 2004). This inhibition appears primarily due to increased production of plasminogen activator inhibitor type-1 (PAI-1) production by endothelial cells, rather than to a decrease in PA production (Blei *et al.*, 1993; Yamamoto *et al.*, 2004). (ii) In contrast, Wang and colleagues showed that decreased invasion (Vitrogen collagen assay) of choroidal endothelial cells (CECs) by triamcinolone acetate was due to an attenuation of MMP-2 activity

(Wang *et al.*, 2002b). Other reports support an inhibitory action of angiostatic steroids on endothelial cell protease activity (Ashino-Fuse *et al.*, 1989; Yamaji *et al.*, 1999), however in these studies, it was not reported whether this was due to decreased PA activity, or increased protease turnover. Indeed, it is possible that a combination of both mechanisms is important *in vivo*.

Following the *in vitro* observations of angiostatic steroid-induced PAI-1 activity, Penn and colleagues provided the first evidence that angiostatic steroids may operate by the same mechanism in pathophysiology (Penn *et al.*, 2001). Retinopathy of prematurity (ROP) is a pathologic condition of the retina in which abnormal angiogenesis can lead to vitreal bleeding and retinal detachment. Increasing incidence of ROP is linked to new neonatal care strategies that enhance the survival of very low birth weight babies by supplementation of arterial oxygen (Phelps, 1993). Surgical intervention has limited success so there is; therefore, a need for preventative therapy. It has been proposed that ROP can be prevented by angiostatic steroid administration (Penn *et al.*, 2001). Using a rat model of ROP (Penn *et al.*, 1994), treatment with angiostatic steroid was shown to produce a significant reduction in the severity of abnormal retinal angiogenesis (Penn *et al.*, 2001) concomitant with an increase in PAI-1 production. The limited effect of anecortave acetate (AA) on normal retinal vasculogenesis (Penn *et al.*, 2001), in contrast to its more profound effect on pathological angiogenesis, further improves its profile as a potential therapeutic agent.

There may be a role for glucocorticoids in the treatment of other angiogenesis-related ocular diseases such as choroidal neovascularisation in AMD (D'Amico *et al.*, 2003). In the developed world, AMD is the leading cause of blindness in individuals 50 years and older. In agreement with their *in vitro* effects (Wang *et al.*, 2002b), steroids (dexamethasone plus triamcinolone acetonide, (TA)) reduced the incidence of experimentally-induced subretinal neovascularisation in monkeys (Ishibashi *et al.*, 1985) and, more recently, in humans (Hooper & Guymer, 2003; Danis *et al.*, 2000). Unlike anecortave acetate, TA has glucocorticoid activity and, consequently, it was speculated that the angiostatic effect of this glucocorticoid *in vivo* was through dual

pathways (inhibition of inflammation and altered vascular endothelial cell ECM turnover) (Hooper & Guymer, 2003). Another report suggests that TA reduces inflammatory cytokine production (IL-6 and IL-8 release from foetal calf serum-stimulated fibroblast cultures) (Oddera *et al.*, 2002), further supporting a dual mode of action for this steroid.

Inhibition of matrix remodelling by steroids may contribute to delayed wound healing since the MMPs are thought to play an important role throughout the entire tissue repair process (Xue *et al.*, 2006). However, excessive MMP activity contributes to the development of chronic wounds and, therefore, selective control of proteolysis may prove to be a valuable therapeutic approach to promote the healing of chronic ulcers. In a rabbit ear model, granulation tissue formation (an early, angiogenesis-dependent stage of wound healing) was not completed when TA was administered at the time of injury (Hashimoto *et al.*, 2002). In contrast, no significant difference in healing was observed when the steroid was administered 10 days after injury. This time-dependence suggests that the effect of TA was due to inhibition of angiogenic growth factor expression (such as VEGF, for example, which becomes prominent 1 to 3 days after injury (Brown *et al.*, 1992)) during the early stages of wound healing, or inhibition of matrix synthesis (Hashimoto *et al.*, 2002). The mode of action of steroids in delayed wound healing, however, has yet to be shown conclusively (and is likely to be multi-factorial).

1.3.2.3 Effects on growth factor production and growth factor receptors

Glucocorticoids may inhibit angiogenesis by suppressing VEGF production, a key stimulus of endothelial cell proliferation and migration. Indeed, pharmacological levels of glucocorticoids have been shown to have anti-mitotic (Beck *et al.*, 1986; Cariou *et al.*, 1988; Kräling *et al.*, 1999; Banciu *et al.*, 2006; Zou *et al.*, 2006) and anti-migratory effects (Blei *et al.*, 1993) on endothelial cells through an unknown mechanism. VEGF from VSMCs is thought to act on endothelial cells in a paracrine fashion to stimulate endothelial cell proliferation and migration. Glucocorticoids inhibit VEGF mRNA expression and stability in different cell culture systems (Nauck *et al.*, 1998; Gille *et al.*, 2001; Wen *et al.*, 2003). In cultures of human aortic

VSMCs, physiological concentrations of the corticosteroids hydrocortisone, cortisone and dexamethasone abolished the PDGF-induced expression and secretion of VEGF (Nauck *et al.*, 1998). In contrast, the cholesterol precursors of these corticosteroids (desoxycorticosterone and pregnenolone) which have no anti-inflammatory activity did not have any significant effect in this concentration range. Thus, in addition to inhibiting the 'classical' mediators of inflammation (such as arachidonic acid, prostaglandins and leukotrienes) glucocorticoids act as a new class of VEGF inhibitors. The field is currently expanding to investigate whether this inhibitory effect is true of other growth factor-induced expression of VEGF, such as bFGF, and in other cells such as primary fibroblasts from human skin and pulmonary arteries (Nauck *et al.*, 1998).

Similar findings were obtained using primary chondrocytes obtained from the tibial growth plates of neonatal pigs (Koedam *et al.*, 2002). This is a model of longitudinal bone growth, a process that is often disturbed in children undergoing treatment with glucocorticoids. VEGF protein secretion from these cells, as well as mRNA transcript, were inhibited by dexamethasone, hydrocortisone and prednisolone. This is strongly suggestive of a GR-mediated effect. In addition, the authors speculated that a post-transcriptional regulatory mechanism is most likely to account for GC-induced VEGF inhibition (Koedam *et al.*, 2002).

Glucocorticoid-mediated inhibition of VEGF could have downstream effects on other angiogenic factors. For example, VEGF up-regulates COX-2 mRNA, protein and enzyme activity levels in human microvascular endothelial cells (HMEC-1) in a dose- and time-dependent manner (Tamura *et al.*, 2002). This effect was abolished by pre-treatment of cells with a selective COX-2 inhibitor, NS-398, suggesting specific up-regulation of COX-2 activity by VEGF. New insights into the COX-2 promoter regulatory region have identified several critical elements including: a cyclic adenosine monophosphate response element (CRE); a nuclear factor for IL-6 expression (NF-IL-6); NF-κB; a polyoma enhancer activator (PEA)-3; and a zinc finger protein family of transcription actors that recognise the consensus (A/T)GATA(A/G) and related sequences (Tamura *et al.*, 2002). Indeed, GATA

binding sites are present in promoters of other genes that enhance endothelial cell function including PECAM-1 (Gumina *et al.*, 1997), endothelial nitric oxide synthase (eNOS) (Zhang *et al.*, 1995) and von Willebrand factor (vWF) (Jahroudi & Lynch, 1994).

Data collected from various animal models have validated these *in vitro* findings. VEGF has been shown to accelerate gastric ulcer healing by enhancing angiogenesis at the ulcer site (Jones *et al.*, 2001) but until recently the mechanism was unclear. Dexamethasone delayed ulcer healing in a rat model, by inhibition of angiogenesis, and this effect was associated with a decrease in prostaglandin E_2 (PGE_2) levels and down-regulation of VEGF (but not bFGF) protein expression (Luo *et al.*, 2004). Supplementation with PGE_2 attenuated the inhibitory action of dexamethasone and reversed the adverse effects on angiogenesis and ulcer healing. However, PGE_2 treatment only partially abrogated the inhibitory action on VEGF expression suggesting that other mechanisms are responsible. Similar findings were obtained in larger animal models using other corticosteroids. For example, in a Göttingen minipig model of osteoporosis, glucocorticoid-associated bone loss in animals that received prednisolone orally for 8 weeks correlated with a reduction in VEGF expression levels (Pufe *et al.*, 2003). VEGF-mediated angiogenesis is considered important in physiological bone turnover and during repair; for example, VEGF is strongly expressed during the process of fracture healing (Pufe *et al.*, 2002). The ability of glucocorticoids to inhibit COX-2 activity (and, hence, PGE_2 production) combined with the ability of PGE_2 to increase VEGF synthesis (Harada *et al.*, 1994), may explain the pathway underlying glucocorticoid-mediated reduction in VEGF activity.

The endogenous oestradiol metabolite, 2-methoxyestradiol (2-ME), has now entered into phase II clinical trials as an anti-tumour agent (Mooberry, 2003). Preclinical studies have demonstrated significant efficacy of this agent against solid tumours and attempts have been made to demonstrate its mechanism of action. In highly angiogenic pituitary tumours in rats, 2-ME inhibited tumour growth and angiogenesis, concomitant with a decrease in VEGF expression (Banerjeei *et al.*,

2000). In support of this, Mabjeesh and colleagues found similar efficacy of 2-ME *in vivo* against human breast cancer cell (MDA-MB-231) xenografts growing in the mammary fat-pad of immunodeficient mice (Mabjeesh *et al.*, 2003). The mechanism of action was further dissected *in vitro* and it was shown that 2-ME inhibits the hypoxia-induced DNA binding and transcriptional activity of HIF suggesting that the regulation of VEGF production may occur at the level of HIF.

The field has now widened to encompass research into glucocorticoid-induced effects on the VEGF receptors and some recent evidence suggests that down-regulation of VEGF-R2 contributes to glucocorticoid-induced angiostasis. In a model of cutaneous wound healing in mice, VEGF-R2 expression was induced in the wound tissue following full-thickness punch wounds (Zhang *et al.*, 2004). Bioluminescence from a luciferase reporter, under the control of the VEGF-R2 promoter, was imaged in real time in live animals to produce quantitative measurements of luciferase activity, and hence VEGF-R2 expression, in the wounds. Dexamethasone-treated mice had significantly less VEGF-R2 expression during the healing period compared to saline-treated controls (Zhang *et al.*, 2004). In addition, histological analysis revealed that VEGF-R2 was predominantly expressed in endothelial cells suggesting that the endothelium itself may be the site of action of glucocorticoids. Macrophage infiltration into the damaged tissue was lower in dexamethasone-treated mice suggesting that anti-inflammatory effects may also contribute to the delayed wound healing in glucocorticoid-treated mice.

In physiology, lung development in humans involves the formation of pulmonary alveoli (also known as septation) during the postnatal period. In rats, there is a critical period for septation to occur, mainly between the 4th and 14th postnatal days, which is impaired upon administration of corticosteroids (Massaro *et al.*, 1985). Until recently, the mechanism through which this effect occurs was unclear. It has now been established that during normal lung development, VEGF and VEGF-R2 production increase in parallel over this period during normal lung development (Bhatt *et al.*, 2000). SU5416, a synthetic VEGF-R2 inhibitor currently in clinical trials (Arora & Scholar, 2005), prevented septation and decreased arterial density

indicating that angiogenesis is important in lung development (Jakkula *et al.*, 2000). In a study by Clerch and colleagues, dexamethasone-induced inhibition of alveolar formation revealed that regulation of VEGF-R2 expression (by DNA microarray analysis of neonatal mouse lung) was central to the angiostatic effects of glucocorticoids in this system (Clerch *et al.*, 2004). In the same study, validation of the array data was achieved with concordant findings relating to VEGF-R2 protein, by Western blotting. The significance of down-regulation of VEGF-R2 during cutaneous wound healing and in lung development alike is likely to be a result of attenuating the downstream effects of VEGF signalling of endothelial cell functions such as survival, migration, proliferation and tube formation, all of which are key components of the angiogenesis cascade.

1.3.2.4 Modulation of cytokine production

Angiogenesis is required for the progression of chronic inflammation and the anti-inflammatory effects of glucocorticoids may, at least in part, explain their angiostatic actions since the 2 processes share many common mediators (Colville-Nash *et al.*, 1995; Dostert & Heinzel, 2004; Roman-Blas & Jiménez, 2006). Common to both processes are vasodilatation, increased vascular permeability and migration of inflammatory cells, which are mediated by NO, TNF-α, TGF-β, IL-6, COX-2 and NF-κB and these are suppressed by large concentrations of glucocorticoids (Hori *et al.*, 1996; Hasan *et al.*, 2000; Beer *et al.*, 2000; Yossuck *et al.*, 2001; Blomme *et al.*, 2003).

Clinical studies in people with Cushing's syndrome and use of experimental animal models suggest that vascular NO metabolism is impaired in the presence of excess glucocorticoids (Saruta, 1996). This has been supported by evidence *in vitro* and the underlying mechanisms are thought to be as a result of a direct effect on inducible NOS (iNOS) enzyme levels, as well as an indirect effect on enzyme activity by impairment of co-factor (tetrahydrobiopterin) and substrate (L-arginine) availability (Whitworth *et al.*, 2002).

Glucocorticoids have also been shown to inhibit TNF-α production by human monocytes *in vitro* (Waage & Bakke, 1988) and astrocytes (Brenner *et al.*, 1993). This is of therapeutic importance since dexamethasone may provide a protection against the development of retinopathy by decreasing the permeability of the blood-retinal barrier and reducing the damaging inflammatory response. Indeed, this hypothesis is supported by *in vivo* evidence; in a mouse model of oxygen-induced retinopathy, dexamethasone decreased the injury response to hyperoxia (Rotschild *et al.*, 1999) and this protective effect on the vasculature correlated with a significant decrease in TNF-α expression (Yossuck *et al.*, 2001).

The differential effects of various angiostatic versus anti-inflammatory steroids on local cytokine production has been investigated in rodents by subcutaneous implantation of sterile polyether sponges which elicit a reproducible neovascular response (Hori *et al.*, 1996). Levels of IL-6 and TNF-α were measured in the sponges at various timepoints. Among the angiostatic steroids tested, U-24067 and tetrahydro-S caused a dose-dependent inhibition of sponge-induced angiogenesis but did not influence the level of cytokine production compared with vehicle and had no effect on spleen or thymus weight. In contrast, dexamethasone inhibited angiogenesis concomitant with a marked reduction in the levels of IL-6 and TNF-α and caused significant spleen and thymus weight loss indicative of a systemic immunosuppressive effect. In the same study, the angiostatic steroids caused a reduction in leukocyte infiltration into the sponges. Since these agents lack 'classical' anti-inflammatory glucocorticoid activity, it is feasible that they inhibit infiltration as a result of their direct inhibition of neovascular growth.

Further evidence has accumulated to support the notion of an angiostatic effect of glucocorticoids within the vessel wall independent of a systemic inflammatory response. For example, in the presence of physiological or pharmacological levels of glucocorticoids, mouse and rat aortic rings embedded in an ECM matrix produced fewer angiogenic sprouts, respectively (Small *et al.*, 2005; Nicosia & Ottinetti, 1990).

1.3.2.5 Effects on the arachidonic acid cascade

Glucocorticoids have been shown to influence other facets of the inflammatory cascade which, in turn may influence angiogenesis. The conversion of arachidonic acid to PGE_2, via COX-2, is induced at the inflammatory site in patients with rheumatoid arthritis (Crofford *et al.*, 1994) and in experimental animal models of inflammation (Masferrer *et al.*, 1994). Ghosh and colleagues analysed the role of COX-2 in angiogenesis, during granulation tissue formation, using the rat air pouch model (Colville-Nash *et al.*, 1995). Sub-anti-inflammatory doses of either cortisone or dexamethasone reduced VEGF protein levels, and inhibited angiogenesis, as measured by an inhibition of the vascular network formation using a vascular casting method. This effect was shown to be mediated via COX-2-derived PGE_2 (Colville-Nash *et al.*, 1995). PGE_1 and PGE_2 are also key mediators of angiogenesis in the rabbit cornea and CAM assays (Form & Auerbach, 1983), which are also sensitive to the effects of angiostatic steroids (Crum *et al.*, 1985). Therefore it is feasible that regulation of prostaglandin synthesis could be a central mechanism for inhibition of angiogenesis by steroids.

Others have investigated the effects of steroidal (*e.g.* betamethasone) and non-steroidal (*e.g.* ketoprofen) anti-inflammatory drugs (SAIDs and NSAIDs) on angiogenesis in the CAM assay. A relatively low concentration (200 nM) of betamethasone inhibited angiogenesis induced by tumour supernatant, indicating a genomically-mediated effect (Lemus *et al.*, 2001). Ketoprofen had a similar angiostatic effect in the subcutaneous sponge implant model but, unlike betamethasone, it was shown to induce apoptosis in stromal and endothelial cells which partially may explain its angiostatic properties (Zuniga *et al.*, 2003). Although the specific mechanism underlying this cell death is not entirely clear, it is possible that cell death receptor pathways (Fas/CD95, caspase 8) are involved (Kogianni *et al.*, 2004). NSAIDs may also act indirectly by regulating the balance of pro- and anti-angiogenic factors locally within tissues. For example, prostaglandins stimulate VEGF and bFGF expression (Cheng *et al.*, 1998) and sulindac (a NSAID which inhibits both COX isoforms) inhibits angiogenesis in implanted sponges in mice (Illanes *et al.*, 2002). However, sulindac also has properties independent of COX

inhibition in reducing growth of polyps and pre-cancerous lesions. Treatment of mice with sulindac, at concentrations that inhibit colon tumour formation, has been shown to increase TSP-1 production (Moon *et al.*, 2005). Consequently, NSAIDs may disrupt the normally tightly regulated balance of angiogenesis factors by up-regulating angiostatic factors as well as inhibiting production of pro-angiogenic factors.

1.3.2.6 Actions on endothelial cell-leukocyte interactions

Studies of leukocyte adhesion to endothelial cells reinforce the notion that separate mechanisms exist for the anti-inflammatory and angiostatic properties of glucocorticoids (Cronstein *et al.*, 1992). The endothelium is known to play a critical role in inflammation by directing circulating leukocytes into extravascular tissues by expressing adhesive surface molecules for leukocytes *e.g.* endothelial-leukocyte adhesion molecule (ELAM)-1, and intercellular adhesion molecule (ICAM)-1 (Bevilacqua *et al.*, 1987; Walz *et al.*, 1990). Pre-incubation of endothelial cells with an inflammatory stimulus such as lipopolysaccharide (LPS) leads to an up-regulation in expression of these markers, and, subsequently, an increase in adherence of leukocytes (Cadepond *et al.*, 1991). This effect was markedly attenuated by treatment with dexamethasone (100 nM) and reversed with the GR antagonist RU38486, indicating that these effects are mediated through GR. Furthermore, in the study by Cadepond and colleagues cortisol, but not inactive THF, inhibited endothelial cell responses to LPS suggesting a GR mediated anti-inflammatory mechanism (Cadepond *et al.*, 1991). However, the absence of a GRE in the gene for ELAM-1 transcription suggests that glucocorticoids must either interfere directly with a regulator of ELAM-1 transcription (such as NF-κB) or a counter-regulatory element (such as IκB) (Montgomery *et al.*, 1991).

In summary, glucocorticoids have well established anti-inflammatory effects which may in part explain some indirect angiostatic effects on the vasculature. It is also apparent, however, that additional mechanisms, independent of anti-inflammatory effects, are of central importance.

1.3.2.7 Effects on vessel maturation and stabilisation

Glucocorticoids may inhibit the stabilisation and maturation of nascent angiogenic sprouts. In support of this hypothesis, an observational study in a sheep model of preterm labour, in which corticosteroids were administered to enhance foetal lung maturation, revealed that the capillary tight junctions in the foetal sheep brain were compromised, via an unknown mechanism (Huang *et al.*, 2001). As described (Section 1.1.2.1), Ang1/Tie2 signalling is important in maintaining inter-endothelial contacts (Thurston *et al.*, 2000) and thus may be important in the regulation of vessel maturation by glucocorticoids. In support of this, Féraud and colleagues investigated Ang1 and Tie2 production in mouse adrenal atrophy whereby administration of dexamethasone resulted in down-regulation of Ang1 and Tie2 mRNA and protein levels (Féraud *et al.*, 2003).

As well as vessel stabilisation via inter-endothelial contacts, deposition of a new basement membrane is another sign of maturation of angiogenic sprouting, and may be a potential target of glucocorticoid action. Indeed, in isolated endothelial cells, treatment with glucocorticoids causes inhibition of MMP protein levels with a concomitant increase in TIMP levels and activity (Kräling *et al.*, 1999). This shift in the proteolytic balance and deposition of a new basement membrane-type matrix has been shown to reproducibly reduce proliferation of human dermal microvascular endothelial cells (Kräling *et al.*, 1999) and thus could explain their angiostatic actions.

1.3.2.8 Non-transcriptional effects of glucocorticoids

Previous attention to glucocorticoid-induced inhibitory actions has mainly focused on transcriptional mechanisms. More recently, post-transcriptional and translational modes that regulate expression have been identified. Importantly, these effects may explain similar angiostatic effects of steroids which do not share the same capacity to activate GR. Glucocorticoids have been shown to play an important role in the destabilisation of COX-2 transcripts during the IL-1-induced expression of COX-2 mRNA (Buttgereit *et al.*, 1995). Furthermore, Gille and colleagues showed that glucocorticoids increase VEGF mRNA turnover in keratinocytes indicating post-transcriptional regulation of gene expression (Gille *et al.*, 2001). In other instances, glucocorticoids have been shown to inhibit gene expression at the level of mRNA half-life (Peppel *et al.*, 1991; Garcia-Gras *et al.*, 2000). These effects were rapid (mRNA was degraded in less than 2 hours) and did not appear to require *de novo* protein synthesis since translation inhibition by cycloheximide did not attenuate or block increased growth factor-induced mRNA turnover caused by dexamethasone. The poly(A) tail and untranslated regions (UTRs) at the 5' and 3' ends of mRNA sequences influence stability and have been shown to be involved in glucocorticoid-mediated mRNA destabilisation in the case of IFN-β (Peppel *et al.*, 1991) and MCP-1 (Poon *et al.*, 1999) which may explain, in part, the anti-inflammatory actions of glucocorticoids. However, similar elements appear to confer stability in the VEGF gene (Gille *et al.*, 2001) and therefore a similar mechanism may exist to regulate angiogenesis.

Following receptor activation by angiogenic factors (*e.g.* VEGF and integrins), multiple signals from transmembrane RTKs form cascades which eventually lead to gene transcription and a network of cross-talks that ultimately determine cellular activity. As described previously (Section 1.1.3.2), the MAPK pathway is of particular importance for transduction of soluble growth factor and ECM-derived signals, in endothelial cells (Muñoz-Chápuli *et al.*, 2004), and MAPK signalling can be modulated by steroids in a rapid non-genomic fashion in endothelial cells (Nuedling *et al.*, 1999; Simoncini *et al.*, 2004). These intracellular pathways, including MAPK, are important in mediating changes in endothelial cells (such as

morphogenesis, migration, proliferation and survival) and consequently, may be a target of glucocorticoid-mediated angiostasis. Signalling molecules downstream of integrin-matrix interactions, such as the Rho guanosine triphosphatases (GTPases; in the Ras superfamily), have been shown to control actin and microtubule cytoskeletal networks (Zhao *et al.*, 2006) and, therefore, regulate cell morphogenesis. Selective glucocorticoid control of Rho kinase isoforms (ROCK1 and ROCK2) regulates cell-cell interactions (Rubenstein *et al.*, 2007) and it has been suggested that steroids may control tight junction dynamics which are likely to be important in stabilisation of angiogenic vessels.

The proposed angiostatic effects of glucocorticoids on endothelial cells are thought to be dependent on stimulation of GR but may not always involve activation or repression of GREs. For example, treatment of human endothelial cells with dexamethasone (100 nM) stimulated eNOS activity, an effect which was blocked by GR antagonism (RU38486), PI3K inhibition (wortmannin and LY294002) or eNOS inhibition (N-nitro-L-arginine methylester, L-NAME), but was not blocked by transcriptional inhibition (actinomycin D) (Hafezi-Moghadam *et al.*, 2002). Again, this indicates that some of the anti-inflammatory effects of corticosteroids occur via a non-transcriptional mechanism. In the study by Hafezi-Moghadam and colleagues, dexamethasone treatment increased the activity of the protein kinase, Akt, indicating activation of downstream targets of PI3K. Akt has previously been shown to directly phosphorylate and activate eNOS (Fulton *et al.*, 1999). At present, it is unclear how GR recruits and activates PI3K and the importance of these non-transcriptional actions of glucocorticoids *in vivo.*

Transgenic mice expressing a mutant GR, GR^{Dim} (by introducing a point mutation into the GR gene), exhibit intact immunosuppressive responses to corticosteroids (Reichardt *et al.*, 1998). In mutant mice, GR cannot bind GRE and therefore impairs GRE-dependent transactivation, whereas cross-talk (protein-protein interactions), which results in the transrepression of AP-1-driven genes, remains intact. Although several physiological functions are impaired in these mice (*e.g.* lung development and depression of the HPA axis) the mutation does not reduce viability (in contrast to

GR$^{-/-}$ mice which die shortly after birth) (Cole *et al.*, 1995). Consequently, these mice offer an opportunity to dissect angiostatic mechanisms *in vivo*. Defining the nuclear and non-nuclear actions of GR will be crucial in the development of selective GR modulators which could distinguish between the beneficial and harmful effects of corticosteroids. Discriminating between nuclear and non-nuclear effects of glucocorticoids in angiogenesis will also be fundamental to understanding their mechanisms of action.

1.3.2.9 Summary

Although there is a vast amount of evidence that glucocorticoids inhibit angiogenesis by influencing anti-inflammatory, anti-migratory, anti-proliferative and anti-basement membrane mechanisms, the relative significance of these interactions requires further clarification. Further, the influence of physiologically-relevant concentrations of glucocorticoids on cell function and molecular signalling pathways has received little attention due to their widespread use as pharmacological anti-inflammatory agents. Some of the evidence for glucocorticoid-mediated angiostasis, introduced in this section, does rely on physiological levels of glucocorticoids and, therefore, serves as a useful basis for investigating their angiostatic mechanisms. Of particular note, 11β-HSD1 activity in the vessel wall influences the angiostatic effects of glucocorticoids by altering the steroid concentrations locally at sites of angiogenesis (Small *et al.*, 2005) demonstrating that endogenous glucocorticoids are also essential inhibitors of angiogenesis. However, again, the cellular and molecular mechanisms that underpin this phenomenon remain unclear. Finally, it is clear that glucocorticoids can influence diverse cell functions including differentiation, proliferation and migration through an equally diverse array of transcriptional and non-transcriptional mechanisms.

1.4 Hypothesis and aims

Glucocorticoids are recognised inhibitors of angiogenesis although their mechanism of action has not been establised. Previous studies have indicated that glucocorticoids can directly alter the function of endothelial cells. Since changes in endothelial cell function and structure are central to angiogenesis, this suggests that glucocorticoids may inhibit angiogenesis by direct modulation of the vascular endothelium.

1.4.1 Hypothesis

It was hypothesised that glucocorticoids act directly on GR on endothelial cells to inhibit key steps of the angiogenesis cascade including; endothelial cell morphogenesis, migration and proliferation. Glucocortioid-induced inhibition of tube formation by endothelial cells is likely be mediated by altered production of pro- and anti-angiogenic factors (including VEGF and TSP-1).

1.4.2 Aims

1) To establish and characterise an *in vitro* model of human endothelial TLS formation and establish methods for assessing endothelial cell migration and proliferation.
2) To investigate whether physiological glucocorticoids inhibit tube formation by direct, GR-dependent action on the endothelium.
3) To determine whether glucocorticoids alter gene expression of pro-angiogenic and angiostatic factors during endothelial TLS formation.
4) To investigate the influence of glucocorticoids on activation of signalling proteins involved in transduction of VEGF and ECM-derived signals.
5) To determine whether physiological glucocorticoids inhibit endothelial cell migration and proliferation.

Chapter 2

Materials and Methods

2.1 Materials

Unless otherwise stated all chemicals, reagents and drugs were purchased from Sigma, Dorset, UK. All radioactivity was purchased from GE Healthcare, Buckinghamshire, UK. HPLC grade solvents were purchased from Rathburn Chemicals, Walkerburn, UK. Enzymes for molecular biology were purchased from Promega, Southampton, UK.

2.2 Buffers and solutions

Bovine serum albumin (BSA) standard: 1 mg/ml in distilled water prepared from lyophilised powder (supplied in D_C Protein Assay Kit II, Bio-Rad, Hertfordshire, UK). 1 ml aliquots stored at -20°C.

DNA/RNA gel loading buffer: 20% Ficoll 400 w/v, 1% sodium dodecyl sulphate (SDS) w/v, 0.25% bromophenol blue w/v, 0.25% xylene cyanol in 0.1M EDTA, pH 8.0.

Mowiol mounting medium: 20% Mowiol-4-88 w/v (Calbiochem-Merck, Nottingham, UK), 50% glycerol v/v in PBS. Incubated (overnight at 50°C) with intermittent vortexing then centrifuged (for 10 minutes at 5000g). Aliquots stored at -20°C.

20x NuPAGE MES-SDS running buffer: 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 7.2, 50 mM Tris-base, 1% SDS v/v, 1 mM EDTA (ready mixed from Invitrogen, Paisley, UK).

20x Plate wash concentrate: PBS, surfactant, 2% chloroacetamide (ready mixed from Calbiochem-Merck).

Phosphate buffered saline (PBS): 0.01 M phosphate buffer with 137 mM NaCl, 2.7 mM KCl in distilled water, pH7.4. Stored at 4°C.

5x Protein gel loading buffer: 300 mM Tris (pH 6.8), 50% glycerol v/v, 10% SDS w/v, 12.5% β-mercaptoethanol v/v, 0.01% bromophenol blue w/v in distilled water. Prepared in fume hood.

Protein lysis buffer: 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.6% Triton X-100 v/v, 10% glycerol v/v in distilled water, stored at 4°C. On day of use, 1 Complete, Mini Protease Inhibitor Cocktail Tablet (Roche, Basel, Switzerland) and 1 mM Na_3VO_4 were added to 50 ml lysis buffer and kept on ice.

Reverse transcription 10x buffer: 0.1 M Tris-HCl (pH 9.0), 0.5 M KCl, 1% Triton X-100 (ready mixed from Promega). Stored at -20°C.

Stripping buffer (for Western blots): 0.2 M glycine (pH 2.5), 1% SDS v/v in distilled water.

10x TBE (Tris/borate/EDTA) buffer: 0.9 M Tris-base, 0.9 M boric acid, 20 mM EDTA (pH 8.0) in distilled water, autoclaved before use.

Thermophilic DNA polymerase 10x reaction buffer: 0.1 M Tris-HCl (pH 9.0), 0.5 M KCl, 1% Triton X-100 v/v (ready mixed from Promega). Stored at -20°C.

TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, autoclaved before use.

5x TNS Tween buffer: 2.5 M NaCl, 100 mM Tris-HCl, 0.5% Tween 20 v/v, in distilled water, autoclaved before use.

Transfer buffer (for Western blots): 25 mM Tris-base, 192 mM glycine, 20% methanol v/v, in distilled water.

2.3 Drugs and radiolabelled steroids used in cell culture studies

[^{3}H]$_4$-Cortisol: 15.2 nM commercial stock solution of [^{3}H]-Cortisol in ethanol, stored at -20°C.

Cortisol: (FW: 362.46) 50 μM hydrocortisone, 0.2% ethanol v/v, 145 mM NaCl in water, sterile-filtered (ready mixed from Sigma). 1 ml aliquots stored at -20°C.

[^{3}H] $_4$-Cortisone: 22.2 nM commercial stock solution of [^{3}H]-Cortisone in ethanol, stored at -20°C.

Cortisone: (FW: 360.44) 36.04 mg cortisone was made up to 10 ml with ethanol (10^{-2} M solution), then 10 μl was added to 10 ml deionised water (10^{-5} M solution). 1 ml aliquots stored at -20°C.

Fibroblast Growth Factor-Basic (bFGF): 25 μg human recombinant fibroblast growth factor-basic (bFGF) was made up to 1 ml with 5 mM Trizma-HCl, pH 7.6 and filtered (0.2 μm, 25 μg/ml solution). 50 μl aliquots stored at -20°C.

PD98059: (FW: 267.28) 5 mg of PD98059 (Calbiochem-Merck) were dissolved in 374 μl of DMSO (50 mM solution). 50 μl aliquots stored at -20°C and protected from light.

Platelet factor 4: 72.4 μM of human platelet factor 4 in 20 mM Tris-HCl, 2 M NaCl, pH 7.4 (Cambridge Biosciences, Cambridge, UK) were diluted in PBS to give 34.5 μM solution. 50 μl aliquots stored at -20°C.

Prostaglandin E_2: (FW: 352.47) 5 mg of prostaglandin E_2 were dissolved in 142 μl of ethanol (10^{-1} M solution), then 2 serial dilutions (1:100) in deionised water (10^{-5} M solution). 1 ml aliquots stored at -20°C.

Prostaglandin $F_{2\alpha}$: (FW: 475.62) 1 mg of prostaglandin $F_{2\alpha}$ tris salt was dissolved in 210 µl of ethanol (10^{-2} M solution), then 10 µl were added to 10 ml deionised water (10^{-5} M solution). 1 ml aliquots stored at -20°C.

RU38486: (FW: 429.59) 42.96 mg RU38486 (mifepristone, a gift from Dr Roger Brown, Endocrinology Unit, University of Edinburgh) were dissolved in 10 ml of ethanol (10^{-2} M solution), then 100 µl were added to 10 ml deionised water (10^{-4} M solution). 1 ml aliquots stored at -20°C.

Spironolactone: (FW: 416.57) 41.66 mg spironolactone were dissolved in 10 ml of ethanol (10^{-2} M solution), then 100 µl were added to 10 ml deionised water (10^{-4} M solution). 1 ml aliquots stored at -20°C.

SU5416: (FW: 238.28) 5 µg of SU5416 were dissolved in 1 ml of DMSO (21 mM solution). 100 µl aliquots stored at -20°C.

Vascular Endothelial Growth Factor (VEGF): 10 µg human recombinant vascular endothelial growth $factor_{165}$ were dissolved in 100 µl of water (100 µg/ml solution). 10 µl aliquots stored at -20°C.

2.4 Cell culture

2.4.1 Transfected Chinese Hamster Ovary cell culture

Chinese Hamster Ovary (CHO) cells transfected with human 11β-HSD1 and 11β-HSD2 (CHO-h11β-HSD1 and CHO-h11β-HSD2, respectively) were a gift from Dr Scott Webster, Endocrinology Unit, University of Edinburgh. These were used as positive controls for 11β-HSD activity experiments. Cells were cultured in Ham's F12 medium containing 10% foetal bovine serum (FBS), penicillin-streptomycin (1 U/ml, 1 µg/ml) and 2 mM L-glutamine (all from Lonza Wokingham, Berkshire, UK). Cell cultures were maintained at 37°C, 5% CO_2 in a humidified atmosphere and growth medium was replaced every 2 days. At 90-95% confluency cells were

washed twice in 4 ml PBS and detached from the flask by incubating with 3 ml trypsin-versene (EDTA) (Lonza) at 37°C for up to 5 minutes with gentle agitation. Trypsin-versene was neutralised by adding 15 ml of medium (containing serum) to the flask and removed by centrifugation (at 1000 rpm for 5 minutes at room temperature) and disposal of supernatant. Cells were resuspended in fresh medium and divided into new 75 cm^2 flasks using split ratios of between 1:10 and 1:15.

2.4.2 Human umbilical vein endothelial cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Promocell, Heidelberg, Germany. Each batch of cells had been pooled from multiple donors by the supplier and arrived as proliferating cultures in 25 cm^2 flasks. Cells had been phenotypically characterised by the supplier and were positive (by immunofluorescence) for endothelial markers (Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1/CD31), von Willebrand Factor (vWF)) and showed uptake of fluorescently-labelled acetylated low density lipoprotein (DiI-Ac-LDL) and were negative for smooth muscle α-actin (α-SMA). Cells were used between passage number (p) 2 and 6 in all experiments discussed in this thesis. Attempts to cryopreserve HUVECs to extend the lifespan of a single batch proved unsuccessful; therefore, cells were discarded after reaching p6. Cell cultures were maintained according to the supplier's recommendations at 37°C, 5% CO_2 in a humidified atmosphere and growth medium was replaced every 2 days. HUVECs were routinely cultured in endothelial cell growth medium-2 (EGM-2) Bullet Kit medium (Lonza) supplemented with heparin, ascorbic acid, GA-1000, 2% FBS, hFGF-B, VEGF, R^3-IGF and hEGF as recommended by the supplier. Hydrocortisone (cortisol), however, was omitted. At 70-80% confluency cells were washed twice in 4 ml HEPES buffered saline solution and detached from the flask by incubating with 3 ml trypsin/EDTA (Reagent Pack subculture Reagent Kit, Lonza) at 37°C for up to 5 minutes with gentle agitation. Trypsin/EDTA was removed and neutralised by adding 4 ml of trypsin neutralising solution (TNS) to the flask. Cells were divided into new 75 cm^2 flasks using split ratios of between 1:4 and 1:10.

2.4.3 Human aortic endothelial cell culture

Human aortic endothelial cell (HAoEC) culture was performed by Miss Sadaf Ali under my supervision. Primary HAoECs were obtained from Promocell. A single batch of cells from a 26 year old, Caucasian male arrived as a proliferating culture in a 25 cm^2 flask. Cells had been phenotypically characterised by the supplier and were positive (by immunofluorescence) for endothelial markers PECAM-1/CD31 and vWF, showed uptake of DiI-Ac-LDL and were negative for α-SMA. Cells were used between p2 and p6. Cell cultures were maintained according to the supplier's recommendations at 37°C, 5% CO_2 in a humidified atmosphere and growth medium was replaced every 2 days. HAoECs were routinely cultured in EGM-MV medium (Promocell) supplemented with 5% FBS, 0.4% endothelial cell growth supplement/heparin (ECGS/H), 10 ng/ml hEGF, 50 μg/ml gentamicin and 50 mg/ml amphotericin included in the supplement pack. Hydrocortisone (cortisol), however, was omitted. At 70-80% confluency cells were washed twice in 4 ml HEPES buffered saline solution and detached from the flask by incubating with 3 ml trypsin/EDTA (Reagent Pack subculture Reagent Kit, Lonza) at 37°C for up to 5 minutes with gentle agitation. Trypsin/EDTA was removed and neutralised by adding 4 ml of trypsin neutralising solution (TNS) to the flask. Cells were divided into new 75 cm^2 flasks using split ratios of between 1:4 and 1:10.

2.4.4 Cell counting

In all cell culture assays cell counting was performed to ensure consistency of cell density between experiments. Briefly, following trypsinisation, cells suspended in TNS were pipetted into a sterile 15 ml Falcon tube centrifuged (at 1000 rpm for 5 minutes at room temperature), the supernatant drained and the pellet resuspended in 1 ml of the appropriate experimental medium (see relevant sections for details). 10 μl of cell suspension were added to 90 μl of 0.4% trypan blue solution, mixed by gentle agitation and approximately 30 μl were placed into a haemocytometer and the number of cells counted. The number of cells per ml was determined using the standard formula:

$$\text{cells/ml} = \frac{\text{number of cells counted x dilution factor}}{\text{number of squares counted x volume of 1 square}}$$

2.4.5 Endothelial tube-like structure formation assay

The endothelial tube-like structure (TLS) assay was chosen since it recapitulates one of the key steps in the angiogenesis cascade, the differentiation of endothelial cells into tubules, and is considered to resemble the *in vivo* process (Lawley & Kubota, 1989). Since the subject of this thesis was to dissect out the mechanisms of glucocorticoid-induced angiostasis, a rapid, easy to perform, quantitative assay amenable to protein and RNA extraction and immunostaining was required. The 2D TLS assay has all of these attributes. Extensive methodological development (discussed in Chapter 3) was undertaken to arrive at the following, final assay conditions.

2.4.5.1 Culture conditions

HUVECs (4 x 10^4 cells/ml/well) resuspended in standard basal medium were seeded into wells pre-coated with 250 µl of standard Matrigel (BD Biosciences, Oxford, UK) in a 24-well, flat-bottomed microtiter plate (Cellstar-Greiner Bio-One, Gloucestershire, UK). Standard basal medium comprised EGM-2 (Lonza) supplemented with ascorbic acid, heparin and GA-1000; however, no growth factors or serum supplied with the bullet kit were added. Growth factors, steroids and established inhibitors were added immediately at the time of cell seeding, where indicated in appropriate results chapters, and well contents were mixed by gentle agitation. Plates were incubated for up to 24 hours at 37°C, 5% CO_2, in a humidified atmosphere.

2.4.5.2 Quantification of tube-like structure formation

Quantification of TLS formation was performed by inverted light microscopy (Carl Zeiss Axiovert 25, Carl Zeiss, Hertfordshire, UK) and image analysis. Images at low magnification (x5) at the centre of each well were captured after 4-5 hours and 21-24 hours of incubation and saved for analysis. Images were captured using the

Microcomputer Imaging Device (MCID) from a live-feed camera (Pentax 250, Pentax, Slough, UK) using MCID Basic v7.0 software (InterFocus Imaging, Linton, UK). Image files were randomised by a different person to ensure quantification was performed by an individual blind to treatment groups to ensure the integrity of the results. Capillary connections, defined as the number of branch points between 2 or more TLSs, were identified and marked using Adobe Photoshop cs (Adobe System Inc, California, USA) software. Total numbers of capillary connections per field of view were counted and tallied using a cell counter. In all experiments, each condition was performed in triplicate wells.

2.4.5.3 Time-course of tube-like structure formation

To determine the time-course of TLS formation, the nature of the endothelial cellular changes, and the mechanism through which cortisol inhibits TLS formation, time-lapse videomicroscopy was used. Technical assistance with time-lapse equipment was provided by Mr Bob Morris and advice on videoclip preparation was provided by Professor Adriano Rossi, Centre for Inflammation Research, University of Edinburgh.

Sterile, polystyrene (9.0 cm^2) SlideFlasks (Nunc, New York, USA) were pre-coated with 750 µl Matrigel and allowed to set for approximately 60 minutes. HUVECs (2×10^5 cells) in 2 ml of endothelial basal medium containing 5 mM HEPES buffered saline solution were seeded onto the Matrigel. Treatments were added at the time of cell seeding and immediately thereafter (within 10 minutes), stage position settings and focus adjustments were made then image acquisition started. Images were acquired with a Leica DM IRBE microscope using a x10 objective and captured with a Leica Q500MC image processing system (Leica Cambridge Ltd., Cambridge, UK). Images were captured from 3 separate positions per flask at 4 minute intervals for 24 hours where possible. TLS counting was used to assess the time course of TLS formation. From time-course graphs, time to reach peak number of TLSs and maximum number of TLSs were determined.

2.4.6 Characterisation of tube-like structures

2.4.6.1 Structural composition of tube-like structures

The structural composition of TLSs was assessed by immunofluorescent staining of filamentous-actin (F-actin) and microtubules (α-tubulin), important components of the cytoskeleton. Antibodies, expertise in immunofluorescence staining protocols, and fluorescence microscopy were provided by Dr Kate Marshall, Centre Cardivascular Science, University of Edinburgh.

HUVECs were resuspended (4×10^4 cells/ml) in 'experimental' medium and 1 ml per well was seeded onto sterilised round coverslips (13 mm; 0 thickness) pre-coated with 30 µl of phenol red-free Matrigel in a 24-well, flat-bottomed microtiter plate (Cellstar-Greiner Bio-One, UK). Experimental medium consisted of EGM-2 medium (Lonza, UK) supplemented with heparin, ascorbic acid, GA-1000, and 2% charcoal-stripped FBS (a gift from Miss Tijana Mitić, Endocrinology Unit, University of Edinburgh); no growth factors from the Bullet kit were added. Cortisol (600 nM) was added immediately at the time of cell seeding, as indicated. After 1, 4, 10 and 22 hours of incubation, medium was removed by gentle aspiration and cultures fixed (for 3 minutes at room temperature) with 4% paraformaldehyde in Dulbecco's PBS v/v. Cultures were rinsed (once for 2 minutes) in 0.05% Triton X 100 in PBS v/v, permeabilised (once for 3 minutes) in 0.1% Triton X 100 in PBS v/v, blocked (for 1 hour at room temperature) in 3% BSA in PBS v/v, then rinsed again (twice for 2 minutes). Cultures were incubated with (1:1000 in 0.3% BSA in PBS v/v) mouse monoclonal anti-α-tubulin (clone B512) antibody (Sigma) for 1 hour at room temperature then rinsed again (twice for 2 minutes). Cultures were incubated with (1:500 in 0.3% BSA in PBS v/v) goat anti-mouse IgG (H+L) Alexa Fluor 594 secondary antibody (Molecular Probes-Invitrogen, Paisley, UK), for 1 hour at room temperature, protected from light then rinsed again (twice for 2 minutes). Direct fluorescent staining of F-actin was achieved with (1:500) phalloidin Alexa Fluor 488 (Molecular Probes-Invitrogen) and staining of DNA with 0.2 µg/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) which were included along with the secondary antibody. Coverslips were carefully removed from the wells with

tweezers, inverted, and mounted on Superfrost Plus microscope slides (VWR International, Leicestershire, UK) using Mowiol mounting medium (approximately 30 µl) and stored protected from light. Slides were viewed by an Olympus AX-70 fluorescence microscope (Olympus Optical Company, London, UK) and images captured with a Hamamatsu Orca-ER digital live-feed camera and controller (Hamamatsu Photonics, Hertfordshire, UK) using Smart Capture 3 software (Digital Scientific, Cambridge, UK).

2.4.6.2 Functional composition of tube-like structures

The endothelial nature of TLSs was assessed by immunocytochemistry for an endothelium-specific cell surface marker (CD31). HUVECs (1×10^4) were cultured in 250 µl of standard basal medium for 5 hours on 8-well permanox chamber slides (Nalge-Nunc, Hereford, UK) pre-coated with 100 µl of standard Matrigel. Staining was performed using Vectastain's avidin: biotinylated (peroxidase) enzyme complex (ABC) Kit (Vector Laboratories, California, USA) as a secondary detection system according to manufacturer's instructions. Briefly, culture medium was gently removed by aspiration, TLSs rinsed with PBS and fixed with 10% formalin. Quenching of endogenous peroxidase activity was achieved by incubating (for 30 minutes at room temperature) with 0.3% hydrogen peroxide in distilled water, v/v. Cultures were incubated (for 30 minutes at room temperature) with diluted rabbit serum then with rat anti-mouse CD31 (1 in 50 dilution, overnight at 4°C) monoclonal antibody (BD Pharmingen, Oxford, UK). For negative control, the primary antibody was omitted. The following day, cultures were rinsed (x3) with PBS then incubated (for 30 minutes at room temperature) with biotinylated rabbit anti-rat secondary antibody then further rinsing (x3) in PBS. Next, slides were incubated (for 30 minutes at room temperature) with Vectastain ABC reagent then further rinsing (x3) in PBS. Finally, slides were incubated for 5 minutes with 3,3'-diaminobenzidine (DAB) (Vector Laboratories) as a chromogenic substrate for the peroxidase moiety with positive structures staining brown. The reaction was stopped with running tap water and coverslips mounted using an aqueous medium. Slides were viewed by light microscopy (Carl Zeiss Axioskop, Carl Zeiss, Hertfordshire, UK) and images

captured using MCID from a live-feed camera (3-CCD, JVC Professional Europe, London, UK).

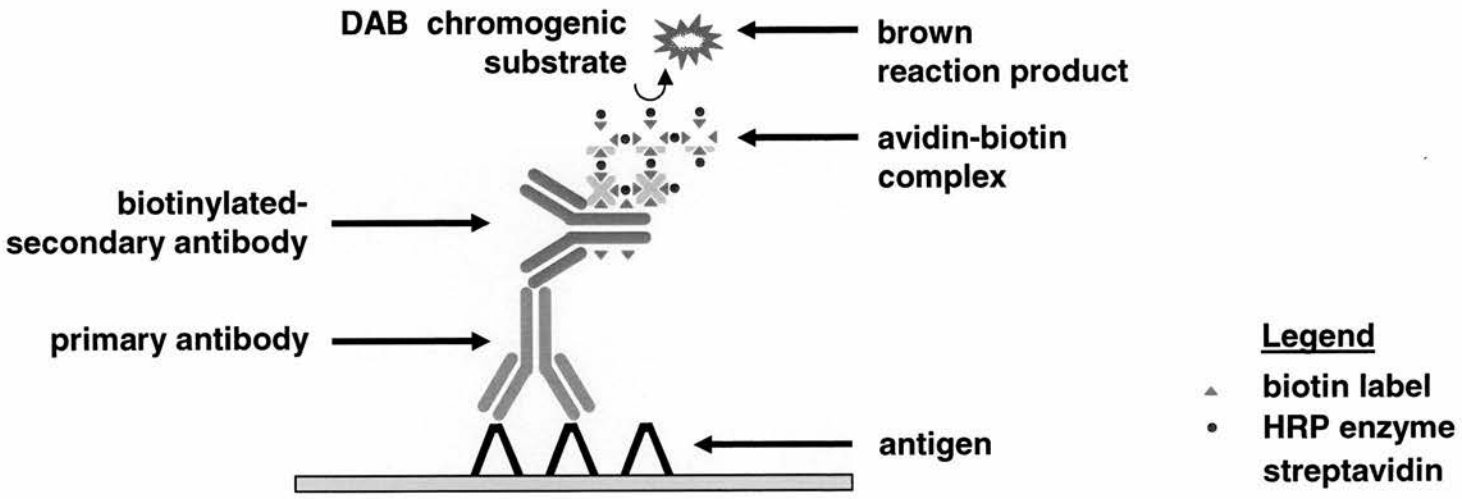


Figure 2.1 Schematic representation of a 3-layer method of immunostaining cells and tissue

In the avidin-biotin complex (ABC) method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex. Streptavidin and avidin both possess four binding sites for biotin and therefore result in amplification of the signal. Horseradish peroxidase (HRP) activity in the presence of an electron donor, in this case, 3,3'-diaminobenzidine (DAB) produces a brown end-product that is highly insoluble in alcohol. Adapted from Immunohistochemical Staining Methods, Fourth Edition (Dako, Cambridgeshire, UK).

2.4.7 Reverse transcription-polymerase chain reaction

Reverse-transcription-polymerase chain reaction (RT-PCR) allows the detection and amplification of specific mRNA species from isolated total RNA and was used to investigate expression of corticosteroid receptors and 11β-HSDs in endothelial cell preparations. Using conditions provided by a commercially available kit (Reverse Transcription System, Promega) the Avian myeloblastosis virus reverse transcriptase (AMV-RT) enzyme transcribes mRNA into single stranded cDNA, the necessary template for *Taq* polymerase (*Taq* bead Hot Start Polymerase, Promega). Subsequent PCR amplification consists of a number of cycles during which the cDNA template is repeatedly denatured, annealed with target-specific primers and replicated using *Taq* polymerase. Following 20-40 cycles the amplified DNA can be analysed for size and quantity using gel electrophoresis.

Only RNase-free, sterile solutions and equipment were used for RT-PCR in order to prevent degradation of target RNA by exogenous RNases and contamination of reactions with exogenous RNA or DNA. All reactions were prepared on ice unless otherwise stated.

2.4.7.1 Tissue preparation

Intact tissues Livers from C57Bl6 mice aged 10-12 weeks were frozen on dry ice immediately after dissection from the animal for use as a positive control for 11β-HSD1 mRNA expression. Tissues were stored at -80°C until required. RNA extracted from human kidney was a gift from Dr Elaine Marshall, Endocrinology Unit, University of Edinburgh.

Undifferentiated cell monolayers Flasks of HUVECs and CHO-transfected cells at 70-95% confluency were rinsed with PBS (2 x 4 ml) and were removed directly from the culture flask by addition of 3-4 ml of TRIzol Reagent (Invitrogen). The contents were transferred to a 15 ml sterile Falcon tube and stored at -80°C until required.

Endothelial tube-like structures The use of MatriSperse Cell Recovery Solution (Matrisperse; BD Biosciences) is recommended by the manufacturer as a highly reproducible method of recovering endothelial cells from Matrigel matrix for subsequent biochemical analysis. Matrisperse depolymerises Matrigel at 2-8°C without enzymatic digestion and/or lengthy incubation periods at high temperatures thereby avoiding biochemical changes and digestion of extracellular portions of cell surface receptors and adhesion molecules. The manufacturer's protocol was followed in a pilot experiment but extremely low RNA yields necessitated method optimisation and fewer PBS washes were used. These modifications improved efficiency of cell recovery and resulted in improved RNA yields.

Briefly, medium was aspirated from TLS cultures on Matrigel-coated, 35 mm cell culture dishes and structures were washed twice with cold PBS. Matrisperse (2 ml) was added to each dish and the contents scraped and transferred to a 50 ml Falcon tube sitting on ice. Dishes were rinsed with a further 3 ml of Matrisperse and contents transferred to the tube. Tubes were inverted periodically (45 minutes to 1 hour) to aid dissolving of gel. After complete release from gel, cells were centrifuged to a pellet (at 1200 rpm for 5 minutes at 4°C), washed once by gentle resuspension in ice cold PBS and centrifugation, and the supernatant discarded. 1 ml of Trizol was added to the cell pellet and the contents transferred to a cryovial for storage at -80°C until required.

Endothelial progenitor cells RNA extracted from endothelial progenitor cells was a gift from Dr John McDermott, National University of Ireland, Galway, Republic of Ireland. RNA was isolated from peripheral blood from 3 healthy human volunteers.

2.4.7.2 RNA extraction

RNA extraction was carried out using Trizol, a mono-phasic solution containing phenol and guanidine isothiocyanate. This reagent maintains RNA integrity whilst disrupting cells and dissolving cell components.

Tissue homogenization Ice-cold Trizol (1 ml) was added to a 50-100 mg piece of frozen liver dissected from the rest of the organ and placed in a 2 ml sterile eppendorf tube. Samples were homogenised on ice using an Ystral mechanical homogeniser (Scientific Instruments Centre, Hampshire, UK). Samples were centrifuged (at 12,000g for 10 minutes at 4°C) to remove insoluble material and small amounts of unhomogenised tissue. The resulting supernatant was removed to a fresh eppendorf.

Phase separation Following homogenisation of tissue, and thawing of cells and TLSs in Trizol, samples were allowed to equilibrate to room temperature then left for 5 minutes to allow complete dissociation of the nucleoprotein complexes. Chloroform (0.25 ml for cells and TLSs, 0.3 ml for liver) was then added to each sample. Samples were mixed vigorously by vortexing (15 seconds) then incubated (at room temperature for 3 minutes). Samples were centrifuged (at 12,000g for 15 minutes at 4°C) resulting in a lower red phenol-containing phase (containing proteins), an interface (containing DNA and denatured proteins) and an upper aqueous phase (containing RNA).

RNA precipitation The upper aqueous phase from each sample was transferred into a fresh eppendorf. RNA was precipitated by addition of 20 µl (for cells and TLSs) and 30 µl (for liver) of well-resuspended RNaid matrix (Anachem, Bedfordshire, UK). Following addition of matrix, samples were vortexed and mixed by gentle agitation by hand for 5 minutes. Samples were centrifuged (at 12,000g for 1 minute at room temperature) and formed an obvious white pellet of matrix containing RNA precipitate.

RNA wash Following centrifugation the supernatant was removed and the pellet was washed with 0.5 ml of RNA wash buffer (containing 37.5% ethanol v/v) by careful mixing with sterile 1 ml filter pipette tips. Once fully resuspended, samples were vortexed for 15 seconds and centrifuged (at 12,000g for 1 minute at room temperature) to pellet. The wash step was repeated a further 2 times and samples were kept on ice, where possible, throughout.

RNA resuspension Following the last RNA wash the pellet was resuspended in 18 µl (for cells and TLSs) and 30 µl (for liver) of nuclease-free water (Promega) containing 10 mM dithiothriotol (DTT) and Recombinant RNasin Ribonuclease Inhibitor (RNasin; Promega, Southampton, UK). Samples were then incubated at 55°C for 12 minutes (vortexing after 6 minutes) and quickly centrifuged (at 12,000g for 1 minute at 4°C) to pellet. The supernatant containing RNA was carefully removed by pipette, avoiding transfer of matrix, to a fresh eppendorf and stored at -80°C until required.

RNA quantification and integrity Before use, RNA was quantified using a GeneQuant RNA/DNA Calculator (Pharmacia Biotech, Uppsala, Sweden). RNA was diluted 1:100 in nuclease-free water and the optical density at λ260 nm and λ280 nm was determined to assess concentration and purity.

To assess RNA integrity, RNA samples were run on a 1.2% agarose gel stained with 0.001% (v/v) of 10 mg/ml ethidium bromide. Intact total RNA run on an agarose gel will have sharp, clear 28S and 18S ribosomal RNA (rRNA) bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band indicating that the RNA is completely intact. All RNA samples were confirmed to have approximately 2:1 ratio (28S:18S) before proceeding with RT-PCR.

2.4.7.3 Reverse transcription

First strand complementary DNA (cDNA) synthesis was performed using the Reverse Transcription System (Promega, Southampton, UK). 0.5 µg of total RNA was reverse transcribed in a reaction mixture containing 5 mM $MgCl_2$, 1x reverse transcription buffer, 1 mM each of dATP, dCTP, dGTP and dTTP, 20 units (U) RNasin, 0.5 µg oligo$(dT)_{15}$ primers and 15U AMV-Reverse Transcriptase made up to 20 µl in nuclease-free water. The reactions were carried out on an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) with a heated lid. The programme consisted of incubation at 42°C for 45 minutes followed by 95°C for 5

minutes and finally chilled to 4°C to inactivate the enzyme and prevent binding to DNA.

Negative control reactions for each experiment were performed in parallel with samples. A reaction was performed as above using an RNA sample chosen at random in the absence of AMV-reverse transcriptase in order to determine genomic DNA contamination. Additionally, a negative control reaction containing nuclease-free water instead of RNA was performed to determine RNA contamination of the Reverse Transcriptase System reagents. Products from the RT reactions (cDNA) were diluted 1:5 with nuclease-free water prior to PCR amplification.

2.4.7.4 Polymerase chain reaction

5 µl of cDNA template was used in each PCR reaction containing 1x Thermophilic DNA polymerase reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix (consisting of dATP, dCTP, dGTP and dTTP), 40 pmol gene-specific upstream primer and 40 pmol gene-specific downstream primer made up to 50 µl in nuclease-free water. One *Taq*Bead (1.25U) was added to each reaction tube before starting the required PCR programme. A negative control reaction containing nuclease-free water rather than cDNA was performed where necessary to determine contamination of PCR reagents.

PCRs were carried out on an Eppendorf Mastercycler Gradient with a heated lid. Samples were heated to 95°C for 5 minutes for initial denaturation then underwent 35 cycles of PCR amplification (denaturation at 90°C for 1 minute, primer annealing at primer-specific temperature for 1 minute and elongation at 72°C for 2 minutes). Upon completion of PCR programme, samples were incubated at 72°C for a further 5 minutes to ensure elongation of products to full length and chilled to 4°C prior to gel electrophoresis. 35 cycles of amplification were used as standard throughout these non-quantitative RT-PCR experiments, and were shown to enable detection of all of the genes examined. Gene specific primer sequences and annealing temperatures are detailed in Table 2.1.

2.4.7.5 Gel electrophoresis

RT-PCR products were analysed by electrophoresis on a 1.2% agarose gel. Gels were prepared by melting 1.2% (w/v) agarose (Lonza) in 0.5x Tris/Borate/EDTA (TBE) buffer and adding 0.001% (v/v) of 10 mg/ml ethidium bromide to stain the gel. After pouring the gel mould with appropriately sized combs in place, gels were allowed to set at room temperature then placed in 0.5x TBE in a gel tank.

20 µl of DNA step ladder containing fragments ranging from 100 base pairs (bp) to 4000 bp in 100 bp increments were loaded into the first well on each gel to allow determination of product size. 20 µl of each RT-PCR product were mixed with 4 µl loading buffer and loaded into individual wells of the gel. Gels were electrophoresed at 100V for 45 minutes-1hour, until the loading buffer migrated approximately 6 cm from the well, and then photographed under UV light at λ260 nm. It should be noted that whilst PCR products were not sequenced due to time constraints, all RT-PCR reactions resulted in the production of a single product which corresponded with size predicted following primer design.

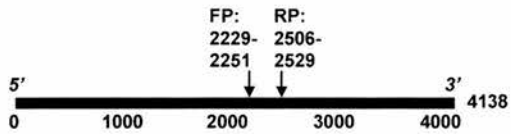
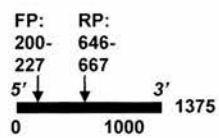
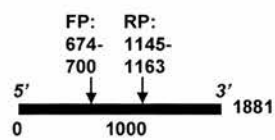
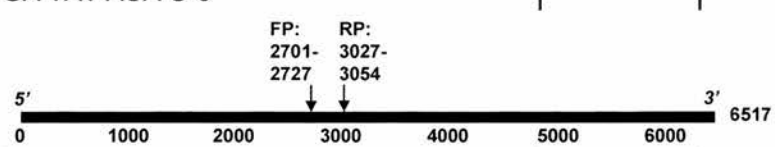
PCR	Primer sequences and position	Annealing temp (°C)	Amplicon length (base pairs)
Tie2	Forward: 5'-TCA CTC CAG TAT CAG CTC AAG GG-3' Reverse: 5'-CAG CTG GTT CTT CCC TCA CGT T-3' 	55	300
11β-HSD1	Forward: 5'-AAA GTG ATT GTC ACW GGG GCC AGC AAA-3' Reverse: 5'-ATC CAR AGC AAA CTT GCT TGC-3' 	60	467
11β-HSD2	Forward: 5'-TAG CTG CAT GGA GGT GAA TTT CTT TGG-3' Reverse: 5'-GAA GTA CAT GAG CCC CAG G-3' 	55	490
GR	Forward: 5'-TGT GGT TTA TAG AGG GCC AAG ACT TGG-3' Reverse: 5'-GGC ACA ACT TCC CTT TTC TGA TAT ACA C-3' 	62	354

Table 2.1 PCR primer sequences, positions and reaction conditions

Human tissue-specific Tie2 primer sequences were taken from Dr Clare Christy's PhD thesis (Endocrinology Unit, University of Edinburgh, 2003). 11β-HSD1 primers were designed by Dr Karen Chapman and GR primers by Dr Roger Brown (both Endocrinology Unit, University of Edinburgh) and detect human and rodent mRNA. Human tissue-specific 11β-HSD2 primers were designed by Dr Roger Brown. The position of the forward primers (FP) and reverse primers (RP) on each gene are shown schematically.

2.4.8 Quantitative real time-polymerase chain reaction

To examine the influence of glucocorticoids and GR blockade on TLS gene expression, quantitative real time-PCR (Qrt-PCR) was used. Qrt-PCR is used to measure the quantity of cDNA present in a sample and is a modification of PCR since the amount of double-stranded DNA product is measured after each round of amplification (in "real-time") using gene sequence-specific primers, a fluorescent reporter probe and light cycler detection system. These data can be analysed by computer software to calculate cDNA copy number with reference to a standard curve based on dilution standards from pooled samples from the same experiment. Theoretically, equal amounts of RNA from each sample are reverse transcribed in the first instance to cDNA. However, in order to correct for differences in abundance and efficiencies in the RT reaction between samples, data are normalised against a stably transcribed gene ("housekeeping gene" or internal control) to precisely measure total amount of sample.

2.4.8.1 TLS preparation and treatment

HUVECs (200,000) in 2 ml of standard basal medium were seeded on 35 mm cell culture dishes pre-coated with Matrigel (500 μl) and incubated in the presence of cortisol (600 nM), cortisol plus RU38486 (1 μM), or left untreated (control). RNA was recovered using Matrisperse solution (Section 2.4.7.1) after 1 hour, 4 hours, 8 hours and 22 hours of incubation to examine the timecourse of TLS gene expression. 1 ml of Trizol was added to the cell pellet and the contents transferred to a cryovial for storage at -80°C until required.

2.4.8.2 RNA extraction and quantification

RNA extraction and quantification was performed using the Trizol method and GeneQuant RNA/DNA Calculator (Pharmacia Biotech) as previously described (Section 2.4.7.2). Due to the large quantity of samples, RNA extraction was performed on batches of up to 40 samples on 2 separate occasions to prevent degradation of RNA. Only RNase-free, sterile solutions and equipment were used

for Qrt-PCR in order to prevent degradation of target RNA by exogenous RNases and contamination of reactions with exogenous RNA or DNA.

2.4.8.3 Reverse transcription

First strand cDNA synthesis was performed using 0.5 μg of total RNA with the Reverse Transcription System (Promega) as described previously (Section 2.4.7.3).

Again, negative control reactions for each batch of samples were performed in parallel with samples. A reaction was performed as above using an RNA sample chosen at random in the absence of AMV-reverse transcriptase in order to determine genomic DNA contamination. Additionally, a negative control reaction containing nuclease-free water instead of RNA was performed to determine RNA contamination of the Reverse Transcriptase System reagents. Products from the RT reaction (cDNA) were diluted 1:4 with nuclease-free water prior to Qrt-PCR amplification based on reaction kinetics of serially-diluted pooled samples (1:4, 1:8, 1:16, 1:32, 1:64, 1:128).

2.4.8.4 Quantitative polymerase chain reaction

2 μl of cDNA template from each sample, pooled standard dilutions (prepared fresh after 2-3 freeze-thaw cycles), and negative controls were added to a 384-well plate (Roche) in triplicate.

Primer-probe master mix was prepared by mixing 5 μl/sample of (2X mix) LightCycler 480 Probes Master (Roche) with 0.5 μl/sample of (20X mix) TaqMan MGB primer probe mix plus 2.5 μl/sample of water (supplied with LightCycler 480 Probes Master, Roche). All TaqMan minor groove binder (MGB) probes and primers supplied (TaqMan Gene Expression Assays, Applied Biosystems, Warrington, UK) had been pre-mixed to a concentration of 18 μM for each primer and 5 μM for the probe resulting in a 20X mix. TaqMan primer probe mixes were supplied as pre-validated ready-to-use assays eliminating the need for primer design or PCR optimisation. All assays were designed for use with human tissue, were exon

spanning and will not detect genomic DNA. All probes had a FAM (carboxyfluorescein) reporter dye at the 5' end and a non-fluorescent quencher at the 3' end and, consequently, were protected from light throughout. Master mix (8 µl/sample) was added to each well using a digital multi-channel pipette to minimise variation in volume added. Thus, final volume in each well was 10 µl. Plates were sealed using a transparent sealing foil (Roche) and the contents of the well brought to the bottom by centrifugation (at 2,000 rpm for 2 minutes at 4°C).

PCRs were carried out on a LightCycler 480 (Roche). Samples were heated to 95°C for 5 minutes for pre-incubation then underwent 50 cycles of PCR amplification (denaturation at 95°C for 10 seconds, primer annealing at 60°C for 30 seconds and elongation at 72°C for 1 second) and finally underwent cooling at 40°C for a further 5 minutes. 50 cycles of amplification were used as standard for all reactions and were shown to enable detection of all of the genes examined. TaqMan Gene Expression Assays used are listed in Table 2.2.

2.4.8.5 Absolute quantification analysis

Absolute quantification analysis uses sample "crossing point" (Cp) to determine the concentration of target DNA in unknown samples after amplification. The Cp value is the point at which the fluorescence of a sample rises above the background fluorescence. Data were analysed by LightCycler 480 software v1.2.0.169 (Roche). Calibration curves were generated for each gene using crossing point (Cp) values as a function of log of target copy number. Compared with expected standard concentrations (1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 arbitrary units), efficiencies of the calibration curves (slope) were in the range 1.500 to 2.171 and error values (mean squared error of the single data points fit to the regression line) were in the range 0.0152 to 0.0728. By determining where the Cp of an unknown sample falls on the standard curve, the initial concentration of target DNA in the samples can be determined by the software. Sample concentrations, expressed as mean of triplicates, were exported to Excel and normalised to corresponding cyclophilin A concentrations, as a ratio, which served as an internal control.

LocusLink symbol for associated gene	LocusLink gene name	Celera Panther protein classification		Amplicon length (base pairs)	Calibration curve	
		Level 1	Level 2		Error	Efficiency
PPIA	Peptidylprolyl isomerase A (cyclophilin A)	Isomerase	Other isomerase	98	0.0152	1.773
VEGF	Vascular endothelial growth factor	Signalling molecule	Growth factor	60	0.0328	1.683
KDR	Kinase insert domain receptor (VEGF-R2)	Receptor	Protein kinase receptor	84	0.0477	1.534
NOTCH1	Notch homolog 1, translocation associated (*Drosophila*)	Signalling molecule	Membrane-bound signalling molecule	80	0.0728	1.500
DLL4	Delta-like 4 (*Drosophila*)	Signalling molecule	Membrane-bound signalling molecule	78	0.0119	1.928
THBS1	Thrombospondin 1	Extracellular matrix	Carbohydrate kinase	109	0.0293	2.016
ITGA6	Integrin, alpha 6	Cell adhesion molecule	Other cell adhesion molecule	68	0.0385	2.171
CAV1	Caveolin 1, caveolar protein	Membrane traffic protein	Membrane traffic protein	66	0.0266	1.921

Table 2.2 TaqMan gene expression primer probe mixes

Human tissue-specific TaqMan primer probe mixes were purchased from Applied Biosystems as ready-to-use assays. Nucleotide sequences of forward and reverse primers were not disclosed by the supplier but all were exon spanning and detected cDNA and not genomic DNA. Assays were designed for use with human tissue. The fluorescent oligonucleotide probes hybridizing to the template between the primers were covalently labelled at their 5' ends with the reporter dye FAM (carboxyfluorescein). Calibration curve error values and efficiencies were determined by absolute quantification analysis from crossing point (Cp) values and were within the acceptable range.

2.4.9 11β-Hydroxysteroid dehydrogenase activity

Although 11β-HSDs are present in the vessel wall their exact cellular localisation remains controversial. To further validate the mRNA expression data, functional activity assays were performed with undifferentiated HUVECs and TLSs to establish 11β-HSD activity in endothelium and ask whether activity is influenced by cell differentiation state. To determine 11β-HSD activity in endothelial cells *in vitro*, a published method was adapted using radio-labelled steroids as substrates for the reactions (Cai *et al.*, 2001).

2.4.9.1 In vitro 11β-HSD activity assay

HUVECs (1.75×10^5 cells per well) were cultured in standard culture medium (EGM-2 containing growth factors and 2% FBS) and standard basal medium (EGM-2 containing only ascorbic acid, heparin and GA-1000) containing ^{3}H-steroid in 2 separate experiments. 11β-HSD1 activity was determined by adding 10 pmol/ml [^{3}H]$_4$-cortisone (GE Healthcare) and 11β-HSD2 activity was determined by adding 10 pmol/ml [^{3}H]$_4$-cortisol (GE Healthcare). Chinese hamster ovary (CHO) cells stably transfected with human 11β-HSD1 (CHO-h11β-HSD1) and 11β-HSD2 (CHO-h11β-HSD2), a kind gift from Dr Scott Webster, Endocrinology Unit, were used as positive controls. CHO cells (1.75×10^5 cells per well) were cultured in Ham's F12 medium (containing 10% FBS, penicillin-streptomycin and 2 mM L-glutamine). Medium alone served as a negative control. 11β-HSD2 activity was determined by adding 10 pmol/ml [^{3}H]$_4$-cortisol (GE Healthcare). HUVECs (1.75×10^5 cells per well) cultured on Matrigel-coated plates were run in parallel experiments to examine activity of both isozymes in TLSs under standard basal conditions. Matrigel and media, without cells, were used as further negative controls. After 5, 24 and 48 hours of incubation, 200 µl samples of media were collected into glass tubes (Fisher Scientific, Leicestershire, UK) and stored at -20°C until required.

2.4.9.2 Radiolabelled steroid extraction from media

Steroids were extracted from media using C_{18} Sep-pak columns (Waters Millipore, MA, USA). Briefly, samples of media were thawed and centrifuged to pellet any loose cellular material. Columns were prepared by elution (under gravity) with 5 ml of methanol (HPLC grade) then washed with 5 ml of water (HPLC grade; Rathburn Chemicals). Samples were then run onto the column, washed with 5 ml of water and eluted into glass tubes with 2 ml of methanol. Steroid-containing methanolic samples were then dried down at 60°C under nitrogen gas.

2.4.9.3 High performance liquid chromatography

The high performance liquid chromatography (HPLC) system used to detect $[^3H]_4$-cortisol and $[^3H]_4$-cortisone in samples was operated by Mrs Eileen Miller (Endocrinology Unit, University of Edinburgh). The system comprised an auto-sampler, mobile phase pump and symmetry shield RP_{18} 5 μm column (Waters, Hertfordshire, UK) and a radioactivity monitor linked to a scintillation fluid pump (Berthold Technologies, Huddersfield, UK). The system was controlled by the Winflow computer programme (JMBS Developments, Grenoble, France).

Briefly, samples were re-suspended in 1 ml mobile phase (60% water, 15% acetonitrile, 25% methanol) and 180 μl of each sample was injected into the system and quantified by on-line liquid scintillation counting (Dover *et al.*, 2007). Radioactive standards were injected at the start of each batch of samples to confirm peak identity. Following chromatography, the area under each peak was integrated using the Winflow software and used to quantify the percentage conversion of $[^3H]_4$-cortisone to $[^3H]_4$-cortisol (11β-reductase activity) or $[^3H]_4$-cortisol to $[^3H]_4$-cortisone (11β-dehydrogenase activity). Enzyme activity was expressed as percentage conversion per 1.75×10^5 cells.

2.4.10 Intracellular signal transduction

To assess the influence of glucocorticoids on focal adhesion kinase (FAK), and the MAPK/ERK signal transduction pathway in endothelial cells, Western blotting was used to detect FAK and phosphorylated (activated) ERK (pERK) in HUVEC protein extracts after incubation with cortisol, a GR antagonist (RU38486) and an established inhibitor of MAPK signal transduction (PD98059). The method utilises SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins by molecular weight. Proteins are transferred from the gel onto a membrane where they are "probed" using specific labelled antibodies to the protein of interest. Quantitative analysis is achieved by densitometry of fluorescent-probed bands and comparison with an internal loading control. Training in methodology and antibodies were provided by Dr Kurt Sales, MRC Human Reproductive Sciences Unit, Edinburgh).

2.4.10.1 Cell preparation

HUVECs (1 x 10^6 cells per dish) were harvested and seeded into 10 cm tissue culture dishes (Cellstar-Greiner Bio-One) in standard culture medium (EGM-2 containing growth factors, 2% FBS, ascorbic acid, heparin and GA-1000) and incubated for 24 hours to allow cells to adhere. The following day medium was replaced with EGM-2 containing 1% FBS, ascorbic acid, heparin and GA-1000 to provide serum-reduced conditions for a further 24 hours incubation. Forty-eight hours after cell seeding, medium was replaced with standard basal medium to provide serum-free conditions for 1 hour prior to treatment and for the duration of the experiment. At this stage, dishes were approximately 90% confluent which resulted in sufficient protein recovery to run Western blots (preliminary findings from pilot experiments). On day 3, HUVECs were treated with 600 nM cortisol for up to 4 hours in time-course experiments. Vehicle (ethanol; 0.004% v/v) treated cells served as a control. For inhibitor studies, HUVECs were pre-treated with inhibitor (RU38486, 1 μM or PD98059, 50 μM) for 1 hour prior to stimulation with 600 nM cortisol for a further 4 hours.

2.4.10.2 Protein extraction

Medium was removed from dishes and cells were washed once with PBS (2 ml) to remove dead cells and cell debris. Fresh lysis buffer (containing a cocktail of protease inhibitors) was prepared on each day of protein extraction and 200 µl were added to each dish (kept on ice throughout) to minimise protein degradation by endogenous proteases. Cells were detached from dishes using a cell scraper to aid cell lysis and kept on ice (10 minutes) apart from occasional vortexing. Thereafter, insoluble material was pelleted by centrifugation (at 13,000 rpm for 10 minutes at 4°C). The clarified lysate was removed to a new tube and stored at -20°C until required for protein quantification and SDS-PAGE.

2.4.10.3 Protein quantification

Prior to Western blotting, it was necessary to determine protein content to ensure equal gel loading. Protein concentration was measured using the Bio-Rad D_C protein assay kit (Bio-Rad), based on the method of Lowry (Lowry *et al.*, 1951). Briefly, the reaction of protein with an alkaline copper tartrate solution and subsequently with Folin reagent produces reduced species with characteristic blue colour.

To determine protein concentration in samples, a standard curve was prepared each time the assay was performed. Bovine serum albumin (BSA, Bio-Rad) standards were prepared by diluting 1 mg/ml stock to 200 µg/ml in distilled water followed by 6 serial dilutions (final concentrations; 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml). Samples were diluted 1:40 in distilled water which ensured protein concentrations were within the range of the standard curve graph. Standards and diluted samples (25 µl each) were added to a 96-well, flat-bottomed microtiter plate (Cellstar-Greiner Bio-One) in duplicate. Reagents were prepared fresh on each occasion according to manufacturer's instructions. Briefly, Reagents A and S were mixed (50:1) and added (25 µl) to each well followed by Reagent B (100 µl). Plates were left for 15 minutes (room temperature) to allow colour to develop then absorbance in each well was measured using a Labsystems Multiskan EX photometer (Thermo LabSystems, Cheshire, UK) at 690 nm. Sample protein concentrations were calculated from

absorbance values using AssayZap v2.5 software (BioSoft, Cambridge, UK) and a standard curve linear plot (Figure 2.2).

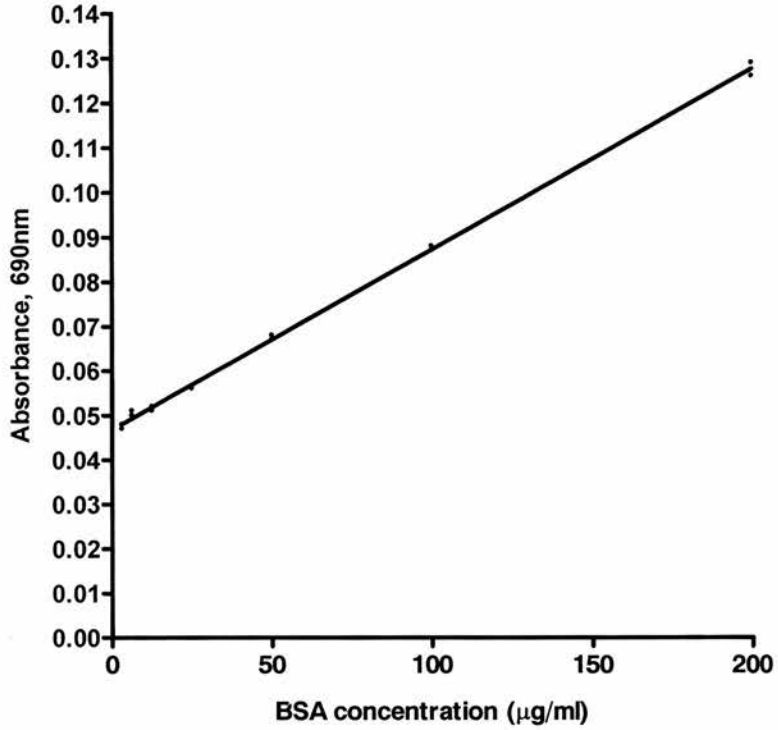


Figure 2.2 Protein assay standard curve

Typical standard curve using Bio-Rad D_C protein assay kit based on the method of Lowry. Representative graph of absorbance (at 690 nm) versus BSA concentration using 7 standards prepared by serial dilution and analysed in duplicate. A linear plot (shown above) was used to estimate protein concentrations in samples from absorbance values using AssayZap software.

2.4.10.4 *Immunoprecipitation and Western blot analysis*

Immunoprecipitation For focal adhesion kinase (FAK) studies in which the protein is of low abundance in cells, immunoprecipitation was used to enrich phosphotyrosine-containing proteins in lysates. Lysates were first pooled from treatment groups to provide sufficient protein for analysis. Equal amounts of total protein (4 mg) in lysis buffer were incubated (overnight at 4°C) with (1:100 in Odyssey blocking buffer) specific mouse monoclonal IgG_{2b} anti-phosphotyrosine antibody, p-Tyr (PY99) (Santa Cruz Biotechnology, California, USA) immobilised onto agarose beads, with gentle rotation. Beads were washed extensively (x3) with lysis buffer and centrifugation (at 15,000 rpm for 10 minutes at 4°C) and immune complexes were eluted in 20 µl of 5x protein gel loading buffer, boiled for 5 minutes, then kept on ice until required for gel electrophoresis.

Gel electrophoresis A total of 30 µg of protein (for MAPK studies) plus 5x protein gel loading buffer were resuspended in 20 µl distilled water, boiled (for 5 minutes), then kept on ice. Proteins were resolved on NuPAGE 4-12% Bis-Tris gels (1.5 mm x 15 wells; Invitrogen). Combs and tape were removed from the gels and wells rinsed with distilled water. The gel apparatus was assembled and 1x NuPAGE MES-SDS running buffer added to the central reservoir to cover the top of the gel and outer reservoir to cover the bottom of the gel. 10 µl of SeeBlue Pre-stained Protein Standard (Invitrogen) containing 9 polypeptides ranging from 4 to 250 kiloDaltons (kDa) were loaded into the first well on each gel to allow determination of sample protein molecular weight. Samples were carefully loaded into wells using a Hamilton syringe with a 22s gauge needle (Hamilton, Nevada, USA). Gels were electrophoresed at 40 mA per gel for approximately 2 hours until the loading dye front had migrated to the bottom of the gel.

Transfer In order to make proteins accessible to antibody detection, they were moved from within the gel to an Immobilon-FL polyvinylidene difluoride (PVDF) membrane (Millipore, Watford, UK) by electroblotting. Briefly, gels were removed from the cassette and the top and bottom of the gels trimmed to a final size of

8 cm x 6 cm. Membranes were soaked in methanol (30 seconds), distilled water (30 seconds), and then soaked in transfer buffer (5 minutes) to equilibrate. Stacks were assembled comprising; 3 layers of Whatman 3MM (0.3 mm) chromatography blotting paper (Fisher Scientific) pre-soaked in transfer buffer, wetted PVDF membrane, gel (containing protein), and finally, another 3 layers of pre-soaked blotting paper. Care was taken to avoid air bubbles between layers during assembly. Stacks were blotted at a constant voltage (14 V for 2 hours) using a 20 cm x 20 cm semi-dry blotting unit (Scie-Plas, Warwickshire, UK). As a result of the "blotting" process, proteins are exposed on a thin surface layer for subsequent detection.

Blocking and detection A two-step detection method was used to detect target proteins. First, to prevent interactions between the membranes and the antibody used for detection, the PVDF membrane was blocked (for 1 hour at room temperature on rocker) in Odyssey blocking buffer (Li-Cor Biosciences, Cambridge, UK) diluted 1:2 in PBS. Membranes were incubated with primary antibody (all 1:1000 in Odyssey blocking buffer containing 0.01% sodium azide) overnight on rocker. For FAK studies, rabbit polyclonal IgG anti-FAK (C-20) antibody (Santa Cruz Biotechnology) was used. For MAPK studies, membranes were co-incubated with rabbit polyclonal IgG anti-phospho-p44/p42 MAP kinase (to detect phosphorylated ERK1 and ERK2) and mouse polyclonal IgG anti-p42 MAP kinase (3A7) (to detect total ERK2 protein) (both Cell Signaling Technologies, Massachusetts, USA). Membranes were washed (3 x 10 minutes at room temperature) in 1x TNS Tween buffer on rocker then incubated (for 1 hour at 4°C on rocker, protected from light) with secondary antibody (both 1:5000 in Odyssey blocking buffer containing 0.01% sodium azide). For FAK studies, goat anti-rabbit IgG (H+L) Alexa Fluor 680 (Molecular Probes-Invitrogen) was used. For MAPK studies, membranes were co-incubated with goat anti-rabbit IgG (H+L) Alexa Fluor 680 and goat anti-mouse IgG (H+L) IR dye 800 (Rockland Immunochemicals, Pennsylvania, USA). Membranes were washed (3 x 10 minutes at room temperature) in 1x TNS Tween buffer on rocker then proteins were revealed and quantified by Odyssey Li-Cor Infrared Imager. Target protein bands, phosphorylated ERK1/2 and FAK, were determined from the relative mobility on SDS-PAGE by comparison with molecular weight standards and were normalised as

a ratio to total ERK2 protein and IgG, respectively, using Odyssey 2.1 software (Li-Cor Biosciences).

2.4.11 Assessment of cell proliferation

Cell proliferation in HUVECs induced by VEGF and bFGF, and the effect of a range of concentrations of cortisol (3 nM to 1 μM) on the action of these growth factors, was assessed by 2 independent methods; by measurement of cell viability and by direct measurement of newly synthesised DNA.

2.4.11.1 Cell viability assay

The CellTiter-Glo Luminescent Cell Viability Assay (Promega) is an indirect method of assessing the number of live cells based on quantitation of ATP present which signals metabolically-active cells. Whether the cells are actively dividing or quiescent is not distinguished but an increase in cell viability indicates cell growth. The assay is designed for use with multi-well formats making it popular as a high-throughput screening (HTS) tool to assess effects of test compounds on cell proliferation and cytotoxicity. The protocol requires addition of a single reagent (CellTiter-Glo Reagent) directly to cells in culture medium. Cell washing, removal of medium or multiple pipetting steps are not required and, therefore, the assay is highly sensitive. CellTiter-Glo Reagent contains recombinant firefly luciferase which catalyses the mono-oxygenation of beetle luciferin to oxyluciferin in the presence of Mg^{2+}, ATP and oxygen in a reaction which emits light. There is a direct relationship between the luminescent signal and the number of cells. Training in methodology, validated culture conditions, and growth factor and reference compound concentrations were provided by Dr Judith McKay and Mrs Julie Kellett, Department of Pharmacology, Aptuit, Edinburgh, UK.

HUVECs were seeded in inner wells (to minimise edge effects) of an opaque 96-well plate (Corning Fisher, Leicestershire, UK) at a density of 1.5×10^3 cells/well and incubated (37°C; 95% O_2:5% CO_2) for 2 hours to allow cells to adhere to the plates. This density was found to be sufficient to produce approximately 70% confluence in

the individual wells after 96 hours of incubation with growth factor stimuli, but was insufficient to produce 100% confluence, thereby avoiding cell-cell contact inhibition of cell proliferation. Cells were incubated in 'experimental' medium consisting of EGM-2 Bullet Kit medium (Lonza) supplemented with heparin, ascorbic acid, GA-1000, and 2% charcoal-stripped FBS. No growth factors from the kit were added. Serum-supplemented medium is necessary for the reaction but charcoal stripping removes the majority of hormones (Leake *et al.*, 1987), thereby preventing influence of endogenous steroids. After the initial 2 hour period, cells were incubated for a further 94 hours in the presence of: VEGF (25 ng/ml) or bFGF (1 ng/ml), VEGF or bFGF plus established inhibitors (SU5416, 1 nM-1 μM; platelet factor 4, 3 nM-1 μM, respectively), or VEGF or bFGF plus cortisol (3 nM-1 μM). The final volume in each well was 100 μl. Drug dilutions were prepared fresh on each day of experimentation. Additionally, cells were incubated in the maximum concentration of drug vehicles (ethanol, 0.004% v/v; DMSO, 0.167% v/v) or medium alone as controls. Consequently it was possible to test the effect of cortisol on VEGF- and bFGF-induced proliferation in two steps. Firstly, comparing the effect of growth factor alone with vehicle control, after establishing whether vehicle had an effect by comparison with medium alone. Secondly, checking for inhibition of growth by the steroid or reference substance by comparing proliferation in those wells with the "growth factor alone" wells. At the end of the 96 hours, incubation plates were equilibrated to room temperature (approximately 30 minutes) and 100 μl of CellTiter-Glo Reagent were added to each well. Plates were incubated for a further 10 minutes at room temperature to stabilise the luminescent signal before being read on a Wallac 1420 VICTOR^2 (Perkin-Elmer, Buckinghamshire, UK) plate reader with a counting time of 1 second/well. Each condition was performed in triplicate.

2.4.11.2 Bromodeoxyuridine incorporation assay

The 5-bromo-2'-deoxyuridine (BrdU) Cell Proliferation Assay (Calbiochem-Merck) allows detection and quantification of BrdU, following partial denaturation of the DNA double strand, by use of a detector monoclonal anti-BrdU antibody to bind to

any BrdU incorporated into dividing cells during the S phase of the cell cycle. Horseradish peroxidase-conjugated goat anti-mouse IgG antibody is then used to bind to the detector antibody and catalyse the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) to form a coloured product that absorbs light at dual wavelengths of 405 nm and 540 nm. The intensity of the coloured product is directly proportional to the amount of incorporated BrdU present in the cells which, in turn, should be directly proportional to the rate of cell division occurring in the sample.

HUVECs were seeded in inner wells of a 96-well, flat-bottomed microtiter plate (Cellstar-Greiner Bio-One) at a density of 3.5 x 10^3 cells/well and incubated (37°C; 95% O_2:5% CO_2) for 2 hours to allow cells to adhere to the plates. Cells were cultured in 'experimental' medium as described above. This density was found to be sufficient to produce approximately 70% confluence in the individual wells after 48 hours of incubation with growth factor stimuli and, again, was insufficient to produce 100% confluence. After an initial 2 hour stabilisation period, cells were incubated for a further 46 hours with the same growth factor and inhibitor conditions previously described (see CellTiter-Glo assay) with the exception that BrdU was added to the wells 1 hour after growth factors and inhibitors were added. The final volume in each well was 125 µl. Drug dilutions were prepared fresh on each day of experimentation. Additionally, cells were incubated in the maximum concentration of drug vehicles (final concentration, 0.004% v/v ethanol or 0.167% v/v DMSO) or medium alone as controls. Additional wells with cells but no BrdU were included to obtain background absorbance measurements. At the end of the 48 hour incubation period, cells were fixed (30 minutes at room temperature) and denatured according to the assay kit instructions prior to incubation (1 hour at room temperature) with the anti-BrdU antibody, followed by incubation (30 minutes at room temperature) with horseradish peroxidase-conjugated goat anti-mouse IgG. Wells were washed (3x300 µl of 1x plate wash, prepared fresh on each occasion in distilled water) between incubations to minimise background with an Anthos Labtec aw1 microplate washer (ASYS Hitech, Eugendorf, Austria). Finally, the cells were incubated (15 minutes at room temperature in light-proof conditions) with TMB and the reaction

was stopped by the addition of sulphuric acid (2.5 M). The optical densities (dual wavelengths at 405 nm and 540 nm) were measured within 30 minutes of adding the stop solution by a Multiskan Ascent plate reader and data captured using Ascent v2.6 software (Thermo LabSystems, UK). The absorbance of cells without the BrdU label and that of the tissue culture medium, were measured and subtracted from each reading. It was possible to test the effect of cortisol on VEGF- and bFGF-induced proliferation by comparing proliferation in those wells with the "growth factor alone" wells after demonstrating growth factor alone had an effect by comparing with vehicle control. Each condition was performed in triplicate.

2.4.12 Assessment of cell migration

Developed in 1962 by Stephen Boyden (Boyden, 1962), the Filter assay is still employed in cell migration studies. This assay involves a two-compartment system where cells may be induced to migrate from an upper compartment through a porous polyethylene terephthalate (PET) membrane into a lower compartment, thus following the gradient of a chemoattractant. Commercially available cell culture inserts (ThinCerts; Greiner Bio-One) were used to test the effects of glucocorticoids on basal-unstimulated, and VEGF-induced, endothelial cell migration. Migratory cells can be rapidly and accurately measured by labelling with calcein-AM, a non-fluorescent cell-permeable derivative of calcein which becomes fluorescent on hydrolysis by cells. The amount of fluorescence and, hence, the number of migratory cells can be measured using a fluorescence plate reader since relative fluorescence units (RFU) correlate linearly with number of cells. An overview of the individual steps of the migration assay is given in Figure 2.3.

Boyden chambers were prepared by immersing 8.0 μm ThinCerts into a 24-well, flat-bottomed microtiter plate (Cellstar-Greiner Bio-One) containing either: 600 μl of standard basal medium (EGM-2 containing only ascorbic acid, heparin and GA-1000) as untreated control; medium with VEGF (10 ng/ml); medium with VEGF plus inhibitor SU5416 (1 μM; positive control); or medium with VEGF plus cortisol (600 nM). HUVECs were harvested as described previously (Section 2.4.2) and

seeded into the upper compartment at a density of 2×10^5 cells/insert in 200 µl of standard basal medium. SU5416 (1 µM) or cortisol (600 nM) were added immediately to the upper compartment to maintain these final concentrations in both compartments whereas VEGF was only present in the lower chamber to produce a gradient of chemoattractant.

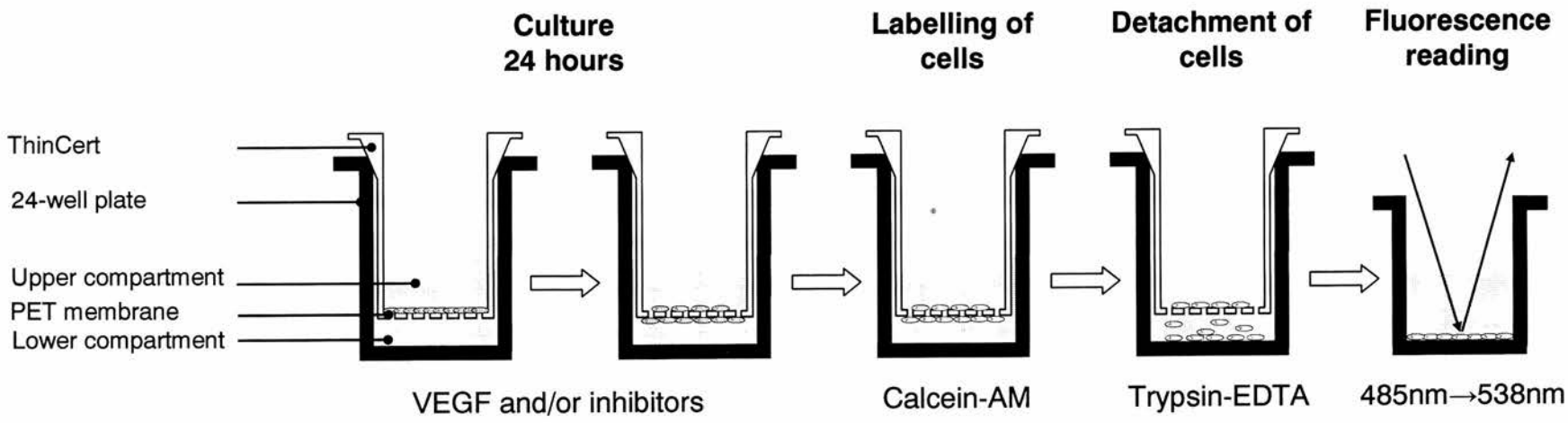


Figure 2.3 Schematic representation of a quantitative cell migration assay

HUVECs seeded in the upper compartment may be induced to migrate actively through the PET membrane towards 10ng/ml VEGF (a chemoattractant) in the lower compartment in the presence or absence of recognised inhibitors (1 μM SU5416, VEGF receptor antagonist) or 600 nM cortisol. Adherent cells remain attached to the underside of the PET membrane. After fluorescently labelling all cells with calcein-AM, the migratory cells are detached from the underside of the membrane by action of trypsin-EDTA. Finally, the migratory cells are quantified in a standard fluorescence microplate reader. The individual steps are illustrated in the diagram. Adapted from Greiner Bio-One protocol, A Quantitative Cell Migration Assay Using ThinCert Cell Culture Inserts (www.gbo.com/bioscience).

Plates were incubated for 24 hours to allow cells to migrate towards VEGF. Early methodological development determined ThinCert pore size and duration of incubation to allow sufficient migration of cells. Final concentration of VEGF chosen was the EC_{50} value determined in pilot experiments. Following incubation with chemoattractant, cell culture medium in the lower compartment was carefully removed by gentle aspiration and replaced with 450 μl of standard basal medium with calcein-AM dye (8 μM) and incubated for a further 45 minutes. Contents of the upper chamber were then removed by gentle aspiration. ThinCerts were then transferred into a freshly prepared 24-well cell culture plate containing 500 μl of pre-warmed Trypsin-EDTA (Reagent Pack subculture Reagent Kit, Lonza) and incubated for 10 minutes with periodic agitation to aid detachment of cells from the underside. ThinCerts were discarded and 200 μl of Trypsin-EDTA solution (now containing the labelled migratory cells) were transferred into a black flat-bottomed 96-well plate (Nalge-Nunc) to minimise background fluorescence between wells. Fluorescence measurements (excitation wavelength, 485 nm; emission wavelength, 538 nm) were made by a Fluoroskan Ascent FL plate reader and data captured using Ascent software (Thermo LabSystems, UK). Consequently, it was possible to test the effect of cortisol on unstimulated migration (by comparing with basal control wells) and VEGF-induced migration (by comparing VEGF plus cortisol with VEGF alone wells after demonstrating VEGF had an effect). Each condition was performed in triplicate.

2.5 Statistics

All data are expressed as mean ± SEM, where '*n*' indicates the number of different assays performed. Treatment conditions in quantitative TLS, proliferation and migration assays were performed in triplicate wells and TLS quantification was performed by an individual blind to treatment groups. Statistical analysis was performed by one-way analysis of variance (ANOVA) as indicated. Dunnett's multiple comparison post hoc tests were used where appropriate. Repeated measures ANOVA was used for the time-lapse imaging and two-way ANOVA for the real-time PCR data-sets. For histological analysis, vessel counting was performed by an individual blind to treatment groups and statistical analysis was performed by unpaired t-test. Differences were considered significant when $p<0.05$.

Chapter 3

Development of an *in vitro* Model of Tube Formation in Human Endothelial Cells to Assess the Anti-Angiogenic Actions of Glucocorticoids

3.1 Introduction

The recognition that glucocorticoids are potent inhibitors of angiogenesis has led to this property being exploited clinically; for example in the treatment of capillary haemangioma (Hasan *et al.*, 2000). Despite extensive research, however, the cellular and molecular mechanisms under-pinning this inhibition of angiogenesis are not well understood. Since it is has been shown that glucocorticoids can alter vascular tone by acting directly on endothelial cells (Ullian, 1999), it is possible that direct interaction with the endothelium is also central to the angiostatic effects of these steroids. Indeed, glucocorticoids can inhibit production of nitric oxide (NO) (Johns *et al.*, 2001), proteolytic activity (Pepper *et al.*, 1994), migration (Wang *et al.*, 2002b) and proliferation (Cariou *et al.*, 1988) in endothelial cells, all of which are key components of angiogenesis.

In vitro models of tube formation by endothelial cells provide one of the most appropriate methods for investigating the action of factors that influence angiogenesis since they: are rapid to perform, are easy to quantify (Auerbach *et al.*, 2003), involve controlled cell populations and do not have confounding factors characteristic of *in vivo* models (such as inflammatory and stromal cells). Advances in isolation techniques have allowed investigators to isolate pure cell populations from human sources (Jaffe *et al.*, 1973) making results more directly applicable to a human setting than those obtained using rodent models. Furthermore, molecular signalling pathways can be dissected using single cell populations. Against this background, we elected to study angiogenesis *in vitro* in the 2-dimensional (2D) model of endothelial tube formation. This produces endothelial tubes with an identifiable lumen and is thought to recapitulate the morphogenic component of tube formation (Benelli & Albini, 1999). Assays that model this process *in vitro* have become increasingly popular in recent years given that the formation of tubules is considered similar to the *in vivo* situation (Lawley & Kubota, 1989). A limited number of studies have investigated the effects of glucocorticoids on endothelial tube formation *in vitro* but these have tended to use pharmacological concentrations of synthetic glucocorticoids (Matsuda *et al.*, 2005) or endothelial cells from non-human species (Wang *et al.*, 2002b). No previous study has addressed the influence of

physiological levels of endogenous glucocorticoids on human endothelial cell morphogenesis.

One potential complicating factor in assessing the impact of glucocorticoids on endothelial cells *in vitro* is the possibility that added glucocorticoids may be inter-converted by the isozymes of 11β-HSD. Indeed, both 11β-HSD1 and 11β-HSD2 are present in the vessel wall (Hadoke *et al.*, 2001; Christy *et al.*, 2003; Brem *et al.*, 1998) and the former has been shown to regulate angiogenesis in tissue explants *ex vivo*, in sponges *in vivo*, and in pathological models (Small *et al.*, 2005). Whether isozymes of 11β-HSD are expressed in the endothelium, however, is still controversial and, furthermore, it is not clear whether repeated passage of endothelial cells (or tube formation itself) will induce expression of these isozymes.

3.1.1 Hypothesis

This study explored the hypothesis that glucocorticoids inhibit 2D tube-like structure (TLS) formation by human endothelial cells cultured on Matrigel.

3.1.2 Aims

The specific aims were:

1) to establish and characterise an *in vitro* model of human endothelial TLS formation.
2) to determine whether 11β-HSD isozyme activity modulates glucocorticoid activity in this system.
3) to investigate whether physiological glucocorticoids inhibit tube formation by direct action on the endothelium.

3.2 Methods

3.2.1 Development and characterisation of an endothelial tube formation assay

Although the 2D model of endothelial tube formation is widely used for investigating angiogenesis the methodology varies greatly between different groups (Table 3.1). Using modifications of protocols from other authors (Cattaneo *et al.*, 2003; Kuzuya & Kinsella, 1994), several different incubation conditions, time courses and methods of quantification were tested before a satisfactory experimental protocol was established. These are outlined in the following sections.

3.2.1.1 Cells

Primary human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAoECs) were obtained from Promocell (Germany) and routinely passaged as previously described (Section 2.4.2). Cells were used between p2 and p6 in all experiments. Attempts to cryopreserve HUVECs to extend the lifespan of a single batch proved unsuccessful; therefore, cells were discarded after reaching p6. Where possible, cells were used at the same level of confluency (70-80%) to ensure reproducibility of TLS formation. Cells were detached from the flasks with trypsin and, in all assays, cell counting was performed to ensure consistency of cell density between experiments (Section 2.4.4).

3.2.1.2 Culture medium

TLSs were incubated in endothelial cell growth medium-2 (EGM-2, Lonza, UK) a specially formulated primary cell culture system developed for endothelial cell growth in a low serum environment. Standard culture medium was prepared according to the supplier's instructions; by adding the supplied growth factors and serum to the basal medium. Although exact concentrations of growth factors supplied with the Bullet kit medium were not revealed by the company for commercial reasons, details regarding the final concentration range of most of the contents have been obtained (Table 3.2).

Preliminary experiments established the optimum medium for TLS formation and defined the standard basal medium conditions to be used in subsequent experiments. This comprised: EGM-2 basal medium supplemented with ascorbic acid, heparin and GA-1000 supplied in the Bullet kit. No growth factors, serum or hydrocortisone (cortisol) were added (Table 3.2). Under these conditions sufficient TLS formation occurred to allow accurate quantification and investigation of the angiostatic effects of glucocorticoids.

Study	Cell type	Passage number	Supporting matrix	Well format	Cell density (cells/well)	Timepoint	Quantification method
(Beckner & Liotta, 1996)	HUVECs	4-6	Matrigel	96	15-50,000	Overnight	Number of tubes intersecting gridlines
(Bompais *et al.*, 2004)	EPCs & HUVECs	N/D	Matrigel	48	40,000	6 hours	Network density
(Bussolati *et al.*, 2001)	PAECs & HUVECs	2-3	GFR-Matrigel	24	50,000	2 hours & 13 days	CD31 staining, TLS length, thickness and number of connections
(Cattaneo *et al.*, 2003)	HUVECs	2-6	Matrigel	24	50,000	12-18 hours	Area of TLS network
(Donovan *et al.*, 2001)	HUVECs & HuDMECs	N/D	GFR-Matrigel, Matrigel	24	30,000	6 hours	H&E stain, TLS length, number of connections and area of network
(Kubota *et al.*, 1988)	HUVECs & HuDMECs	2-4	Fibronectin, laminin, collagen I+IV & Matrigel	24	N/D	N/D	Qualitative assessment
(Kuzuya & Kinsella, 1994)	HUVECs, BAECs & BBCECs	3-6	Matrigel	24	35-45,000	12 hours	Network length
(Montanez *et al.*, 2002)	HUVECs	2-9	Matrigel	24	85,000	24 hours	TLS number, length and width

Table 3.1 2-Dimensional endothelial tube formation assays in the literature

Variations of the 2-dimensional (2D) endothelial tube formation assay from selected original articles. Some details were not disclosed (N/D). Continued on next page.

Study	Cell type	Passage number	Supporting matrix	Well format	Cell density (cells/well)	Timepoint	Quantification method
(Morales *et al.*, 1995)	HUVECs	4-8	Matrigel	24	40,000	Overnight	Diff-Quick stain, area of TLS network
(Rafiee *et al.*, 2004)	HIMECs	8-12	Matrigel	24	50,000	16 hours	Qualitative assessment
(Sanz *et al.*, 2002)	HUVECs & HMEC-1s	N/D	Matrigel	384 & 1536	2-6,000	12-14 hours	Automated, Angiogenic Index (AI) = TLS length/surface area X 1000
(Tan *et al.*, 2004)	HUVECs	3-7	Collagen	96	15,000	24 hours	TLS number
(Wang *et al.*, 2002b)	BCECs	3-7	Vitrogen	24	100,000	5 days	Total TLS length
(Wiedermann *et al.*, 1996)	HUVECs	4-8	Matrigel	24	40,000	6 hours	Semi-quantitative, degree of TLS formation graded
(Wilasrusmee *et al.*, 2003)	HAoECs	3-8	Matrigel	96	1-4,000	24 & 48 hours	Number of connections
(Yamagishi *et al.*, 1999)	HuDMECs	5-10	Matrigel	24	40,000	6 hours	TLS length

Table 3.1 2-Dimensional endothelial tube formation assays in the literature (Continued)

Variations of the 2-dimensional (2D) endothelial tube formation assay from selected original articles. Bovine aortic endothelial cells (BAECs), bovine brain capillary endothelial cells (BBCECs), bovine coronary endothelial cells (BCECs), endothelial progenitor cells (EPCs), human aortic endothelial cells (HAoECs), human dermal microvascular endothelial cells (HuDMECs), human intestinal mucosal microvascular endothelial cells (HIMECs), immortalised human dermal microvascular endothelial cells (HMEC-1s),human umbilical vein endothelial cells (HUVECs), porcine aortic endothelial cells (PAECs). Some details were not disclosed (N/D).

EGM-2 Bullet kit contents	EGM-2 final concentration	Standard culture medium (for routine maintenance)	Standard basal medium (for TLS assay)
Ascorbic acid	<200 ng/ml	√	√
Heparin	1-5 μg/ml	√	√
GA-1000	30 μg/ml gentamicin, 15 μg/ml amphotericin	√	√
FBS	2%	√	X
hFGF-B	5-20 ng/ml	√	X
VEGF	<100 ng/ml	√	X
R^3-IGF	5-50 ng/ml	√	X
hEGF	<30 ng/ml	√	X
Hydrocortisone	<50 mg/ml	X	X

Table 3.2 Contents of endothelial cell growth medium

Lonza (UK) revealed concentration ranges of Bullet kit components under commercial constraints. Gentamicin and amphotericin B (GA-1000), foetal bovine serum (FBS), human fibroblast growth factor-B (hFGF-B), vascular endothelial growth factor (VEGF), human recombinant insulin like growth factor-1 (R^3-IGF-1), human epidermal growth factor (hEGF). (√) indicates addition of factor to medium, whereas (X) indicates factor was not added.

3.2.1.3 Supporting matrix

A commercially available (BD Biosciences, UK) solubilised basement membrane preparation (Matrigel) was used to support growth of endothelial cells into TLSs. Matrigel is a gelatinous mixture of extracellular and basement membrane proteins derived from the mouse Engelbreth-Holm-Swarm sarcoma.

A comparison was made of TLS formation on a solidified layer of Matrigel (2D) versus cells dispersed throughout Matrigel and allowed to solidify (3-dimensional, 3D). As a result of this pilot study the 2D assay was used in subsequent investigations. Based on previous reports (Table 3.1), tissue culture plates (24-well format) were coated with 250 μl Matrigel per well which formed an even, solid layer to act as substrate for TLS formation.

3.2.1.4 Quantification of tube-like structure formation

Quantification of TLS formation was performed by inverted light microscopy (Carl Zeiss Axiovert 25) and image analysis. One image at low magnification (x5) at the centre of each well was captured and saved for analysis. Images were captured from a live-feed camera (Pentax 250, Pentax UK Ltd., UK) using MCID software. Image files were randomised to ensure quantification was performed by an individual blind to treatment groups to ensure integrity of the results. Three established methods previously described in the literature were assessed to determine their accuracy and consistency:

1) Capillary connections, defined as the number of branch points between 2 or more TLSs, were identified and marked using Adobe Photoshop cs (Adobe System Inc, USA) software. Total numbers of capillary connections per field of view were counted and tallied using a cell counter (Figure 3.1A). During method development, data were initially expressed as the mean absolute number of connections (Donovan *et al.*, 2001; Bussolati *et al.*, 2001). For the final, established method, data were expressed as a percentage of the number of connections generated in concomitant control

wells (Wilasrusmee *et al.*, 2003). Consequently, control wells were run on each plate.

2) Measurements of TLS length (Montanez *et al.*, 2002; Yamagishi *et al.*, 1999) were made digitally (in pixels) using Adobe Photoshop cs software. Average length of TLSs intersecting gridlines in each field of view was calculated (Figure 3.1B).

3) TLS density measurements (Grant *et al.*, 1989) were derived by computer analysis of pixel density using MCID software. Measurement of area occupied by TLS networks was obtained using greyscale pixel threshold (total grain count function) in each image.

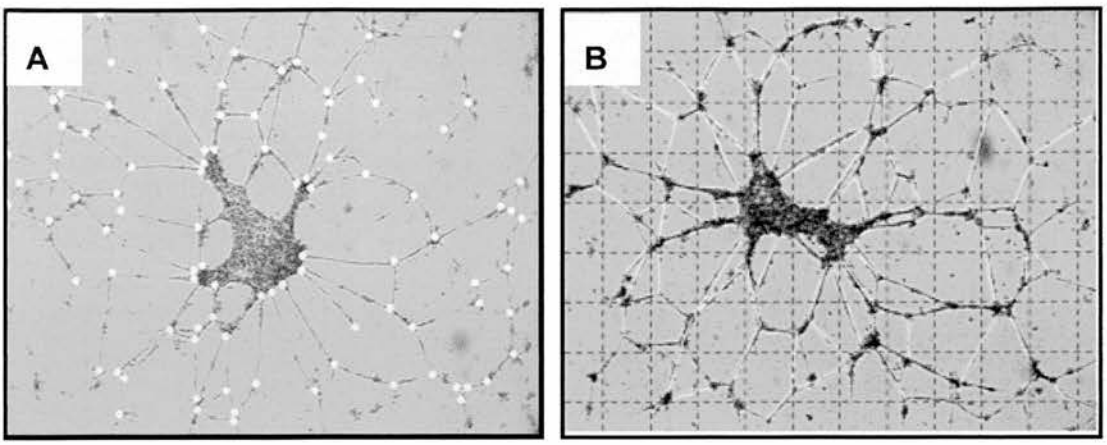


Figure 3.1 Methods of quantification of TLS formation

Light micrographs of TLSs demonstrating 2 different published methods of quantification. In these images individual capillary connections (branch points) are marked with a white dot (a) for counting and lengths of TLSs intersecting gridlines are measured (in pixels) and identified with a white line, (b).

3.2.1.5 Time-course of TLS formation

To determine the time-course of TLS formation, time-lapse video microscopy was used. Technical assistance with time-lapse equipment was provided by Mr Bob Morris and advice on movie-clip preparation was provided by Professor Adriano Rossi, both Centre for Inflammation Research, University of Edinburgh.

SlideFlasks (Nunc, USA) were coated with 750 µl Matrigel and allowed to set for 60 minutes. HUVECs (200,000) in 2 ml standard basal medium containing 5 mM Hepes buffered saline solution (Lonza, UK) were seeded onto Matrigel. Preliminary experiments determined that these culture conditions were necessary to buffer against pH changes in the flasks. Cells were incubated with 10 ng/ml VEGF, 600 nM cortisol, or both. Immediately (within 10 minutes) after cell seeding onto Matrigel, images were acquired with a Leica DM IRBE microscope using a x10 objective and captured with a Leica Q500MC image processing system (Leica Cambridge Ltd., UK). Images were captured from 3 separate positions per flask at 4 minute intervals for 24 hours, where possible. Movie-clips were reconstructed using Apple QuickTime Player version 7.0 (Apple Inc., USA). Numbers of tubes per field of view were counted at 2 hour interval in reconstructed movie-clips, and plotted versus time in line graphs. From time-course graphs, time to reach peak TLS number and maximum TLS number were determined and plotted as bar charts.

Based on this information, TLS formation was assessed at 4 hours, 8 hours and 22 hours after seeding, to test the influence of added factors. This is consistent with time-points selected by others described in the literature (Bussolati *et al.*, 2001; Donovan *et al.*, 2001; Lansink *et al.*, 1998).

3.2.1.6 Characterisation of tube-like structures

The endothelial nature of TLSs was assessed using immunohistochemistry (detailed in 2.4.6.1). Briefly, HUVECs (10,000) were cultured in 250 µl of standard basal medium for 5 hours on 8-well permanox chamber slides (Nunc, USA) pre-coated with 100 µl Matrigel. Culture medium was gently removed and TLSs rinsed once

with PBS and fixed with 10% formalin. Staining was performed using the Vectastain ABC Kit (Vector Lab., USA) according to the manufacturer's instructions in combination with rat anti-mouse CD31 (1 in 50 dilution) monoclonal antibody (BD Pharmingen, USA). Slides were incubated at room temperature for 5 minutes with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, USA) as substrate with positive structures staining brown. For negative control, the primary antibody was omitted. Slides were viewed by light microscopy (Carl Zeiss Axioskop, Carl Zeiss MicroImaging, Inc., USA) and images captured from a live-feed camera (3-CCD, JVC Professional Europe Ltd, UK) using the Microcomputer Imaging Device (MCID; InterFocus Imaging Ltd, UK).

The structural composition of TLSs was assessed by immunofluorescent staining of F-actin and α-tubulin; important components of the cytoskeleton. Antibodies, expertise in immunofluorescence staining protocols, and fluorescence microscopy were provided by Dr Kate Marshall, Centre for Cardiovascular Science, University of Edinburgh.

Briefly, HUVECs were resuspended (4×10^4 cells/ml) in experimental medium and 1 ml per well was seeded onto sterilised round coverslips (13 mm; 0 thickness) pre-coated with 30 µl of phenol red-free Matrigel (to overcome the problem of signal interference during immunofluorescence detection caused by standard Matrigel; Dr Kate Marshall, personal communication) in a 24-well, flat-bottomed microtiter plate (Cellstar-Greiner Bio-One, UK). Experimental medium consisted of EGM-2 medium (Lonza, UK) supplemented with heparin, ascorbic acid, GA-1000, and 2% charcoal-stripped FBS; no growth factors from the Bullet kit were added. Preliminary experiments determined that standard basal medium was not sufficient to instigate formation of TLS networks in this set-up. Cortisol (600 nM) was added immediately at the time of cell seeding. In time-course experiments, media were removed after 1, 4, 10 and 22 hours of incubation and cultures fixed and stained as described in (Section 2.4.6.2). Slides were viewed by an Olympus AX-70 fluorescence microscope (Olympus Optical Company, UK) and images captured

with a Hamamatsu Orca-ER digital live-feed camera and controller (Hamamatsu Photonics, UK) using Smart Capture 3 software (Digital Scientific, UK).

3.2.2 11β-Hydroxysteroid dehydrogenases in endothelial cells and tube-like structures

To further characterise the model it was important to determine 11β-HSD activity. Since the subject of this thesis was to test the influence of glucocorticoids on endothelial cells, it was important to determine whether glucocorticoids are converted endogenously in the model between active and inactive forms by the action of 11β-HSD isozymes. Furthermore, the differentiation state of some cells may determine the expression pattern of 11β-HSDs; for example human monocyte differentiation into macrophages causes an up-regulation of 11β-HSD1 (Thieringer *et al.*, 2001). Consequently, the presence of 11β-HSDs in endothelial cells from 3 different stages of development; endothelial precursor cells, undifferentiated HUVECs and mature endothelial TLSs was investigated.

3.2.2.1 11β-HSD1 and 11β-HSD2 mRNA expression

Expression of 11β-HSD1 and 11β-HSD2 mRNA was investigated in undifferentiated HUVECs and mature TLSs by RT-PCR (Section 2.4.7). Mouse liver and human kidney were used as positive controls for 11β-HSD1 and 11β-HSD2 expression, respectively. Tie2 is a common endothelial cell surface marker expressed almost exclusively by endothelial cells (Dumont *et al.*, 1992) and was included as a positive control for the PCR reaction. To extend the expression profiling to an immature endothelial precursor, 11β-HSD1 and 11β-HSD2 expression was investigated by RT-PCR in endothelial progenitor cells (EPCs) isolated from healthy human volunteers (RNA was a gift from Dr John McDermott, National University of Ireland, Galway, Republic of Ireland).

3.2.2.2 11β-HSD1 and 11β-HSD2 activity

To determine 11β-HSD activity in undifferentiated HUVECs and mature TLSs a previously described method (Cai *et al.*, 2001) was adapted. HUVECs (1.75×10^5 cells per well) in 2 ml of standard basal medium were seeded in 6 well-plates directly on plastic and in dishes pre-coated with Matrigel. 11β-HSD activity was established by the conversion of ^{3}H-steroid. 11β-reductase activity was determined by incubating cells in the presence of 10 pmol/ml $[^3H]_4$-cortisone. Chinese hamster ovary (CHO) cells stably transfected with human 11β-HSD1 served as a positive control. 11β-dehydrogenase activity was determined by incubating cells in the presence of 10 pmol/ml $[^3H]_4$-cortisol. CHO cells stably transfected with human 11β-HSD2 served as a positive control. Media alone, and Matrigel with medium added but no cells, served as negative controls.

After 5 hours, 24 hours and 48 hours incubation, samples of media were taken and stored at -20°C until analysis. Time-points to assess conversion for CHO cells were in the range of 24 to 48 hours as recommended by Dr Scott Webster, Endocrinology Unit (personal communication). An earlier time-point (5 hours) was also included in view of the striking morphological changes observed in HUVECs on Matrigel at this time. Steroids were purified from media samples by Sep Pak extraction and analysed by HPLC (Section 2.4.9).

3.2.3 Influence of glucocorticoids on endothelial tube formation

Prior to experiments exploring the influence of glucocorticoids on endothelial tube formation, the model was used to examine the effects of recognised pro-angiogenic stimuli as positive controls to demonstrate responsiveness of the model under these conditions, and to further validate the quantification method.

3.2.3.1 VEGF and prostanoid influence on endothelial tube formation

Studies were performed to examine the influence of the pro-angiogenic factors VEGF (0.5, 5, 50, 500 ng/ml) (Ferrara & Henzel, 1989) and 2 members of the prostanoid family, $PGF_{2\alpha}$ and PGE_2. (10 nM, 100 nM, 500 nM, 1 μM) (Majima *et*

al., 2000) in standard basal medium conditions. Conditions were performed in triplicate wells.

3.2.3.2 Influence of glucocorticoids on endothelial tube formation

The angiostatic effect of glucocorticoids was examined by incubating HUVECs on Matrigel under standard basal medium conditions in the presence or absence of cortisol (300 nM, 600 nM or 1200 nM) or cortisone (300 nM, 600 nM or 1200 nM). As steroids had been dissolved in ethanol and then diluted in aqueous solutions, vehicle control conditions were established that contained equivalent aqueous dilutions of ethanol (final concentration, 0.004% v/v). Vehicle or steroid was added to the culture medium at time of cell seeding onto Matrigel. Conditions were performed in triplicate wells.

3.2.3.3 Influence of glucocorticoids on endothelial tube formation by human aortic endothelial cells

The influence of glucocorticoids on tube formation by another type of endothelial cell was investigated using human aortic endothelial cells (HAoECs). These studies were performed by a Pharmacology Honours project student, Miss Sadaf Ali, under my supervision. Briefly, HAoECs were utilised in the TLS assay using the same conditions previously described (Section 2.4.5.1). The angiostatic effect of glucocorticoids was examined by incubating HAoECs on Matrigel under standard basal medium conditions (control) or in the presence of cortisol (600 nM). To determine the receptors responsible for glucocorticoid-induced angiostasis, TLSs were incubated with the glucocorticoid receptor (GR) selective, synthetic glucocorticoid, dexamethasone (600 nM). To examine the effects of glucocorticoids on VEGF-stimulated TLS formation, HUVECs and HAoECs were incubated with VEGF (10 ng/ml) alone, or with VEGF in combination with cortisol or dexamethasone (both 600 nM).

3.2.3.4 Identification of the receptor responsible for glucocorticoid-induced angiostasis

To further investigate whether glucocorticoid-induced angiostasis was mediated by glucocorticoid or mineralocorticoid receptors, HUVECs were cultured on Matrigel under standard basal conditions in the presence of cortisol (600 nM) and either the GR antagonist RU38486 (1 μM) or the mineralocorticoid receptor (MR) antagonist spironolactone (1 μM). In control wells, HUVECs were cultured with either antagonist in the absence of cortisol.

3.2.4 Statistics

Data are expressed as mean ± standard error. "*n*" refers to number of different experiments performed on separate occasions using different batches of HUVECs; where possible all measurements of tube formation were made in triplicate wells and the mean used for further analysis. Comparisons at selected timepoints were made by one-way ANOVA and Dunnett's post hoc test. Inter-assay and intra-assay coefficients of variation were 43% and 24% for number of connections after 4-5 hours in culture and 73% and 38% after 20-23 hours in culture (n=27). Time-lapse data were analysed by repeated measures ANOVA and least squares difference post hoc test (n=6).

3.3 Results

3.3.1 Development and characterisation of an endothelial tube formation assay

3.3.1.1 Cells

Initial observations indicated that HUVECs beyond p6 failed to form adequate networks of TLSs (data not shown) and therefore cells beyond p6 were not used in these studies. Untreated control cells between p2 and p6 showed similar, reproducible tube formation after 4-5 hours and 21-23 hours on Matrigel (Figure 3.2). Therefore, cells were used between these passages in all further studies.

3.3.1.2 Culture medium

Initial experiments performed to determine the optimal conditions for tube formation *in vitro* compared TLS formation by HUVECs cultured under basal conditions or in the presence of growth factors (from the EGM-2 Bullet kit) and/or serum (see Table 3.2). TLS formation occurred in basal conditions and was enhanced by addition of growth factors and serum (Figure 3.3). In the presence of serum and growth factors, however, addition of further pro-angiogenic growth factor (VEGF in the range 25-75 ng/ml, exact concentration not disclosed by supplier) had no apparent effect on TLS formation compared with controls. This suggests that HUVECs cultured under these conditions were already maximally stimulated. In addition, under these conditions, cortisol (300 nM) did not impair TLS formation (Figure 3.4). Further method refinement investigated the effects of glucocorticoids on TLS formation in the absence of growth factors (hFGF-B, VEGF, R^3-IGF-1, hEGF) or serum in EGM-2 containing only ascorbic acid, heparin and GA1000. These conditions produced sufficient, quantifiable TLS formation; ~150 connections were counted after 4-5 hours and ~35 connections after 21-23 hours in culture. In addition, in the presence of pro-angiogenic factors TLS formation was stimulated (Figure 3.5) and in the presence of cortisol (300 nM to 1200 nM) the number of connections was reduced (compared with controls after 5 hours and 22-24 hours incubation TLS formation was impaired) (Figure 3.15). Therefore, these conditions, "standard basal

medium" were adopted as standard incubating medium in all further TLS studies (see Table 3.2).

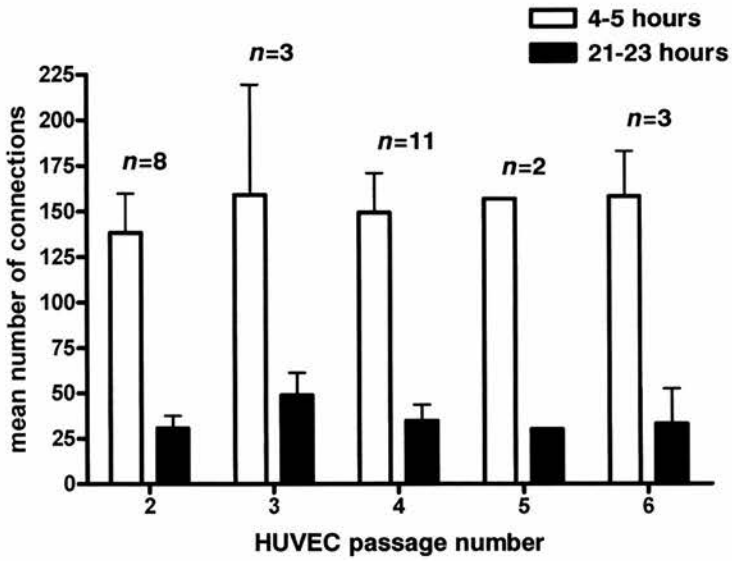


Figure 3.2 Influence of HUVEC passage number on TLS formation

HUVECs seeded on Matrigel in standard basal medium in the absence of growth factors or inhibitors. TLS formation was quantified at 2 time-points by counting the number of connections between TLSs. Time-point 1 was 4-5 hours incubation; time-point 2 was 21-23 hours incubation. Data represent mean ± SEM (number of experiments as noted on bar chart), no significant difference in TLS formation using HUVECs in this passage range was detected using one-way ANOVA.

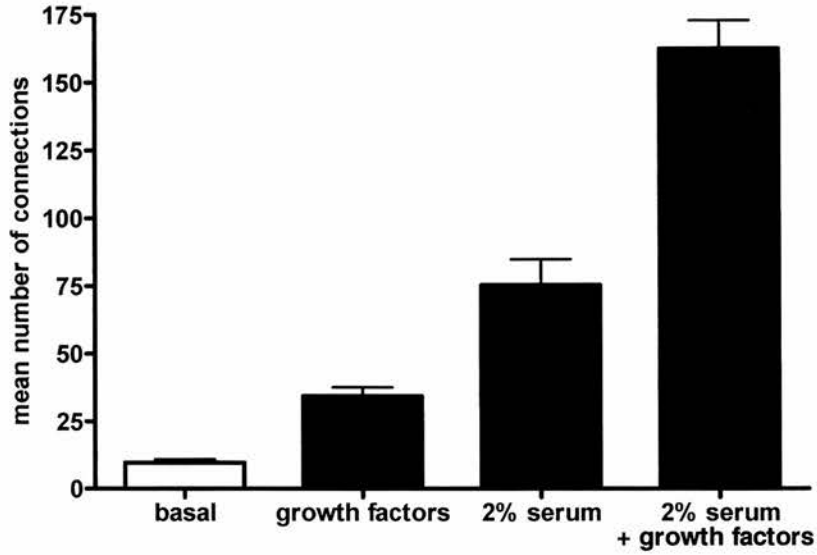


Figure 3.3 Influence of culture conditions on TLS formation

HUVECs seeded on Matrigel for 21 hours in either standard basal medium (containing no growth factors or serum), with growth factors; human fibroblast growth factor-B (hFGF-B), vascular endothelial growth factor (VEGF), human recombinant insulin like growth factor-1 (R^3-IGF-1), human epidermal growth factor (hEGF), with 2% foetal bovine serum (FBS) or with 2% FBS plus growth factors. Tube formation was quantified as the number of connections between tube-like structures (TLSs). Data represent mean ± SEM (n=1, each condition was performed in triplicate wells).

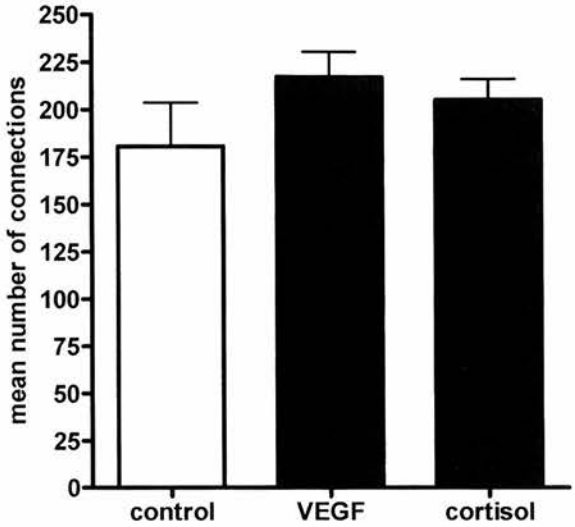


Figure 3.4 TLS formation in the presence of exogenous growth factors and serum: Influence of VEGF and cortisol

HUVECs seeded on Matrigel for 21 hours in endothelial cell growth medium-2 (EGM-2) supplemented with Bullet kit growth factors (human fibroblast growth factor-B (hFGF-B), vascular endothelial growth factor (VEGF), human recombinant insulin like growth factor-1 (R^3-IGF-1), human epidermal growth factor (hEGF)) and 2% foetal bovine serum (FBS) but excluding hydrocortisone (cortisol). Tube formation was quantified as the number of connections between tube-like structures (TLSs). A physiologically relevant concentration of cortisol (300 nM), and of the pro-angiogenic factor, vascular endothelial growth factor (VEGF; 25-75 ng/ml, exact concentration not disclosed by supplier) had no effect on TLS formation under these conditions. Data represent mean ± SEM (n=1 experiment, each condition was performed in triplicate wells).

3.3.1.3 Supporting matrix

Initial results obtained with standard Matrigel indicated that this was suitable for generation of a practical number of TLSs by HUVECs. Previous studies using this assay (Table 3.1) in 24-well plates have used 250-320 μl Matrigel, consistent with the 250 μl used in these investigations.

3.3.1.4 Quantification of tube-like structure formation

Methods for quantification were assessed for reliability, reproducibility and consistency. Computer generated measurement of TLS density proved to be unreliable since undifferentiated cells and cell debris were indistinguishable from TLS networks and, therefore, this method was discontinued at an early stage. Quantification of TLS formation by measuring length or by counting connections was more reliable and both methods detected enhanced angiogenesis in response to VEGF. However, whereas measurement of TLS length only detected a modest increase in response to VEGF (Figure 3.5B), counting TLS connections identified a clear, consistent, concentration-dependent augmentation (Figure 3.5A). In addition, TLS length was not altered in the presence of glucocorticoids (300 nM, 600 nM and 1200 nM cortisol; n=3; data not shown). Therefore, since counting connections identified changes in TLS formation, was reproducible and was technically less demanding and less time-consuming, it was adopted for measurement of angiogenesis in subsequent investigations.

3.3.1.5 Time-course of TLS formation

To understand the dynamic nature of the processes involved in TLS formation, cultures were monitored repeatedly over 24 hours using time-lapse imaging. There was little evidence from these movie-clips of cell migration or proliferation and TLS formation consisted mainly of cell morphogenesis (see enclosed movie-clip).

Two distinct phases of TLS formation were identified in untreated control flasks using this method (Figure 3.6A). Phase I consists of network assembly; TLS number increased most rapidly during the first 4 hours after cell seeding, and continued to

increase reaching a maximum number of TLSs after 8 hours on Matrigel in control flasks. Phase II involves the degradation of the TLS network whereby cells detach from the substratum and lift off, resulting in a decrease in TLS number from 8 hours onwards. Most of the network was degraded after 24 hours under these conditions. VEGF (10 ng/ml) caused an increase in the maximum effect (Emax; 14.4 ± 1.2 TLSs versus 11.6 ± 1.5 TLSs in controls; Figure 3.6B) but this did not reach statistical significance. Furthermore, VEGF also caused a significant ($p<0.05$) decrease in the time to reach peak TLS number (2.5 ± 0.5 hours versus 6.0 ± 0.5 hours in controls; Figure 3.6C). Cortisol (600 nM) blocked the effects of 10 ng/ml VEGF (Emax, 9.6 ± 0.9 TLSs; time to peak, 4.8 ± 1.0 hours) when in combination. Cortisol (600 nM) alone caused a reduction in the amplitude of the TLS formation curve ($p<0.01$, repeated measures ANOVA, effect of treatment) and a reduction in time to reach peak number (4.4 ± 1.2 hours versus 6.0 ± 0.5 hours in controls; Figure 3.6C).

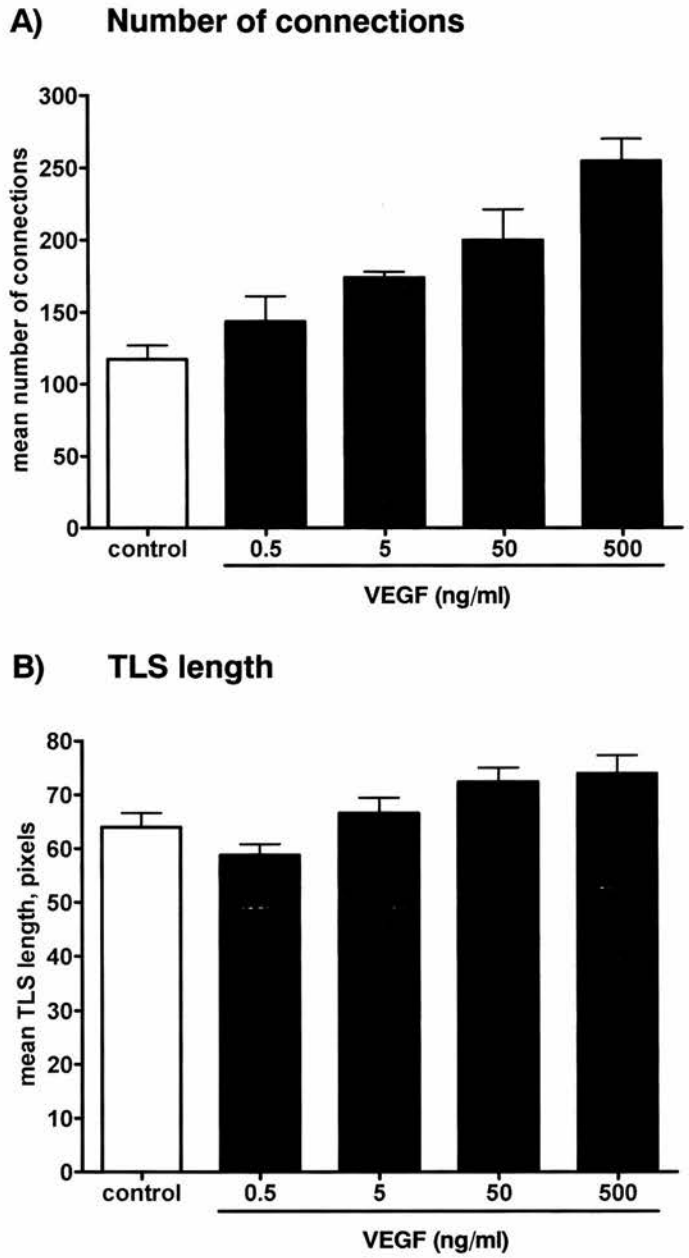


Figure 3.5 Comparison of quantification methods using the pro-angiogenic effects of VEGF

HUVECs incubated on Matrigel in standard basal conditions for 5 hours in the presence of vehicle (control) or a range (0.5-500 ng/ml) of concentrations of pro-angiogenic VEGF (vascular endothelial growth factor). VEGF stimulated TLS formation consistently using 2 methods of quantification: (a) counting capillary connections (branch-points), or (b) measuring TLS length. Data represent mean ± SEM (n=1 experiment, each condition was performed in triplicate wells).

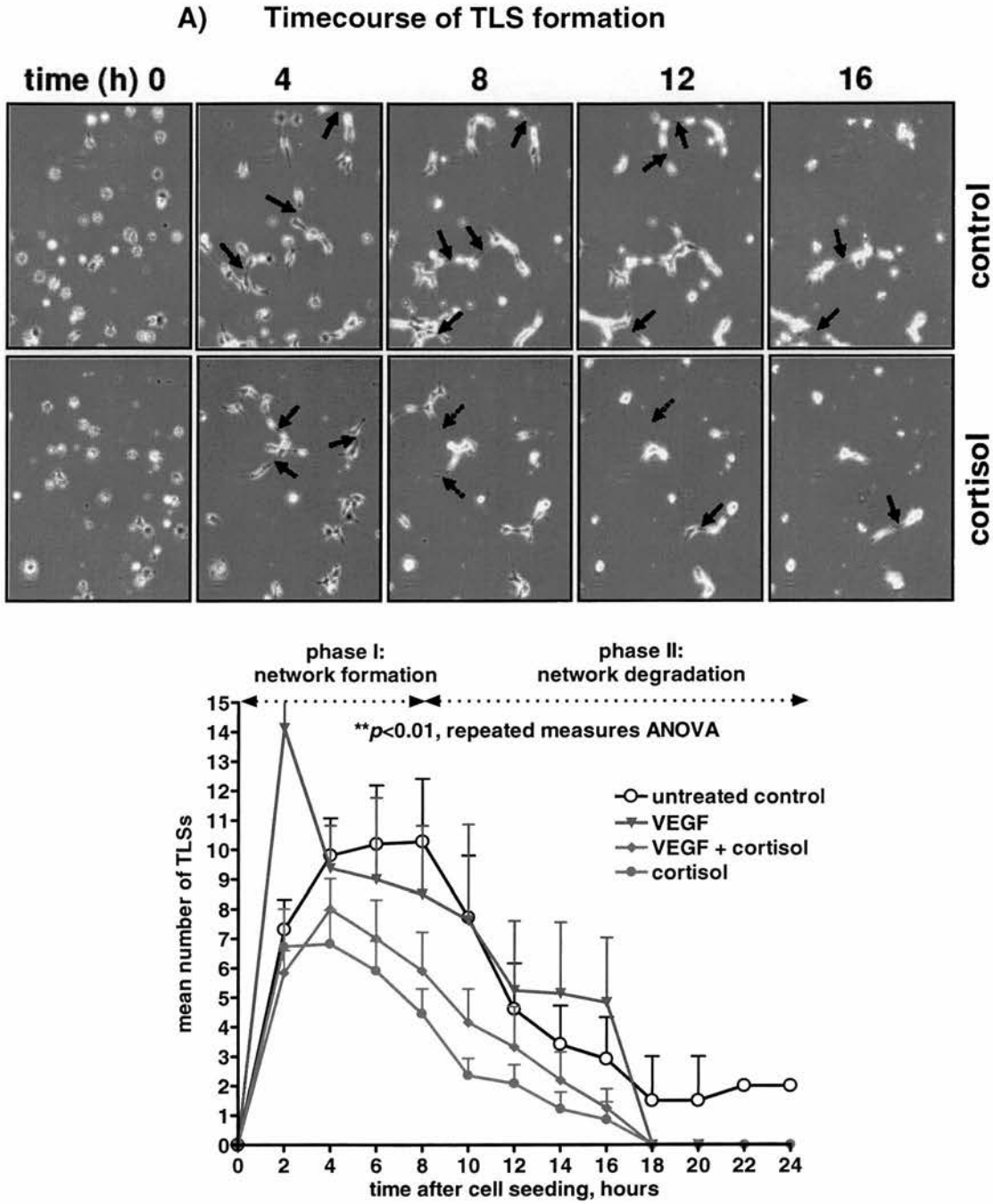


Figure 3.6 Dynamic nature of TLS formation identified by time-lapse imaging

HUVECs incubated on Matrigel in SlideFlasks in standard basal conditions for up to 24 hours in the presence of 10 ng/ml vascular endothelial growth factor (VEGF), 600 nM cortisol, or both. Images captured (3 positions per flask, 4 minute intervals) using time-lapse video microscopy. (a) Tube formation was quantified by counting the number of TLSs per frame, indicated by arrows, in reconstructed movie-clips. Profiles of TLS formation were developed plotting TLS number over time. Figure continued on next page.

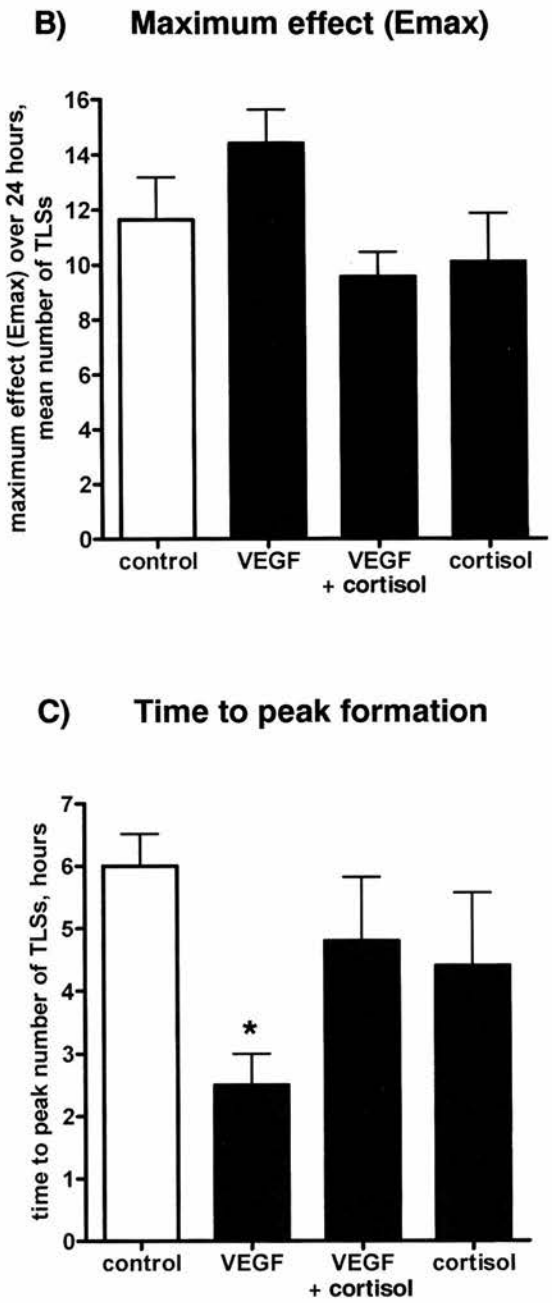


Figure 3.6 Dynamic nature of TLS formation identified by time-lapse imaging (Continued)

From the time-lapse data set (a), the maximum effects of treatment, Emax (b), and time to reach peak number of TLSs, (c), were determined. Data represent mean ± SEM (n=6 experiments). *p<0.05

3.3.1.6 Characterisation of tube-like structures

Undifferentiated HUVEC monolayers (data not shown) and mature TLSs both produced positive staining for the endothelial marker CD31 (Figure 3.7).

TLS staining of actin filaments (F-actin) and α-tubulin microtubules produced specific staining of fibres in undifferentiated HUVECs that appeared as well-formed, parallel stress fibres throughout the cytoplasm and peri-nuclear space and was highly concentrated just beneath the cell membrane. A similar staining pattern of fibres was observed in TLSs which became apparent after 4 hours on Matrigel and was highly concentrated in filopodia-like extensions after 10 hours (Figure 3.8). Staining for F-actin, but not α-tubulin, appeared more diffuse and homogeneous after cortisol treatment (Figure 3.9) and correlated with a loss of TLS network integrity. Positive nuclei were observed using DAPI in both undifferentiated control cells and TLSs.

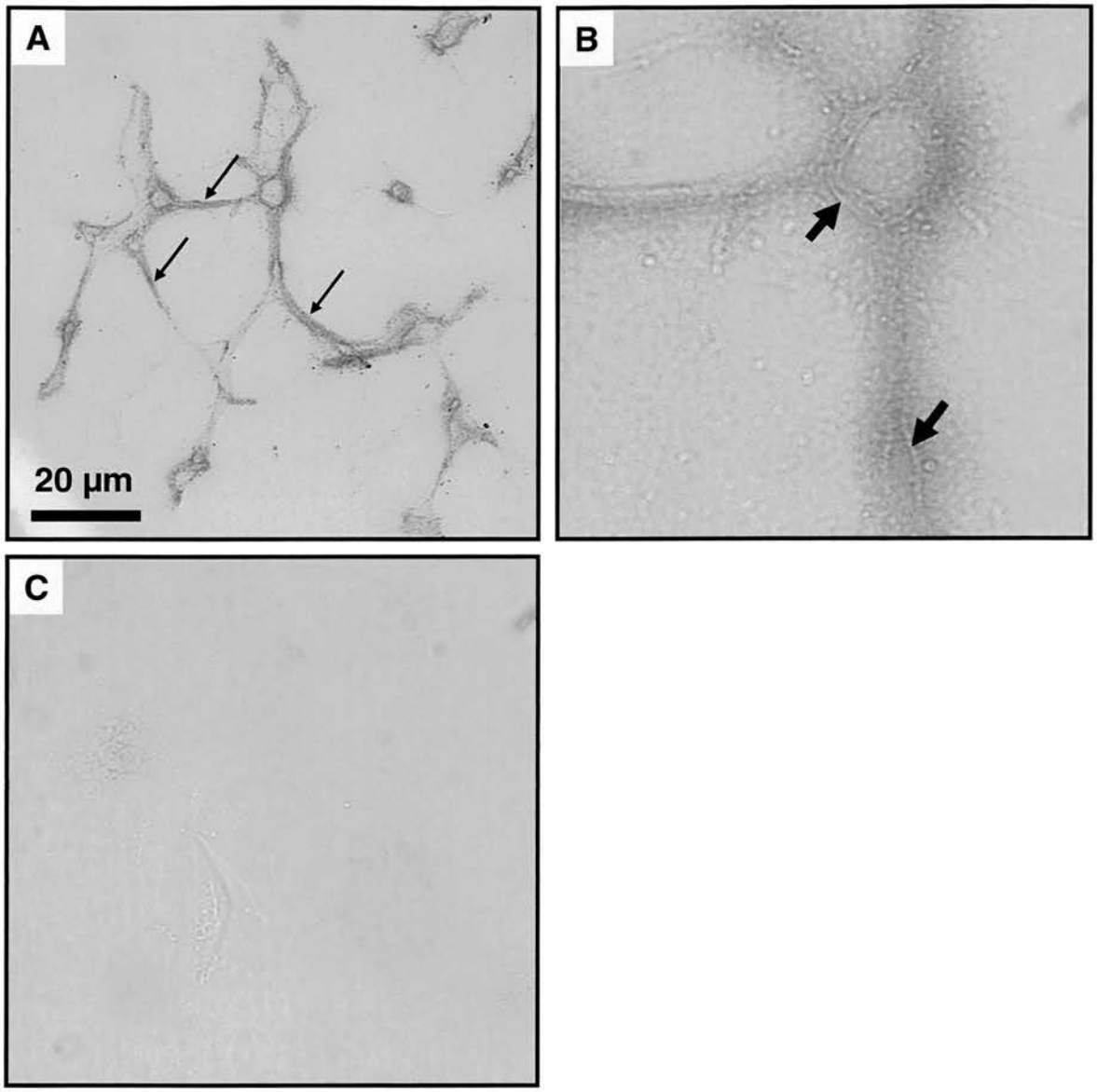


Figure 3.7 Characterisation of TLSs by immunostaining with an endothelial surface marker

Immunohistochemical confirmation that HUVECs retain an endothelial cell phenotype upon differentiation into TLSs. HUVECs cultured on Matrigel for 4 hours. (a) Staining for CD31 immunoreactivity, x10 magnification. TLSs are indicated by thin arrows. (b) At x40 magnification, endothelial cell membranes are visible (thick arrows). (c) Negative control, without primary antibody.

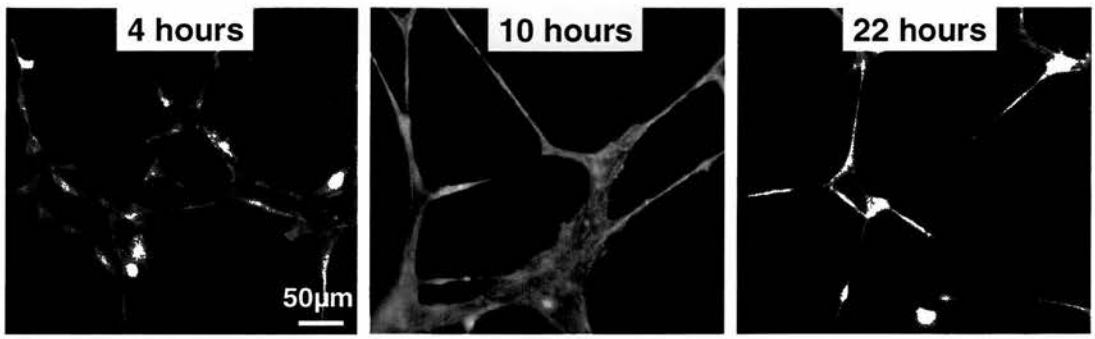


Figure 3.8 Characterisation of TLSs composition by staining the cytoskeleton

HUVECs cultured on Matrigel-coated cover slips for up to 22 hours. Direct nuclear staining of DNA with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, blue), Filamentous-actin with phalloidin-488 (green) and α-tubulin with goat anti-mouse IgG Alexa Fluor 594 secondary antibody (red). TLSs consist of adjoining filopodia-like extensions that form cell-cell contacts between neighbouring cells. Networks are evident from 4 hours and reach maturity after 10 hours. After 22 hours, cells begin to lift off the Matrigel and the networks break up.

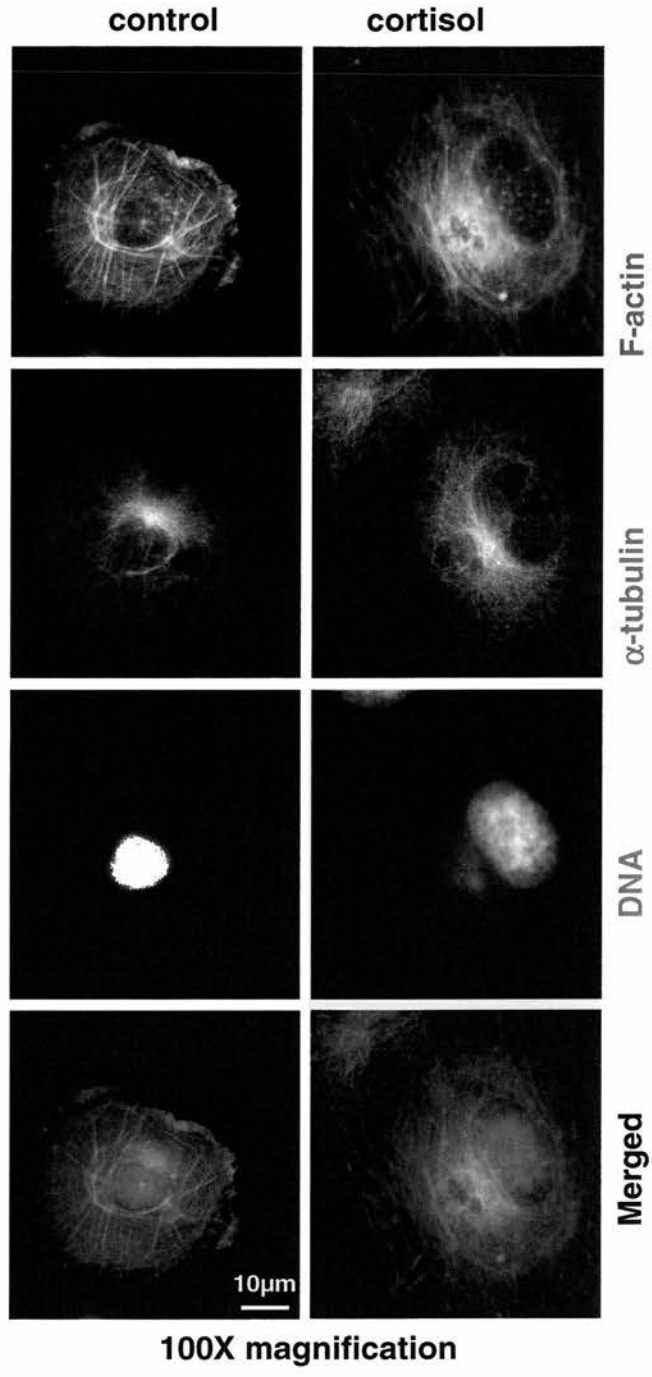


Figure 3.9 Glucocorticoids and the endothelial TLS cytoskeleton

HUVECs were cultured on Matrigel-coated cover slips for 1 hour and stained for DNA (blue), filamentous (F)-actin (green) and α-tubulin (red). Exposure to cortisol (600 nM) had no apparent effect on microtubule staining but induced a more diffuse and homogenous distribution of F-actin throughout the cells.

3.3.2 11β-Hydroxysteroid dehydrogenases in endothelial cells and tube-like structures

3.3.2.1 11β-HSD1 and 11β-HSD2 mRNA expression

Neither 11β-HSD1 nor 11β-HSD2 mRNA expression (n=5) were detected by RT-PCR in undifferentiated cultured HUVECs, mature TLSs or EPCs (n=3) isolated from human blood. Mouse liver strongly expressed 11β-HSD1; human kidney and CHO cells transfected with human 11β-HSD2 both expressed 11β-HSD2 as positive controls for the PCR reaction. Expression of Tie2 and GR served as positive controls for RNA integrity and both were detected in undifferentiated cultured HUVECs (n=2), mature TLSs (n=5) and EPCs (n=3) isolated from human blood (Figure 3.10). Furthermore, expression of the endothelial marker Tie2 was detected in HUVECs and TLSs (data not shown).

3.3.2.2 11β-HSD1 and 11β-HSD2 activity

Neither 11β-reductase nor 11β-dehydrogenase activity were detected in either undifferentiated HUVECs or TLSs after 5 hours, 24 hours or 48 hours in culture as indicated by the failure to inter-convert $[^3H]_4$-cortisone and $[^3H]_4$-cortisol (n=2-3). This is consistent with a lack of mRNA expression for either of the isozymes of 11β-HSD. Positive control (CHO) cells transfected with 11β-HSD1 converted $[^3H]_4$-cortisone to $[^3H]_4$-cortisol (Figure 3.11) and $[^3H]_4$-cortisol to $[^3H]_4$-cortisone (Figure 3.11), demonstrating bi-directional activity, with the reductase activity being predominant (10-fold higher). CHO cells transfected with 11β-HSD2 converted $[^3H]_4$-cortisol to $[^3H]_4$-cortisone (Figure 3.12), but not the reverse reaction, demonstrating exclusive dehydrogenase activity (n=2).

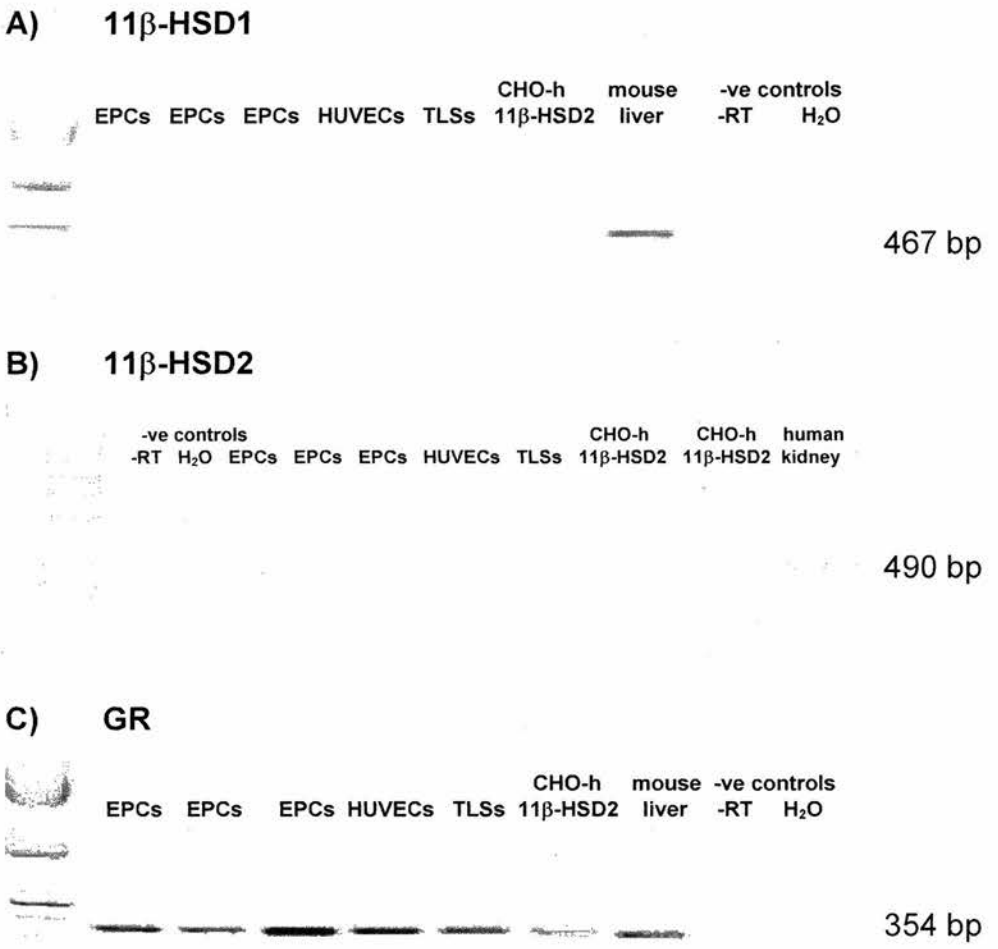


Figure 3.10 Expression of 11β-HSD1, 11β-HSD2 and GR in endothelial cell preparations

Specific primers were used to detect expression of (a) 11β-HSD1, (b) 11β-HSD2 and (c) GR mRNA in human umbilical vein endothelial cells (HUVECs), endothelial tube-like structures (TLSs) and human endothelial progenitor cells (EPC). Mouse liver, human kidney and Chinese Hamster Ovary cells transfected with human 11β-HSD2 (CHO-h11β-HSD2) were used as positive controls. 0.5 μg of total RNA were isolated from HUVECs, TLSs, mouse liver, human kidney and CHO-h11β-HSD2 whilst 0.075-0.245 μg of total RNA were isolated from EPCs for RT-PCR. RT-PCR products were analysed by gel electrophoresis of 10 μl of each sample (20 μl of each in 11β-HSD2 reaction) and were of the expected size (Table 2.1). Negative controls included a reaction carried out in the absence of the RT enzyme (-RT) and an RT reaction containing no RNA (H_20). Image is a typical representation of multiple gels.

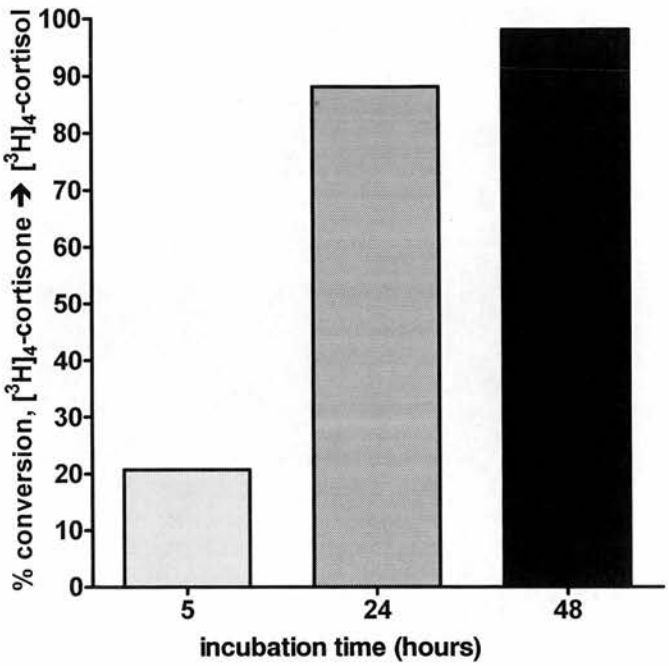


Figure 3.11 11β-reductase activity in 11β-HSD1-transfected Chinese hamster ovary cells

11β-HSD activity is expressed as the percentage conversion of $[^3H]_4$-cortisone to $[^3H]_4$-cortisol by 1.75×10^5 cells. 11β-HSD activity was measured after 5 hours, 24 hours and 48 hours incubation. No conversion was detected in HUVECs growing on tissue culture plastic (n=3) or HUVECs seeded on Matrigel in standard basal conditions (n=2) at any time-point (data not shown). In contrast, Chinese hamster ovary cells transfected with human 11β-HSD1 (CHO-h11β-HSD1, positive control) converted 21 ± 11% after 5 hours, 88 ± 7% after 24 hours and 98 ± 3% after 48 hours incubation (n=2). Chinese hamster ovary cells transfected with human 11β-HSD2 (CHO-h11β-HSD2) did not convert $[^3H]_4$-cortisone to $[^3H]_4$-cortisol (n=3, data not shown). Results shown are the mean of 2 experiments.

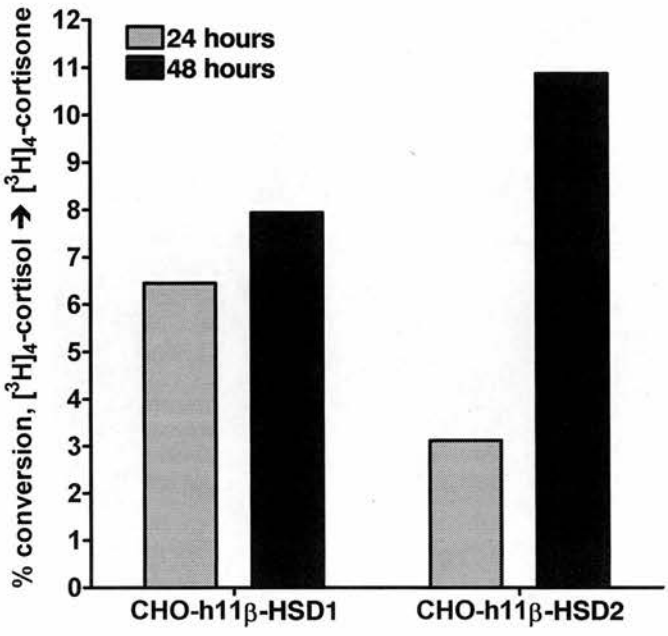


Figure 3.12 11β-dehydrogenase activity in 11β-HSD1 and 11β-HSD2-transfected Chinese hamster ovary cells

11β-HSD activity is expressed as the percentage conversion of $[^3H]_4$-cortisol to $[^3H]_4$-cortisone by 1.75×10^5 cells. 11β-HSD activity was measured after 5 hours (data not shown), 24 hours and 48 hours incubation. No conversion was detected in HUVECs growing on tissue culture plastic (n=3) or HUVECs seeded on Matrigel in standard basal conditions (n=2) at any time-point (data not shown). Chinese hamster ovary cells transfected with human 11β-HSD1 (CHO-h11β-HSD1) converted 6 ± 3% after 24 hours and 8% after 48 hours incubation (n=2). Chinese hamster ovary cells transfected with human 11β-HSD2 (CHO-h11β-HSD2, positive control) converted 3% after 24 hours and 11 ± 5% after 48 hours incubation (n=2). Results shown are the mean of 2 experiments.

3.3.3 Influence of glucocorticoids on endothelial tube formation

3.3.3.1 VEGF and prostanoids enhance endothelial tube formation

As described, TLS formation was stimulated in a concentration-dependent manner with VEGF (0.5 ng/ml, 143.5 ± 17.5; 5 ng/ml, 174.0 ± 4.0; 50 ng/ml, 200.0 ± 21.4 and 500 ng/ml 255.0 ± 15.3 connections) compared with control (117.3 ± 9.6 connections) (Figure 3.5A) after 5 hours incubation (n=1). Following 22 hours of incubation the number of connections were lower in all wells (including control) compared with 5 hours, however, VEGF stimulated TLS formation at the lower concentrations (0.5 ng/ml, 120.3 ± 4.3; 5 ng/ml, 107.7 ± 4.8 connections) compared to control (88.0 ± 9.0 connections). At 50 ng/ml, the level of TLS formation was similar to that of control (93.3 ± 5.5 connections) and had reduced with supra-physiological levels, 500 ng/ml (70.3 ± 10.3 connections).

Similarly, TLS formation was stimulated in a concentration-dependent manner with both prostanoids. $PGF_{2\alpha}$ at concentrations across the physiological range (10 nM to 1 μM) stimulated TLS formation after 4-5 hours (10 nM, 128 ± 25%; 100 nM, 140 ± 25%; 500 nM, 153 ± 33% and 1 μM, 167 ± 30% compared with control, 100%; Figure 3.13A) although this did not reach significance (n=5). After 20-23 hours, a similar concentration-dependent increase was observed that reached significance (p<0.05) at 1 μM (10 nM, 124 ± 18%; 100 nM, 208 ± 52%; 500 nM, 240 ± 44% and 1 μM, 309 ± 74% compared with control, 100%; Figure 3.13B).

Similar trends were observed with PGE_2 at concentrations (10 nM to 1 μM) across the physiological range (Figure 3.14). PGE_2 stimulated TLS formation after 4-5 hours (10 nM, 98 ± 18%; 100 nM, 136 ± 23%; 500 nM, 146 ± 21% and 1 μM, 143 ± 21%) and after 20-23 hours (10 nM, 111 ± 36%; 100 nM, 251 ± 98%; 500 nM, 376 ± 155% and 1 μM, 332 ± 144%) and reached a maximum effect at 500 nM although this did not reach significance (n=4-5).

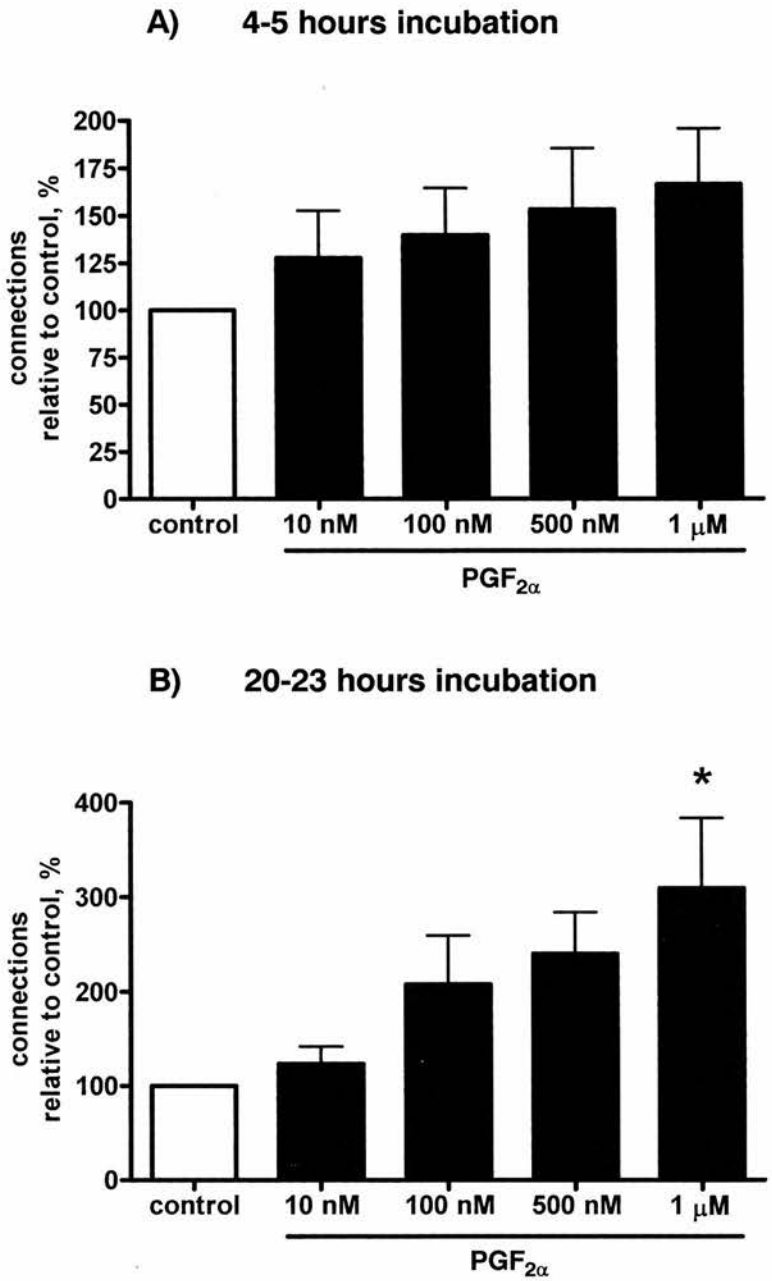


Figure 3.13 Influence of prostaglandin $F_{2\alpha}$ on TLS formation

HUVECs incubated on Matrigel in standard basal conditions (control) or in the presence of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 nM-1 μM). $PGF_{2\alpha}$ stimulated TLS formation in a concentration-dependent manner with maximum effect observed at 1 μM consistently (a) after 4-5 hours (although this did not reach significance) and (b) after 20-23 hours of incubation *p<0.05. Data represent mean ± SEM (n=5, each condition was performed in triplicate wells).

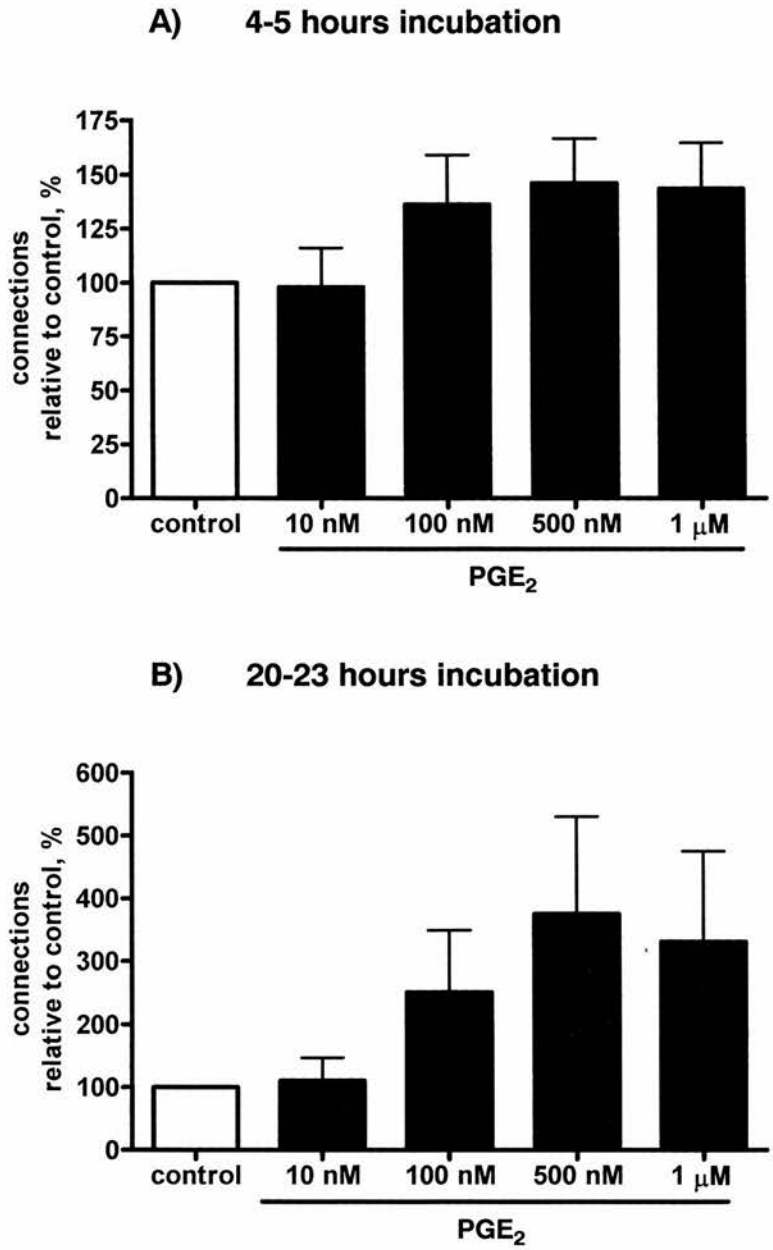


Figure 3.14 Influence of prostaglandin E_2 on TLS formation

HUVECs incubated on Matrigel in standard basal conditions (control) or in the presence of prostaglandin E_2 (PGE_2, 10 nM-1 μM). PGE_2 stimulated TLS formation in a concentration-dependent manner with maximum effect observed at 500 nM consistently (a) after 4-5 hours and (b) after 20-23 hours incubation although this did not reach statistical significance. Data represent mean ± SEM (n=4-5, each condition was performed in triplicate wells).

3.3.3.2 Cortisol, but not cortisone, inhibits endothelial tube formation in HUVECs

Incubation of TLSs with active (cortisol), but not inactive (cortisone), glucocorticoids inhibited TLS formation. At physiological concentrations cortisol (300 nM to 1200 nM), reduced the number of connections compared with controls after 5 hours (300 nM, 85 ± 4%; 600 nM, 86 ± 7% and 1 μM, 84 ± 9%) and 22-24 hours (300 nM, 73 ± 8%; 600 nM, 59 ± 5% and 1 μM, 64 ± 12%) incubation compared with control (100%). The angiostatic effect of cortisol was greatest ($p<0.01$) after 22-24 hours of incubation with 600 nM cortisol (Figure 3.15). There were no significant differences in mean TLS length between the different treatment groups (data not shown). In contrast, the biologically inactive metabolite of cortisol, cortisone, failed to inhibit TLS formation across a range (300 nM to 1200 nM) of physiological concentrations (Figure 3.16). These data are consistent with lack of 11β-HSD1 mRNA expression and 11β-reductase activity in TLSs.

3.3.3.3 Cortisol and dexamethasone inhibit basal, and VEGF-stimulated, endothelial tube formation in HUVECs and HAoECs

Extending these findings to another human endothelial cell type, TLS formation by HAoECs on Matrigel was inhibited to a similar degree as HUVECs with 600 nM cortisol (4 hours, 54 ± 5%; 22 hours 45 ± 5%) and with the synthetic GR selective glucocorticoid, dexamethasone (600 nM; 4 hours, 52 ± 3%; 22 hours 53 ± 6%) compared with control (100%). Whereas the angiostatic effect of cortisol on HUVEC TLS formation was greatest after 22-24 hours in culture, the angiostatic effect on HAoEC TLS formation was of similar magnitude and statistically significant ($p<0.01$) after 4 hours (Figure 3.17A) and 22 hours (Figure 3.17B) in culture.

Under VEGF-stimulated conditions, TLS formation by HUVECs (data not shown) and HAoECs (Figure 3.17C&D) were inhibited by co-incubation with 600 nM cortisol or dexamethasone. Again, these effects were of similar magnitude and significant ($p<0.01$) after 4 hours and 22 hours in culture.

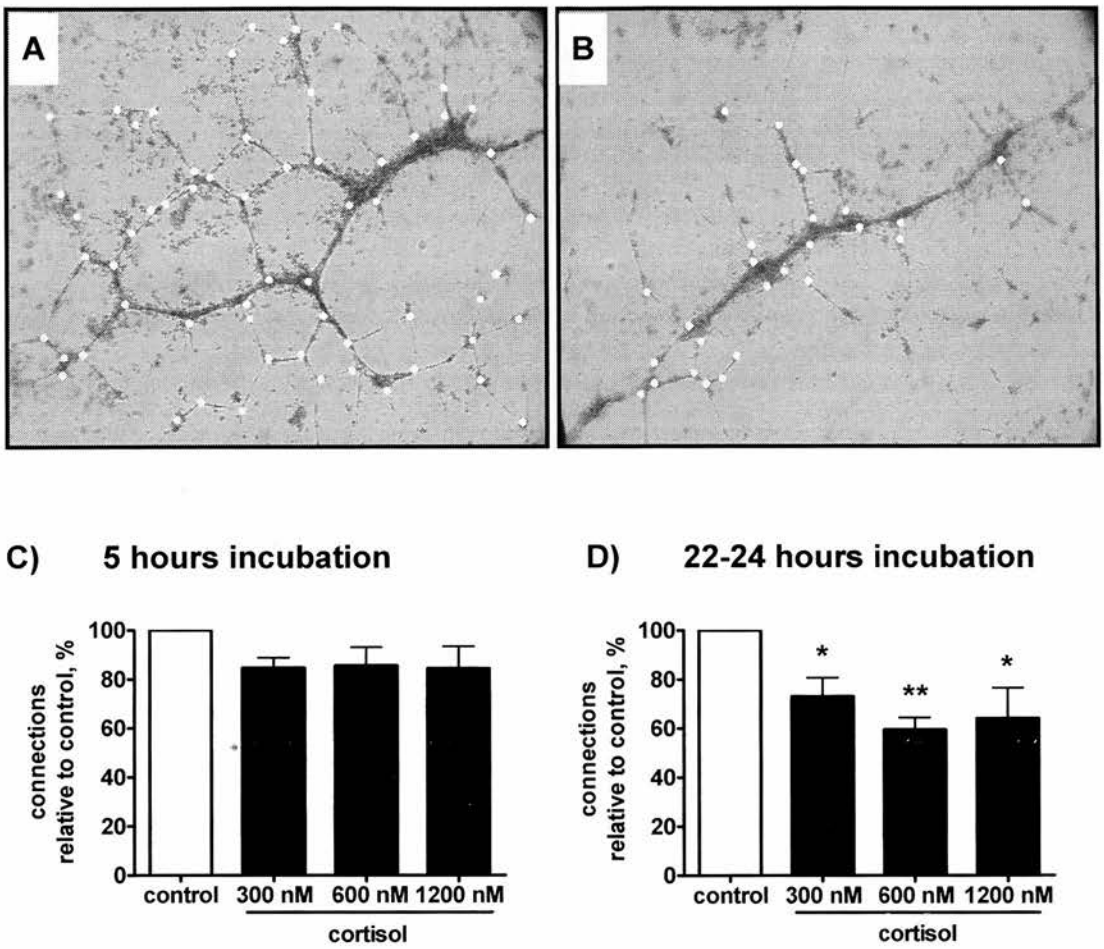


Figure 3.15 Influence of cortisol on TLS formation

Light micrographs of HUVECs incubated on Matrigel under standard basal conditions (control) or in the presence of cortisol (600 nM) for 22 hours. In comparison with untreated control wells, cortisol (300-1200 nM) inhibited TLS formation after (a) 5 hours and (b) 22-24 hours incubation. The angiostatic effect of active cortisol was greatest at the later time-point (*p<0.05, **p<0.01). Data represent mean ± SEM (n=6, each condition was performed in triplicate wells).

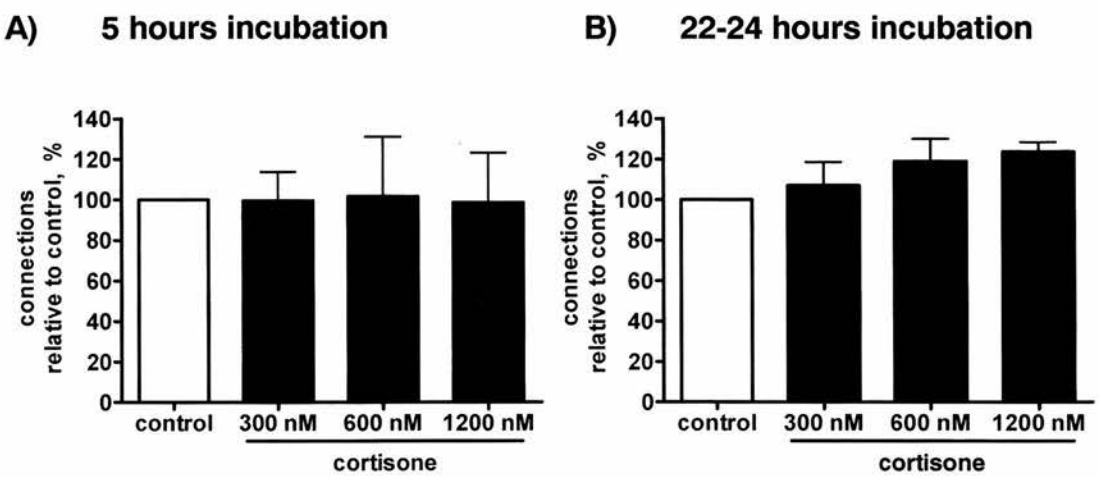


Figure 3.16 Influence of cortisone on TLS formation

HUVECs incubated on Matrigel in standard basal conditions (control) or in the presence of cortisone for (a) 5 hours and (b) 22-24 hours. Unlike active cortisol, its biologically inert metabolite, cortisone (300-1200 nM) had no effect on TLS formation compared with control wells. Data represent mean ± SEM (n=3, each condition was performed in triplicate wells).

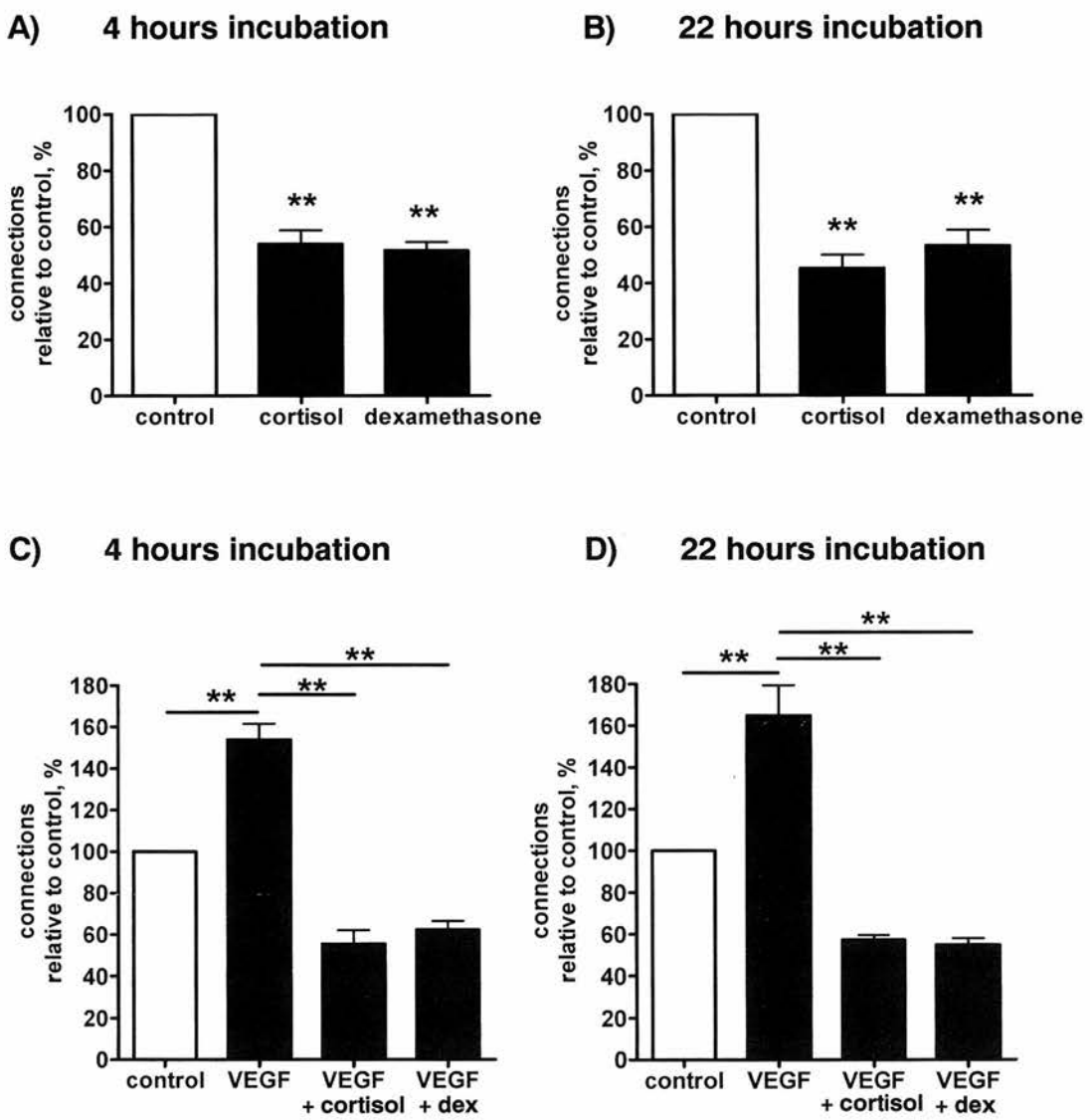


Figure 3.17 Influence of glucocorticoids on basal and VEGF-stimulated TLS formation in human aortic endothelial cells

Human aortic endothelial cells (HAoECs) incubated on Matrigel in standard basal conditions in the presence of cortisol (600 nM) or dexamethasone (600 nM) for (a) 4 hours and (b) 22 hours. Both steroids caused a significant (**p<0.01) inhibition of TLS formation. Vascular endothelial growth factor (VEGF; 10 ng/ml) stimulated TLS formation (**p<0.01) compared with control, an effect that was blocked in the presence of cortisol (600 nM) or dexamethasone (600 nM; **p<0.01 compared with VEGF alone) (c) after 4 hours and (d) after 22 hours. Data represent mean ± SEM (n=5, each condition was performed in triplicate wells).

3.3.3.4 *Identification of the receptor responsible for glucocorticoid-mediated inhibition of tube formation*

Cortisol (600 nM) caused a 30% reduction in mean number of connections in GR and MR antagonism experiments that did not reach statistical significance (69 ± 7% compared with untreated control, 100%, n=4). The angiostatic effect of cortisol (600 nM) was reversed by co-incubation with the GR antagonist RU38486 (p<0.05, n=4, Figure 3.18A) and there was a trend towards a similar effect by the MR antagonist spironolactone (Figure 3.18B), however, the latter did not reach significance (p=0.16). RU38486, spironolactone and vehicle did not influence TLS formation in comparison to untreated control.

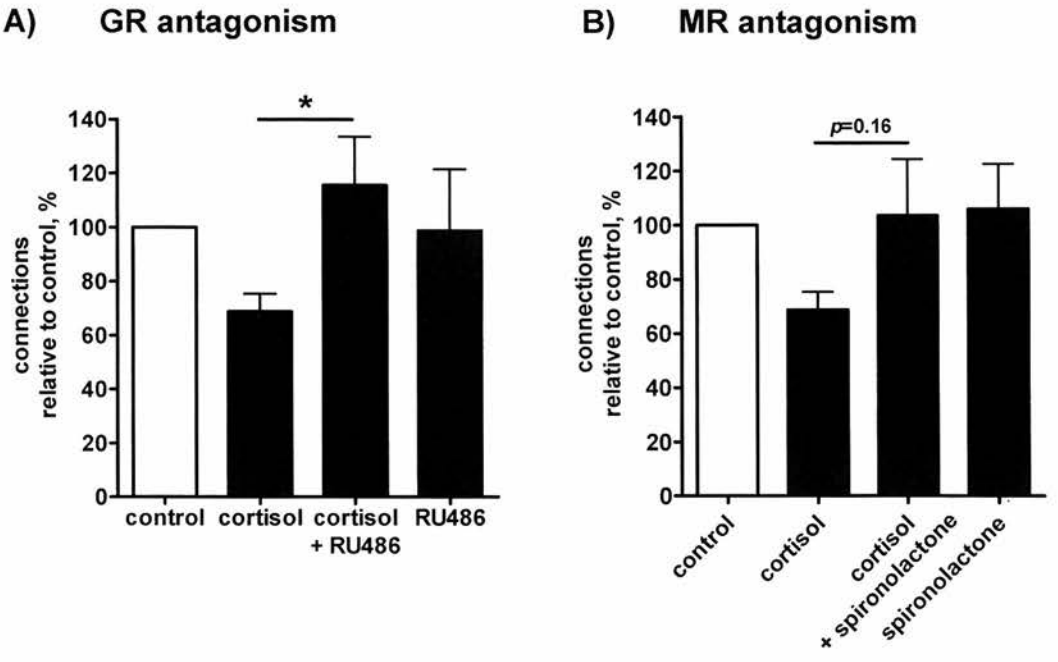


Figure 3.18 Glucocorticoid-induced angiostasis: Effects of GR and MR antagonism

HUVECs incubated on Matrigel in standard basal conditions in the presence of cortisol (600 nM) alone, or cortisol and the glucocorticoid receptor antagonist RU38486 (1 μM) or the mineralocorticoid receptor antagonist spironolactone (1 μM) or either antagonist alone. The angiostatic effect of cortisol was reversed by GR antagonism ($p<0.05$; cortisol plus RU38486 versus cortisol) and by spironolactone, although the latter was not significant (cortisol plus spironolactone versus cortisol, $p=0.16$). RU38486, spironolactone and vehicle control (data not shown) had no effect on TLS formation compared with untreated control wells. Data represent mean ± SEM ($n=4$, each condition was performed in triplicate wells).

3.4 Discussion

In this chapter, through the development of an *in vitro* model of endothelial tube formation, it was determined that physiologically-relevant concentrations of active (but not inactive) glucocorticoids act directly on endothelial cells to inhibit a key component of the angiogenesis cascade. Furthermore, evidence presented here suggests that glucocorticoids inhibit angiogenesis by GR-dependent action on tube formation by the endothelium.

3.4.1 Development of the endothelial TLS model

Endogenous glucocorticoids generated in the vessel wall by 11β-reductase activity are known to control angiogenesis by regulating local glucocorticoid availability (Small *et al.*, 2005), however it is not clear how they act. A major aim of the present study was to develop an *in vitro* model to address this.

In vitro angiogenesis assays offer the opportunity to investigate angiogenic mechanisms with speed and simplicity that cannot be achieved using *in vivo* assays (Vailhe *et al.*, 2001). Experiments involving controlled cell populations have end-points that are easy to quantify, reproducible, and do not have confounding factors of *in vivo* models such as stromal and inflammatory cells. For mechanistic studies, recovery of cells to study gene and protein expression is another advantage. A single cell population assay was chosen to investigate primarily whether glucocorticoids have a direct effect on the endothelium rather than acting via an intermediary cell type such as neighbouring SMCs. In the extensive literature on angiogenesis assays (Auerbach *et al.*, 2003), it is acknowledged that culturing endothelial cells on basement membrane proteins promotes their morphologic differentiation into mature capillary or tube-like structures (CLSs or TLSs) versus the typical "cobblestone" appearance that they assume when grown on plastic (Lawley & Kubota, 1989). Therefore the endothelial TLS assay offers the opportunity to study the endothelial cell in a situation that more closely approximates its conformation during angiogenesis *in vivo*.

The endothelial tube formation model was selected because it is thought to recapitulate the morphogenesis step of angiogenesis alone (Vailhe *et al.*, 2001). Potential effects of glucocorticoids on this step of angiogenesis in isolation could therefore be examined. According to the literature, TLS formation occurs as a result of endothelial cell differentiation and occurs in the absence of cell proliferation (Kubota *et al.*, 1988) and migration (Manoussaki *et al.*, 1996).

One of the key requirements of the TLS model was that it should involve human endothelial cells to extend the findings reported by Small and colleagues (Small *et al.*, 2005), which were restricted to mouse models. Strategies to stimulate, and inhibit, angiogenesis have demonstrated efficacy in mice but fail to provide similar results in humans (Rajagopalan *et al.*, 2003; Oehler & Bicknell, 2000). Consequently, it is best to use human cells or tissues, wherever possible, to avoid species differences (Mestas & Hughes, 2004). An important consideration in the choice of cell is that all endothelial cells are not alike, demonstrating phenotypic differences. For example, differences in protease activity have been described between large vessel-derived endothelial cells and endothelial cells of microvascular origin (Jackson & Nguyen, 1997). Therefore, wherever possible, *in vitro* assays should be carried out using endothelial cells from more than one source. In the present study, HUVECs were used predominantly due to the availability of batches from pooled donors, their rapid availability, and low cost compared with other sources. For these reasons, HUVECs are by far the most common choice of cell used in this assay (Table 3.1) and other *in vitro* endothelial cell assays. The observation in the present study, that glucocorticoids inhibit TLS formation, was originally made in HUVECs. Key observations were subsequently confirmed in another human endothelial cell type, HAoECs, demonstrating that these findings are not limited to a single vascular territory and extend the findings reported here to an arterial endothelial cell-type. Furthermore, the angiostatic effects of physiological concentrations of glucocorticoids in the TLS model have been described using human cells of microvascular origin (by Dr Michael Rae, Centre for Reproductive Biology, personal communication) and are significant since angiogenesis *in vivo* occurs in microvascular beds.

Attempts to extend the lifespan of each batch of cells by cryopreservation proved unsuccessful as, although cells appeared healthy and underwent normal cell cycle division upon revival, they failed to form TLS networks in the assay compared with non-frozen cells. This suggests that irreversible damage to the cell is caused by either the freezing process and/or storage conditions. It is not clear from the literature whether cryopreservation of endothelial cells for extended use in the TLS assay is routine but anecdotal evidence suggests that it is. A key advantage of using multiple fresh batches of HUVECs as opposed to the extended use of a single batch is that observations are made from a larger population base thereby minimising the possibility of individual donor effects. Batch-specific variation in the degree of control TLS formation was evident in these studies given the mean inter-assay coefficients of variation; however, this was circumvented by performing control groups on each occasion and normalising data to control.

Cell culture provides an approximation of the *in vivo* environment. For example, it is documented that cells growing in isolated tissue culture for extended periods display altered growth characteristics compared to those growing *in situ* in live tissues (Cervenkova *et al.*, 2001; Shi *et al.*, 2004). To address whether this was an issue in the present study, experiments were performed to assess the expression of CD31 (PECAM-1, platelet endothelial cell adhesion molecule-1) and Tie2, endothelial cell surface markers involved in cell-cell interactions (Dumont *et al.*, 1992; Newman *et al.*, 1990). Both cultured HUVECs and TLSs retained expression of both markers after sub-culturing, suggesting that they do retain functional and biochemical characteristics of endothelial cells in their normal environment. Furthermore, the use of HUVECs within a similar passage range (p2-p6) to that used here is commonplace according to the literature (Beckner & Liotta, 1996; Kuzuya & Kinsella, 1994; Cattaneo *et al.*, 2003); however, the basis for this choice is not made clear in these reports. In the present study, the degree of TLS formation, and the presence of markers of key endothelial functions, was found to be consistent within this passage range and therefore use of cells within this range was reasonably justified.

In this study, a 2D model of TLS formation was developed in which the TLSs form directly upon the surface of the Matrigel. 2D models lend themselves more easily to quantification than 3D models, in which cells or tissue explants are dispersed through, or sandwiched between, layers of matrix since the latter model consists of sprouting in multiple focal planes. Also, 3D models are more time-consuming and may require several days or weeks for networks to develop (Nicosia & Ottinetti, 1990; Lansink *et al.*, 1998; Nakatsu *et al.*, 2003); in contrast endothelial cells plated on Matrigel form TLSs within several hours as reported here and by others (Beckner & Liotta, 1996; Cattaneo *et al.*, 2003; Kuzuya & Kinsella, 1994). These were important considerations in the final choice of assay since rapid and accurate quantitation were prerequisites of the model. During method refinement, cells dispersed throughout the matrix did not form adequate networks compared with cells seeded on a solidified layer of Matrigel. Furthermore, recovery of cellular material from TLSs in the 2D model was possible and allowed successful extraction of RNA and protein for gene expression and signal transduction studies. In contrast, it has been shown previously that aortic rings embedded in Matrigel do not allow for successful recovery of RNA for gene expression studies (Mr Louis Chablis, unpublished findings) and resistance of Matrigel to penetration by antibodies (Dr Gary Small PhD thesis, University of Edinburgh 2006) precluded immunohistochemical identification of component cells. An important facet of the present study was to characterise the structural and functional components of TLSs by direct staining methods. Consequently the 2D model was adopted for use in these studies.

The use of EGM-2 culture medium in the TLS assay has been described by others (Donovan *et al.*, 2001; Wilasrusmee *et al.*, 2003) but specific culture conditions with regard to the inclusion of Bullet kit growth factors is not commonly reported. Consequently, growth factor requirements for TLS formation were carefully evaluated in the course of method development. Conditions were optimised to produce sufficient networks that could be easily quantified and more importantly, were responsive to angiogenic stimuli and inhibition. Preliminary studies revealed that TLS formation in the presence of Bullet kit growth factors and serum was too

prolific and further augmentation of growth with VEGF (positive control) could not be achieved. Evidence from the literature suggests that further augmentation of TLS formation with VEGF (100 ng/ml) *can* be achieved in the presence of serum, but only at lower levels (1%) than those used in the present study (Kawasaki *et al.*, 2000). Furthermore, the angiostatic effect of cortisol (300 nM) was not observed under growth factor rich conditions and therefore any inhibitory effect is likely to have been masked by the prolific network formation. This is consistent with a previous report that dexamethasone (at micromolar concentrations) failed to inhibit TLS formation under similar conditions (Wilasrusmee *et al.*, 2003). It is feasible that exogenous addition of factors was able to overcome glucocorticoid-induced impairment of growth factor production and suggests an interaction between glucocorticoids and growth factors. Under standard basal conditions (containing only EGM-2 plus ascorbic acid, antibiotics and heparin), a level of TLS growth was achieved that could be stimulated by growth factors and inhibited by glucocorticoids. Ascorbic acid was included because of reports that it promotes angiogenesis in aortic ring cultures and maintains new vessel structure (Nicosia *et al.*, 1991) and heparin was included since it is necessary for glucocorticoids to inhibit angiogenesis (Folkman *et al.*, 1983; Crum *et al.*, 1985) in the CAM assay.

Choice of basement membrane matrices was decided as a result of careful consideration of the literature. Matrigel has previously been shown to cause the differentiation of endothelial cells into TLSs (Lawley & Kubota, 1989). Although the composition of Matrigel is not completely characterised it appears highly consistent since the results from Matrigel TLS assays are reproducible allowing inter-lab experimental comparisons. Consequently, it has allowed the screening of several anti-angiogenic molecules (Beckner & Liotta, 1996; Kuzuya & Kinsella, 1994; Morales *et al.*, 1995; Stoltz *et al.*, 1996). It has been determined that one of the main signals responsible for TLS formation is laminin (Kubota *et al.*, 1988) which is the primary constituent of Matrigel, followed by collagen IV, heparin sulphate proteoglycans, entactin and nidogen (Kleinman *et al.*, 1982). However TLS formation does not occur on laminin or collagen IV either alone or in combination, but does when combined with collagen I, therefore interactions

between the endothelium and multiple basement proteins is necessary to induce TLS formation. Matrigel is an ideal choice of preparation since it is a preparation extracted from a mouse tumour (EHS sarcoma) and contains the entire essential extracellular matrix proteins involved in angiogenesis *in vivo*. Consequently, Matrigel has been used in the TLS assay for almost 20 years (Lawley & Kubota, 1989) and is by far the most frequently used matrix to support growth.

Lack of a standard quantification method of TLS formation prompted a comprehensive comparison of the most commonly described methods. Computer analysis techniques have been used infrequently to count vessels in the TLS assay (Sanz *et al.*, 2002). In the present study, vessel density was not reliable or consistent since it was not possible to set a binary threshold function to accurately distinguish undifferentiated cells and cell debris from TLS networks as previously described (Lansink *et al.*, 1998; Donovan *et al.*, 2001). This resulted in highly variable results between replicate wells and, therefore, this method was excluded early on. Counting capillary connections (or branch-points) and measurement of TLS length showed a similar pattern of TLS formation in response to a pro-angiogenic stimulus (VEGF) but counting connections was more sensitive. VEGF (10 ng/ml) has previously been shown (Donovan *et al.*, 2001) to have a significant effect on TLS length but only on a growth-factor reduced substrate. This effect was not manifest on standard Matrigel (Donovan *et al.*, 2001), or in the present study. Thus, augmentation of TLS length appears to be dependent on matrix type and could explain the lack of stimulation by VEGF in the present study. Furthermore, TLS length was not altered in the presence of glucocorticoids indicating that modulation of TLS length is not as sensitive to modulation by exogenous angiogenic factors as counting connections. Counting connections was reliable, technically less demanding, less time consuming, and most importantly detected stimulation (VEGF; prostanoids) and inhibition (cortisol, dexamethasone) by exogenous factors. These advantages led to the adoption of counting connections as the standard method for quantification of TLS formation. Since connection counting was performed by an individual blind to the treatments the integrity of the final data was ens-ed.

It is clear from the literature that early TLS formation is followed by detachment and degradation of the network. Here, a pattern of TLS formation consistent with other reports using similar conditions was observed (Kuzuya & Kinsella, 1994; Wiedermann *et al.*, 1996; Morales *et al.*, 1995; Bussolati *et al.*, 2001) Briefly, after 1 to 2 hours on Matrigel, HUVECs attached to the matrix underwent elongation and began to align with each other. After 4 to 5 hours, anastomosing networks were obvious; after 8 to 10 hours, networks appeared to reach maturity and TLSs were visible. During the regression phase, TLS networks disintegrated and cells began to detach and lift off. By 24 hours, most of the TLS network had degraded and all that remained were aggregates of cells that resembled apoptotic bodies although this was not tested in this study. To address which phase, if any, was influenced by glucocorticoids and to begin to explore potential angiostatic mechanisms, TLS formation was initially assessed after 4 to 5 hours, and again after 20-24 hours of incubation. This was consistent with time-points chosen to quantify TLS formation in other studies (Morales *et al.*, 1995; Tan *et al.*, 2004; Wiedermann *et al.*, 1996) but the reasons for using these time-points are not made clear in these reports.

At the time these studies were performed quantitative studies in the literature were limited to static morphometric approaches. However, consistent with the findings from our own studies, a report has recently emerged describing extensive characterisation of the dynamic process of TLS formation (on fibrin gels) using similar methodology (Stephanou *et al.*, 2006). Time-lapse studies gave a more dynamic insight into the time-course of TLS formation and proved invaluable in improving our understanding of the processes involved in the Matrigel model. These studies revealed that the time course of TLS formation was consistent with those previously observed at isolated time-points (4 hours and 22 hours) and enhanced our understanding of the process by revealing that maximal TLS formation occurs at 8 hours. Indeed, this served as an important basis to plan gene expression studies (explored in Chapter 4) and provide a useful justification for including 8 hour time-points in future studies using this model. Time-lapse imaging also demonstrated that TLS formation occurs as a result of changes in cell morphology with the absence of obvious signs of proliferation or migration, confirming claims

that this serves as a useful model of endothelial cell differentiation in isolation from these other processes (Taraboletti & Giavazzi, 2004). Furthermore, the effects of glucocorticoids on the dynamic process of TLS formation have now been revealed. 'Snap-shots' data only revealed that fewer connections were present after glucocorticoid treatment but it was not clear whether this was due to reduced network formation or enhanced degradation. The time-lapse imaging studies indicate that glucocorticoids can affect both; by reducing the magnitude of the initial formation phase, and, accelerating the network degradation phase thus providing further insight into their mechanism of action.

To validate the resemblance of TLSs to angiogenic networks *in vivo*, several studies have examined their ultra-structural integrity. The presence of a lumen within TLSs is considered evidence that the model recapitulates cell morphological changes that lead to capillary formation *in vivo* (Taraboletti & Giavazzi, 2004) but this is an area of controversy. Using TLS models similar to that described in this thesis (culturing HUVECs on Matrigel), Lawley and Kubota reported hollow, lumen-like endothelial tubes surrounded by cells in cross sections of TLSs (Lawley & Kubota, 1989). Others have reported structures that are more like narrow clefts (Montanez *et al.*, 2002). In some instances lumen formation appeared to take place within individual cells and in others involved remodelling of groups of cells; a pattern that is consistent with that observed in the cytoskeleton studies reported in this chapter. The formation of tight junctions between endothelial cells in the TLS assay has been described by others (Auerbach *et al.*, 2003), resembling those *in vivo*, further validating the relevance of the model used in the present study.

3.4.2 11β-Hydroxysteroid dehydrogenases in endothelial cells and tube-like structures

The rationale for determining the presence of 11β-HSDs in this model was that both isozymes are present in the vessel wall (Hadoke *et al.*, 2001; Christy *et al.*, 2003; Brem *et al.*, 1998) although the cellular localisation is controversial. Since 11β-HSD1 has been shown to have a role in regulating angiogenesis *in vitro* and *in*

vivo (Small *et al.*, 2005), determining the source of this activity in the vessel wall is important. Studies have demonstrated expression of both isozymes in primary human vascular SMC cultures (Cai *et al.*, 2001) and in primary cultures of rat aortic endothelial cells (Brem *et al.*, 1998) whereas histological studies in rat vessels suggest that immunoreactivity for 11β-HSD1 is restricted to vascular smooth muscle (Walker *et al.*, 1991). Other studies have revealed that 11β-HSD2 is localised to the endothelium in human renal artery (Kataoka *et al.*, 2002) and in mouse aortic media (Miss Danielle Armour, unpublished findings). Direct comparison of studies is difficult, given the use of vessels from different species and anatomical locations combined with a variety of techniques for detecting 11β-HSDs. Another confounding factor to explain disparities in expression is that they may vary according to culture conditions. For instance, the expression of 11β-HSD1 in hepatocytes growing in culture is sensitive to culture conditions (Jamieson *et al.*, 1995). Furthermore, 11β-HSD1 is induced upon differentiation of human monocytes into macrophages (Thieringer *et al.*, 2001) and in adipocytes (Napolitano *et al.*, 1998). This indicates that 11β-HSD expression, and activity, in isolated cells growing in culture may vary depending on culture condition. Thus it was necessary to determine 11β-HSD status in the TLS assay prior to embarking on studies exploring the effects of substrates for these enzymes (*i.e.* glucocorticoid effects).

In the present study neither of the 11β-HSD isozymes was expressed or active in undifferentiated HUVECs and the expression and activity profiles did not change upon differentiation of cells into mature TLSs. In contrast, stably transfected CHO cells acted as robust positive controls with reductase and dehydrogenase activity detected in line with previous *in vitro* studies from our department (Dr Scott Webster, personal communication). A published study demonstrated 11β-reductase activity in cultured vascular smooth muscle cells incubated with substrate over the same time period (Cai *et al.*, 2001). The finding that 11β-HSD1 acted as both a reductase and dehydrogenase in transfected CHO cells is not surprising given that this has been previously reported (Bujalska *et al.*, 2005). Indeed, in most cell models, stably transfected with human 11β-HSD1 cDNA into HEK293 cells

(Bujalska *et al.*, 1997) or transiently transfected with rat 11β-HSD1 into CHO cells (Agarwal *et al.*, 1989), both reductase and dehydrogenase activity were reported. Further, there is some evidence that 11β-HSD1 has bi-directional capability in intact vascular tissue; 11β-dehydrogenase was detected in endothelium-denuded aorta and smooth muscle cells apparently devoid of 11β-HSD2 expression (Brem *et al.*, 1995). Absence of expression of 11β-HSDs from isolated human circulating endothelial precursor cells in the present study is consistent with these findings from isolated resident endothelial cells. This suggests that the expression of these isozymes is not determined by developmental stage. However, there are data to suggest that activity of 11β-HSDs in the vasculature is territory-dependent as higher activity was reported in rat mesenteric arteries compared with aorta (Walker *et al.*, 1991). Consequently, it will be important in future studies to validate these findings using human endothelial cells from other physiologically relevant microvascular sources (*e.g.* human dermal microvascular endothelial cells, HuDMECs).

A role for intra-vascular generation of endogenous glucocorticoids in modulation of angiogenesis was indicated recently by our demonstration that 11β-HSD1 in vascular smooth muscle catalyses local production of active glucocorticoid (Dover *et al.*, 2007; Small *et al.*, 2005). This process was maintained *in vivo* and influenced the angiogenic response to healing in the myocardium and cutaneous wounds (Small *et al.*, 2005). It was not clear, however, whether glucocorticoids generated by medial 11β-HSD1 were inhibiting angiogenesis by an autocrine action on the vascular smooth muscle or a paracrine action on the endothelium. In the present study, a lack of 11β-reductase activity in endothelial TLSs suggests that 11β-HSD1 regulates angiogenesis by active glucocorticoids generated by another cell type in the vessel wall such as neighbouring SMCs (Cai *et al.*, 2001). Indeed, a lack of endogenous glucocorticoid-generating capacity in endothelial tubes may negate a possible role for this isozyme in regulating angiogenesis autonomously in the endothelium. Alternatively, activated macrophages have also been shown to express 11β-HSD1 (Thieringer *et al.*, 2001) suggesting that generation of active glucocorticoids within these cells may contribute to regulation of inflammation. However, these findings could not be confirmed in an *in vivo* setting (Dover *et al.*, 2007). These are

hypotheses that need to be addressed more rigorously and future projects are planned to adapt the TLS model to a co-culture system. Alternatively, HUVECs are amenable to transfection (Ear *et al.*, 2001) and therefore11β-HSD activity could be added into the model in this way. In summary, 11β-HSDs are not expressed in this system and therefore the effects of glucocorticoids could be dissected without the confounding factor of local metabolism, but the role of 11β-HSDs could not be dissected further in this model.

3.4.3 Influence of glucocorticoids on TLS formation

Prior to testing the effects of glucocorticoids in this system, assessment of the responsiveness of the model was demonstrated first of all using pro-angiogenic stimuli as positive controls. VEGF and prostaglandins all reproducibly stimulated TLS formation in keeping with their physiological role in stimulating angiogenesis *in vitro* and *in vivo* (Bussolati *et al.*, 2001; Ferrara & Henzel, 1989; Majima *et al.*, 2000; Tamura *et al.*, 2006). The pro-angiogenic effects of prostaglandins may be due to enhanced VEGF synthesis which has been demonstrated *in vitro* (Pai *et al.*, 2001; Fukuda *et al.*, 2003) and *in vivo*. Thus, since the precursor to endogenous prostaglandin synthesis, (COX-2) is down-regulated by glucocorticoids (Crofford *et al.*, 1994; Ghosh *et al.*, 2000); this may represent part of the angiostatic molecular mechanism of glucocorticoids. The stimulatory effect of VEGF on TLS formation was observed after only 4 to 5 hours of incubation compared with both prostaglandins which exerted maximal effects at the later time-point of 20 to 23 hours incubation. Differing modes of action to induce TLS formation may underpin the difference in timescales. TLS formation was consistently enhanced by 3 different angiogenic factors demonstrating the responsiveness of the model to exogenous stimuli and further validating the quantification method.

Physiologically-relevant concentrations of cortisol inhibited TLS formation (Figure 3.15), demonstrating that cortisol has a direct inhibitory effect on endothelial tube formation in the absence of peri-vascular cell types. Glucocorticoid-mediated inhibition of VEGF-induced TLS formation (Figure 3.17) suggests an inhibitory

effect on the downstream signalling events elicited by the action of VEGF on the endothelium and supports a previous study which demonstrated a role for glucocorticoids inhibiting VEGF-induced calcium flux in HUVECs (Dr Gary Small, unpublished findings). Cortisol blocked VEGF effects in order that, in the presence of cortisol, VEGF had no effect. Consequently, there may be elements of cortisol action which are VEGF independent, or alternatively, that there is endogenous VEGF being blocked by cortisol. These angiostatic actions of cortisol in the TLS assay are consistent with, and extend, the angiostatic effects of the rodent equivalent, corticosterone (30 to 600 nM), in the aortic ring model (Small *et al.*, 2005; Nicosia & Ottinetti, 1990) and in a model of cutaneous wound repair (Beer *et al.*, 2000). Preliminary data from the cytoskeleton studies would suggest that glucocorticoids may alter the cytoskeleton of endothelial TLSs; however, further investigation is required to confirm this. This hypothesis is supported by the literature in endothelial cell monolayers (Criscuolo & Balledux, 1996) and interfering with microfilaments or microtubules suppresses key angiogenic cellular responses including migration and rearrangement into TLSs (Grant *et al.*, 1991; Shen *et al.*, 2007). However, a direct effect of glucocorticoids on the cytoskeleton of TLSs has not been reported thus far and would be a novel finding.

From the initial observation of endothelial cells exposed to pharmacological concentrations of steroids a description of cellular "rounding-up" with retraction of cytoplasmic extensions and detachment from the basement membrane was reported (Folkman & Ingber, 1987). A patho-physiological concentration (1.2 μM) of cortisol did not exceed the magnitude of inhibition in the present study using 600 nM and is consistent with studies in the chick chorioallantoic membrane (CAM) assay in which various steroids generated bell-shaped concentration-response curves (Crum *et al.*, 1985). In addition, findings from the CAM assay (Crum *et al.*, 1985), mouse aortic rings (Small *et al.*, 2005), and the data presented in this chapter all support a role for glucocorticoid suppression of angiogenesis independent of inflammation and immunity. The inhibitory effect of physiological glucocorticoids reported here is significant as it correlates with human fasting plasma cortisol levels (Wake *et al.*, 2003). Although the angiostatic effect of endogenous glucocorticoids in human

pathophysiology has not been investigated, impaired wound healing (an angiogenesis associated process) is seen in Cushing's disease patients with excess endogenous glucocorticoids (Gordon *et al.*, 1994) and in patients on extended glucocorticoid therapy (Green, 1965).

In contrast to the angiostatic effect of cortisol, the inability of cortisone at similar concentrations to inhibit TLS formation contrasts with the angiostatic effects seen in *in vivo* angiogenesis models (Folkman *et al.*, 1983; Maragoudakis *et al.*, 1989; Small *et al.*, 2005). A likely explanation for this is due to lack of 11β-HSD1 activity in TLSs and is further evidence that active glucocorticoids are not generated in the endothelium. Cortisone *does* inhibit VEGF production in cultured human SMCs (Nauck *et al.*, 1998), which also possess 11β-HSD1 activity (Cai *et al.*, 2001). Consequently cortisone may confer angiostasis in the vessel wall following regeneration to cortisol, by 11β-HSD1, and blocking the action of VEGF.

Cortisol may inhibit tube formation via GR or MR, both of which may be expressed in endothelial cells. GR are expressed in endothelial cells (Yang & Zhang, 2004) but levels may vary between endothelial cell types (Inoue *et al.*, 1999). MR expression has previously been reported in HUVECs (Oberleithner *et al.*, 2003) but the expression of MR in TLSs is not clear and would be worth checking in future studies. Expression of GR was confirmed in the present study in undifferentiated HUVECs and expression was not altered upon differentiation into mature TLSs. At relevant concentrations neither GR nor MR antagonism influenced TLS formation, consistent with previous reports (Yamamoto *et al.*, 1994). The angiostatic effect of glucocorticoids on TLS formation appears to be mediated via GR since inhibition occurred with the GR selective glucocorticoid dexamethasone. The receptor antagonism studies were also indicative of GR dependency since the inhibitory effect of cortisol was reversed by GR antagonism. However, since MR antagonism also tended to reverse the cortisol effect, albeit not statistically significantly, MR may also be involved and further studies aimed at elucidating its presence in TLSs will be required to resolve this issue. Since progestogens were not added to the assay, the progesterone receptor-blocking effects of RU38486 (Schreiber *et al.*, 1983) cannot

account for this mechanism. The GR-mediated inhibition of endothelial tube formation is of pathophysiological significance since wound repair is impaired in mice with a defective GR mutation (Grose *et al.*, 2002).

3.4.4 Conclusion

The data in this chapter provide evidence for the establishment of an *in vitro* assay of endothelial tube formation as a useful tool to examine the influence of glucocorticoids on the endothelium and to dissect angiostatic mechanisms independently of systemic anti-inflammatory and metabolic changes. Given that 11β-HSD activity is absent from this system, the influence of exogenous glucocorticoids at physiologically relevant concentrations could be faithfully tested. These studies demonstrated that endogenous and synthetic glucocorticoids inhibit endothelial tube formation and that this effect occurs via glucocorticoid receptors. This may represent one of the key components of glucocorticoid-mediated angiostasis *in vivo* and in pathological situations. The TLS model will serve as a useful tool to examine the molecular signalling pathways through which glucocorticoids inhibit endothelial tube formation.

Chapter 4

Influence of Glucocorticoids on Molecular Signalling During Endothelial Tube Formation

4.1 Introduction

The demonstration that glucocorticoids inhibit tube formation by endothelial cells *in vitro* (Chapter 3) suggests that their anti-angiogenic action *in vivo* may be due, at least in part, to direct interaction with the endothelium. These studies have not shown, however, the mechanism(s) through which this glucocorticoid receptor-dependent inhibition is mediated. A careful review of the literature suggests that regulation of angiogenesis by glucocorticoids is due to: effects on growth factor production and their receptors, inhibition of endothelial cell-ECM interactions, degradation of the basement membrane, and/or effects on the arachidonic acid cascade (Section 1.3.2). Moreover, the time-lapse studies from Chapter 3 highlight altered VEGF signalling as a likely mechanism since VEGF loses its stimulatory effect in the presence of glucocorticoids (Section 3.3.1.5). In addition, the literature suggests that altered intracellular signalling cascades might play a role.

4.1.1 VEGF signalling

Glucocorticoids inhibit TLS formation in the presence of VEGF (Chapter 3) and this is consistent with other studies in the literature describing glucocorticoid effects on angiogenesis. Glucocorticoids can influence VEGF production (Nauck *et al.*, 1998), stability (Gille *et al.*, 2001) and action (Matsuda *et al.*, 2005; Wu *et al.*, 2006). In isolated HUVECs, VEGF-induced intracellular calcium flux was inhibited by cortisol treatment (Small *et al.*, unpublished findings) suggesting a role in glucocorticoid-mediated angiostasis. *In vivo*, patterns of expression of VEGF and VEGF receptors in steroidogenic, and steroid responsive, tissues in mice, suggest a role in hormonally-regulated angiogenesis *in vivo* (Shweiki *et al.*, 1993). Also, in various disease models, glucocorticoid treatment suppresses VEGF production (Pufe *et al.*, 2003; Luo *et al.*, 2004).

Notch signalling plays a fundamental role in the determination of cell fate (*e.g.* cell proliferation or differentiation) (Artavanis-Tsakonas *et al.*, 1995) and is known to be involved in VEGF signalling. The Notch pathway is highly conserved and is known to play important roles in development in both invertebrates and in vertebrate tissues,

recently reviewed by Roca & Adams, 2007. However, the angiogenic signalling that controls Notch and its ligands' activity is only partially understood (Liu *et al.*, 2003). A series of studies have shown that one ligand for the Notch receptors, Dll4, is normally induced by VEGF and is a negative-feedback regulator that restrains vascular sprouting and branching (Hellstrom *et al.*, 2007; Siekmann & Lawson, 2007; Leslie *et al.*, 2007). Consistent with this role, the deletion or inhibition of Dll4 results in excessive, non-productive angiogenesis in tumours (Thurston *et al.*, 2007).

4.1.2 Endothelial cell-ECM interactions

The extracellular matrix (ECM) provides guidance cues for the establishment of endothelial tubes in the absence of cell-cell contacts via the matrix-integrin-cytoskeletal signalling axis (Davis & Senger, 2005). The involvement of particular integrins is dependent on the matrix environment to which endothelial cells are exposed (Davis *et al.*, 2002). In laminin-rich matrices, such as Matrigel, $\alpha 6$ and $\beta 1$ subunits are thought to play a critical role in regulating tube formation (Bauer *et al.*, 1992; Davis & Camarillo, 1995; Da Silva *et al.*, 2003). It is not entirely clear from the literature how expression of integrins translates into cues for endothelial tube formation but over-expression of $\beta 1$ subunits, when unligated, is enough to induce apoptosis (Stupack *et al.*, 2001).

Matricellular proteins, such as TSP-1 are secreted proteins that influence cell function by modulating cell-matrix interactions (Bornstein, 2001). TSP-1, and fragments of TSP-1, can act directly on endothelial cells to inhibit angiogenesis by interactions with cell surface proteins CD36 (a member of the class B scavenger receptor family of proteins), CD47 (which is involved in the increase of intracellular calcium upon cell adhesion to the ECM) and integrins on the surface of the cell. Binding of TSP-1 to CD36 mediates inhibition of endothelial cell migration (Dawson *et al.*, 1997) and tube formation (Iruela-Arispe *et al.*, 1991; DiPietro *et al.*, 1994) and leads to induction of apoptosis (Jiménez *et al.*, 2000).

Caveolins are prominently expressed in most adherent mammalian cells and their main function is in the formation of invaginations, termed caveolae, in the plasma membrane (Williams & Lisanti, 2004). Caveloae have a role in signal transduction (via the ERK-MAPK pathway) and caveolin-1 has been shown to play a role in integrin signalling (Wary *et al.*, 1998) and is, therefore, important in cell-matrix interactions. Recent studies have proposed a role for caveolin-1 in the regulation of angiogenesis; over-expression of caveolin-1 accelerates capillary tube formation by endothelial cells in 3D models (Liu *et al.*, 2002) and *vice versa* (Griffoni *et al.*, 2000). However, it is not fully understood what regulates caveolin expression. Glucocorticoids induce caveolin-1 expression and the formation of caveolae, via GR, in isolated epithelial cells (Barar *et al.*, 2007), but no study to date has investigated glucocorticoid effects on caveolin-1 expression in the endothelium.

4.1.3 Intra-cellular signal transduction

Intra-cellular signal transduction pathways convey the necessary signals which determine the final behaviour of endothelial cells during angiogenesis; including proliferation, migration and differentiation (Muñoz-Chápuli *et al.*, 2004).

4.1.3.1 Focal adhesion kinase

FAK is a cytoplasmic tyrosine kinase that plays a key role in integrin-mediated signal transduction (Parsons, 2003) and has an important role in endothelial cell activities associated with angiogenesis including proliferation, migration and differentiation (Ilic *et al.*, 2003; Shen *et al.*, 2005). Members of the FAK family link transmembrane glycoproteins to the actin cytoskeleton and, therefore, have a crucial role in mediating cell shape and motility induced by cell-matrix interactions. Other studies have implicated FAK in the transduction of soluble signals including VEGF (Qi & Claesson-Welsh, 2001). There is a limited literature on rapid, non-transcriptional activation of FAK by glucocorticoids (Koukouritaki *et al.*, 1999; Koukouritaki & Lianos, 1999) while other studies have demonstrated that glucocorticoids attenuate cytokine induced FAK expression (Jee *et al.*, 2007), at least in non-endothelial cell types.

4.1.3.2 Mitogen-activated protein kinases

Further downstream of receptor tyrosine kinases and the FAK relay, multiple signals from transmembrane receptors (such as VEGF-R and integrins) form cascades which eventually lead to gene transcription and a network of cross-talks that ultimately determine cellular activity. The Ras-ERK-MAPK pathway is of particular importance in the transduction of differentiation/morphogenesis signals in endothelial cells (Meadows *et al.*, 2001) and involves a series of phosphorylation steps which confer activity of the pathway. Since previous studies have shown that the phosphorylation state of ERK1/2 is reduced during endothelial tube formation, in the absence of significant changes in their transcript levels (Grove *et al.*, 2002), post-translational modification is just as important for investigating influence of glucocorticoids on angiogenesis as changes in gene transcription. Also, glucocorticoid-mediated inhibition of intracellular calcium flux in HUVECs (Small *et al.*, unpublished findings) implicates the MAPK pathway since calcium flux is known to play an important role in the activation of ERK/MAPK signalling (Munaron, 2002; Kupzig *et al.*, 2005).

4.1.4 Regulation of angiogenic molecular signalling by glucocorticoids

Glucocorticoids exert some of their actions through binding to receptors that are hormone-regulated transcription factors that may activate or repress gene expression (Webster & Cidlowski, 1999). A careful review of the existing literature suggested that GC-mediated inhibition of angiogenesis is due to a reduced generation or action of pro-angiogenic factors (*e.g.* VEGF), increased activation of angiostatic factors (*e.g.* TSP-1, Notch signalling) and/or altered endothelial cell-extracellular matrix (ECM) interactions (*e.g.* integrins, caveolae). A relatively new concept of non-transcriptional action of glucocorticoids has emerged where activated GR initiate signalling without a direct effect on gene transcription (Limbourg & Liao, 2003) and inhibition of MAPK signalling (*e.g.* ERK, JNK) has been implicated in the regulation of angiogenesis.

Previous investigations of glucocorticoid effects on angiogenic factor gene expression have tended to use; pharmacological, synthetic glucocorticoids (*e.g.* dexamethasone) (Wu *et al.*, 2006); a variety of human (Tong *et al.*, 2006) and non-human (Machein *et al.*, 1999), endothelial (Gallicchio *et al.*, 2006) and non-endothelial cell types (Wu *et al.*, 2006). Furthermore, these previous studies were limited to isolated, proliferating cells growing on tissue culture plastic. These studies demonstrated that glucocorticoids tended to decrease the synthesis of pro-angiogenic factors such as VEGF (Koedam *et al.*, 2002; Tong *et al.*, 2006), whilst they induce expression of angiostatic factors such as TSP-1 (Flugel-Koch *et al.*, 2004) and tissue inhibitors of metalloproteases (TIMPs) (Forster *et al.*, 2007); thus shifting the balance of factors in the direction of angiostasis. Similarly, pharmacological levels of synthetic glucocorticoids have been suggested to inhibit activation of MAPK proteins (JNK, ERK and p38) (Pelaia *et al.*, 2001; González *et al.*, 1999) in isolated endothelial cell monolayers.

Together, these studies have given some insight into the molecular mechanism(s) of action of glucocorticoids in the regulation of angiogenesis. It is not clear, however, whether regulation of transcription of these factors, or regulation of activated protein levels can account for glucocorticoid-mediated inhibition of endothelial tube formation and angiogenesis. Lastly, it is not clear whether any modulation of molecular signalling, in this way, is dependent on glucocorticoid receptors.

4.1.5 Hypothesis

The investigations described in this chapter address the hypotheses that glucocorticoids inhibit endothelial tube formation by down-regulation of the expression of pro-angiogenic factors (VEGF, VEGF-R2, α6-integrin, caveolin-1, FAK, ERK, p38, JNK and Akt) and/or up-regulation of the expression of angiostatic factors (TSP-1, Notch-1 and Dll4). These studies also addressed the hypothesis that glucocorticoid-mediated changes in endothelial gene, and protein, expression levels are mediated via GR.

4.1.6 Aims

The specific aims were:

1) to determine whether glucocorticoids alter gene expression of VEGF, VEGF-R2, TSP-1, α6-integrin, Notch-1, Dll4 and caveolin-1 during endothelial TLS formation.
2) to investigate the influence of glucocorticoids on activation of FAK, ERK, p38, JNK and Akt proteins in endothelial cell monolayers to provide a tool for investigating signal transduction in endothelial TLSs.

4.2 Methods

4.2.1 Influence of glucocorticoids on angiogenic factor gene expression in TLSs

The influence of glucocorticoids on the expression of angiogenic factors during TLS formation was examined using quantitative real-time PCR (QrtPCR). This required recovery of tube-like structures from Matrigel, isolation of RNA, reverse transcription and QrtPCR using primers for genes of interest. Since few studies to date have addressed modulation of gene expression in endothelial TLSs, some method development was necessary. Pharmacological GR antagonism was used to confirm, and extend, the demonstration (Chapter 3) that glucocorticoid-mediated inhibition of TLS formation is dependent on GR binding.

4.2.1.1 TLS preparation and glucocorticoid treatment

Initial experiments demonstrated that using the 24-well plate set-up for TLS formation did not yield sufficient cells for isolation of RNA. Consequently, TLS formation was expanded to enable tube generation in a 35 mm dish. Briefly, HUVECs (200,000) in 2 ml of standard basal medium were seeded on 35 mm cell culture dishes pre-coated with Matrigel (500 µl) and incubated in the presence of cortisol (600 nM), cortisol plus RU38486 (1 µM), or left untreated (control). RNA was recovered using Matrisperse solution (Section 2.4.7.1) after incubation for 1, 4, 8 or 22 hours to examine both the time-course of gene expression in TLSs and effects of treatment on the expression of these genes. Trizol (1 ml) was added to the cell pellet and the contents transferred to a cryovial for storage at -80°C until required.

4.2.1.2 RNA extraction and reverse transcription

RNA extraction (Section 2.4.7.2) and reverse transcription (Section 2.4.7.3) were performed as described previously. Due to the large number of samples, RNA extraction was performed on batches of up to 40 samples on 2 separate occasions to prevent degradation of RNA. Only RNase-free, sterile solutions and equipment were used in order to prevent degradation of target RNA by exogenous RNases and contamination of reactions with exogenous RNA or DNA. Briefly, first strand

cDNA synthesis was performed using 0.5 μg of RNA with the Reverse Transcription System (Promega, UK) in a reaction containing 5 mM $MgCl_2$, 1x reverse transcription buffer, 1 mM each of dATP, dCTP, dGTP and dTTP, 20U RNasin, 0.5 μg oligo$(dT)_{15}$ primers and 15U AMV-Reverse Transcriptase. Oligo$(dT)_{15}$ primers were used to prime RT reactions, rather than short random oligonucleotides to ensure that poly (A) containing mRNAs were selectively reverse transcribed; thereby ensuring less variation in the QrtPCR results (Hembruff *et al.*, 2005). The reactions were carried out on an Eppendorf Mastercycler Gradient (Eppendorf, Germany) consisting of incubation at 42°C for 45 minutes followed by 95°C for 5 minutes and finally chilled to 4°C. Negative control reactions (no RT enzyme; no RNA) were performed in parallel for each batch of samples. Products from the RT reaction (cDNA) were diluted 1:4 with nuclease-free water prior to Qrt-PCR amplification based on reaction kinetics of serially-diluted, pooled samples from the gene expression experiment. Prior to running large numbers of samples on real-time plates, randomly selected cDNA samples and negative controls, were run on standard PCR using Tie2 primers (Table 2.1) to establish quality of cDNA and determine potential contamination.

4.2.1.3 Quantitative real-time polymerase chain reaction

QrtPCR was used to determine the relative concentration of mRNA present in samples using primers for genes of interest (Table 2.2) as detailed in Section 2.4.8. Cyclophilin A was used as an internal (loading) control (Horiuchi *et al.*, 2006; Maresh *et al.*, 2004). TaqMan primer probe mixes were supplied as pre-validated ready-to-use assays. All assays were designed for use with human tissue, were exon spanning and will not detect genomic DNA. All probes had a FAM reporter dye at the 5' end and a non-fluorescent quencher at the 3' end and, consequently, required protection from light throughout the procedure.

Briefly, 2 μl of cDNA template from each sample, pooled standard dilutions (prepared fresh after 2 to 3 freeze-thaw cycles), and negative controls were added to a 384-well plate (Roche, Switzerland) in triplicate wells. Primer-probe master mix was prepared by mixing a 5 μl/sample of LightCycler 480 Probes Master (Roche)

with 0.5 µl/sample of TaqMan MGB primer probe mix plus 2.5 µl/sample of water. Master mix (8 µl/sample) was added to each well using a digital multi-channel pipette to minimise variation in volume added. Thus, the final volume in each well was 10 µl. PCRs were carried out on a LightCycler 480 (Roche) as detailed previously (Section 2.4.8.4).

To determine the concentration of target DNA in unknown samples after PCR amplification, absolute quantification analysis was used (as described in Section 2.4.8.5). Data were analysed by LightCycler 480 software v1.2.0.169 (Roche). By determining where the Cp value of an unknown sample falls on the standard curve, the initial concentration of target DNA in the samples were determined by the software. Sample concentrations, expressed as a mean of triplicates, were exported to Excel and normalised to corresponding cyclophilin A concentrations, as a ratio, which served as an internal control.

4.2.2 Influence of glucocorticoids on VEGF protein production during TLS formation

Preliminary investigations in the Endocrinology Unit (University of Edinburgh), using a VEGF ELISA kit, demonstrated production of soluble VEGF ($VEGF_{121}$ and $VEGF_{165}$) protein by mouse aortic rings cultured in Matrigel (Small *et al.*, unpublished findings). The possibility that glucocorticoids inhibit TLS formation by inhibiting VEGF production by endothelial cells was addressed using a similar approach. Briefly, HUVECs (4×10^4 cells/ml) in standard basal medium (EGM-2 with no growth factors or FBS) in the presence of cortisol (600 nM), vehicle (ethanol, 0.0006% v/v) or untreated (control) were seeded directly onto uncoated, and Matrigel-coated, 24-well tissue culture plates. Conditioned media were collected 1, 5 and 22 hours after cell seeding, and soluble VEGF protein levels determined using a human VEGF ELISA kit (Calbiochem-Merck, UK) according to the manufacturer's instructions. Plates were read using a spectrophotometric plate reader at dual wavelengths of 450 nm and 540 nm (MRX, Dynatech Laboratories, UK).

4.2.3 Influence of glucocorticoids on activation of signal transduction proteins in endothelial cells

The influence of glucocorticoids on signal transduction pathways in endothelial cells was assessed using a biochemical approach. Western blotting was used to detect FAK and phosphorylated (activated) ERK (pERK) in HUVEC protein extracts to examine the influence of cortisol, GR antagonism (RU38486) and an established inhibitor of MAPK signal transduction (PD98059). Training in methodology and antibodies were provided by Dr Kurt Sales, MRC Human Reproductive Sciences Unit, Edinburgh).

4.2.3.1 Optimisation of culture conditions

Developing an *in vitro* system with which to determine the effects of glucocorticoids on signal transduction in endothelial cells required extensive development of existing methodology being used in endometrial carcinoma (Ishikawa) cells (Sales *et al.*, 2005). To reduce background MAPK activity, serum withdrawal from culture medium is routine prior to stimulation of cells (Russell *et al.*, 2000; Wang *et al.*, 2002a). Furthermore, since confluence of isolated endothelial cells in culture induces cell cycle exit by inhibiting MAPK activity (Viñals & Pouysségur, 1999), sub-confluent cultures were required.

Pilot experiments revealed that complete serum starvation overnight (serum-free conditions) resulted in prolific cell death and poor protein yields. Consequently, HUVECs were harvested and initially cultured in standard culture medium (EGM-2 containing growth factors, 2% FBS, ascorbic acid, heparin and GA-1000) for 24 hours to allow cells (1×10^6 cells per dish) to adhere. The following day medium was replaced with EGM-2 containing 1% FBS, ascorbic acid, heparin and GA-1000 to provide serum-reduced conditions for a further 24 hours incubation. Forty-eight hours after cell seeding, medium was replaced with standard basal medium (EGM-2 with no growth factors or FBS) to provide serum-free conditions for 1 hour prior to treatment and for the duration of the experiment. At this stage, dishes were approximately 90% confluent which resulted in sufficient protein recovery to run

Western blots. During the refinement of the final protocol, it was necessary to vary the number of cells, size of cell culture dish, and volume of lysis buffer to provide sufficient protein for analysis. The final conditions are described in Section (Section 2.4.10.1).

4.2.3.2 Time-course of effects of glucocorticoids on activation of signal transduction proteins

To determine the effects of glucocorticoids on phosphorylation of signal transduction proteins, HUVECs were treated (on day 3 following serum reduction and serum starvation) with cortisol (600 nM), or vehicle (ethanol; 0.004% v/v) for 5, 10, 15, 30, 60, 120, 180 and 240 minutes. Incubation times were consistent with MAPK signalling studies in Ishikawa (Sales *et al.*, 2005) and endothelial (González *et al.*, 1999; Pelaia *et al.*, 2001) cells.

4.2.3.3 ERK1/2 activation by glucocorticoid treatment and blockade by pathway and receptor inhibitors

To further dissect the mechanism through which cortisol stimulates phosphorylation of p44/p42 MAPK (ERK1/2) activity, HUVECs were pre-treated for 1 hour with the GR antagonist, RU38486 (1 μM), or with the specific p44/p42 MAPK (MEK) inhibitor, PD98059 (50 μM), for 1 hour prior to stimulation with cortisol (600 nM) for a further 4 hours. For negative control, cells received either inhibitor or antagonist alone for 5 hours. As a further control, cells received cortisol (600 nM) or vehicle (ethanol, 0.004% v/v), for 4 hours, to validate the previous time-course findings.

4.2.3.4 Western blotting for signal transduction proteins

Protein extraction Following treatment with cortisol and/or inhibitors, protein was extracted from cell monolayers as described previously (Section 2.4.10.2). Briefly, 200 μl of fresh lysis buffer (containing a cocktail of protease inhibitors; Complete Protease Inhibitor Tablet, Roche, Switzerland) were added to each dish (kept on ice throughout) to prevent protein degradation by endogenous proteases.

Cells were detached from dishes using a cell scraper to aid cell lysis. Thereafter, insoluble material was pelleted by centrifugation. The clarified lysate was removed to a new tube and stored at -20°C until required for protein quantification and SDS-PAGE.

Protein quantification To ensure equal gel loading, protein concentration was measured using the Bio-Rad D_C protein assay kit (Bio-Rad, UK), based on the method of Lowry (Lowry *et al.*, 1951), as described previously (Section 2.4.10.3).

Immunoprecipitation For focal adhesion kinase (FAK) studies, in which the protein is of low abundance in cells, immunoprecipitation was used to enrich phosphotyrosine-containing proteins in lysates (Section 2.4.10.4). Briefly, lysates were first pooled from treatment groups to provide sufficient protein for analysis (from 9 experiments). Equal amounts of total protein in lysis buffer were incubated (overnight) with specific mouse monoclonal IgG_{2b} anti-phosphotyrosine antibody, p-Tyr (PY99) (Santa Cruz Biotechnology, USA) immobilised onto agarose beads. Beads were washed extensively with lysis buffer and immune complexes were eluted in 20 µl of 5x protein gel loading buffer, boiled for 5 minutes, then kept on ice until required for gel electrophoresis.

Gel electrophoresis Pilot experiments revealed that loading of 15 and 20 µg of protein did not result in detectable bands. Consequently, a total of 30 µg protein were resolved (Section 2.4.10.4) on NuPAGE 4-12% Bis-Tris gels (Invitrogen, UK). SeeBlue Pre-stained Protein Standard (Invitrogen) was loaded into the first well on each gel to allow determination of sample protein molecular weight. Gels were electrophoresed at 40 mA per gel for approximately 2 hours until the loading dye front had migrated to the bottom of the gel.

Transfer In order to make proteins accessible to antibody detection, they were moved from within the gel to an Immobilon-FL polyvinylidene difluoride (PVDF) membrane (Millipore, UK) by electroblotting (Section 2.4.10.4). Briefly, stacks were assembled comprising; 3 layers of Whatman chromatography blotting paper

(Fisher Scientific) pre-soaked in transfer buffer, wetted PVDF membrane, gel (containing protein), and finally, another 3 layers of pre-soaked blotting paper. Care was taken to avoid air bubbles between layers during assembly. Stacks were blotted at a constant voltage (14 V for 2 hours) using a semi-dry blotting unit (Scie-Plas).

Secondary probing and chemiluminescence-based detection Initially, proteins of interest were detected using a multi-step chemiluminescent-based method as previously described (Sales *et al.*, 2004). Briefly, gels were blotted onto a PVDF membrane (Immobilon-P, Millipore, UK) and blocked for 1 hour in 4% BSA in TBS Tween and 0.1% sodium azide. Blots were then incubated with primary antibodies against phosphorylated (activated) forms of MAPK subgroups; p44/p42 (ERK1/2), p38 and c-jun N-terminal kinase (JNK), and the cell survival protein, protein kinase B (Akt). All primary antibodies were diluted 1:1000 (in 4% BSA) and were made in rabbit. Membranes were washed (in TNS Tween) then incubated with secondary antibody (1:30,000, goat anti-rabbit alkaline phosphatase, Sigma, UK). Membranes were washed again, then immunoreactive proteins were visualised using the ECF chemiluminescence system (GE healthcare, UK) following the manufacturer's instructions. Proteins were revealed and quantified by Typhoon 9400 PhosphorImager analysis (Amersham Biosciences, UK). Blots were then "stripped" to remove immune complexes and substrate (in 40% methanol for 1 hour followed by 45 minutes in stripping buffer on rocker) before being re-probed with primary antibodies (1:1000, made in rabbit) against total ERK1/2, p38, JNK and Akt to act as a loading control. Blots were then incubated with the same secondary antibody as before, and detected in the same manner. Target protein bands; phosphorylated ERK1/2, p38, JNK and Akt, were determined from the relative mobility on SDS-PAGE by comparison with molecular weight standards and were normalised to total ERK2, p38, JNK and Akt proteins, as a ratio, using the STORM 860 system (Molecular Dynamics, Amersham Pharmacia Biotech, Little Chalfont, UK).

Two-step Fluorescence-based detection During the present studies, a move towards a fluorescence-based method was made where the secondary antibody is directly conjugated to a fluorescent probe. Since primary antibodies made in

different species are available, the protein of interest and the loading control can be detected simultaneously; thereby removing the membrane-stripping and re-probing stages. A two-step detection method was used to detect target proteins as detailed in Section 2.4.10.4. Briefly, gels were blotted onto a PVDF membrane (Immobilon-FL, Millipore, UK) and blocked in Odyssey blocking buffer (Li-Cor Biosciences, UK) before being incubated with primary antibody (1:1000). For FAK studies, rabbit polyclonal IgG anti-FAK (C-20) antibody (Santa Cruz Biotechnology, USA) was used. For MAPK studies, membranes were co-incubated with rabbit polyclonal IgG anti-phospho-p44/p42 MAPK and mouse polyclonal IgG anti-p42 MAP kinase (3A7) (both Cell Signaling Technologies, USA). Membranes were washed (in TNS Tween) then incubated with secondary antibody (1:5000). For FAK studies, goat anti-rabbit IgG (H+L) Alexa Fluor 680 (Molecular Probes-Invitrogen, UK) was used. For MAPK studies, membranes were co-incubated with goat anti-rabbit IgG (H+L) Alexa Fluor 680 and goat anti-mouse IgG (H+L) IR dye 800 (Rockland Immunochemicals, USA). Membranes were washed again and proteins were revealed and quantified by Odyssey Li-Cor Infrared Imager. Target protein bands, phosphorylated ERK1/2 and FAK were determined from the relative mobility on SDS-PAGE by comparison with molecular weight standards and were normalised as a ratio to total ERK2 protein and IgG, respectively, using Odyssey v2.1 software (Li-Cor Biosciences).

4.2.4 Statistics

Data are expressed as mean ± standard error. "*n*" refers to number of different experiments performed on separate occasions using different batches of HUVECs. For ELISA and QrtPCR studies, all conditions were performed in triplicate wells. Comparisons at selected timepoints were made by one-way ANOVA and Dunnett's post hoc tests. Comparisons of treatment with time were made by two-way ANOVA.

4.3 Results

4.3.1 Influence of glucocorticoids on angiogenic factor gene expression in endothelial tube-like structures

4.3.1.1 Cyclophilin A expression is not modulated by treatment but does change with time

Expression of cyclophilin A mRNA was not modulated significantly by treatment with cortisol (600 nM) but there was a trend towards a decrease in expression after 8 hours (Figure 4.1). Cyclophilin A expression decreased with time in treatment and control groups (p=0.005, two-way ANOVA, effect of time). Changes in expression levels with time of all of the angiogenic factors examined were observed (TSP-1, p<0.05; α6-integrin, p<0.005, Figure 4.2; VEGF, p<0.05; VEGF-R2, p<0.0001, Figure 4.3; Notch-1, p<0.05; Dll4, p=0.0001, Figure 4.4 and caveolin-1, p<0.01, Figure 4.5).

4.3.1.2 Cortisol up-regulates expression of thrombospondin-1 during TLS formation

QrtPCR measurements indicated a significant (p<0.05) 2.3-fold increase in thromobospondin-1 mRNA level in TLSs 8 hours following treatment with cortisol (600 nM) (Figure 4.2A). No differences in expression levels of thrombospondin-1 mRNA were observed following cortisol treatment at earlier (1 hour and 4 hours) or later (22 hours) timepoints.

4.3.1.3 Cortisol does not regulate expression of other key angiogenic factors during TLS formation

There were no significant differences in mRNA levels of the other angiogenic factors examined (α6-integrin, Figure 4.2B; VEGF, Figure 4.3A; VEGF-R2, Figure 4.3B; Dll-4, Figure 4.4A; Notch-1, Figure 4.4B; caveolin-1,Figure 4.5) in response to treatment with cortisol (600 nM).

4.3.1.4 Effects of cortisol and GR antagonism on gene expression

The number of replicates in the cortisol plus RU38486 treatment groups were low (n=1-3). However, the effect of cortisol on TSP-1 expression (5.2 ± 1.1 arbitrary units, AU) was blocked by GR antagonism since thrombospondin-1 mRNA returned to a level similar to that of control (2.3 ± 0.4 AU) in the presence of RU38486 (1.7 ± 0.3 AU) p=0.11, n=3). Cortisol plus RU38486 resulted in increased expression of VEGF (1.5-fold, p<0.05, n=3), VEGF-R2 (2.0-fold, p<0.01, n=3) and Notch-1 (1.9-fold, p<0.01, n=3) after 1 hour, and decreased expression of caveolin-1 (7.1-fold, p<0.05, n=3) after 22 hours, versus cortisol treatment alone. The effects of RU38486 alone (control) were not tested in this study.

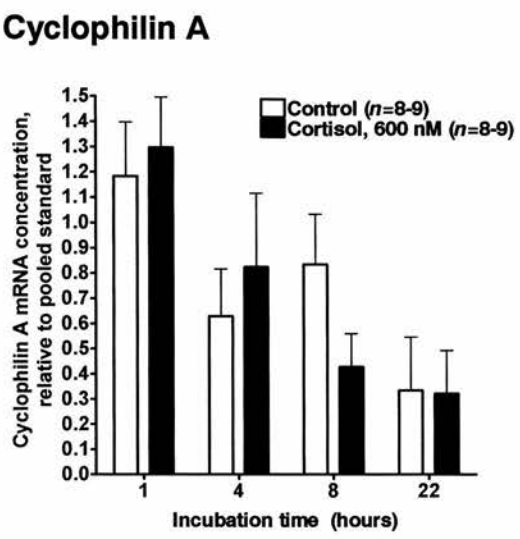


Figure 4.1 Cyclophilin A expression in endothelial TLSs

Cyclophilin A mRNA expression in human umbilical vein endothelial cells (HUVECs) on Matrigel during tube-like structure (TLS) formation. Cortisol (600 nM) was added to the culture medium at time of cell seeding, or left untreated (control). Quantitative real-time PCR (QrtPCR) measurements (mean concentration ± SEM) were determined from a standard curve (data not shown) using crossing point (Cp) values. Significant ($p<0.01$) effects of time, but not treatment, were observed ($n=8$-9, each condition was performed in triplicate wells) which is consistent with changes in expression of all the genes examined, with time. Since the key question was whether cortisol treatment affects gene expression (rather than an effect of time) cyclophilin A was used as internal control.

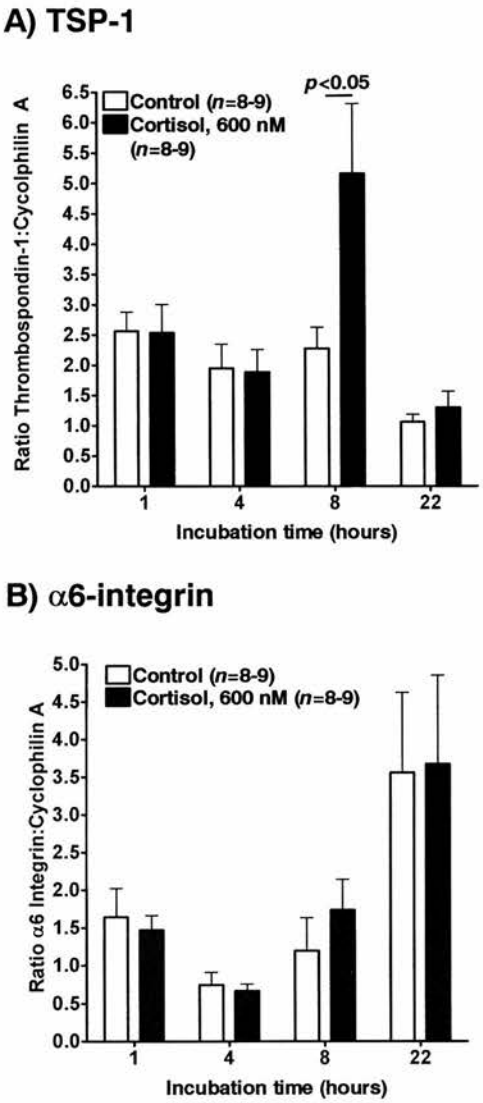


Figure 4.2 TSP-1 and α6-integrin expression studies in endothelial TLSs and the influence of cortisol

Effects of cortisol on (a) thrombospondin-1 (TSP-1) and (b) α6-integrin mRNA expression in human umbilical vein endothelial cells (HUVECs) on Matrigel during tube-like structure (TLS) formation. Cortisol (600 nM) was added to the culture medium at time of cell seeding, or left untreated (control). Quantitative real-time PCR (QrtPCR) measurements (mean concentration ± SEM) were expressed as a ratio of concentration of mRNA of gene of interest to internal control (cyclophilin A). Significant effects of time ($p<0.05$, TSP-1; $p<0.005$, α6-integrin) and cortisol treatment ($p<0.05$, TSP-1, 8 hours) were observed ($n=8$-9, each condition was performed in triplicate wells).

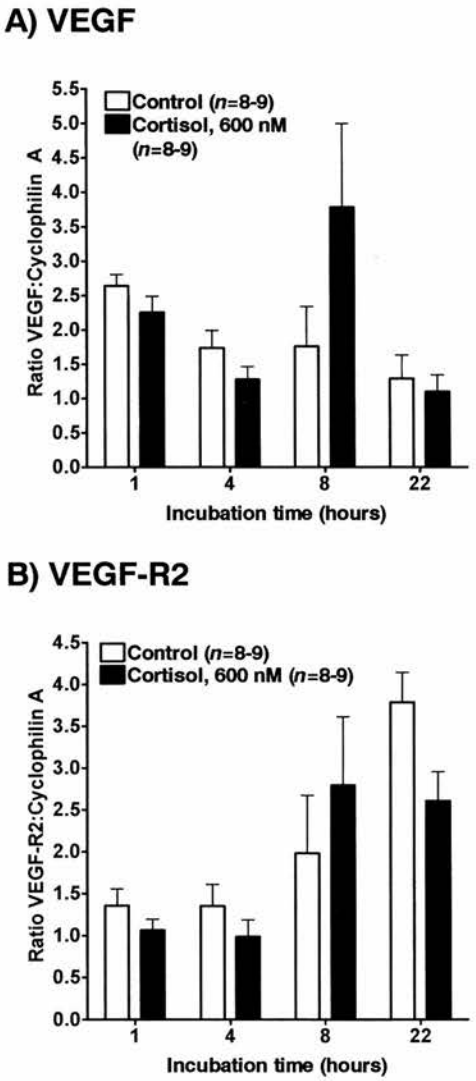


Figure 4.3 VEGF and VEGF-R2 expression studies in endothelial TLSs and the influence of cortisol

Effects of cortisol on (a) vascular endothelial growth factor A (VEGF-A, VEGF) and (b) VEGF receptor 2 (VEGF-R2) mRNA expression in human umbilical vein endothelial cells (HUVECs) on Matrigel during tube-like structure (TLS) formation. Cortisol (600 nM) was added to the culture medium at time of cell seeding, or left untreated (control). Quantitative real-time PCR (QrtPCR) measurements (mean concentration ± SEM) were expressed as a ratio of concentration of gene of interest to internal control (cyclophilin A). Significant effects of time ($p<0.05$, VEGF; $p<0.0001$, VEGF-R2) but no effects of cortisol were observed ($n=8$-9, each condition was performed in triplicate wells).

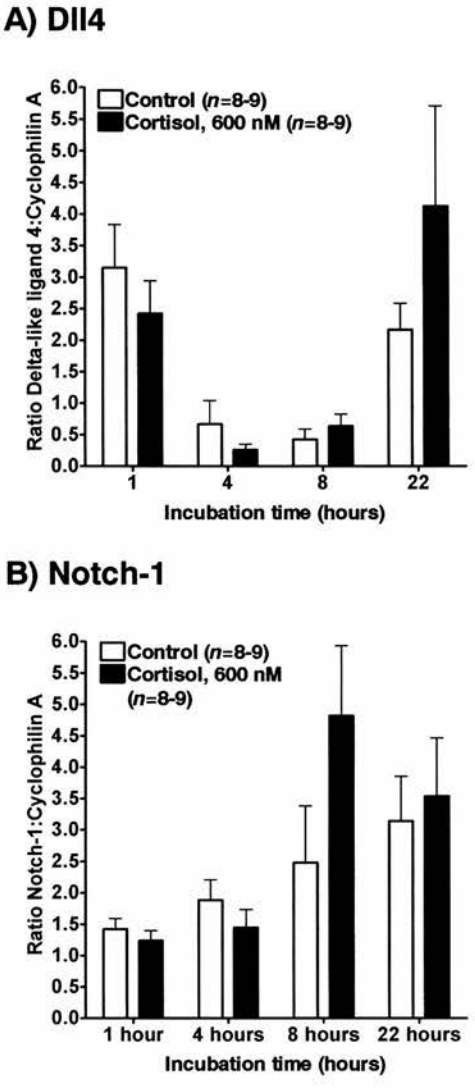


Figure 4.4 Dll4 and Notch-1 expression studies in endothelial TLSs and the influence of cortisol

Effects of cortisol on (a) delta-like ligand 4 (DLL-4) and (b) Notch-1 mRNA expression in human umbilical vein endothelial cells (HUVECs) on Matrigel during tube-like structure (TLS) formation. Cortisol (600 nM) was added to the culture medium at time of cell seeding, or left untreated (control). Quantitative real-time PCR (QrtPCR) measurements (mean concentration ± SEM) were expressed as a ratio of concentration of gene of interest to internal control (cyclophilin A). Significant effects of time ($p<0.05$, Notch-1; $p=0.0001$, Dll4) but no effects of cortisol were observed ($n=8$-9, each condition was performed in triplicate wells).

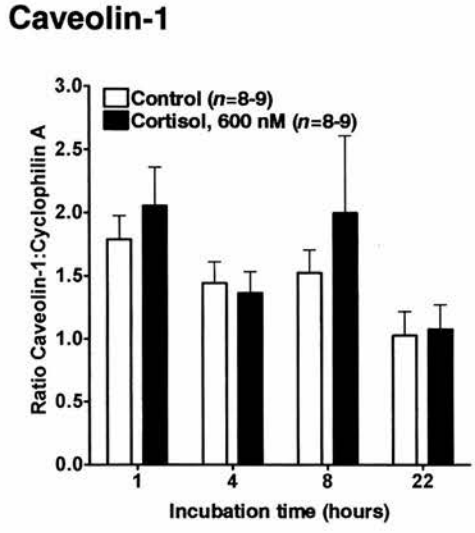


Figure 4.5 Caveolin-1 expression studies in endothelial TLSs and the influence of cortisol

Effects of cortisol on caveolin-1 mRNA expression in human umbilical vein endothelial cells (HUVECs) on Matrigel during tube-like structure (TLS) formation. Cortisol (600 nM) or cortisol and RU38486 (1 μM, GR antagonist) were added to the culture medium at time of cell seeding, or left untreated (control). Quantitative real-time PCR (QrtPCR) measurements (mean concentration ± SEM) were expressed as a ratio of concentration of gene of interest to internal control (cyclophilin A). A significant effect of time ($p<0.01$) but no effects of cortisol was observed (n=8-9, each condition was performed in triplicate wells).

4.3.2 Influence of glucocorticoids on VEGF protein production during TLS formation

In this pilot experiment, no VEGF was detected in any of the sample media despite detection of VEGF standards included in the kit (data not shown).

4.3.3 Influence of glucocorticoids on activation of signal transduction proteins in endothelial cells

4.3.3.1 Immunoprecipitation and Western blotting for FAK in HUVECs

FAK protein expression was detected (Figure 4.6A) in enriched phosphotyrosine-containing protein lysates (from 9 experiments) by a strong band at 125 kDa. Densitometry measurements (Figure 4.6B) indicated a 2-fold increase in FAK expression after 4 hours of vehicle treatment versus untreated control (no incubation). There was an increase in expression of FAK in the presence of cortisol (600 nM) but less so compared with 4 hour vehicle-treated cells. A 4 hour control of untreated cells was absent from this study, to determine whether the effect of vehicle *was* due to vehicle or an environmental effect.

4.3.3.2 Western blotting for activation of signal transduction proteins in HUVECs

Expression of phosphorylated and total p44/p42 (ERK1/2) protein was detected (Figure 4.8A) in lysates with strong dual bands at 44 and 42 kDa. In contrast, reliable bands could not be detected for p38, JNK, and Akt, at the expected molecular weights, even after high exposure times (Figure 4.7).

4.3.3.3 Cortisol induces activation of ERK after 3 to 4 hours in HUVECs

Densitometry measurements (Figure 4.8B) indicated an increase in phosphorylation of ERK with time, in both vehicle and cortisol-treated cells ($p<0.005$). There were 1.5 to 2.3-fold increases in phosphorylation of ERK between 5 and 15 minutes of incubation with either vehicle or cortisol treatment. However, no differences were observed between cortisol and vehicle treatment groups at these early time-points

(data not shown). In contrast, there was a 1.5-fold increase in phosphorylation of ERK in cortisol-treated cells versus vehicle-treated cells, after 3 hours and 4 hours, although this difference did not reach statistical significance (p=0.1).

4.3.3.4 ERK activation by glucocorticoid treatment and blockade by pathway and receptor inhibitors

The trend towards an increase in phosphorylation of ERK in cortisol-treated cells (600 nM, 4 hours) (p=0.1 versus vehicle-treated cells) was inhibited by co-incubation with the MEK inhibitor, PD98059 (50 μM) (p<0.01, Figure 4.9A), or with the GR antagonist, RU38486 (1 μM) (p<0.01, (Figure 4.9B) versus cortisol treatment alone. Phosphorylation of ERK with the inhibitor or antagonist alone was not different to either untreated or vehicle controls (p=not significant).

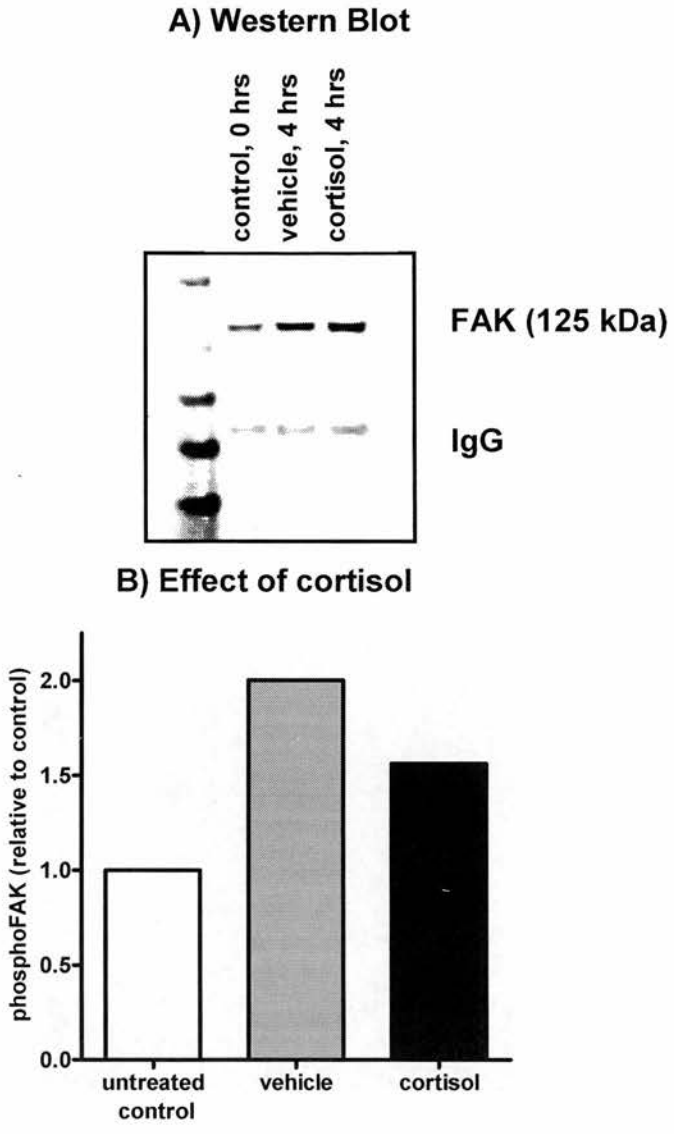


Figure 4.6 Influence of cortisol on focal adhesion kinase (FAK) protein in HUVECs

Effect of cortisol on focal adhesion kinase (FAK) in isolated human umbilical vein endothelial cells (HUVECs). Cortisol (600 nM) or vehicle (ethanol; 0.004% v/v) were added to HUVECs in serum-free endothelial cell growth medium-2 (EGM-2), incubated for 4 hours, or left untreated (control). Cell lysates were subject to immunoprecipitation with a phosphotyrosine antibody and immunoblot analysis using an antibody against FAK. (a) A representative Western blot is shown. (b) Relative density in immunoblots was calculated by dividing the value obtained from the FAK band by the value obtained from the IgG band and expressed as fold above untreated control. Data are from 1 Western blot using pooled lysates from 9 experiments.

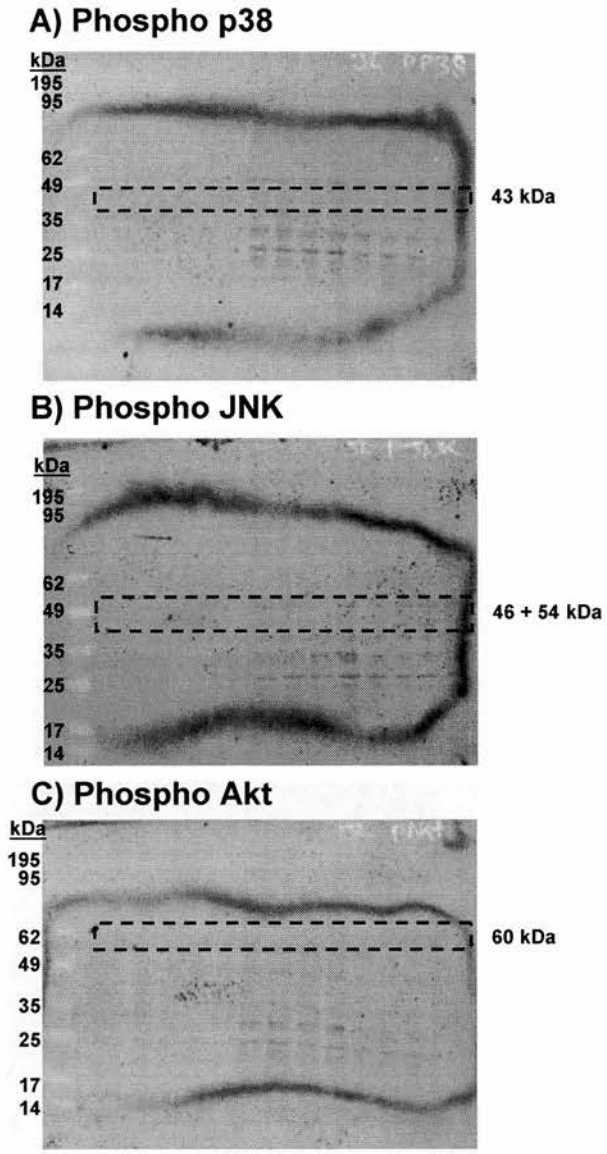


Figure 4.7 Lack of a specific detection method for phosphorylation of p38, JNK and Akt proteins in HUVECs

Cell lysates from cortisol (600 nM), vehicle (ethanol; 0.004% v/v) or untreated HUVECs (15 minutes to 4 hours) were subject to immunoblot analysis using polyclonal (made in rabbit, all 1:1000 dilution) antibodies against phosphorylated (activated) (a) p38, (b) c-jun N-terminal kinase (JNK) and (c) Akt proteins. Secondary probing was performed using goat anti-rabbit alkaline phosphatase antibody (1:30,000) then immunoreactive proteins were attempted to be visualised using the ECF chemiluminescence system (GE healthcare, UK). Bands at the expected molecular weight, as indicated in the immunoblots, could not be detected.

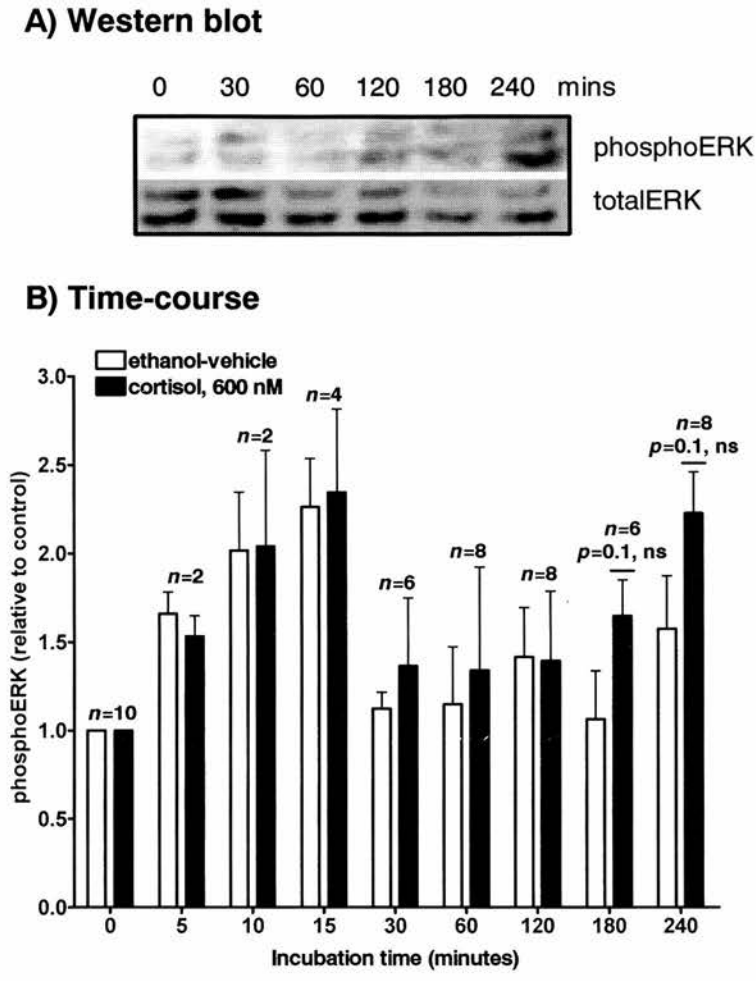


Figure 4.8 Influence of cortisol on phosphorylation of p44/p42 (ERK1/2) protein in HUVECs

Effect of cortisol on extracellular signal-regulated kinase 1/2 (ERK1/2) signalling in isolated human umbilical vein endothelial cells (HUVECs). Cortisol (600 nM) or vehicle (ethanol; 0.004% v/v) were added to HUVECs in serum-free endothelial cell growth medium-2 (EGM-2), incubated for up to 4 hours, or left untreated (control). Cell lysates were subject to immunoblot analysis using an antibody against phosphorylated (activated) ERK1/2 and total ERK (as a loading control). (a) A representative Western blot of ERK expression is shown. (b) Relative density in immunoblots was calculated by dividing the value obtained from the phosphorylated blots by the value obtained from total protein blots and expressed as fold above untreated control. Data are mean ± SEM; n=6-10 where indicated on the bar chart. A significant effect of time (p<0.005) was observed and there was a trend towards an effect of cortisol treatment after 180 minutes (p=0.1) and 240 minutes (p=0.1) although this did not reach statistical significance (ns).

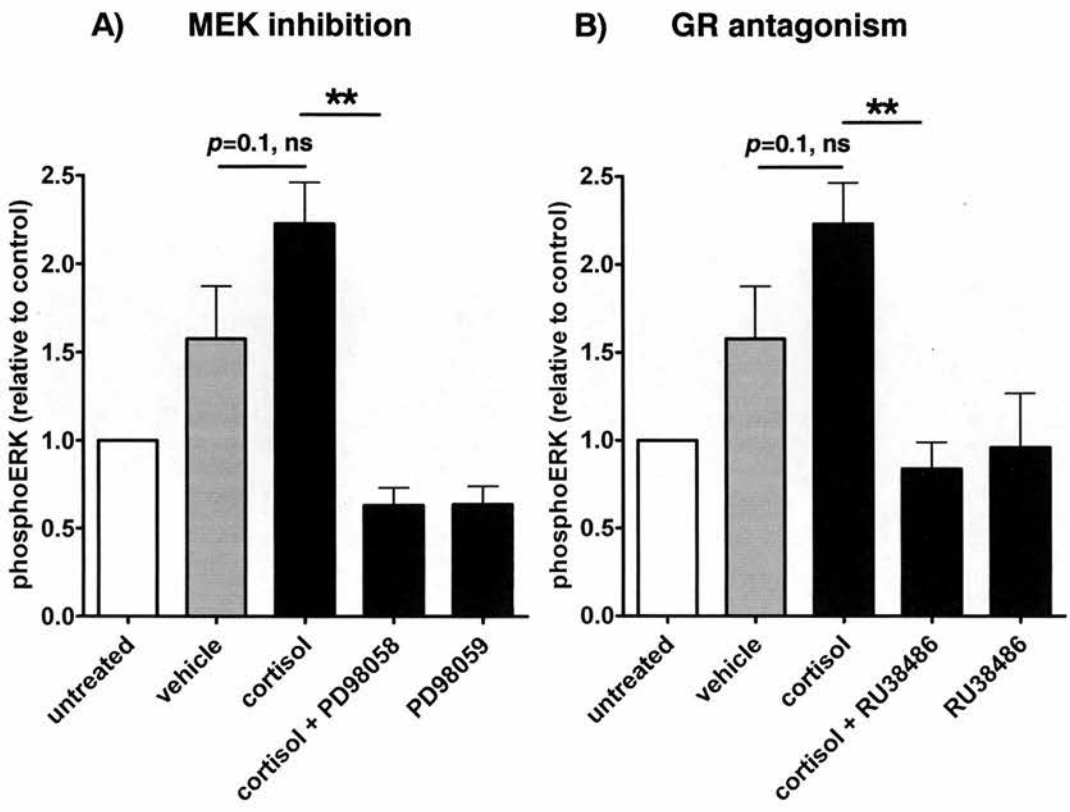


Figure 4.9 Glucocorticoid-induced activation of p44/p42 (ERK1/2): Effects of a pathway inhibitor and GR antagonist

HUVECs incubated in serum-free endothelial cell growth medium-2 (EGM-2) for 4 hours in the presence of vehicle (ethanol; 0.004% v/v), cortisol (600 nM), cortisol and the MAPK/ERK kinase (MEK) inhibitor PD98059 (50 μM), cortisol and the glucocorticoid receptor antagonist RU38486 (1 μM), or either inhibitor/antagonist alone. Activation of ERK1/2 was determined by immunoblot analysis using antibodies against phosphorylated (activated) ERK1/2 and total ERK (as a loading control). The stimulatory effect of cortisol on phosphorylation of ERK (p=0.1 versus vehicle control) was abolished with (a) a MAPK inhibitor (p<0.01; cortisol plus PD98059 versus cortisol), and by (b) GR antagonism (p<0.01; cortisol plus RU38486 versus cortisol). PD98059 and RU38486 alone had no effect but vehicle caused a (non-significant) 1.5-fold increase in phosphorylation of ERK versus untreated control. Data represent mean ± SEM (n=8, control, vehicle and cortisol, n=3, cortisol plus inhibitor/antagonist).

4.4 Discussion

The intention of these studies was to investigate the molecular mechanisms through which endogenous glucocorticoids inhibit endothelial tube formation. Using the *in vitro* model developed for these investigations (Chapter 3), it was determined that glucocorticoids up-regulate expression of angiostatic thrombospondin-1, which occurs concurrently with the degradation of TLS networks described previously (Chapter 3). Consequently, glucocorticoids may serve to inhibit angiogenesis *in vivo* by favouring a net generation of angiostatic factors which inhibit cellular morphogenesis. In addition, it was shown that glucocorticoids directly stimulate MAPK signalling, via the ERK pathway, in undifferentiated endothelial cells raising the possibility that this pathway is activated as a stress response mechanism. This method development will serve as a tool to determine whether similar stimulation of the ERK pathway by cortisol occurs during inhibition of endothelial tube formation and angiogenesis. Modulation of molecular signalling by glucocorticoids, during endothelial tube formation, was dependent on GR, consistent with a role for these receptors in glucocorticoid-mediated inhibition of tube formation.

It is known that tube formation by endothelial cells requires gene transcription and *de novo* protein synthesis (Grove *et al.*, 2002; Grant *et al.*, 1991). Furthermore, it is important to study gene expression and signal transduction during endothelial tube formation since endothelial cells growing on Matrigel are likely to be synthesising many other specific proteins distinct from those synthesised on plastic (Grant *et al.*, 1991). The 2D model of TLS formation is an ideal model to study the influence of glucocorticoids on molecular signalling during endothelial tube formation since cells resemble their conformational state *in vivo* (Lawley & Kubota, 1989) whilst they can be recovered from the substrate to isolate RNA and protein. Also, soluble growth factors (*e.g.* VEGF), secreted from developing endothelial tubes into the culture medium, can also be easily analysed. Consequently, quantitative PCR, Western blotting and ELISA are the most appropriate technologies to study changes in gene and protein expression, and soluble protein production, respectively.

4.4.1 Influence of glucocorticoids on angiogenic factor gene expression in TLSs

QrtPCR is, at present, the most sensitive method for the detection of changes in abundance of mRNAs (Bustin *et al.*, 2005). This proved to be a sensitive method to detect changes in mRNA expression, in the present study, given the low intra-assay variation between replicate samples. The reactions were specific for the genes of interest since all primers were exon spanning and, therefore, will not detect genomic DNA. For QrtPCR, there is a general consensus on using a single control gene for normalisation purposes (Vandesompele *et al.*, 2002) but the choice of internal control must be made after careful consideration. Ideally, the abundance of internal control mRNA should not vary in the tissues or cells under investigation, or in response to experimental treatment to correct for possible differences in RNA quantity or quality across experimental samples. However, according to the literature, very few house-keeping genes fit this criterion as all are regulated to some extent and many vary in expression levels across a range of different conditions (reviewed in (Thellin *et al.*, 1999; Bustin, 2000; Nolan *et al.*, 2006)). There is no consensus in the literature on which internal control to use for primary human endothelial cell gene expression studies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Forster *et al.*, 2007; Murthi *et al.*, 2007), β-actin (Matsuda *et al.*, 2005; Yan *et al.*, 2005) and cyclophilin A (Zlot *et al.*, 2003; Lucerna *et al.*, 2007) have all been documented in the literature, with the latter reported for use in HUVEC studies. Ribosomal RNAs (rRNAs), are another popular choice of internal control in QrtPCR studies but, as rRNA subunits are not poly-adenylated, they cannot be used when dealing with cDNA derived from total RNA using oligo(dT) primers in the RT reaction (Nicot *et al.*, 2005). Another drawback to the use of rRNA as an internal standard is its high abundance compared with target RNA transcripts making it difficult to subtract the baseline value in data analysis accurately (Vandesompele *et al.*, 2002). In the present study, cyclophilin A was of similar abundance to the other genes of interest (similar Cp values) and was, therefore, a good internal control candidate. Although cyclophilin A expression varied with time, it did not vary with the effects of treatment apart from a non-significant change at 8 hours in the cortisol treatment

group. These apparent changes in genes expression are more likely due to changes in RNA stability due to the culture conditions (*e.g.* serum starvation), differences in starting amounts of RNA due to inherent errors in the RNA quantification method, and differences in efficiencies of the RT step. Since one of the primary aims of the study was to determine the influence of cortisol on TLS formation at selected time-points, the use of cyclophilin A was justified on this basis.

4.4.1.1 Endothelial cell-ECM interactions

TSP-1 was the only factor to be significantly regulated by cortisol out of all those examined in the present study. A significant decrease in TSP-1 mRNA over time in untreated control TLSs is consistent with the literature (Lane *et al.*, 1992) and fits with a role for TSP-1 as a negative regulator of endothelial tube formation (Iruela-Arispe *et al.*, 1991; DiPietro *et al.*, 1994). The induction of TSP-1 mRNA expression in TLSs, by cortisol, could explain the mechanism through which glucocorticoids inhibit TLS formation.

The time-course of induction of TSP-1 synthesis by cortisol is consistent with the time-course of morphological changes observed by time-lapse imaging (Chapter 3) and is also consistent with a previous study (100 nM dexamethasone; trabecular meshwork cells which have vascular endothelial cell properties) (Flugel-Koch *et al.*, 2004). The highest level of TSP-1 mRNA occurred after 8 hours of cortisol treatment which coincides with the time at which the greatest effect of cortisol on TLS number was observed in time-lapse imaging studies (Chapter 3).

TSP-1, and fragments of TSP-1, can act directly on endothelial cells to inhibit angiogenesis by interactions with CD36, CD47 and integrins on the surface of the cell. Binding of TSP-1 to CD36 mediates inhibition of endothelial cell migration (Dawson *et al.*, 1997) and tube formation (Iruela-Arispe *et al.*, 1991; DiPietro *et al.*, 1994) and leads to induction of apoptosis (Jiménez *et al.*, 2000). Thus, the delay between peak induction of TSP-1 mRNA (at 8 hours) by cortisol, and the complete degradation of the TLS network observed at 18 hours (Figure 3.6), may explain the

time required for 1) the cells' translational machinery to be employed, 2) the protein to be secreted, 3) it to act through its cell receptor, and 4) activation of intracellular signalling cascades and, ultimately, tube degradation. In addition, cortisol does not induce TSP-1 mRNA earlier than 8 hours and this may explain why cortisol (600 nM) does not completely prevent the formation of TLS networks. These data support the hypothesis that glucocorticoids accelerate the degradation phase of TLS formation, consistent with studies in the literature where TSP-1 is counter-adhesive when added to fibronectin and causes disassembly of focal adhesions and stress fibres formed by vascular endothelial cells (Greenwood & Murphy-Ullrich, 1998). Indeed, glucocorticoid-mediated inhibition of tube formation was shown to occur with morphological changes in stress fibres (Chapter 3) and there is preliminary evidence to support a role for FAK (Figure 4.6).

The magnitude of induction of TSP-1 transcription induced by glucocorticoids, in the present study, is similar to a previous report in isolated trabecular meshwork cells (Flugel-Koch *et al.*, 2004). The increase in TSP-1 mRNA levels induced by cortisol treatment was blocked in the presence of RU38486 (although small numbers) suggesting that glucocorticoid-mediated regulation of TSP-1 is dependent on glucocorticoid receptors. Again, this is consistent with the GR-dependent effects of cortisol on inhibition of TLS formation described in Chapter 3. The potential effect of RU38486 alone on TSP-1 levels during TLS formation was not tested in these studies, however, in other studies, RU38486 (1-50 μm) treatment did not alter TSP-1 mRNA expression in isolated endometrial epithelial (Ishikawa) (Mirkin & Archer, 2004) or stromal (Iruela-Arispe *et al.*, 1996) cells. The presence of GREs on the TSP-1 promoter has not been reported so far but analysis of the human TSP-1 gene has revealed the presence of 2 progesterone-responsive elements in the progesterone receptor promoter (Iruela-Arispe *et al.*, 1996). Since the glucocorticoid and progesterone receptors share the same nucleotide binding sequence (Beato, 1989) it is likely that the TSP-1 gene may also contain GREs and this will be addressed in a future study.

In support of a role for TSP-1 in aberrant angiogenesis *in vivo*, overexpression of TSP-1 results in abnormal wound angiogenesis in mice (Streit *et al.*, 2000), when tissue remodelling is necessary. The possibility that the TSP-1 gene might be responsive to steroids *in vivo* has not yet been fully elucidated and the literature to date is limited to a few studies on progesterone and reproductive function (Iruela-Arispe *et al.*, 1996; Espey *et al.*, 2000) or dexamethasone in glaucomatous eyes (Flugel-Koch *et al.*, 2004) and the influence on angiogenesis *per se* in these studies is difficult to ascertain.

Any full assessment of the biological consequences of variable mRNA levels must include information regarding regulatory RNAs, protein levels and protein activity (Nolan *et al.*, 2006). Lack of protein availability prevented studies investigating the possibility that the increase in TSP-1 mRNA synthesis by TLSs is translated into increased protein production. However, very recent evidence now suggests that glucocorticoids (nM) exert a direct angiostatic effect in the endometrium concurrently with an increase in TSP-1 production (Rae *et al.*, manuscript submitted).

The reciprocal pattern of integrin expression and tube formation in the present study; where high expression is seen during peak tube formation and low expression during degradation, may be critical for tube formation *per se*. The fact that cortisol was without effect on α6-integrin expression suggests that this is not an important facet of glucocorticoid-mediated inhibition of tube formation. Also, it is more likely that multiple integrins act together in regulating endothelial tube formation (Davis & Senger, 2005; Davis *et al.*, 2002). Furthermore, signalling molecules downstream of integrin-matrix interactions, such as the Rho guanosine triphosphatases (GTPases; in the Ras superfamily), have been shown to control actin and microtubule cytoskeletal networks (Zhao *et al.*, 2006) and, therefore, regulate cell morphogenesis. It might be interesting to follow up on this lead in future studies. Therefore, on balance, there is insufficient evidence presented here to conclude that glucocorticoids do not regulate the integrin signalling axis but regulation of α6-integrin at the level of gene expression can now be ruled out.

Glucocorticoids did not significantly alter levels of caveolin-1 mRNA suggesting that this does not play a significant role in glucocorticoid-mediated inhibition of TLS formation in this system. It is important to note that caveolin-1 can be tyrosine phosphorylated under certain conditions such as oxidative stress (Aoki *et al.*, 1999), and post-translational modification may play an important role in regulation of active protein levels.

4.4.1.2 VEGF signalling

In the present study, glucocorticoids do not appear to regulate levels of VEGF mRNA during TLS formation. It is feasible that glucocorticoids regulate levels of specific VEGF-A isoforms but this was not examined in the present study. For example, triamcinolone acetate caused a significant decrease specifically in $VEGF_{165}$ levels in HUVEC monolayers (Tong *et al.*, 2006). Nonetheless, $VEGF_{165}$ is the most abundantly expressed VEGF isoform (Houck *et al.*, 1992) and would have been detected using the current primers (along with the other isoforms), and so substantial changes in its expression level should have been detected in the present study.

There may be different mechanisms of regulation of VEGF gene expression and these may differ between cell types. For example, glucocorticoids were shown to decrease growth factor-regulated VEGF gene expression, via GR, in keratinocytes (Gille *et al.*, 2001), chondrocytes (Koedam *et al.*, 2002) and vascular smooth muscle cells (Nauck *et al.*, 1998). In these previous studies, however, cells were stimulated with other angiogenic stimuli (HGF, FCS and PDGF, respectively), in contrast to the present study which examined effects on basal, unstimulated VEGF expression. This is supported by the fact that, hypoxia-induced VEGF mRNA levels are less sensitive to glucocorticoid-mediated inhibition than in normoxic conditions (Machein *et al.*, 1999). Consequently, it seems unlikely that glucocorticoids regulate expression of endogenous VEGF production when the only stimulus is the endothelial cells' interaction with the matrix.

The lack of effect of cortisol on VEGF-R2 mRNA levels, in the present study, is consistent with a previous study in cell monolayers using dexamethasone (Machein *et al.*, 1999). However, during cutaneous wound healing (Zhang *et al.*, 2004) and in neonatal lung development (Clerch *et al.*, 2004), VEGF-R2 expression is suppressed by administration of glucocorticoids. This difference between the present *in vitro* and previous *in vivo* findings may be explained by different regulatory mechanisms depending on the context of the surrounding tissue microenvironment. Nevertheless, the results presented here demonstrate that modulation of VEGF and VEGF-R2 expression does not play a significant role in glucocorticoid-mediated inhibition of endothelial tube formation.

In conclusion, the present evidence indicates that glucocorticoids are more likely to block the downstream actions of VEGF following receptor activation, during tube formation, rather than altering its synthesis within the endothelium. The fact that glucocorticoids inhibit the expression of VEGF in human vascular smooth muscle cells (Nauck *et al.*, 1998), but not in endothelial cells, also supports the notion of a paracrine mechanism of inhibition. Co-cultures and conditioned media studies support this hypothesis since glucocorticoids suppress VEGF production (by fibroblasts and epithelial cells) and TLS formation by HUVECs cultured on Matrigel (Matsuda *et al.*, 2005; Wu *et al.*, 2006). This is also supported by the fact that glucocorticoids blocked VEGF-induced TLS formation (Chapter 3).

The relatively low abundance of Dll4 in control TLSs at 4 and 8 hours, when TLS number peaks, is consistent with a role of Dll4 as a negative regulator of angiogenesis. In support of this, Dll4-induced expression in HUVECs inhibited vessel sprouting in a 3D TLS assay (Harrington *et al.*, 2007). It is less obvious why Notch-1 expression increased during TLS formation, in the present study (in a time-dependent manner) in the absence of exogenous VEGF. It is possible that Notch-1 production is switched on as a negative feedback regulator of endogenous VEGF production.

The lack of effect of cortisol on transcription of either Dll4 or Notch-1 demonstrates that these are not likely to play an important role in glucocorticoid-mediated inhibition of TLS formation. Since these studies were performed another report has described the influence of glucocorticoids on Notch signalling (in endothelial cell monolayers); expression of Notch-4 was induced by cortisol but only in the presence of growth factors (EGF and bFGF) (Wu & Bresnick, 2007). The complexity of Notch signalling in endothelial cells was highlighted with the finding that Notch has effects on the cell cycle independent of VEGF activity (Sainson *et al.*, 2005). Consequently, it is likely that Matrigel contains soluble growth factors sufficient to induce expression of Notch-1. Alternatively, this pathway may be regulated by signals from direct endothelial cell-ECM interaction (*e.g.* integrins, TSP-1). In conclusion, glucocorticoids might regulate Notch signalling in the endothelium but this is strongly dependent on the environmental conditions and does not appear to be important in the present system.

In summary, these studies have highlighted the complexity of examining regulation of angiogenic factor gene expression during TLS formation. Nevertheless, these studies have implicated the involvement of TSP-1 induction by glucocorticoids which could explain the molecular basis of their angiostatic effect on tube formation.

4.4.2 Influence of glucocorticoids on VEGF protein production in HUVECs

The failure to detect VEGF protein in unstimulated HUVECs using an ELISA is most likely due to levels below the limits of detection of the assay. This may be in part due to the serum-free conditions used as serum is recommended by the manufacturer for the stability of the protein. Undetectable protein production from HUVECs is consistent with previous studies (Dupuy *et al.*, 2003; Nanobashvili *et al.*, 2003). In other studies where VEGF was detected (Belgore *et al.*, 2003; Yoon *et al.*, 2006), the levels were very low and towards the lower limit of detection. In the present study, VEGF protein production did not appear to be stimulated by culturing cells on Matrigel and this is consistent with the relatively low levels of mRNA detected in QrtPCR studies. Consequently, it is likely that cell-matrix interaction

alone is not a sufficient stimulus to promote VEGF secretion. Method refinement such as increasing the number of cells, inclusion of (charcoal-stripped) serum and/or stimulation of cells with a growth factor (*e.g.* bFGF) or hypoxia may prove useful in future studies. However, since glucocorticoids have already been shown to block the stimulatory effect of VEGF on TLS formation under the same, serum-free conditions (Chapter 3), it seems more likely that cortisol blocks the downstream signalling actions of exogenous VEGF on the endothelium rather than preventing synthesis of new protein and attenuating an autocrine effect of VEGF.

4.4.3 Influence of glucocorticoids on activation of signal transduction proteins in endothelial cells

4.4.3.1 Focal adhesion kinase

In the present study, preliminary data suggest there may be a slight inhibitory effect of cortisol on activation of FAK protein in endothelial cells. However the low abundance of this protein required samples to be pooled from multiple experiments which prevented statistical analysis to strengthen interpretations. These data offer some insight into an important relay mechanism that may well be a target of glucocorticoid-induced inhibition of endothelial tube formation. This positive method development will provide a useful basis for future studies investigating glucocorticoid effects on FAK activation during endothelial tube formation. The possibility of regulation of FAK by cortisol would fit with a mechanistic role in inhibition of tube formation, since FAK activation acts as a signal relay between endothelial cell contacts and the ECM (Burridge *et al.*, 1992).

4.4.3.2 Mitogen-activated protein kinases

The failure to reliably detect p38, JNK, and Akt protein in the present study most likely reflects the need for further method optimisation (*e.g.* increasing antibody titration, different primary antibody) since all 3 proteins have been detected previously in HUVECs (Pelaia *et al.*, 2001; González *et al.*, 1999; Hermann *et al.*, 2000). Alternatively, it is possible that under the conditions used in the present study, these proteins are not constitutively active. In contrast, the reproducible and

quantifiable detection of both phosphorylated and total forms of ERK protein, in untreated and cortisol-treated cell lysates, demonstrates that this is a useful system to study glucocorticoid-mediated regulation of ERK activation in isolated endothelial cells.

Activation of ERK by cortisol treatment may indicate the cellular response to stress in the system. Indeed, MAPK cascades are key components of the signalling networks that sense cellular exposure to environmental stress (Kyriakis & Avruch, 2001) and in some cellular systems, activation of the ERK pathway confers protection against stress and apoptosis (Cuda *et al.*, 2002). An effect of vehicle (ethanol) was apparent in these studies but it could not be determined whether this was due to vehicle *per se*, or whether it was an environmental effect; for example, from the stress of serum-starvation. Consequently, it would not be surprising if there was a basal activation of this pathway by serum starvation which was exacerbated, to a lesser extent by vehicle, and by a greater extent to the stress hormone, cortisol.

Glucocorticoids induce oxidative stress in the intact vasculature (Schäfer *et al.*, 2005) and ERK1/2 has been proposed as a key regulator of the signalling cascade triggered by oxidative stress (Cuda *et al.*, 2002). There is a limited literature on glucocorticoids and ERK signalling in endothelial cells. However, the present findings are fully consistent with a previous study (in trabecular meshwork cells) where dexamethasone treatment caused activation of the ERK/MAPK pathway, which was reversed by GR antagonism and MEK inhibition (Jeon *et al.*, 2003). The present findings contrast with previous studies in endothelial cells where glucocorticoids have been shown to inhibit ERK (Pelaia *et al.*, 2001; González *et al.*, 1999). In these 2 published studies, however, cells were pre-treated with steroid then activated by VEGF or pro-inflammatory cytokines (IL-1β and TNF-α); whereas the present study investigated cortisol effects on basal-unstimulated ERK activation. Other studies demonstrated no effect of synthetic corticosteroids; triamcinolone acetonide (Ebrahem *et al.*, 2006) or dexamethasone (Kasselman *et al.*, 2007) on VEGF-induced phosphorylation in HUVECs. In conclusion, direct ERK activation by cortisol is probably not responsible for attenuating responses to VEGF, since in

the presence of VEGF, blockade rather than potentiation of the ERK response was observed (Chapter 3).

Although MAPK signalling can be modulated by steroids in a rapid non-transcriptional way in endothelial cells (Nuedling *et al.*, 1999; Simoncini *et al.*, 2004), the time-course of cortisol-induced induction of ERK protein expression in the present study is likely to be due to a transcriptional effect of glucocorticoid-activated GR. This is supported by the fact that GR antagonism blocked cortisol-induced activation of ERK. This has been described previously *in vitro* in a non-endothelial cell type (Revest *et al.*, 2005) and was supported by studies *in vivo* where ERK has been shown to be activated concomitantly with GR after behavioural stress (Meller *et al.*, 2003; Yang *et al.*, 2004).

In the present study, transcriptional activation of this pathway by glucocorticoids is likely to have occurred either at the level of ERK itself, or by upstream components of the cascade such as Ras or Raf-1 (Figure 1.2). Pharmacological blockade of MEK (with PD98059), which is immediately upstream of ERK1/2, blocked the effect of cortisol suggesting that induction of this pathway occurs either at the level of Ras/Raf-1; or further upstream *e.g.* at the level of phospholipase C (PLC), protein kinase C (PKC) or, phosphoinositide 3-kinase (PI3K). The former is supported by findings that activated GR can stimulate the transcription of Ras and Raf-1 directly and glucocorticoid-responsive elements (GREs) have been identified on the promoters of Ras (Strawhecker *et al.*, 1989) and Raf (Lee *et al.*, 1996) family members. However, it is also worth noting that TSP-1 signalling of focal adhesion disassembly and reorganisation of the endothelial actin cytoskeleton requires activation of PI3K (Greenwood *et al.*, 1998; Short *et al.*, 2005). Consequently, glucocorticoid-induced TSP-1 synthesis, and glucocorticoid-activation of MAPK signalling, may feature as 2 components of the same mechanism of inhibition of endothelial tube formation.

In summary, these novel data provide a solid foundation for future investigation of the levels and activation state of other members of the ERK/MAPK pathway to further dissect out the effect of glucocorticoids on endothelial cell signal transduction during tube formation and angiogenesis.

4.4.4 Conclusion

The studies described in this Chapter implicate altered gene expression (TSP-1) and signal transduction pathways (ERK/MAPK) as possible targets of glucocorticoid-mediated inhibition of tube formation by endothelial cells. Furthermore, inhibition of VEGF mRNA and protein synthesis by endothelial cells undergoing this morphogenesis can now be excluded as an angiostatic mechanism. This supports the contention that VEGF action on the endothelium, rather than production, may be attenuated by glucocorticoids.

Since VEGF can also facilitate other key angiogenic changes in endothelial cells; such as proliferation and migration, it is possible that these other processes, in addition to TLS formation, are sensitive to the angiostatic effects of glucocorticoids.

Chapter 5

Influence of Glucocorticoids on Endothelial Cell Migration and Proliferation

5.1 Introduction

The work in Chapter 3 showed that glucocorticoids can inhibit tube formation by direct interaction with the endothelial cells. In this 2-dimensional model, inhibition of tube formation appeared to be due to an alteration in endothelial cell morphogenesis, rather than to changes in cellular proliferation or migration. This, however, does not exclude the possibility that direct modulation of endothelial cell proliferation and migration may contribute to glucocorticoid-mediated inhibition of angiogenesis *in vivo*. Indeed, assessment of the intra-cellular mechanism(s) through which glucocorticoids alter endothelial cells (Chapter 4) suggested aberrant growth factor signalling. It was important, therefore, to use alternative approaches to determine whether inhibition of endothelial cell proliferation or migration may contribute to the angiostatic effect of these steroids.

The literature on glucocorticoids and endothelial cell migration and proliferation is limited. An inhibitory effect of angiostatic steroids on cell protease activity (Blei *et al.*, 1993) suggests that glucocorticoids could potentially inhibit endothelial cell migration since degradation of the ECM is a pre-requisite for cell motility *in vivo*. This indirect evidence was supported by a study using the more direct Boyden chamber assay (migration of cells through pores towards a chemoattractant) with high concentrations of triamcinolone acetonide (Wang *et al.*, 2002b) but could not be supported in another study using (400 nM) dexamethasone (Polytarchou & Papadimitriou, 2005). In the latter study, however, only effects of glucocorticoids on undirected (*i.e.* no stimulus) cell migration were tested. No previous study has tested the effects of physiological glucocorticoids on endothelial cell migration in response to VEGF.

Previous studies of glucocorticoid influence on cell proliferation have been difficult to interpret, collectively, since a variety of cell proliferation assays exist (both direct and indirect) and a variety of different species of endothelial cells have been used. The ability of synthetic glucocorticoids to inhibit cellular proliferation (Berk *et al.*, 1988; Longenecker *et al.*, 1982; Longenecker *et al.*, 1984) and migration (Pross *et al.*, 2002b) has been demonstrated using rat and bovine vascular smooth muscle

cells. These properties have prompted their assessment as anti-atherosclerotic (Cavallero *et al.*, 1976) and anti-restenotic (Berk *et al.*, 1991; Versaci *et al.*, 2002) agents. The fact that these inhibitory effects have not translated well into the clinic highlights the existence of important species-specific responses to glucocorticoids. Pharmacological levels of synthetic corticosteroids inhibit human endothelial cell proliferation *in vitro* (Kräling *et al.*, 1999; Banciu *et al.*, 2006; Zou *et al.*, 2006). In contrast, dexamethasone (10^{-10} to 10^{-5} M) has no effect on bovine corneal endothelial cell proliferation under basal, unstimulated conditions (Chen *et al.*, 2006). The effects of physiological levels of glucocorticoids on endothelial cell proliferation, driven by VEGF or bFGF, which are known to be important endothelial cell mitogens *in vivo*, have thus far not been explored.

5.1.1 Hypothesis

This study explored the hypotheses that glucocorticoids inhibit endothelial cell migration and proliferation and block the stimulatory actions of VEGF and/or bFGF on these key angiogenic processes.

5.1.2 Aims

The specific aims were to:

1) establish assays of endothelial cell migration and proliferation using isolated human endothelial cells.
2) investigate the influence of glucocorticoids on endothelial cell migration and proliferation in response to VEGF and bFGF.

5.2 Methods

5.2.1 Cells

Primary HUVECs were obtained from Promocell (Germany) and routinely passaged, as described previously (Section 2.4.2). Cells were used between p2 and p6 in all migration and proliferation experiments. Where possible, cells were used at the same level of confluency (70-80%) to ensure reproducibility of results. Cells were detached from the flasks with trypsin and, in all assays, cell counting was performed to ensure consistency of cell density between experiments (Section 2.4.4).

5.2.2 Development of an endothelial cell migration assay

To test the effects of glucocorticoids on basal (unstimulated) and VEGF-induced endothelial cell migration, commercially available cell culture inserts (ThinCerts; Cellstar-Greiner Bio-One, UK) were used. Based on the Boyden Filter assay, this involves a two-compartment system where cells may be induced to migrate from an upper compartment through a porous membrane into a lower compartment, thus following the gradient of a chemoattractant (Boyden, 1962). Migratory cells can measured by labelling with a fluorescent dye (calcein-AM) and measured using a fluorescence plate reader since relative fluorescence units (RFU) correlate linearly with number of cells. Some degree of method development was required to generate the final protocol as described in the following sections.

5.2.2.1 Culture conditions and pore size

Pore size and duration of incubation have previously been shown to be important determinants of endothelial cell (Matsuda & Nakayama, 1996) and neutrophil (Stein *et al.*, 1983) migration. Therefore, a pilot study was performed; comparing 3.0 μm versus 8.0 μm porous ThinCerts (Cellstar-Greiner Bio-One, UK), and comparing 5 hours versus 24 hours of incubation.

5.2.2.2 Determination of EC_{50} of VEGF as a chemoattractant

VEGF is commonly used as a chemoattractant in the Boyden chamber assay but there is no consensus on the ideal concentration. Yoshida and colleagues used 1 ng/ml VEGF (Yoshida *et al.*, 1996), whilst others have reported using higher concentrations; *e.g.* 10 ng/ml (Gallicchio *et al.*, 2005) and 50 ng/ml (Cattaneo *et al.*, 2003). Therefore, in pilot studies, concentration-response curves were generated using VEGF. To determine the half-maximal effective concentration (EC_{50}) of VEGF, HUVECs in standard basal medium (EGM-2 medium containing only ascorbic acid, heparin and GA-1000) were seeded into the upper chamber of cell culture inserts (8.0 μm pores). The lower chamber contained standard basal medium with VEGF as a chemoattractant at a range of concentrations; 0.5 ng/ml to 500 ng/ml and 7.5 ng/ml to 60 ng/ml in 2 separate experiments. After 24 hours, migratory cells were quantified by labelling with calcein-AM (8 μM) and the amount of fluorescence was measured in a plate reader (Section 2.4.12). EC_{50} was determined by non-linear regression with sigmoidal concentration: response curves shown generated using GraphPad Prism v4.02 (GraphPad Software, USA). The EC_{50} values from 2 experiments were determined and the average value was used in the final protocol. Each condition was performed in triplicate wells.

5.2.3 Influence of glucocorticoids on endothelial cell migration

The effects of cortisol on basal-unstimulated, and VEGF-induced, endothelial cell migration were tested using the final protocol detailed in Section 2.4.12. Briefly, Boyden chambers were prepared by immersing 8.0 μm ThinCerts into a 24-well plate containing either standard basal medium (untreated control), medium with VEGF (10 ng/ml), medium with VEGF plus inhibitor, SU5416 (1 μM; positive control), medium with VEGF plus cortisol (600 nM) or medium with SU5416 or cortisol. HUVECs were harvested and seeded into the upper compartment at a density of 2×10^5 cells/insert. SU5416 or cortisol were added immediately to the upper compartment to maintain these final concentrations in both compartments whereas VEGF was only present in the lower chamber to produce a gradient of chemoattractant. Plates were incubated for 24 hours to allow cells to migrate, then

labelled with calcein-AM dye (8 μM). Contents of the upper chamber were then removed by gentle aspiration. ThinCerts were then transferred into a new 24-well cell culture plate and the labelled migratory cells were detached from the underside using Trypsin-EDTA with periodic agitation. Trypsin-EDTA solution (200 μl) containing the labelled, migratory cells, was transferred into a black 96-well plate to minimise background fluorescence between wells. Fluorescence measurements were made using a Fluoroskan Ascent FL plate reader and data captured using Ascent software (Thermo LabSystems, UK). Consequently, it was possible to test the effect of cortisol on unstimulated migration (by comparing with basal control wells) and VEGF-induced migration (by comparing VEGF plus cortisol with VEGF alone wells after demonstrating VEGF had an effect). Each condition was performed in triplicate wells.

5.2.4 Influence of glucocorticoids on endothelial cell proliferation

Cell proliferation induced by VEGF and bFGF in HUVECs, plus the ability of cortisol (3 nM to 1 μM) to inhibit the action of these growth factors, was assessed using two independent methods; (1) measurement of cell viability and (2) direct measurement of newly synthesised DNA. Both methods involved the use of commercially available kits and involved some degree of method optimisation, discussed in the following sections, to generate the final protocols.

5.2.4.1 Bromodeoxyuridine (BrdU) incorporation assay (direct method): Optimisation of culture conditions

The BrdU Cell Proliferation Assay (Calbiochem-Merck, UK) allows detection and quantification of BrdU, following partial denaturation of the DNA double strand by use of a detector monoclonal anti-BrdU antibody to bind to any BrdU incorporated into dividing cells. An enzyme-linked immunosorbent assay (ELISA) is then used to form a coloured product that absorbs light at dual wavelengths. The intensity of the coloured product is directly proportional to the amount of incorporated BrdU present in the cells.

The manufacturer's instructions were followed (as described in Section 2.4.11.2) with some method refinement during the initial experiments. To produce an optimal level of cell confluency (around 70-90% at fixation stage); cell density (1.5×10^3 cells/well to 5×10^3 cells/well) and total duration of incubation (24 to 96 hours) were varied. In addition, duration of BrdU incubation (3 to 72 hours) was varied.

5.2.4.2 Influence of glucocorticoids on BrdU incorporation

As detailed in Section 2.4.11.2, HUVECs were seeded in inner wells of a 96-well plate at a density of 3.5×10^3 cells/well and incubated for 2 hours to allow cells to adhere to the plates. Cells were cultured in 'experimental' medium consisting of EGM-2 Bullet Kit medium (Lonza, UK) supplemented with heparin, ascorbic acid, GA-1000, and 2% charcoal-stripped FBS. No growth factors from the kit were added. This density was found to be sufficient to produce approximately 70% confluency in the individual wells after 48 hours of incubation with growth factor stimuli and was insufficient to produce 100% confluency, thereby avoiding cell-cell contact inhibition of proliferation. After an initial 2 hour stabilisation period, cells were incubated for a further 46 hours in the presence of; VEGF (25 ng/ml) or bFGF (1 ng/ml), VEGF or bFGF plus established inhibitors (SU5416, 1 nM-1 μM; platelet factor 4, 3 nM-1 μM, respectively), or VEGF or bFGF plus cortisol (3 nM-1 μM). BrdU was added to the wells 1 hour after growth factors and inhibitors were added. Fresh drug dilutions were prepared on each day of experimentation. Additionally, control cells were incubated in the maximum concentration of vehicle (ethanol, 0.004% v/v; DMSO, 0.167% v/v) or in medium alone. Additional wells with cells but with no BrdU were included to obtain background absorbance measurements. At the end of the 48 hour incubation period, cells were fixed and denatured according to the assay kit instructions prior to incubation with the anti-BrdU antibody, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG. Wells were washed (1x plate wash) between incubations to minimise background with an Anthos Labtec aw1 microplate washer (ASYS Hitech, Austria). Finally, the cells were incubated with TMB and the reaction was stopped by the addition of sulphuric acid. Optical densities were measured using a Multiskan Ascent plate reader and data captured using Ascent v2.6 software (Thermo LabSystems, UK). The

absorbance of cells without the BrdU label and of the tissue culture medium, were measured and subtracted from each reading. It was possible to test the effect of cortisol on VEGF- and bFGF-induced proliferation by comparing proliferation in those wells with the "growth factor alone" wells after demonstrating growth factor alone had an effect by comparing with vehicle control. Each condition was performed in triplicate wells.

5.2.4.3 Cell viability assay (indirect method)

The CellTiter-Glo Luminescent Cell Viability Assay (Promega, UK) is an indirect method of assessing the number of live cells based on quantitation of ATP which indicates metabolically-active cells. Whether the cells are actively dividing or quiescent is not distinguished but an increase in cell viability indicates cell growth. CellTiter-Glo Reagent contains recombinant firefly luciferase which catalyses the mono-oxygenation of luciferin to oxyluciferin in the presence of Mg^{2+}, ATP and oxygen in a reaction which emits light. There is a direct relationship between the luminescent signal and the number of cells. Training in methodology, validated culture conditions, and growth factor and reference compound concentrations were provided by Dr Judith McKay and Mrs Julie Kellett, Department of Pharmacology, Aptuit, Edinburgh, UK, therefore limited method development was required.

5.2.4.4 Influence of glucocorticoids on cell viability

As detailed in Section 2.4.11.1, HUVECs were seeded in a 96-well plate at a density of 1.5 x 10^3 cells/well and incubated for 2 hours to allow cells to adhere to the plates. This density was found to be sufficient to produce approximately 70% confluency in the individual wells after 96 hours of incubation with growth factor stimuli, but was insufficient to produce 100% confluency. Cells were incubated in 'experimental' medium consisting of EGM-2 Bullet Kit medium (as above). Serum-supplemented medium is necessary for the reaction but charcoal stripping removes the majority of hormones (Leake *et al.*, 1987), thereby preventing influence of endogenous steroids. After the initial 2 hour period, cells were incubated for a further 94 hours in the presence of growth factors and inhibitors, as described for the BrdU method. Fresh

drug dilutions were prepared on each day of experimentation. Additionally, cells were incubated in the maximum concentration of vehicle or in medium alone to act as controls. At the end of the 96 hours, incubation plates were equilibrated to room temperature and CellTiter-Glo Reagent (100 μl) was added to each well. Plates were incubated for a further 10 minutes at room temperature to stabilise the luminescent signal before being read on a Wallac 1420 VICTOR^2 (Perkin-Elmer, UK) plate reader. Each condition was performed in triplicate wells.

5.2.5 Statistics

Data are expressed as mean ± standard error. "n" refers to number of different experiments performed on separate occasions using different batches of HUVECs; where possible all measurements of migration and proliferation were made in triplicate wells. During method development, cell migration data were initially expressed as the mean relative fluorescence units (RFU). For the final established method, data were expressed as a percentage of the RFU generated in concomitant control wells. Comparisons were made using Student's t-test and one way ANOVA followed by Dunnett's post hoc test, where appropriate. For migration assays, inter-assay and intra-assay coefficients of variation were 49% and 19% respectively (n=16). For proliferation assays, inter-assay and intra-assay coefficients of variation were 75% and 26%, respectively, (n=6) using the BrdU assay, and 68% and 16%, respectively, (n=6) using the cell viability assay.

5.3 Results

5.3.1 Cells

Preliminary data from control wells containing cells between p2 and p6 showed similar, reproducible migration after 24 hours (Figure 5.1). There was a trend, however, towards a correlation of enhanced migration with lower passage number (p=0.09). Consequently, cells were used at the earliest passage number (p2-p3) where possible. To maintain consistency with the migration assays, and with the TLS assay, proliferation assays were performed with cells between passages 2 and 6. The number of proliferation assays performed (n=6 of each) was insufficient for statistical analysis of correlation of proliferation with passage number.

5.3.2 Development of an endothelial cell migration assay

5.3.2.1 Culture conditions and pore size

Pilot studies demonstrated that VEGF-induced endothelial cell migration was not apparent after 5 or 24 hours of incubation using inserts with 3.0 μm pores (Figure 5.2). In contrast, whilst no migration was observed after 5 hours incubation using 8.0 μm pores, a 3.9-fold increase in VEGF (10 ng/ml)-induced migration was observed (VEGF, 28.4 ± 0.8 RFU; versus control, 7.2 ± 0.4 RFU) after 24 hours of incubation.

To determine whether glucocorticoids inhibit VEGF-induced HUVEC migration, preliminary experiments used concentrations of VEGF consistent with previous studies; 25 ng/ml (Kim *et al.*, 2002) and 50 ng/ml (Ali *et al.*, 2005; Cattaneo *et al.*, 2003), as a chemoattractant. Under these conditions, cortisol (600 nM) induced modest reductions (18.5% at 25 ng/ml and 15.0% at 50 ng/ml VEGF) in the degree of migration but these differences were not significant (p=0.82 and p=0.68, respectively; Figure 5.3).

5.3.2.2 Determination of EC_{50} of VEGF as a chemoattractant

EC_{50} values for VEGF were determined as 5 ng/ml and 15 ng/ml from 2 independent experiments (Figure 5.4). The mean of these values, 10 ng/ml, was used in the final protocol.

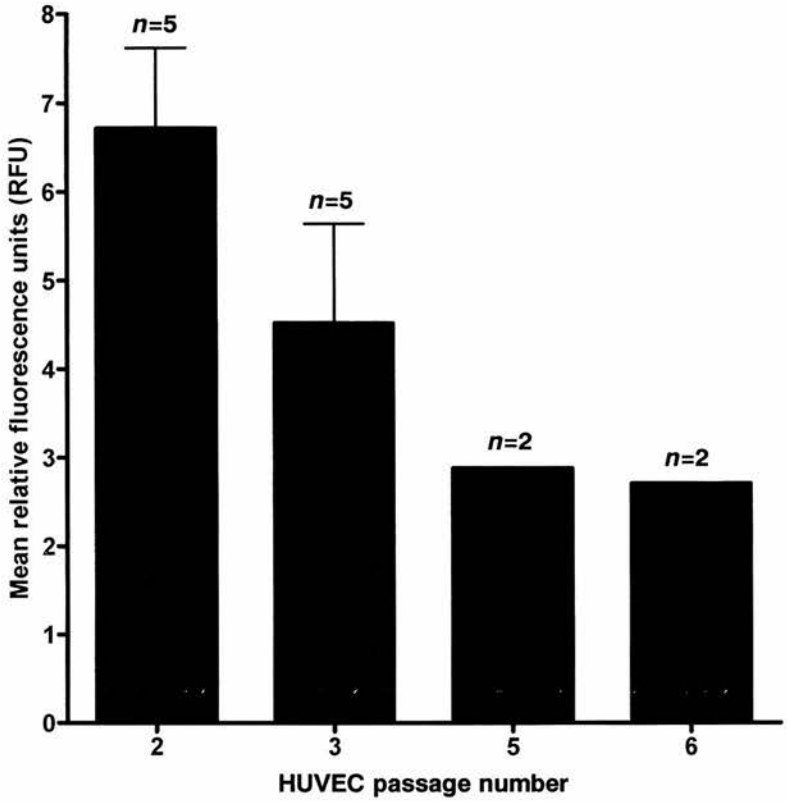


Figure 5.1 Influence of HUVEC passage number on cell migration

Human umbilical vein endothelial cells (HUVECs) in standard basal medium (endothelial cell growth medium-2, EGM-2) were seeded into the upper chamber of cell culture inserts (8.0 μm pores) in a Boyden chamber assay of cell migration. After 24 hours, migratory cells were quantified by labelling with calcein-AM and the amount of fluorescence (relative to cell number) was measured in a plate reader. Data represent mean ± SEM (number of experiments noted on bar chart), each condition was performed in triplicate wells. HUVECs at relatively low passage number demonstrated a greater migratory potential (p=0.09), therefore cells at low passage number (p2 or p3) were used where possible.

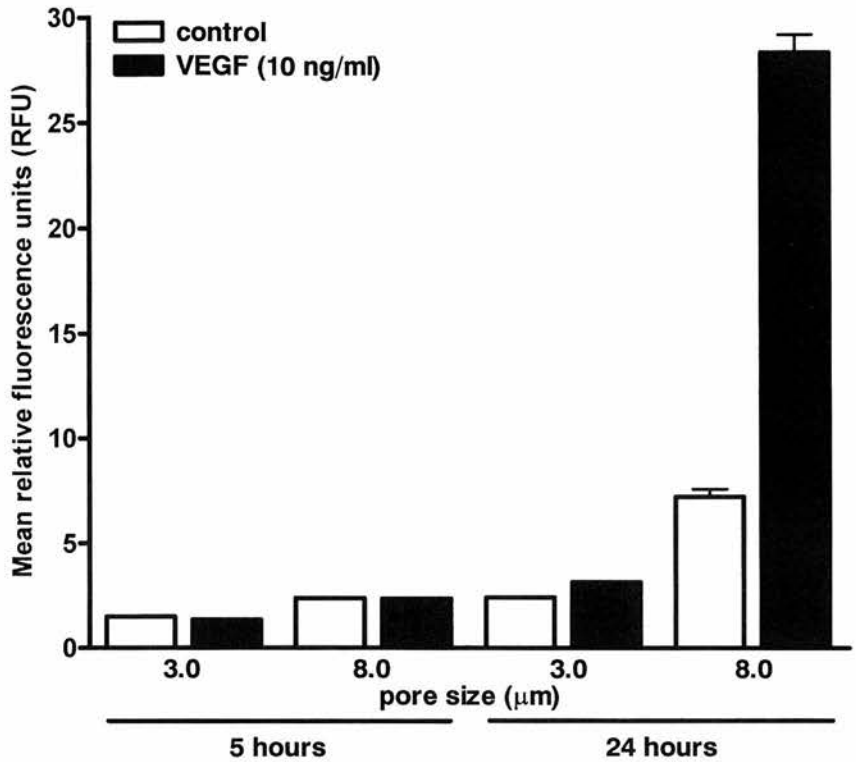


Figure 5.2 Development of a cell migration assay: Effects of pore size and incubation time

Human umbilical vein endothelial cells (HUVECs) in standard basal medium (endothelial cell growth medium-2, EGM-2) were seeded into the upper chamber of cell culture inserts (3.0 μm or 8.0 μm pores). The lower chamber contained EGM-2 (control), or vascular endothelial growth factor (VEGF, 10 ng/ml) as a chemoattractant. After 5 hours or 24 hours, migratory cells were quantified by labelling with calcein-AM and the amount of fluorescence (relative to cell number) was measured in a plate reader. 3.0 μm pores and 5 hours of incubation were both insufficient to produce a stimulatory effect of VEGF. Consequently, 8.0 μm pores and 24 hours of incubation were used in the final protocol. Data are from a single pilot experiment (where error bars represent SEM where each condition was performed in triplicate wells).

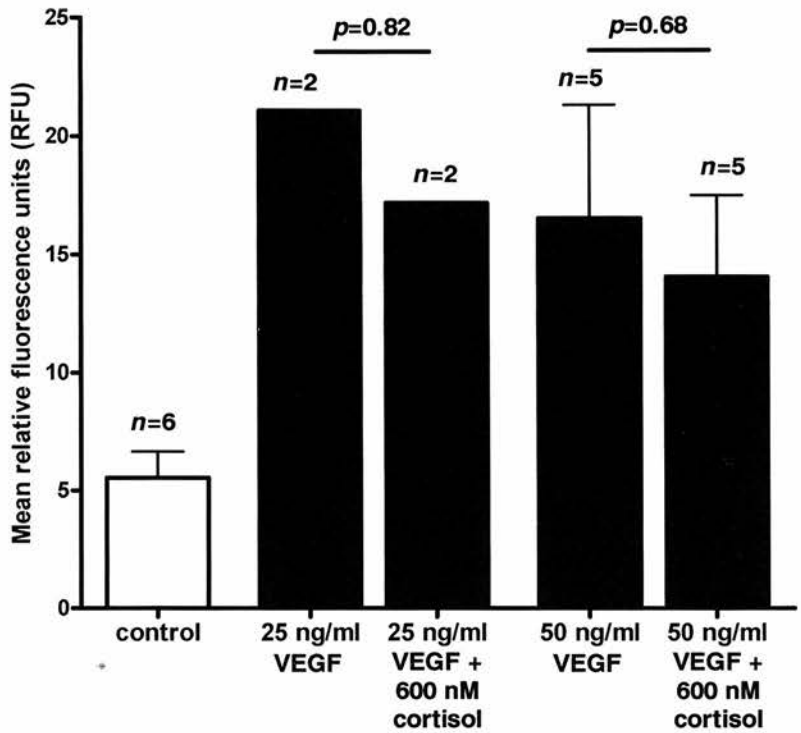


Figure 5.3 Development of a cell migration assay: Effects of VEGF and cortisol

HUVECs in standard basal medium (endothelial cell growth medium-2, EGM-2) were seeded into the upper chamber of cell culture inserts (8.0 μm pores). The lower chamber contained EGM-2 (control) or vascular endothelial growth factor (VEGF, 25 ng/ml or 50 ng/ml) as a chemoattractant. Upper and lower chambers contained cortisol (600 nM) where indicated. After 24 hours, migratory cells were quantified by labelling with calcein-AM and the amount of fluorescence was measured in a plate reader. No significant differences were observed between cortisol and VEGF wells (p=0.82, 25 ng/ml and p=0.68, 50 ng/ml VEGF and cortisol versus VEGF alone). Data represent mean ± SEM (n=2-6), each condition was performed in triplicate wells.

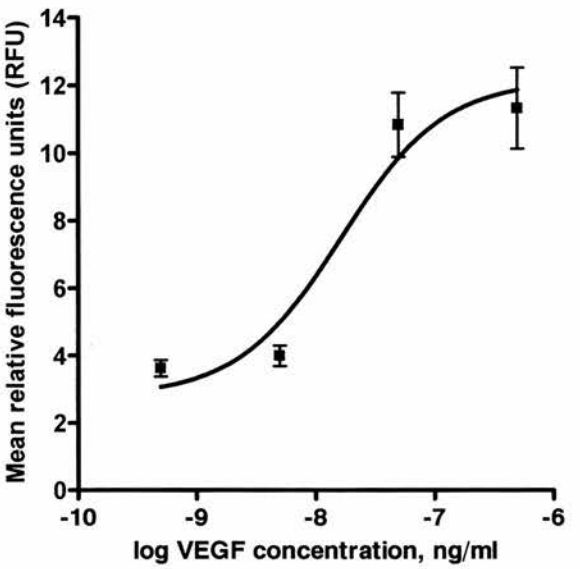


Figure 5.4 Development of a cell migration assay: Determination of EC_{50} of VEGF

HUVECs in standard basal medium (endothelial cell growth medium-2, EGM-2) were seeded into the upper chamber of cell culture inserts (8.0 μm pores). The lower chamber contained vascular endothelial growth factor (VEGF) as a chemoattractant at a range of concentrations; 0.5 ng/ml to 500 ng/ml and 7.5 ng/ml to 60 ng/ml in 2 separate experiments. After 24 hours, migratory cells were quantified by labelling with calcein-AM and the amount of fluorescence was measured in a plate reader. The half-maximal effective concentration (EC_{50}) was determined by non-linear regression with a representative sigmoidal concentration: response curve shown above. The EC_{50} values from 2 experiments were determined and the average value (10 ng/ml) was used in the final protocol. Data represent mean ± SEM from 1 experiment, each condition was performed in triplicate wells.

5.3.3 Influence of glucocorticoids on endothelial cell migration

Endothelial cell migration was stimulated with 10 ng/ml VEGF (262 ± 59%; $p<0.05$), compared with control (100%); Figure 5.5). This VEGF-induced migration was blocked (91 ± 4%) with the VEGF receptor inhibitor SU5416 (1 μM; positive control; $p<0.01$; VEGF plus SU5416 versus VEGF) whereas this compound had only a modest effect (86 ± 5%; p=not significant) on basal-unstimulated migration (Figure 5.5A). In contrast, cortisol (600 nM) had no effect on either VEGF-induced (258 ± 61%; p=0.95) or unstimulated-basal (103 ± 8%; p=not significant) migration (Figure 5.6B).

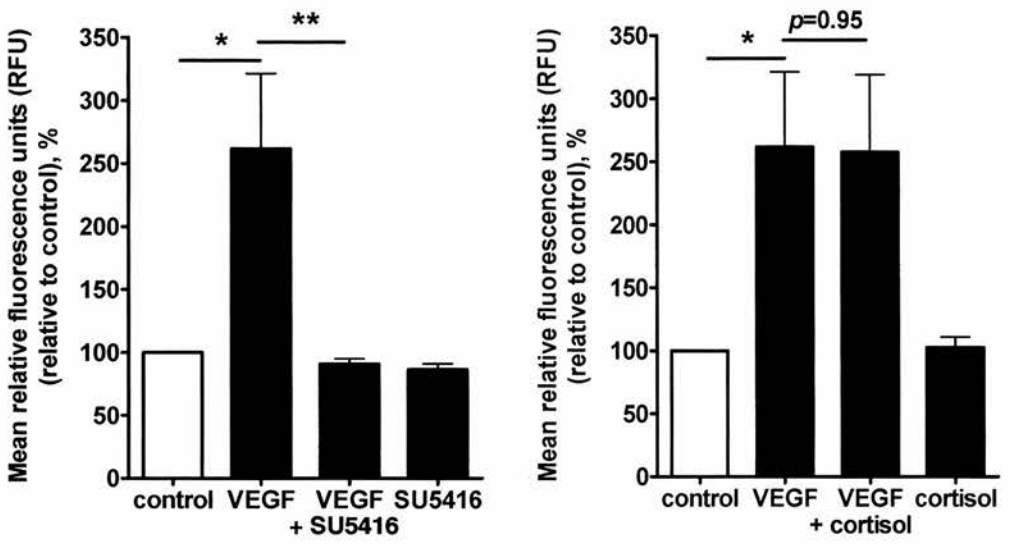


Figure 5.5 Effect of cortisol on endothelial cell migration

HUVECs in standard basal medium (endothelial cell growth medium-2, EGM-2) were seeded into the upper chamber of cell culture inserts (8.0 μm pores). The lower chamber contained vascular endothelial growth factor (VEGF, 10 ng/ml) as a chemoattractant. Upper and lower chambers contained the selective VEGF receptor inhibitor, SU5416 (1 μM, positive control), or cortisol (600 nM), or EGM-2 medium (negative control). After 24 hours, migratory cells were quantified by labelling with calcein-AM and the amount of fluorescence was measured in a plate reader. The stimulatory effect of VEGF (*p<0.05) was (a) blocked by co-incubation with SU5416 (**p<0.01 VEGF and SU5416 versus VEGF) whilst (b) cortisol had no effect (p=0.95). SU5416 or cortisol alone had no effect on cell migration. Data represent mean ± SEM; n=6. Each condition was performed in triplicate wells.

5.3.4 Influence of glucocorticoids on endothelial cell proliferation

5.3.4.1 Bromodeoxyuridine (BrdU) incorporation assay (direct method): Optimisation of culture conditions

Cell seeding density was found to be an important factor in the sensitivity of the assay; 1.5 x 10^3 cells/well resulted in only a modest augmentation of proliferation by 25 ng/ml VEGF (140% compared with vehicle-treated control), whereas 5 x 10^3 cells/well caused cultures to overgrow with little or no effect of growth factor detected. Cultures incubated for a total of 24 hours, or with BrdU during the final 3 hours of the protocol, exhibited insufficient proliferation and low absorbance readings in control wells. Cultures incubated for up to 96 hours resulted in overgrowth of cells. This important method refinement culminated in the final, established culture conditions described in Section 2.4.11.2.

5.3.4.2 Influence of glucocorticoids on BrdU incorporation

Drug vehicles (ethanol, 0.004% v/v; DMSO, 0.167% v/v) had no impact on cell proliferation compared with untreated control wells (p=0.97 and p=0.93, respectively; Figure 5.6).

HUVEC proliferation was induced by incubation with either growth factor; VEGF (25 ng/ml) stimulated 1.6 to 1.9-fold increases (p<0.01) in relative luminescence (Figure 5.7); bFGF (1 ng/ml) stimulated 2.0 to 2.7-fold increases (p<0.05; Figure 5.8).

VEGF-induced cell proliferation was inhibited in the presence of SU5416 (positive control) in a concentration-dependent manner (100 nM, 114 ± 14%, p<0.05; 300 nM, 98 ± 10%, p<0.01 and 1 μM, 71 ± 10%, p<0.01 compared with VEGF plus vehicle-treated control, 185 ± 24%) (Figure 5.7A). Platelet factor 4 (positive control) had little effect on bFGF-induced cell proliferation within a 3 nM to 100 nM range but proliferation was inhibited at 300 nM (181 ± 34%) and 1 μM (103 ± 17%, p<0.05) compared with bFGF-treated control, 273 ± 42%) (Figure 5.8A).

In contrast to the described effects of the positive controls, cortisol (3 nM to 1 μM) had no effect on cell proliferation induced by either growth factor (Figure 5.7B and Figure 5.8B).

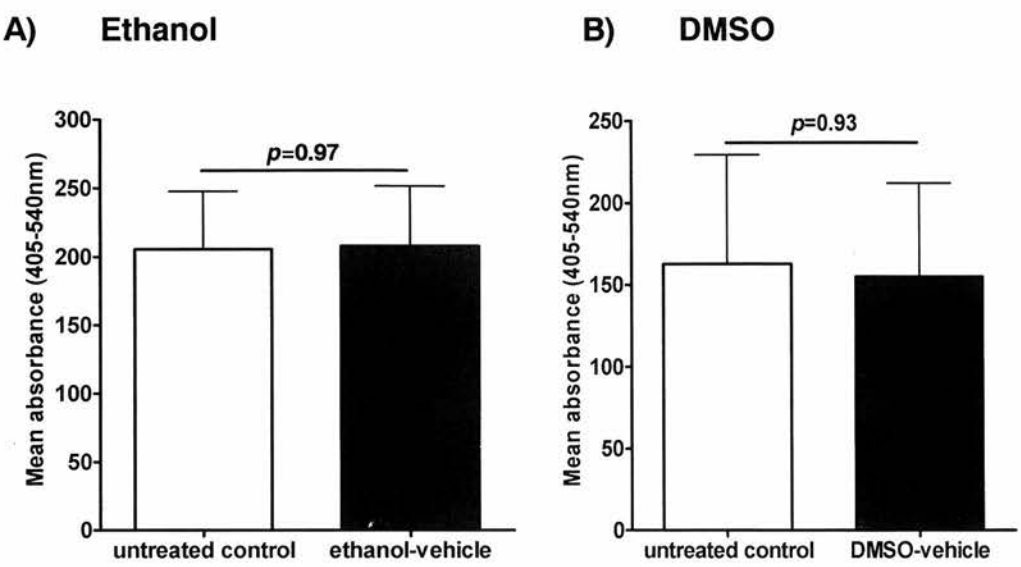


Figure 5.6 Effects of ethanol and DMSO (drug vehicles) on endothelial cell proliferation

HUVECs were seeded into 96-well plates in standard experimental medium (endothelial cell growth medium-2, EGM-2, plus 2% charcoal-stripped foetal bovine serum) or in the presence of: drug vehicles (ethanol, 0.004% v/v; dimethyl sulfoxide, DMSO, 0.167% v/v). Bromodeoxyuridine (BrdU) was added to the wells, and after 48 hours of incubation, BrdU incorporation (relative to the amount of cell proliferation) was quantified by enzyme-linked immunosorbent assay (ELISA) and absorbance was measured in a plate reader. Ethanol and DMSO vehicles had no effect on HUVEC proliferation (p=0.97 and p=0.93, respectively. Data represent mean ± SEM; n=6-13. Each condition was performed in triplicate wells.

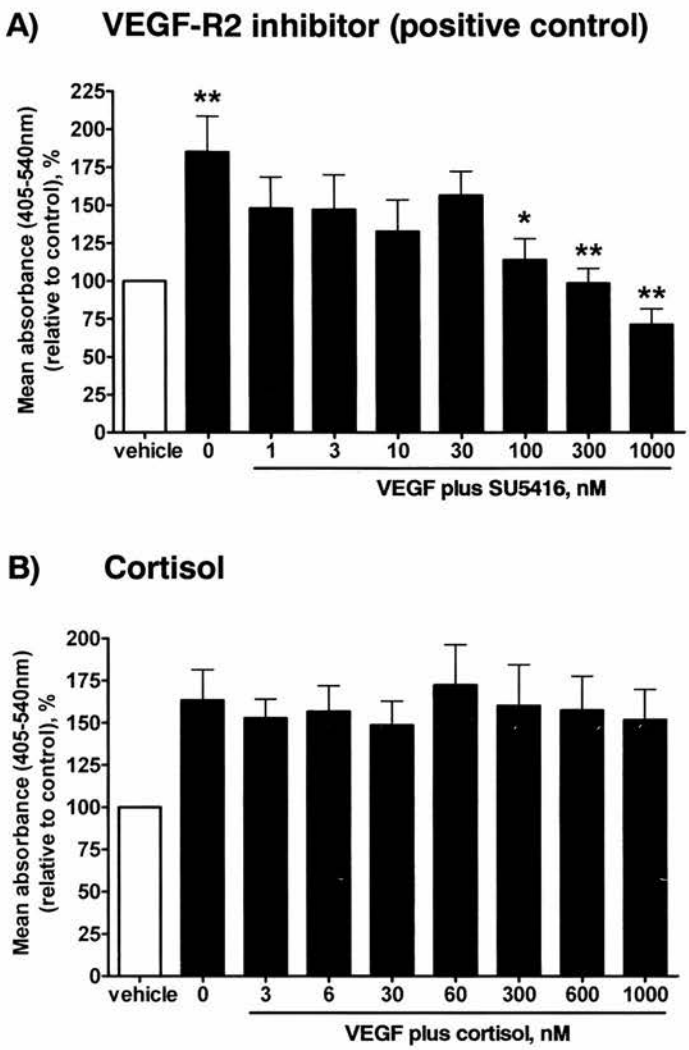


Figure 5.7 Effect of cortisol on VEGF-induced endothelial cell proliferation (BrdU method)

HUVECs in standard experimental medium (endothelial cell growth medium-2, EGM-2, plus 2% charcoal-stripped foetal bovine serum) were seeded into 96-well plates in the presence of: vascular endothelial growth factor (VEGF, 25 ng/ml), VEGF plus an established inhibitor (SU5416, 1 nM-1 μM), or VEGF plus cortisol (3 nM-1 μM). Cells were incubated in drug vehicles (final concentration, 0.004% v/v ethanol or 0.167% v/v DMSO) or medium alone as controls. Bromodeoxyuridine (BrdU) was added to the wells, and after 48 hours of incubation, BrdU incorporation (relative to the amount of cell proliferation) was quantified by enzyme-linked immunosorbent assay (ELISA) and absorbance was measured in a plate reader. The stimulatory effect of VEGF (**p<0.01) was (a) blocked by co-incubation with SU5416 (100 nM, *p<0.05; 300 nM and 1 μM, **p<0.01), while (b) cortisol had no effect on VEGF-induced HUVEC proliferation. Data represent mean ± SEM; n=6. Each condition was performed in triplicate wells.

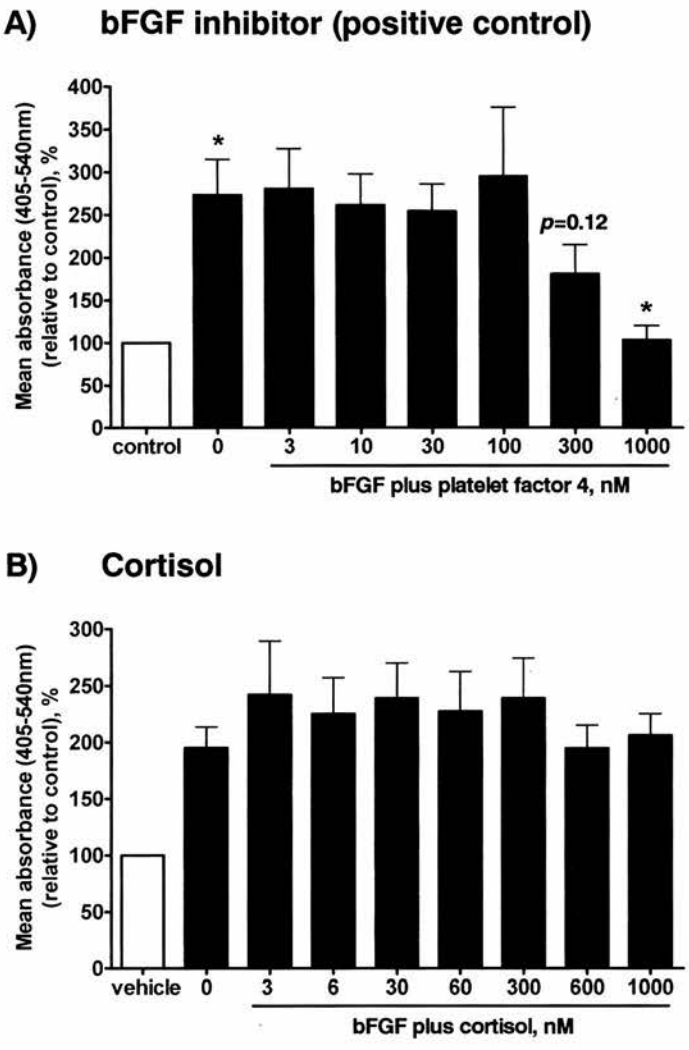


Figure 5.8 Effect of cortisol on bFGF-induced endothelial cell proliferation (BrdU method)

HUVECs in standard experimental medium (endothelial cell growth medium-2, EGM-2, plus 2% charcoal-stripped foetal bovine serum) were seeded into 96-well plates in the presence of: basic fibroblast growth factor (bFGF, 1 ng/ml), bFGF plus an established inhibitor (platelet factor 4, 3 nM-1 μM), or bFGF plus cortisol (3 nM-1 μM). Cells were incubated in vehicle (final concentration, 0.004% v/v ethanol) or in medium alone as controls. Bromodeoxyuridine (BrdU) was added to the wells and, after 48 hours of incubation, BrdU incorporation (relative to the amount of cell proliferation) was quantified by enzyme-linked immunosorbent assay (ELISA) and absorbance was measured in a plate reader. The stimulatory effect of bFGF (*$p<0.05$) was (a) blocked by co-incubation with platelet factor 4 (300 nM, $p<0.12$; 1 μM, *$p<0.05$), whilst (b) cortisol had no effect on bFGF-induced HUVEC proliferation. Data represent mean ± SEM; $n=5$. Each condition was performed in triplicate wells.

5.3.4.3 Influence of glucocorticoids on cell viability

HUVEC proliferation was induced with both growth factors; VEGF (25 ng/ml) resulted in 2.5 to 4.4-fold increases ($p<0.01$) in relative luminescence (Figure 5.9); bFGF (1 ng/ml) resulted in 3.8 to 6.7-fold increases (Figure 5.10).

VEGF-induced cell proliferation was inhibited in the presence of SU5416 (positive control) in a concentration-dependent manner (300 nM, 100 ± 6%, $p<0.01$ and 1000 nM, 54 ± 9%, $p<0.01$ compared with VEGF plus vehicle-treated control, 438 ± 82%) (Figure 5.9A). Basic-FGF-induced cell proliferation was augmented in the presence of 3 nM and 10 nM platelet factor 4 (positive control) and was inhibited at the higher concentrations (ANOVA, $p<0.01$; Dunnett's post hoc test, 300 nM, 90 ± 8%, $p=0.07$ and 1 μM, 86 ± 18%, $p=0.07$ compared with bFGF-treated control, 669 ± 281%) (Figure 5.10A).

In contrast to the described effects of the positive controls, cortisol had little effect on cell proliferation induced by either growth factor at 3 nM to 600 nM. With 1 μM cortisol, however, there was a trend towards inhibition of bFGF-induced proliferation but this did not reach statistical significance (bFGF and cortisol, 190 ± 57% compared with bFGF plus vehicle-treated control, 383 ± 84%, $p=0.09$) (Figure 5.9B and Figure 5.10B).

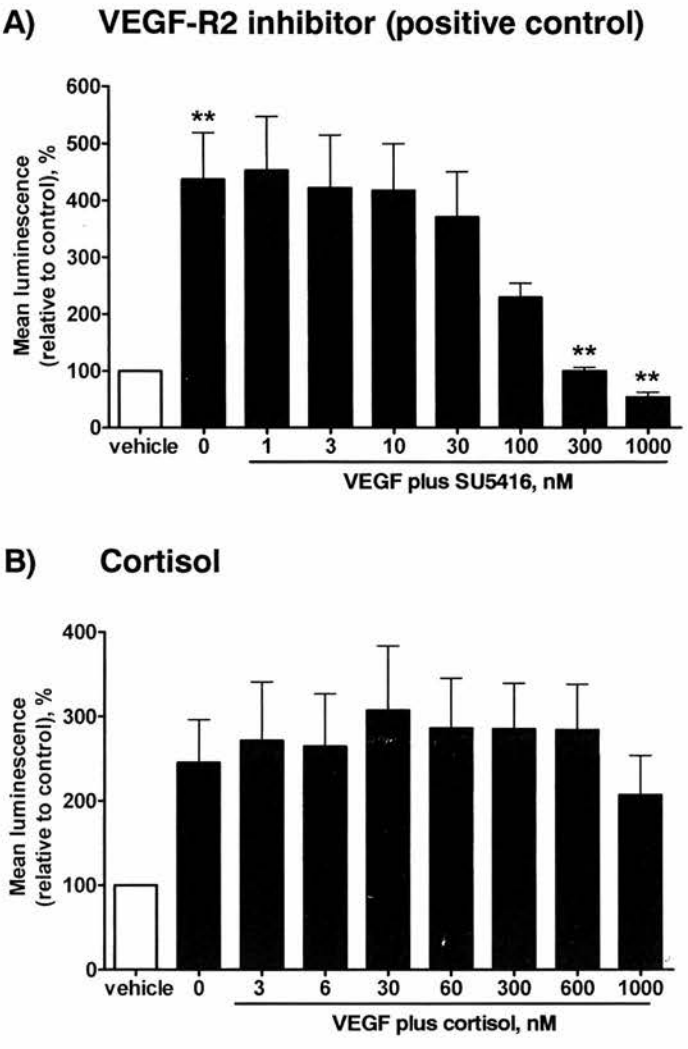


Figure 5.9 Effect of cortisol on VEGF-induced endothelial cell proliferation (cell viability method)

HUVECs in standard experimental medium (endothelial cell growth medium-2, EGM-2, plus 2% charcoal-stripped foetal bovine serum) were seeded into 96-well plates in the presence of: vascular endothelial growth factor (VEGF, 25 ng/ml), VEGF plus an established inhibitor (SU5416, 1 nM-1 μM), or VEGF plus cortisol (3 nM-1 μM). Cells were incubated in vehicles (ethanol, 0.004% v/v; DMSO, 0.167% v/v) or medium alone as controls. After 96 hours of incubation, Cell-Titer Glo reagent (Promega) was added to the wells, and ATP production (relative to the number of viable cells) was measured in a plate reader by luminescence. The stimulatory effect of VEGF was (a) blocked by co-incubation with SU5416 (300 nM and 1 μM, $^{**}p<0.01$), while (b) cortisol had no effect on VEGF-induced HUVEC proliferation. Data represent mean ± SEM; $n=6$. Each condition was performed in triplicate wells.

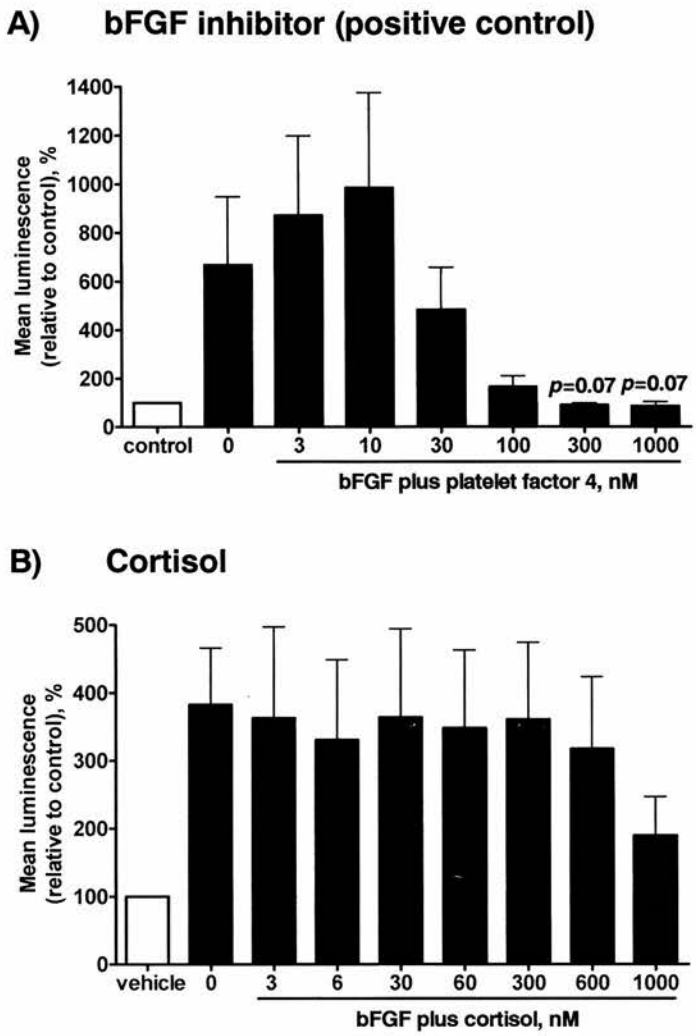


Figure 5.10 Effect of cortisol on bFGF-induced endothelial cell proliferation (cell viability method)

HUVECs in standard experimental medium (endothelial cell growth medium-2, EGM-2 plus 2% charcoal-stripped foetal bovine serum) were seeded into 96-well plates in the presence of: basic fibroblast growth factor (bFGF, 1 ng/ml), bFGF plus an established inhibitor (platelet factor 4, 3 nM-1 μM), or bFGF plus cortisol (3 nM-1 μM). Cells were incubated in vehicle (ethanol, 0.004% v/v) or medium alone as controls. After 96 hours of incubation, Cell-Titer Glo reagent (Promega) was added to the wells, and ATP production (relative to the number of viable cells) was measured in a plate reader by luminescence. The stimulatory effect of bFGF was (a) blocked by co-incubation with platelet factor 4 ($p<0.01$). In contrast, cortisol had little effect in the 3 nM-600 nM range (b), however at 1 μM, cortisol caused a 50% inhibition in proliferation, but again, this did not reach significance ($p=0.09$). Data represent mean ± SEM; $n=6$. Each condition was performed in triplicate wells.

5.4 Discussion

The intention of these studies was to determine whether glucocorticoids inhibit endothelial cell migration and proliferation, and whether they do so by inhibiting cellular responses to angiogenic growth factors relevant to these two processes. The results suggest that physiologically-relevant concentrations of glucocorticoids do not inhibit endothelial cell migration or proliferation *in vitro*. Indeed this is consistent with the fact that glucocorticoids inhibit TLS formation which does not encompass cell migration or proliferation. It is unlikely, therefore, that glucocorticoid-mediated inhibition of angiogenesis *in vivo* is due to direct inhibition of endothelial cell migration or proliferation.

5.4.1 Development of an endothelial cell migration assay

A wide spectrum of assays exist for assaying cell migration; from simple 2D surface migration assays such as phagokinetic tracks (Albrecht-Buehler, 1977) (measuring displacement of colloidal gold by cell movement), to more qualitative methods which include time-lapse imaging of cells migrating in 3D matrices, reviewed in (Entschladen *et al.*, 2005). The 2 most popular methods for assaying endothelial cell migration include the Boyden chamber (or filter) and scratch (or 'wound' healing) assays. In the Boyden chamber assay (Boyden, 1962) endothelial cells move along a gradient of angiogenesis-inducing factors such as VEGF. In the scratch assay a 'wound' is created in a monolayer of confluent cells and the motility of endothelial cells on a flat (2D) surface is analysed (Taraboletti & Giavazzi, 2004). In the scratch assay, the repair process is considered to involve a combination of migration and proliferation (Coomber & Gotlieb, 1990; Zahm *et al.*, 1997; Yarrow *et al.*, 2004) which, thus, limits its use in mechanistic studies where the action of an agent on the migration step alone is required. Since a major aim of these studies was to examine the influence of glucocorticoids on directed cell migration, the Boyden chamber assay was most appropriate. Furthermore, in the scratch migration assay, problems are associated with the requirement to run control and experimental groups under identical growth conditions and levels of confluency. These concerns can be avoided with the Boyden chamber assay where the same population of cells (from the same

flask) is analysed in all treatment groups (Auerbach *et al.*, 1991). Another major advantage of the Boyden chamber assay over other migration assays is the high sensitivity to small differences in concentration gradients (Falk *et al.*, 1980; Staton *et al.*, 2004) making it a useful tool to examine potentially subtle effects of physiological concentrations of glucocorticoids.

The Boyden chamber assay lends itself to testing concentrations gradients and, thus, may reflect the conditions that operate *in vivo* (Auerbach *et al.*, 2003). However, *in vitro* assays are only a rough approximation of the *in vivo* situation. It has been suggested that equilibrium of the chemoattractant between the two chambers is quickly formed, thus, making it difficult to discriminate between chemotaxis (*i.e.* directed cell movement) and chemokinesis (*i.e.* undirected increase of migratory activity) (Entschladen *et al.*, 2005). This has been circumvented by checkerboard analysis studies (Zigmond & Hirsch, 1973). These distinguish chemotaxis (increased migration towards a chemoattractant in the lower compartment) from chemokinesis (with the same concentration of attractant applied to the upper, or the upper and lower, compartments) (Entschladen *et al.*, 2005). Chemokinesis would produce increased cell migration regardless of whether the chemoattractant was placed in the upper, lower or both compartments (Entschladen *et al.*, 2005). VEGF has been shown to have predominantly chemotactic properties in this assay, whereas bFGF has been shown to produce a predominantly chemokinetic pattern (Yoshida *et al.*, 1996). Consequently, in the present study, it was possible to test the effects of glucocorticoids on directed cell migration.

Evaluation of the effects of cell passage number on basal migration levels demonstrated that early passage cells (p2-p3) have greater chemokinetic ability than later passage cells (p4-p6) and suggests they are more likely to be responsive to an angiogenic stimulus. These findings contrast with a lack of effect of passage number across the p2-p6 range in the TLS assay and suggest that migration is more sensitive to passage than TLS formation. For this reason, cells at p2 and p3 were used in cell migration assays, where possible. This is consistent with other reports in the literature which have used endothelial cells at p1 (Polytarchou & Papadimitriou,

2005), p2 (Herve *et al.*, 2005) or p2-p3 (Ahmad & Ahmed, 2004). In these studies, however, the rationale for using cells at low passage was not explained. In contrast, several studies have used endothelial cells as high as p6 (Cattaneo *et al.*, 2003; Ali *et al.*, 2005) and even p8 (Isenberg *et al.*, 2006). In the present study, 5 fresh batches of pooled HUVECs from multiple donors were used; thereby minimising the possibility of individual donor effects. Batch-specific variation in the degree of basal (control) migration was evident in these studies given the mean inter-assay coefficients of variation; this was circumvented, however, by performing control groups on each occasion and normalising data to control.

Relative to the size of the investigated cells, the size of the pores in the filter membrane must be small enough to avoid the passive passage of cells, but large enough to allow their active migration. The most common pore size used in the Boyden chamber assay with HUVECs is 8.0 μm (Hotchkiss *et al.*, 2002; Cattaneo *et al.*, 2003), but there have been studies using 5.0 μm pores (Nissen *et al.*, 1998; Gallicchio *et al.*, 2005). Furthermore, whilst 4-6 hours of incubation is commonly used for this assay (Kim *et al.*, 2002; Ali *et al.*, 2005), longer incubation times (16 hours) have also been reported (Herve *et al.*, 2005). Consequently, a lack of standard experimental conditions prompted an evaluation of the effects of pore size and duration of incubation during method refinement. The fact that relatively little migration was observed with 3.0 μm pores in response to VEGF indicates that the pore size was too small for HUVEC trans-migration. A lack of effect of VEGF on cell migration after 5 hours of incubation contrasts with previous reports but these studies used between 2-fold (Kim *et al.*, 2002; Ischenko *et al.*, 2007) and 5-fold (Ali *et al.*, 2005) higher concentrations of VEGF. Furthermore, the use of serum-free medium in the present study contrasts with most previous reports which generally use serum-reduced media (Lee *et al.*, 2000; Kim *et al.*, 2002; Gallicchio *et al.*, 2005). Although it was not tested, it is possible that the combination of chemoattractant, along with growth factors present in serum, may act synergistically to induce a quicker chemotactic response. This could explain the longer duration of incubation required in the present study (24 hours) compared with previous studies (4-6 hours) (Kim *et al.*, 2002; Ali *et al.*, 2005). To maintain consistency with the endothelial

TLS formation studies, and to exclude effects from undetermined factors present in serum as possible confounding factors, basal (serum and growth factor-free) medium was used in the present study. This combination of pore size, culture conditions and duration of incubation proved sufficient to produce a measurable effect of chemoattractant on HUVEC chemotaxis, thereby providing a useful tool to test the effects of glucocorticoids.

Given the observations in previous chapters, VEGF was an obvious choice as a chemoattractant in these studies. The use of VEGF as a chemoattractant in this assay is common (Cattaneo *et al.*, 2003; Gallicchio *et al.*, 2005; Herve *et al.*, 2005) probably mainly due to its recognised chemotactic properties in physiological (Autiero *et al.*, 2005) and pathophysiological (Nissen *et al.*, 1998) models. The most abundant VEGF-A splice variant, $VEGF_{165}$, was used in the present study since it has been shown to have stronger migration-inducing effects than longer isoforms (*e.g.* $VEGF_{189}$) which require protease activation and cleavage *in vivo* (Ferrara & Davis-Smyth, 1997). Studies to determine the EC_{50} value of VEGF were prompted by a lack of consensus in the literature with quoted values ranging from 1 ng/ml (Ahmad & Ahmed, 2004) up to 50 ng/ml (Cattaneo *et al.*, 2003). It is also likely that the concentration required to produce a sub-maximal chemotactic response will vary depending on the culture conditions used. In preliminary studies, a lack of significant inhibitory effect of cortisol using 25 and 50 ng/ml VEGF could be interpreted as over-stimulation of the cells which could mask any potential effect of glucocorticoids. Accordingly, determination of the EC_{50} value for VEGF was an important step of method development.

Fluorescence-based measurements proved rapid, reliable and not technically demanding; advantages which led to the adoption of this method of quantification. In the original assay, developed in 1962, quantification was performed by counting, under a microscope, 5 to 10 random fields for each filter. Various alternatives have been developed subsequently including; metabolic labelling with X-gal substrate or similar compounds (Klemke *et al.*, 1998), colorimetric detection (Saito *et al.*, 1997), or fluorescence-based detection of stained cells. The results obtained using a dye

method of quantification are said to vary less than those obtained from counting cells directly (Grotendorst, 1987) but the reasons for this are unclear. A possible explanation is that dye methods take into account the total number of cells that migrate through the pores, whereas the counting method measures a sample of cells in random fields that might not be representative of the whole filter. Also, assays which involve a plate reader for end-point analysis are less prone to investigator bias than cell counting which requires selection of fields for enumeration (Frevert *et al.*, 1998; Wang *et al.*, 2007). In comparative studies using leukocytes, the fluorescence end-point assay was shown to be more rapid and more sensitive than the counting assay (De Gendt *et al.*, 1996; Frevert *et al.*, 1998). Importantly, others have shown that relative fluorescence intensity correlates significantly with cell counts (Sunder-Plassmann *et al.*, 1996) and that calcein-AM does not influence cell functions, such as chemotaxis, at least in leukocytes (De Clerck *et al.*, 1994). In the present study, method development demonstrated that the fluorescence-based quantification method was sensitive in detecting stimulation of cell migration using narrow and wide VEGF concentration ranges on different occasions. Consistent with other studies using plate-reader measurements (Klemke *et al.*, 1998; Erzurum *et al.*, 2003), each condition was performed in triplicate and data were expressed as a percentage relative to control wells, to avoid intra- and inter-assay variability, respectively.

5.4.2 Influence of glucocorticoids on endothelial cell migration

Glucocorticoids can inhibit migration of leukocytes (Yamada *et al.*, 1993; Yamaguchi *et al.*, 1994; Hofbauer *et al.*, 1999) and smooth muscle cells (Pross *et al.*, 2002b). There is at least indirect evidence to suggest that glucocorticoids and synthetic angiostatic steroids may modulate endothelial cell migration, for example by inhibiting growth factor-induced proteolytic activity (Blei *et al.*, 1993; Pepper *et al.*, 1994), which is intimately related to cell motility and essential for angiogenesis (Blasi, 1997). Furthermore, a possible effect of glucocorticoids on the endothelial cytoskeleton (Chapter 3) is suggestive of a potential effect on VEGF-induced motility since regulation of actin reorganisation and cytoskeleton remodelling is responsible for this process (Rousseau *et al.*, 2000). Any direct effect of

physiological glucocorticoids on growth factor-induced endothelial cell migration *per se* remained to be determined.

The demonstration that glucocorticoids do not inhibit endothelial cell migration, either in the presence or absence of angiogenic stimuli suggests that this is not a mechanism through which they inhibit angiogenesis. Demonstration of a lack of effect of glucocorticoids in this model were strengthened by the blockade of VEGF action by the specific VEGF signalling inhibitor, SU5416, which acted as a robust positive control. SU5416 has previously been shown to inhibit endothelial cell migration (Hirata *et al.*, 2002; Abdollahi *et al.*, 2003; Dolle *et al.*, 2005). Only one other study to date has examined the influence of glucocorticoids on endothelial cell migration, in the absence of additional chemoattractant, showing, surprisingly, that dexamethasone (400 nM) stimulated HUVEC migration (Polytarchou & Papadimitriou, 2005). However, the presence of high (15%) serum levels in the culture medium, which in itself is chemotactic (English *et al.*, 2000) could potentially have overcome any inhibitory effect of dexamethasone (Polytarchou & Papadimitriou, 2005). Interestingly, dexamethasone-mediated stimulation of migration in this previous study (Polytarchou & Papadimitriou, 2005) was accompanied by activation of eNOS which is consistent with activation of the ERK/MAPK signalling pathway (Chapter 4). This suggests that culture conditions are likely to play a pivotal role in the interpretation of these data.

5.4.3 Influence of glucocorticoids on endothelial cell proliferation

Endothelial cells are normally quiescent in the adult vasculature but readily undergo rapid proliferation during angiogenesis. This process is recapitulated, to some extent, by growing isolated cells on tissue culture plastic. Cell proliferation assays are easy to perform, highly reproducible, and lend themselves to precise quantification and high sample throughput (Auerbach *et al.*, 1991). There are two major classes of proliferation assay: those that determine net cell number (*i.e.* indirect) and those that evaluate cell-cycle kinetics (*i.e.* direct). Cell number can be determined by direct cell counting or by cell viability measurements (*e.g.* ATP or

tetrazolium salts) as an indicator for healthy cells. Whether the cells are actively dividing or quiescent is not distinguished in cell viability assays but a decrease in cell viability may indicate cytotoxicity, rather than anti-proliferative activity, of an agent. In contrast, direct proliferation assays (such as BrdU or [^{3}H]-thymidine incorporation) measure actively dividing cells, since incorporation of the labelled precursor into DNA is directly proportional to the rate of cell division (Dean *et al.*, 1984). Quiescent, non-dividing, but otherwise healthy cells, are not detected by cell proliferation assays. Combining data from multiple different types of proliferation assay will yield a more accurate picture of endothelial cell responses to a given agent (Staton *et al.*, 2004). Thus, in the present study, two commercially-available kits were used to determine the influence of glucocorticoids on both endothelial cell proliferation and viability. Finally, it is important to bear in mind that inhibition of cell proliferation is not a requisite for anti-angiogenic activity of an angiostatic agent and a lack of anti-proliferative activity could indicate an alternative mode of action (*e.g.* inhibition of cell migration, invasiveness *etc.*) (Taraboletti & Giavazzi, 2004).

Both the BrdU (Nanobashvili *et al.*, 2004; Park *et al.*, 2006) and the Cell-Titer Glo (Mendoza *et al.*, 2002) assays have previously been described in the literature. The Cell-Titer Glo assay has been validated for commercial use by our collaborators in the pharmaceutical industry; thus reducing the need for method development. To maintain consistency with the TLS and migration assays, and in keeping with the literature (Dou *et al.*, 2004; Nanobashvili *et al.*, 2004), HUVECs were used at relatively low passage number (up to p4 in 75% of the assays). Method refinement demonstrated that cells were more responsive to angiogenic stimuli at lower passage number (data not shown). It was necessary to vary cell seeding densities and incubation times for each assay, in order to produce a level of confluency at a point of logarithmic cell growth, thus mimicking endothelial cells undergoing angiogenic proliferation.

The influence of cortisol on the action of VEGF and bFGF-induced proliferation were tested since glucocorticoids can interfere with both of these signalling pathways, and importantly, both are important mediators of angiogenesis *in vivo*

(Bohnsack & Hirschi, 2004). Although 2.5-fold higher concentrations of VEGF were required to produce the desired mitogenic effect compared with the chemotactic response, this is consistent with another report where 10-fold higher levels of VEGF were required in proliferation, compared with migration, assays (Kanno *et al.*, 2000). The reason for this is not clear but may reflect different mechanisms of regulation of these endothelial cell processes which involve overlapping but distinct signalling pathways (Becker *et al.*, 2001). However, the concentrations of VEGF and bFGF used in the current experiments were sufficient to reliably produce significant stimulation of HUVEC proliferation in both systems to allow the influence of glucocorticoids to be tested.

The inclusion of serum in the culture medium was necessary for the reaction to measure cell viability, according to the manufacturer's instructions, and was included in the BrdU assay to maintain consistency between proliferation assays. The inclusion of serum is also necessary for experiments which rely on extended periods of culture (several days) to prevent apoptosis induced by serum starvation. Indeed the inclusion of serum in these assays is commonplace in the literature with between 5% (Marks *et al.*, 2002) and 10% (Nanobashvili *et al.*, 2004) reported whilst maintaining significant stimulatory effects with exogenous stimuli. To avoid the possibility of steroid hormones present in FBS masking any effect of exogenous cortisol treatments, charcoal-stripped serum was used: charcoal stripping removes the majority of hormones from serum (Leake *et al.*, 1987).

Findings from the present study consistently demonstrate that endogenous glucocorticoids, across a physiological range of concentrations, do not influence mitogen-induced HUVEC proliferation. This interpretation was strengthened by the fact that the same results were obtained using 2 different methods of assaying proliferation, and that proliferation was inhibited using relevant positive controls. SU5416 and platelet factor 4 have previously been shown to inhibit HUVEC proliferation in response to VEGF, (Mendel *et al.*, 2000) and bFGF (Yoshida *et al.*, 1996; Gentilini *et al.*, 1999), respectively, using similar experimental conditions.

There may be species differences in the vascular response to glucocorticoids. For example, glucocorticoids have well-documented anti-proliferative effects on rodent vascular smooth muscle cells (Berk *et al.*, 1988) and endothelial cells (Heffernan *et al.*, 1978; Derbyshire *et al.*, 1996) which have not translated well into the clinic as potential anti-atherosclerotic and anti-restenotic agents (Hadoke *et al.*, 2006). There have been mixed reports *in vitro* of the effects of glucocorticoids on endothelial cell proliferation. For example, inhibitory effects (Cariou *et al.*, 1988; Kräling *et al.*, 1999; Banciu *et al.*, 2006; Zou *et al.*, 2006), stimulatory effects (Polytarchou & Papadimitriou, 2005) and no effect on unstimulated (Chen *et al.*, 2006) or TNFα-induced (Yamamoto *et al.*, 2004), human endothelial cell proliferation have been reported. The reasons for these conflicting data may reflect differences in; endothelial cell type (species and vascular territory), steroid (synthetic or endogenous; concentration), culture conditions (presence of serum and/or growth factors; charcoal stripping; duration of incubation), and the method used to assay for proliferation (*e.g.* direct cell counting, MTT, BrdU or [^{3}H]-thymidine incorporation). The fact that at the same concentration and culture conditions, cortisol has divergent effects on endothelial cell proliferation (present study) versus endothelial sprouting (which comprises cell proliferation) (Small *et al.*, 2005), highlights the differential effects of physiological glucocorticoids on the individual components of the angiogenesis cascade. This is supported by the fact that cortisol (600 nM) inhibits TLS formation (Chapter 3) without an apparent effect on cell viability, findings which parallel that of another study in which blockade of β1-integrin function destroys the integrity of capillary tubes without a loss of cell viability (Da Silva *et al.*, 2003). In other words, cell death is not a pre-requisite for disruption of endothelial tube formation.

The trend towards an inhibitory effect of 1 μM cortisol on bFGF-induced HUVEC proliferation suggests that, in contrast with physiological levels of glucocorticoids, pharmacological levels might exert direct anti-proliferative effects on isolated endothelial cells. Indeed, this may be one element of their actions *in vivo e.g.* when administered therapeutically for the treatment of capillary haemangioma. For example, bFGF is elevated in the lesions (Takahashi *et al.*, 1994) and urine (Marler

et al., 2005) of infants with proliferating haemangioma, however, transcript or protein levels do not decrease with signs of response to corticosteroids (Hasan *et al.*, 2000). Therefore, inhibition of bFGF action on endothelial cell proliferation (*e.g.* by inhibition of signal transduction), despite unaltered bFGF levels, may account in part for the angiostatic actions of pharmacological steroids. Further studies are required to confirm this possibility.

5.4.4 Conclusion

The data in this chapter provide evidence for the establishment of *in vitro* assays of endothelial cell migration and proliferation as useful tools to examine the influence of glucocorticoids on the endothelium and to dissect angiostatic mechanisms. The results presented in this Chapter clearly demonstrate that concentrations of cortisol which cause the disruption of endothelial tube formation, in contrast, do not inhibit endothelial cell migration or proliferation. This supports the contention that the ability of glucocorticoids to inhibit angiogenesis through direct interaction with endothelial cells is due, at least in part, to a reduction of tube formation, rather than inhibition of proliferation or migration.

Chapter 6

Conclusions

Against the background that pharmacological glucocorticoids are known to inhibit angiogenesis (Folkman *et al.*, 1983; Nicosia & Ottinetti, 1990), and that physiological concentrations of endogenous glucocorticoids generated by 11β-HSD1 contribute to regulation of angiogenesis *in vivo* (Small *et al.*, 2005), the potential role of glucocorticoids in the pathophysiology and therapy of deregulated angiogenesis is highly topical and important. The mechanisms through which glucocorticoids inhibit angiogenesis, however, remain obscure. Advances in our understanding of the nature of the interaction between glucocorticoids and the vascular wall make it possible to propose mechanisms that explain the anti-angiogenic actions of these steroids. At a cellular level, for example, glucocorticoids can alter vascular tone by acting directly on endothelial cells suggesting that direct interaction with the endothelium might also underpin their angiostatic effects. Indeed, this is plausible as endothelial cell morphogenesis, migration and proliferation are key stages in the multi-step angiogenic pathway, and furthermore, glucocorticoids (at least at pharmacological levels) can interfere with each of these functions. The mechanism(s) underlying these anti-angiogenic effects of glucocorticoids likely centre on their ability to regulate the expression and/or action of angiogenic factors (*e.g.* VEGF and TSP-1) and intra-cellular signalling cascades (*e.g.* MAPK) within the endothelium. Thus, the major aims of this thesis were to examine whether physiological glucocorticoids can act directly on endothelial cells to account for their angiostatic effects *in vivo* and to explore the cellular and molecular mechanism through which this phenomenon occurs.

6.1 Methodology

Fundamental to the work described in this thesis was the necessity to use *in vitro* models to investigate possible mechanisms of glucocorticoid-mediated angiostasis. Firstly, this approach allows the use of isolated endothelial cells, thus enabling the investigation of direct effects of glucocorticoids in the absence of confounding influences of other cell types (such as SMCs, perivascular cells and inflammatory cells). Secondly, the models described in this thesis produced results which were consistently reliable (in terms of response to positive controls), reproducible, and readily quantifiable. The use of HUVECs as the predominant endothelial cell type of

choice was based predominantly on their widespread use in the relevant literature (which, in turn reflects their ready availability, low cost and relative robust nature in cell cultures). Thus use of HUVECs was important as it enabled direct comparison of data produced during the course of the PhD with published results. It has to be acknowledged, however, that results obtained in HUVECs cannot necessarily be extrapolated to systemic endothelial cells making it necessary to validate these data using a more representative cell type. Human aortic endothelial cells were chosen for reproducing key observations made in HUVECs, as they are perhaps more relevant to adult physiology and pathophysiology. By examining various structural and functional markers of healthy, intact endothelial cells (*e.g.* CD31 and Tie2 expression), and demonstrating that these did not alter with limited passaging, or by the process of differentiation, this also validated the cell culture models used. Furthermore, in each of the studies presented here careful consideration of the choices of *in vitro* assay, culture conditions, time-points and quantification methods were made by first consulting the literature and then testing the influence of each of these different parameters during the course of method refinement to produce well-reasoned protocols.

A possible complicating factor in examining the effects of glucocorticoids on cell function is the action of the isozymes of 11β-HSD which exert an important regulatory control mechanism of glucocorticoid-mediated inhibition of angiogenesis locally in the vessel wall (Small *et al.*, 2005). The contentious issue of the precise cellular localisation of these isozymes within the vascular wall, however, made it necessary to investigate their presence within both undifferentiated HUVECs and the TLS model. The absence of 11β-HSDs in HUVECs and TLSs meant that both single cells and the tube-formation assay could be used to test the influence of exogenous glucocorticoids on endothelial cell function without complication by steroid metabolism within the cells. However, it precluded further investigation of the role of 11β-HSDs in modulating this process.

6.2 Application of methods

6.2.1 Glucocorticoids influence endothelial cell morphogenesis

The observations described in Chapter 3 advance the field of glucocorticoid-mediated inhibition of angiogenesis by firstly, demonstrating that physiological glucocorticoids can act directly on the endothelium, via GR, and secondly, by demonstrating how they inhibit angiogenesis with regard to their effects on cellular function. One of the key steps of angiogenesis *in vivo* is the process of endothelial tube formation which is recapitulated in the cell morphogenesis (TLS formation) model. The studies in Chapter 3 explored the hypothesis that glucocorticoids inhibit angiogenesis by altering the remodelling of undifferentiated and, otherwise uniform, endothelial cells into a mature network of endothelial tubes that resembles a new capillary bed *in vivo*. Limited evidence in the literature suggested that pharmacological levels of glucocorticoids are angiostatic in this model but no studies to date had examined the influence of physiological glucocorticoids or focussed on human endothelial cells in any detail. In addition to the demonstration in the present study that glucocorticoids inhibit TLS formation in two different endothelial cell types (HUVECs and HAoECs), collaboration with other groups has led to similar observations in primary EPCs (McDermott *et al.*, unpublished findings) and endometrial endothelial cells (Rae *et al.*, manuscript submitted). Therefore, a growing body of evidence now suggests that physiological glucocorticoids might exert a tonic angiostatic effect *in vivo*, via GR, by influencing endothelial cell morphogenesis.

When the time-lapse studies were performed, no other reports had previously described the use of this methodology to examine endothelial TLS formation. These studies were useful not only for confirming the 'snap-shot' data that glucocorticoids inhibit the formation of new tubules, but also in advancing our understanding of the nature of this inhibition. Time-lapse sequences demonstrated that TLS formation occurs in the absence of obvious cell migration or proliferation (which is evident in some other endothelial tube formation models), confirming that this methodology provides a model of endothelial cell morphogenesis. Furthermore, these studies

demonstrated that glucocorticoids reduce TLS formation by both inhibiting formation of new tubes and by actively degrading the existing network. This demonstrates a complexity in the angiostatic effect of glucocorticoids not previously apparent from data obtained at single time-points. Although the use of time-lapse imaging is not widespread at present, this dynamic approach to investigating the mechanism of TLS formation represents an important tool for future investigations. These studies provided a useful basis for planning time-points in subsequent studies (Chapter 4). Furthermore, time-lapse data were also complementary with the cytoskeleton studies which, although preliminary in nature, point towards an attenuation of cell-matrix interactions and subsequent downstream matrix-integrin-cytoskeleton signalling pathway (*e.g.* FAK, ERK, p38, JNK) that led to the studies described in Chapter 4.

The fact that physiological glucocorticoid concentrations inhibit TLS formation under basal (unstimulated) conditions as well us under growth-factor stimulated conditions reveals other facets of their angiostatic mechanisms of action. Firstly, it demonstrates that there are likely to be multiple molecular mechanisms of action *e.g.* inhibition of growth factor signalling, either by altering the balance of pro- to anti-angiogenic factor generation within the endothelium, by influencing the action of angiogenic factors (both soluble and ECM-based) on TLS formation, and/or by inhibition of downstream post-receptor signalling pathways. Secondly, it demonstrates that although glucocorticoids can alter TLS formation directly within endothelial cells themselves, it also strongly suggests that other cell types are likely to be important in glucocorticoid-mediated inhibition of angiogenesis. For example, blocking the action of exogenous VEGF on TLS formation suggests that other cell types (*e.g.* SMCs, macrophages) as a source of these factors, may be important *in vivo* and points towards a paracrine mechanism of action. Indeed, this mechanism of regulation of angiogenesis is well accepted in the contexts of physiological angiogenesis in wound healing and in tumour pathophysiology (Hanahan & Folkman, 1996; Carmeliet, 2000).

Although glucocorticoids can influence endothelial TLS formation directly, the idea of an additional paracrine mechanism of action *in vivo* is supported by the studies (Chapter 3) assessing the presence of 11β-HSDs. Whilst it is feasible that endothelial cells could maintain 11β-HSDs activity in the intact vessel wall, the inability of cortisone to inhibit TLS formation, despite the efficacy of cortisone to inhibit angiogenesis *e.g.* in aortic rings (Small *et al.*, 2005), is significant as it suggests that glucocorticoid regeneration probably occurs in another vascular cell type. Although cortisone can inhibit angiogenesis *in vivo* via the action of 11β-HSD1 (Small *et al.*, 2005), the lack of expression and activity of 11β-HSD1 (in isolated endothelial cells, EPCs and TLSs), and lack of an effect of cortisone in TLS preparations, when taken together demonstrates that an alternative source of glucocorticoid regeneration in the vessel wall exists outside the endothelium. This is important as it advances our current understanding of a role for these enzymes as it has previously been suggested that they act as a mechanism for controlling intracellular glucocorticoid action. However, these findings now suggest that the 11β-HSDs can control glucocorticoid action in the vasculature *between* different cell types. Whether or not physical contact between cell types is required and the mechanism of glucocorticoid secretion and action on neighbouring cell types are both important questions for future studies which could be addressed by co-culture experiments (Section 6.4.2).

6.2.2 Glucocorticoids stimulate generation of an angiostatic factor during endothelial tube formation

The studies in Chapter 4 extend the observation of glucocorticoid-mediated inhibition of endothelial tube formation, via GR, by implicating the possible involvement of the angiostatic factor TSP-1. Although the present studies were limited to mRNA expression levels, the results are supported by evidence that cortisol, at 600 nM, causes up-regulation of TSP-1 protein levels that translates into an angiostatic effect in *ex vivo* (primary endometrial tissue) explants in Matrigel (Rae *et al.*, manuscript submitted). Furthermore, in the studies by Rae and colleagues, the angiostatic effect of cortisol was shown, using a small interfering (si)RNA approach,

to be a direct result of TSP-1 up-regulation. Emerging evidence from the literature, concerning the regulation of angiogenesis *in vivo* and in pathological models by glucocorticoids, suggests that they act by altering the balance of pro-angiogenic (*e.g.* VEGF) (Pufe *et al.*, 2003; Luo *et al.*, 2004) to anti-angiogenic (*e.g.* TSP-1 and TIMP-1) factors (Flugel-Koch *et al.*, 2004; Forster *et al.*, 2007). The data presented here are consistent with this idea and extend our current knowledge by demonstrating that endothelial cells themselves are a source of TSP-1. This might be important for drug delivery strategies aimed at targeting aberrant angiogenesis in disease.

Gene selection (such as VEGF, α6-integrin) for the current work was based on preliminary evidence from Small and colleagues (altered calcium influx in HUVECs; Small *et al.*, unpublished findings) and careful evaluation of the literature. This does not, however, exclude the possibility that interaction with additional genes may contribute to the angiostatic effects of glucocorticoids on endothelial cells.

Lack of a significant effect of cortisol on VEGF and VEGF-R2 mRNA levels should not be taken as a lack of involvement of VEGF signalling in glucocorticoid-mediated inhibition of TLS formation. On the contrary, TLS formation driven by VEGF is blocked by glucocorticoids (Chapter 3), suggesting that it may play a role. However, it is likely that a post-receptor signal transduction mechanism is involved. Limited *in vitro* evidence suggests that MAPK signalling is inhibited in endothelial cells by pharmacological glucocorticoids in angiogenic (Ebrahem *et al.*, 2006) and inflammatory situations (Pelaia *et al.*, 2001). Consequently, it was hypothesised that physiological levels of cortisol would attenuate activation of ERK. However, trends were observed suggesting activation of this pathway by physiological levels of glucocorticoids in unstimulated, undifferentiated cells (Chapter 4), demonstrating the complexity of regulation of this pathway by glucocorticoids. The sensitivity of ERK signalling to the extra-cellular environment, which is the physiological purpose of this pathway, necessitates close consideration of the culture conditions when interpreting these novel data. Whether or not activation of ERK/MAPK is important during the process of tube formation will require studies to be performed on protein recovered from TLSs on Matrigel. The difficulty in obtaining sufficient protein from

TLSs for this purpose highlights the importance of this methodological development. The preliminary work described in this thesis will serve as a useful basis for planning future studies since protocols (*e.g.* for measurement of pERK) are now well established. In conclusion, whether or not activation of ERK signalling is involved in glucocorticoid-mediated angiostasis can be determined in future studies now that relevant, validated tools are available.

6.2.3 Glucocorticoids do not inhibit angiogenesis by influencing endothelial cell migration or proliferation

As described in the introduction (Chapter 1) angiogenesis is a multi-step process, prompting investigation into the effects of glucocorticoids on endothelial cell migration and proliferation. Although it was demonstrated that glucocorticoids consistently inhibit endothelial cell morphogenesis (Chapter 3) additional investigations (Chapter 5) showed that they do not inhibit proliferation or migration of these cells. Pharmacological levels of glucocorticoids are known to inhibit migration (Pross *et al.*, 2002a) and proliferation (Berk *et al.*, 1988) of vascular smooth muscle and endothelial cells in rodents, but whether they inhibit these processes at physiological levels in human endothelial cells was unclear. Furthermore, since glucocorticoids block some of the actions of VEGF on the endothelium in other *in vitro* systems, and VEGF-induced migration and proliferation are required during angiogenesis *in vivo*, these studies addressed the hypotheses that glucocorticoids inhibit VEGF-induced migration and proliferation. Since no effects on either migration or proliferation were noted in these studies, despite using a range of well validated, relevant models, it was concluded that glucocorticoid-mediated inhibition of angiogenesis is not a result of effects on either of these endothelial cell processes. Indeed, this is consistent with the fact that TLS formation occurs in the absence of any obvious signs of cell proliferation or migration. This is important because a similar physiological level of cortisol inhibits endothelial tube formation in the intact vessel wall (Small *et al.*, 2005) (which encompass endothelial cell morphogenesis, migration and proliferation), in spite of a possible lack of effect on either migration of proliferation (Figure 6.1).

6.3 Summary

In summary, the research presented in this thesis demonstrates that physiological levels of glucocorticoids, via GR, are sufficient to inhibit tube formation by direct interaction with human endothelial cells. Exposure of endothelial cells to glucocorticoids undergoing this morphogenesis (or remodelling) revealed some new insights into their molecular mechanisms of action, however, glucocorticoid interactions with the endothelium may be even more complex than previously appreciated. Furthermore, the work in this thesis does not exclude the possibility that glucocorticoid-mediated inhibition of angiogenesis *in vivo* may also include effects on inflammatory and smooth muscle cells. Finally, since physiological glucocorticoids inhibit tube formation in the absence of an effect on either endothelial cell migration or proliferation, this demonstrates that glucocorticoids act selectively in mediating their angiostatic effects.

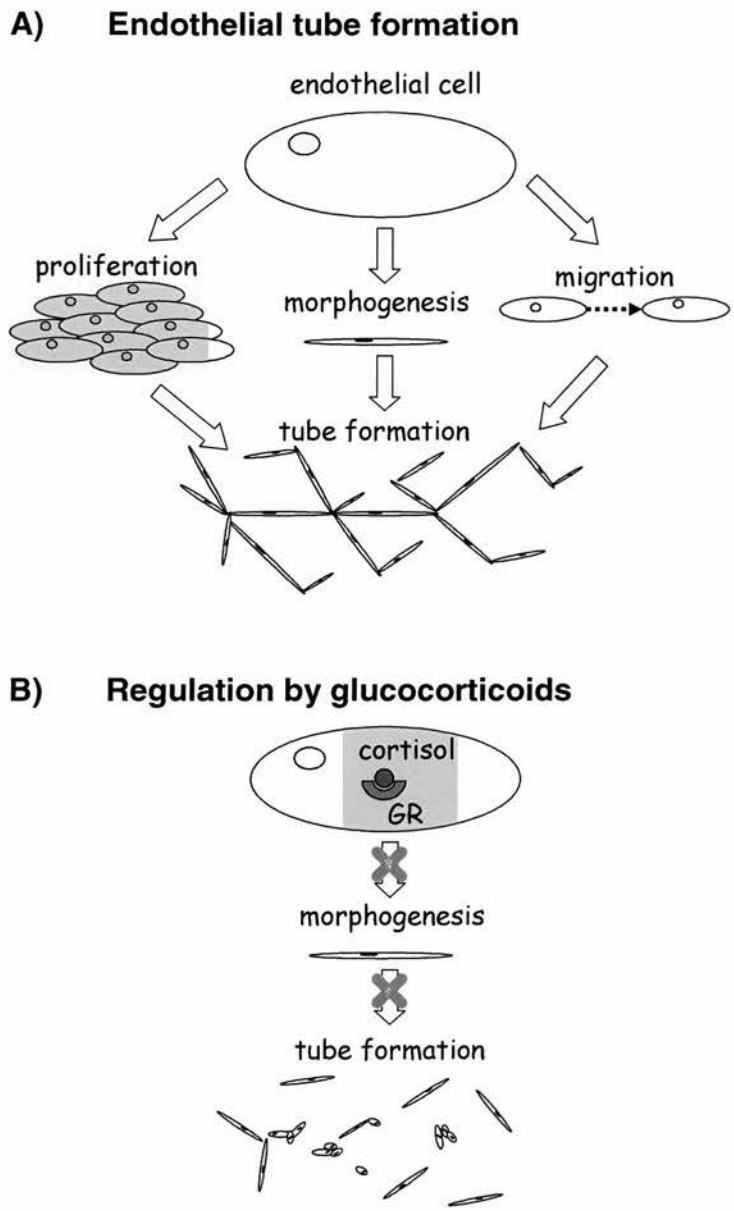


Figure 6.1 Schematic of proposed cellular mechanism of glucocorticoid-mediated inhibition of endothelial tube formation

The proposed mechanism of action of glucocorticoids on angiogenesis (note, diagram is not to scale). A) Endothelial tube formation, a key component of angiogenesis *in vivo* is comprised of multiple cell functions including; endothelial cell morphogenesis, migration and proliferation. These processes are regulated by various angiogenic signals from the surrounding microenvironment including vascular endothelial growth factor, thrombospondin-1, integrins and other unknown factors. B) The findings from this thesis demonstrate that glucocorticoids, via GR, regulate endothelial cell morphogenesis, but not the migration or proliferation steps.

6.4 Future work

Further studies are planned, as a basis for a new PhD project, with the aim of advancing some of the key findings from this thesis. Some of the key questions arising from the present work, and the relevant methodologies to address these, are proposed as follows:

6.4.1 Characterisation of endothelial TLS integrity and influence of glucocorticoids

- *Which components of endothelial cell morphogenesis (e.g. cell polarisation or lumen formation) are influenced by glucocorticoids?*

The influence of glucocorticoids on the ultra-structural integrity of TLSs could be addressed with the use of electron microscopy. This would allow examination of the presence of a lumen in TLSs that would add to the body of evidence reported in this study that the model recapitulates, to an extent, endothelial tube formation *in vivo*. Electron microscopy studies of the cytoplasmic (filopodia-like) extensions that contribute to TLSs, will allow a more qualitative analysis of the cell-cell and cell-matrix interactions.

6.4.2 Glucocorticoid effects on physiological endothelial tube formation

- *Do glucocorticoids inhibit tube formation during invasive sprouting angiogenesis?*
- *Is tube formation inhibited by glucocorticoids when driven by physiological stimuli?*
- *Do glucocorticoids influence the production of angiogenic factors by neighbouring smooth muscle cells, and if so, does this in turn have an influence on endothelial TLS formation?*
- *Is the inter-conversion of cortisone to cortisol by 11β-HSD1-active cells sufficient to confer angiostatic effects on nearby tubes?*

Glucocorticoid-mediated inhibition of TLS formation could be investigated in more physiologically-relevant, 'qualitative', variations of the existing model. For example, the influence of glucocorticoids on endothelial cell invasion in combination with cell morphogenesis, migration and proliferation could be investigated using a 3D system in which endothelial cell-coated beads cultured within fibrin gels have been described as producing branching tubes with 'tip cells' resembling angiogenic sprouts *in vivo* (Nakatsu *et al.*, 2003). Alternatively, culturing endothelial tubes in a hypoxia chamber (Ben-Yosef *et al.*, 2005), or co-culturing endothelial cells directly onto confluent monolayers of matrix-producing cells (*e.g.* fibroblasts) (Donovan *et al.*, 2001) has been suggested to produce a more heterogeneous pattern of TLS formation (long and short TLSs) that more closely resembles capillaries *in vivo*. Given that circulating EPCs contribute to angiogenesis *in vivo* (Asahara *et al.*, 1997) it would be worthwhile testing the possible influence of glucocorticoids on isolated precursor cells in a variation of the TLS model. Preliminary evidence suggests that exposure of EPCs to a physiological concentration of cortisol inhibits TLS formation through an unknown mechanism (McDermott *et al.*, unpublished findings) which is tantalizing and warrants further investigation.

The influence of 11β-HSD1 on endothelial tube formation will be an important issue to address since it is known to regulate endothelial tube formation by the intact vessel wall (Small *et al.*, 2005). Consequently, introducing 11β-HSD1 back into an *in vitro* system will allow the opportunity to study communication between human vascular cell types and to address further a possible paracrine mechanism of action. This could be achieved using cell culture inserts and culturing 11β-HSD1-active cells (*e.g.* activated smooth muscle cells (Cai *et al.*, 2001) or macrophages (Thieringer *et al.*, 2001)) and endothelial cells together, by transfection of endothelial cells, or using conditioned media from 11β-HSD1-expressing cells.

6.4.3 Molecular mechanisms of glucocorticoid-mediated angiostasis

- *Is up-regulation of TSP-1 the cause of glucocorticoid-mediated inhibition of endothelial tube formation?*
- *How important is VEGF signalling in glucocorticoid-mediated inhibition of endothelial tube formation?*
- *Are there other molecular mediators central to the angiostatic action of glucocorticoids?*

The functional consequence of up-regulated TSP-1 could by explored by testing whether blockade (*e.g.* by blocking with antibodies (Desai *et al.*, 2005) or siRNA) of TSP-1, or its receptor (CD36), counteracts the angiostatic effect of glucocorticoids on TLS formation. In addition, it will be important to determine some of the downstream actions of switching-on TSP-1. The literature suggests that the counter-adhesive properties of TSP-1 lead to disassembly of focal adhesions and stress fibres, and eventually apoptosis. This could be addressed by immunocytochemical staining and fluorescence microscopy using markers of focal adhesion kinase (FAK), stress fibres (F-actin) and apoptosis (caspases). A firm foundation for these studies is already in place with the preliminary work described in Chapter 3.

Similarly, blocking VEGF signalling *e.g.* using VEGF-Trap or a similar neutralising antibody would allow determination of whether the angiostatic effect of glucocorticoids is mediated by aberrant VEGF signalling.

More global approaches to studying changes in gene expression (*e.g.* by RNA microarrays) during endothelial TLS formation are emerging in the literature (Glesne *et al.*, 2006). Since angiogenesis is regulated by an enormous number of factors, and likewise, glucocorticoids regulate expression of various factors, this technology offers an efficient means of identifying new targets. No studies to date, however, have used microarrays to examine glucocorticoid effects on angiogenic factors in endothelial tubes and this technology offers an area of opportunity for the future.

Finally, with each of these strategies of identifying molecular targets of glucocorticoids on angiogenesis, it will be important to take these findings and test them in angiogenesis models *in vivo* (*e.g.* subcutaneous sponges (Hori *et al.*, 1996) or cutaneous wound healing (Blomme *et al.*, 2003)) to determine their impact in physiology.

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Appendix 1

Supplementary Material

Supplementary Figure 1 Dynamic nature of TLS formation identified by time-lapse imaging

(Time-lapse movie-clip on enclosed CD)

HUVECs incubated on Matrigel in SlideFlasks in standard basal conditions for up to 24 hours and images were captured at 3 separate positions per flask at 4 minute intervals using time-lapse video microscopy. Movies were reconstructed using Windows Media Player software and 1 second of clip is equivalent to 1 hour of real time. Two distinct phases of TLS formation were identified in untreated control flasks using this method. Phase I consists of network assembly; TLS number increased most rapidly during the first 4 hours after cell seeding, and continued to increase reaching a maximum number of TLSs after 8 hours on Matrigel in control flasks. Phase II involves the degradation of the TLS network whereby cells detach from the substratum and lift off, resulting in a decrease in TLS number from 8 hours onwards. Most of the network was degraded after 24 hours under these conditions. There was little evidence from these movie-clips of cell migration or proliferation and TLS formation consisted mainly of cell morphogenesis. Experiments were performed on 6 separate occasions.

Appendix 2

Publications

From: James J Logie [mailto:james.logie@ed.ac.uk]
Date: Monday, 23. July 2007 12:16pm
To: CMLSEdit
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Cellular and Molecular Life Sciences

Review

Intra-vascular glucocorticoid metabolism as a modulator of vascular structure and function

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Abstract. The ability of glucocorticoids to directly alter arterial function, structure and the inflammatory response to vascular injury may contribute to their well-established link with the development of cardiovascular disease. Recent studies have emphasised the importance of tissue-specific regulation of glucocorticoid availability by the 11β-hydroxysteroid dehydrogenase (11HSD) isozymes, which inter-convert active glucocorticoids and their inactive metabolites. The expression of both type 1 and type 2 11HSDs in the arterial wall suggests that pre-receptor metabolism of glucocorticoids may have a direct impact on vascular physiology. Indeed there is evidence that 11HSDs influence glucocorticoid-mediated changes in vascular contractility, vascular structure, the inflammatory response to injury and the growth of new blood vessels. Hence, inhibition of 11HSD isozymes may provide a novel therapeutic target in vascular disease.

Key words. 11β-Hydroxysteroid dehydrogenase; inflammation; vascular contractility; angiogenesis; cardiovascular disease.

Introduction

There is increasing evidence that direct interaction of glucocorticoids with the vascular wall [1, 2] contributes to their association with increased risk of cardiovascular disease [3, 4]. Certainly, glucocorticoids can interact both with endothelial (EC) and with vascular smooth muscle (VSMC) cells, and furthermore, glucocorticoid-mediated enhancement of vascular contractility has been implicated in the development of hypertension [5]. In addition, glucocorticoids may directly modify new blood vessel formation and vascular lesion development by inhibiting inflammation, proliferation and angiogenic pathways in the arterial wall [6, 7].

Interaction of glucocorticoids with the vasculature is unlikely to be regulated solely by circulating concentrations of these steroids; pre-receptor metabolism within target tissues also has a profound influence on glucocorticoid activity. Such tissue-specific modulation of glucocorticoid activity, regulated by the isozymes of 11β-hydroxysteroid dehydrogenase (11HSD) [8], has a key role, for example, in the development of hypertension, obesity and the metabolic syndrome [9–11]. It is likely that pre-receptor metabolism of glucocorticoids influences steroid action within the vessel wall since both isozymes of 11HSD are expressed in vascular cells [12]. This article reviews the current evidence that vascular 11HSD expression influences glucocorticoid-mediated changes in vascular growth, function, structure and the inflammatory response to vascular injury.

Glucocorticoid signalling in the vascular wall

Glucocorticoids (cortisol in man, corticosterone in rodents) are predominantly synthesised in, and released

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from, the adrenal cortex. Circulating concentrations of these steroids are under the control of the hypothalamic-pituitary-adrenal (HPA) axis, whilst their bioavailability is regulated by interaction with corticosteroid-binding globulin (CBG) and albumin in the plasma. The small proportion of unbound, circulating hormone is able to cross the cell wall and interact with corticosteroid receptors. Classically, glucocorticoids interact with the cytosolic glucocorticoid receptor (GR, or corticosteroid receptor type II). As described below, glucocorticoids may also activate mineralocorticoid receptors (MR, or corticosteroid receptor type I), but this occurs only in a few tissues. GR and MR are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors [13]. Activation of GR results in binding of receptor homodimers to glucocorticoid response elements in target genes, leading to initiation or repression of transcription. There is also increasing evidence that glucocorticoids exert specific, non-genomic actions. Examples exist of rapid glucocorticoid-induced changes to phospholipase A_2 (PLA_2) and phosphoinositide-3-kinase-mediated endothelial nitric-oxide synthase (eNOS) release that are blocked by GR antagonism but not by inhibition of transcription [14, 15]. These non-genomic effects are thought to be mediated by membrane-bound GR [16] (mGR; although the specific signalling pathways associated with these receptors have not been established) or by cytosolic GR (cGR) without requirement for either nuclear translocation or effects on transcription. In the latter case, chaperones or co-chaperones (such as Src) act as signalling components and, therefore, mediators of glucocorticoid-induced effects [17].

Corticosteroid receptors are present in the cells of the vascular wall, supporting the idea that glucocorticoids interact directly with the vasculature. Cytosolic MR and GR have both been demonstrated in freshly isolated vessels [18, 19] and in cultured vascular cells (VSMCs [20, 21] and ECs [22–26]) from a variety of species. The distribution of these receptors may vary with vascular territory, as MR were detected in rabbit aortic and pulmonary VSMCs but not in small arteries [27]. Vascular GR are known to be active as antagonism (with RU38486) blocked dexamethasone-mediated induction of ACE activity in rat aortic ECs [28]. Similarly, activity of MR is demonstrated by their contribution to angiotensin II-induced hypertrophy of VSMCs [29] and aldosterone-induced swelling of ECs [22]. It has not been established whether membrane binding sites for corticosteroids are present, or have a role, in the vascular wall.

The downstream effects of GR activation within the arterial wall, and their influence on cardiovascular risk factors (such as hypertension), are imperfectly understood [5]. Glucocorticoids are essential for maintenance of blood pressure in healthy individuals [1], whilst their ability to increase peripheral vascular resistance in animals devoid of renal mass indicates that a non-renal mechanism contributes to glucocorticoid-induced hypertension [30]. A considerable body of evidence suggests that this non-renal mechanism may involve direct glucocorticoid-mediated alteration of EC and VSMC function [1]. Consequently, regulation of glucocorticoid availability by 11HSDs within the vascular wall may be an important influence on cardiovascular physiology and pathology.

Tissue-specific metabolism of glucocorticoids by 11β-hydroxysteroid dehydrogenases

The 11HSDs, microsomal enzymes of the short-chain alcohol dehydrogenase superfamily [8], interconvert active glucocorticoids and their inert 11-keto forms [31]. Two isozymes, 11HSD1 and 11HSD2, have been identified: 11HSD1 is a low-affinity NADP(H)-dependent, predominant reductase *in vivo*. Dehydrogenase activity of this isozyme is generally not seen in intact cells or organs (including liver [32–34], adipose tissue [35], neurons [36] and vascular smooth muscle [37]); early suggestions of 11HSD1 dehydrogenase activity in vascular smooth muscle [38] are probably attributable to 11HSD2 [37]. 11HSD1 dehydrogenase activity observed in some preparations *in vitro* [39] is probably attributable to release of enzyme from damaged or dying cells, with dissociation from hexose-6-phosphate dehydrogenase, which is thought to maintain the high NADPH concentrations required for reductase activity [40]. 11HSD1 has a Km in the micromolar range for both cortisol and corticosterone [41] and is widely expressed in glucocorticoid-target tissues (including liver, lung, adipose tissue, brain, vascular smooth muscle, skeletal muscle, anterior pituitary, gonads and adrenal cortex [8]), where its role is to amplify local glucocorticoid concentrations [42]. Regulatory control of 11HSD1 is complex, with its synthesis and activity influenced by a variety of factors (such as glucocorticoids [43–45], stress [46, 47], sex steroids [48], growth hormone [49], cytokines [50] and peroxisome proliferator-activated receptor agonists [8]) and its activity driven in the reductase direction through local generation of NADPH by hexose-6-phosphate dehydrogenase [51]. Other factors that may drive 11HSD1 activity in the reductase direction include the cellular environment, co-factor availability, redox potential and substrate concentration.

11HSD2, by contrast, is a high-affinity NAD-dependent, exclusive dehydrogenase, which converts active glucocorticoids into inactive 11-ketosteroids and has a Km for cortisol and corticosterone in the nanomolar range. It is found primarily in mineralocorticoid target tissues, such as the kidney, sweat glands, salivary glands and colon 8], where it is constitutively active and serves to protect MR

from illicit occupation by glucocorticoids. Inhibition of 11HSD2 with liquorice or its derivatives results in glucocorticoid-dependent 'apparent' mineralocorticoid excess and hypertension [52]. Similarly, transgenic disruption of 11HSD2 [9] in mice, or congenital deficiency in man [53], recapitulates the major features of the syndrome of apparent mineralocorticoid excess (SAME). The importance of 11HSD2 in SAME was demonstrated by the description of a defect in cortisol metabolism in children with this syndrome [54]; this was later shown to be the result of mutations in the 11HSD2 gene [55, 56]. 11HSD2 is also expressed in tissues which are not classic MR targets, including the lung, lymph nodes, heart, blood vessel wall and placenta) [57–59]. In the placenta 11HSD2 acts to protect the foetus from excessive exposure to maternal glucocorticoids [60, 61], whereas cardiac 11HSD2 activity may have a role in preventing fibrosis resulting from stimulation of MR by glucocorticoids [62].

The influence of 11HSD isozyme activity on cardiovascular physiology and pathophysiology is well recognised (see Krozowski and Chai for review [63]), but details of the role of 11HSDs within the vessel wall have emerged only recently and remain somewhat uncertain.

Intra-vascular glucocorticoid metabolism

Both isozymes of 11HSD are expressed in the blood vessel wall, suggesting that they could influence vascular function by regulating local availability of active glucocorticoids [1, 64]. The cellular distribution of vascular 11HSD1 and 11HSD2 is not completely clear. Our studies using mouse and rat aorta suggest that 11HSD2 is localised to ECs, whereas 11HSD1 is predominantly in the VSMC (fig. 1) [18, 65]. Others, in contrast, have reported activity of both enzymes in the VSMC [37, 50] and also in the EC [66], it should be noted that the latter investigation [66] demonstrated only 11HSD1 in rat VSMC and indicated that 11HSD1 was the predominant isozyme in the endothelium. Direct comparison of studies is often difficult, given the use of arteries from different species and anatomical locations combined with a variety of techniques for detecting 11HSDs. The balance of the literature suggests that cellular distribution of 11HSD isozymes differs in vessels from distinct anatomical locations and that 11HSD activity increases as artery diameter diminishes; in the rat 11HSD, activity was greater in resistance (mesenteric) arteries than in conduit vessels (aorta) [65] and in the mouse 11β-reductase activity was higher in iliofemoral arteries than in the aorta [A. R. Dover et al., unpublished data]. These variations in cellular distribution and activity suggest that the role of intravascular glucocorticoid metabolism is not the same in all blood vessels.

There is increasing evidence that interconversion of active and inactive glucocorticoids by vascular cells may influence glucocorticoid-mediated modulation of vascular function, structure, growth and inflammation.

Glucocorticoids, 11HSDs and vascular function

Although it is well established that glucocorticoids contribute to maintenance of vascular tone *in vivo,* the mechanisms have been difficult to establish. A variety of interactions contribute to homeostasis, including glucocorticoid-mediated regulation of cardiac output and fluid and electrolyte balance, with salt and water handling modulated both directly [67] and indirectly by influences on the production of angiotensinogen (liver), arginine vasopressin (AVP; hypothalamus) [68] and atrial natriuretic peptide (ANP; cardiac myocytes) [69]. It is apparent, however, that these cardiac and renal effects cannot account totally for the glucocorticoid-mediated increase in blood pressure, and there is evidence that a component of hypertension arises from enhanced contractility of the vascular wall [70–72]. For example, reversal of adrenocorticotophin-dependent hypertension by administration of l-arginine (the substrate for nitric oxide synthase) suggests that nitric oxide deficiency contributes to the elevation of blood pressure [73, 74].

Glucocorticoid-dependent potentiation of noradrenaline- and angiotensin II-mediated vasoconstriction has been at-

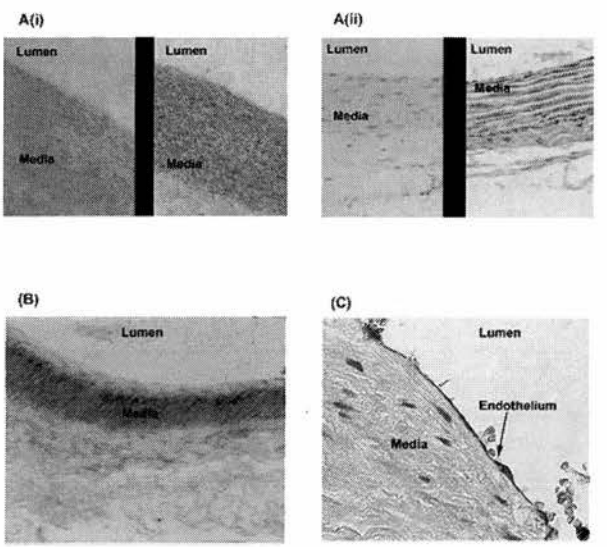

Figure 1. Presence and distribution of 11HSD isozymes in the vascular wall. *In situ* hybridisation (*Ai*) and immunohistochemistry (*Aii*) confirming both expression and activity of 11HSD1 within the rat aortic wall; the enzyme was predominantly localised to medial smooth muscle cells (left-hand panel, sense/control; right hand-panel, antisense/antibody to 11HSD1). Immunohistochemistry demonstrating (*B*) 11HSD1 in rat mesenteric artery smooth muscle and (*C*) 11HSD2 in human intra-renal artery endothelium [unpublished]. Reproduced from [65] with permission. © The Endocrine Society, 1991.

tributed to alterations within the VSMC and the EC (reviewed in Walker and Williams [2] and Ullian [1]). Given that glucocorticoids can act on both MR and GR, the increased contractility observed in many studies may be secondary to increased stimulation of either receptor. Alterations identified within the VSMC (including upregulation of contractile receptors, altered intracellular second messenger activation and modulation of the activity and synthesis of vasoactive substances) result in a direct enhancement of contraction [1]. In contrast, changes in the endothelium can increase contractility in two distinct ways: by increased release of vasoconstrictor compounds (e.g. angiotensin II, endothelin-1 [75, 76]) from the ECs and by impaired endothelium-mediated relaxation. Loss of endothelium-mediated relaxation [77], caused by impaired activity of vasodilators (e.g. prostaglandins, nitric oxide) [78–80], [reduces the ability of the endothelium to modulate contraction.

An alternative mechanism through which glucocorticoids may regulate vascular function is 'foetal programming' of physiological responses [81]. Exposure of the foetus to excess maternal glucocorticoid (either by direct infusion or by inhibition of placental 11HSD2) causes reduced birth weight [82], an outcome associated with increased risk of cardiovascular and metabolic disease in adulthood [83]. Two major causes of low birth weight, maternal dietary restriction and maternal stress, may also be glucocorticoid-dependent [84, 85]. In the ovine foetus, glucocorticoid infusion elevates blood pressure and alters vascular contractility in foetal sheep [86]; this may be significant, as one outcome of foetal programming is elevated blood pressure in adult offspring [87]. However, although enhanced vascular contractility has been demonstrated in rats with programmed hypertension, it is not clear whether this contributes to the elevation of blood pressure [88, 89]. Furthermore, the mechanisms through which pre-natal exposure to excess glucocorticoid programme enhance contractility in adult offspring have not been established.

Influence of 11HSDs on vascular function

In SAME, 11HSD2 deficiency results in sodium retention and severe hypertension, mediated in part by glucocorticoid-dependent activation of MR in the distal nephron [90]. There is, however, a considerable literature to suggest that changes in 11HSD activity within the vascular wall also contribute to elevation of blood pressure. A clear example of this is the demonstration that 11HSD activity is impaired in arteries taken from rat models of hypertension [91–93]. A role for altered vascular function is supported by reports that 11HSD inhibition (with glycyrrhetinic acid) in rats produced an elevation of blood pressure which, whilst mediated by MR activation, was blocked by antagonists of the endothelin-1 system [94, 95]. Moreover, studies of dermal vasoconstriction in patients exposed to liquorice, and in a single individual with SAME [53, 96], demonstrated enhanced cortisol-mediated constriction (fig. 2). The possibility that this is due to changes in glucocorticoid metabolism within the vascular wall, rather than indirect systemic effects of sodium retention, gained further credence with *in vitro* studies which showed that bile acids (e.g. chenodeoxycholic acid), which are endogenous inhibitors of 11HSD [97], pharmacological inhibitors of 11HSD (carbenoxolone, glycyrrhetinic acid) [98, 99] and isozyme selective antisense oligonucleotides [100] alter corticosterone-mediated enhancement of vasoconstriction. Furthermore, 11HSD inhibition (with glycyrrhetinic acid) augmented corticosterone-induced dysfunction in cultured human ECs, indicating both a role for intra-cellular 11HSD and independence from blood pressure elevation *in vivo* [95]. Care is required in interpreting these results, however, as some 11HSD inhibitors can directly alter contractile function by damaging the endothelium [101].

These pharmacological studies have been extended by the use of arteries from 11HSD knockout mice. Aortic function (and blood pressure) are unaltered in $11HSD1^{-/-}$ mice suggesting that intravascular regeneration of active glucocorticoids has no effect on vascular contractility [102, 103]. This indicates that despite the ability of glucocorticoids to enhance vascular contraction, impaired corticosterone generation in the arterial wall does not re-

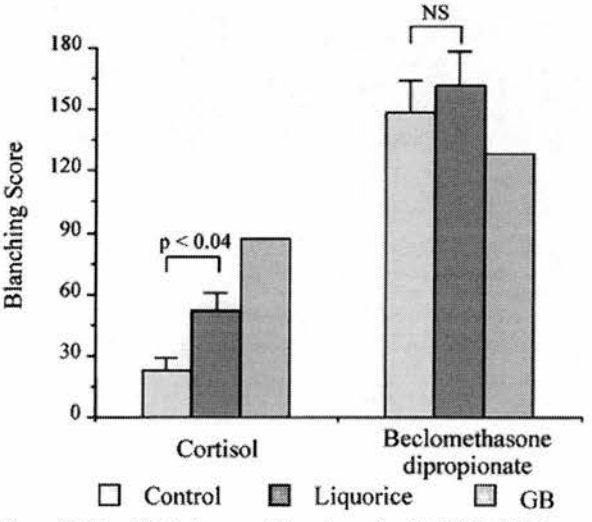

Figure 2. The effect of congenital and acquired 11HSD deficiency on dermal vasoconstrictor sensitivity to cortisol and beclomethasone dipropionate. Inhibition of 11HSD activity with liquorice-enhanced dermal vasoconstriction (measured by skin blanching) in response to cortisol but not to beclomethasone diproprionate. A similar result was obtained in a patient (GB) with the syndrome of apparent mineralocorticoid excess type 1 (11HSD2 deficiency). These data indicate that local regulation of glucocorticoid activity in the vascular wall contributes to contractile tone. Bars are s.e. NS, not significant. Reproduced from [53] with permission. © The Biochemical Society, 1992.

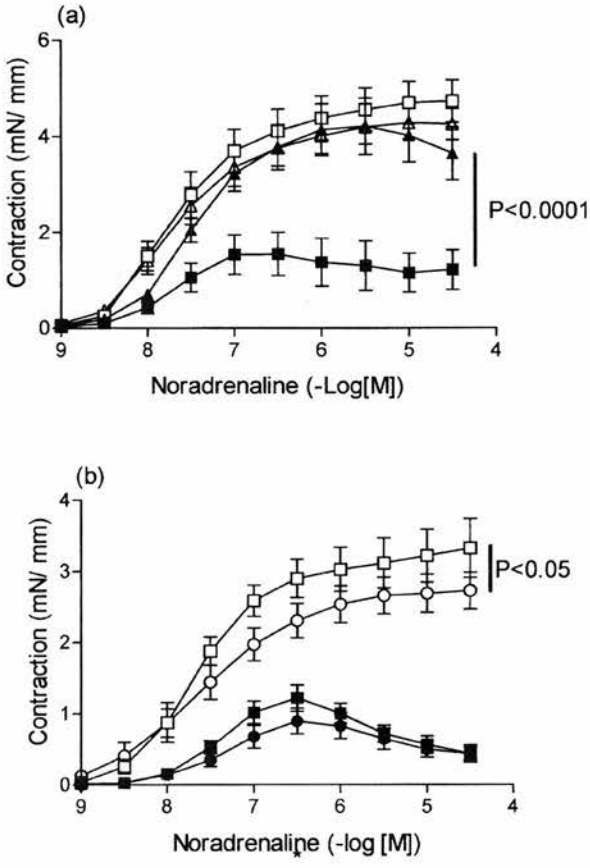

Figure 3. Effect of (*a*) transgenic deletion of 11HSD2 and (*b*) exposure to glucocorticoids on mouse aortic endothelial cell function. In aortic rings isolated from control mice (squares), release of endothelium-derived nitric oxide acts as a physiological antagonist of noradrenaline-mediated contraction; thus, removal of the endothelium (open symbols) results in enhanced contraction. In aortae from $11HSD2^{-/-}$ mice (*a*; triangles) this ability of the endothelium to modulate contraction has been lost, suggesting glucocorticoid-mediated impairment of endothelial cell function. This is contested, however, by the demonstration that (*b*) *in vitro* incubation (24 h, 37 °C; 10^{-7} M corticosterone) of aortic rings from control animals with glucocorticoids (circles) did not produce a similar endothelial cell dysfunction. Values are mean ± s.e. mean; n=8. Adapted with permission from [103] and [18]. © Lippincott, Williams and Wilkins.

duce contractile function. In contrast, enhanced contractility was demonstrated in thoracic aortae from mice lacking 11HSD2 as a result of impaired endothelium-derived NO activity [103], rather than changes in the VSMC (fig. 3a). This suggests that 11HSD2 activity in the endothelium may serve to protect endothelium-dependent relaxation from the detrimental effects of glucocorticoids. This appears to be consistent with *in vivo* studies which suggest that non-selective inhibitors of both isozymes of 11HSDs, such as liquorice derivatives, potentiate rather than impair the vascular actions of glucocorticoids, suggesting inhibition of inactivation rather than reactivation of steroids within the vessel wall. More recent data, however, suggest that the concept of protection of ECs by 11HSD2 may be an oversimplification. In mouse aortic rings, we could not induce EC dysfunction by incubating isolated rings with glucocorticoids (fig. 3b), even in the absence of 11HSD2 [18]; whether dehydrogenase activity of 11HSD1 [38] served to protect the endothelium is not clear, although our data suggest that 11HSD1 does not act as a dehydrogenase in intact arteries. This raises the possibility that *in vivo* differences in vascular function in 11HSD2 knockout mice are dependent on indirect mechanisms, e.g. related to hypertension or sodium retention. Cell-specific manipulation of 11HSDs would be the most attractive means to dissect this biology further, but has yet to be reported for vascular cells.

Glucocorticoids, 11HSDs and vascular inflammation

Whereas studies in transgenic mice have suggested that 11HSD2 activity may influence vascular function, whilst 11HSD1 does not, a much clearer role for 11HSD1 has been identified in regulation of vascular inflammation. The anti-inflammatory and immunosuppressive effects of glucocorticoids, which account for their most common therapeutic applications, are due to GR-mediated interactions with blood vessels, inflammatory cells and mediators of inflammatory responses [104]. For example, glucocorticoids decrease expression of adhesion factors, cytokines and chemokines, and so alter the recruitment of immune cells such as neutrophils and macrophages to sites of inflammation. This also results in a decrease in leukocyte activation and proliferation. Furthermore, the glucocorticoid receptor mediates lymphocyte apoptosis [105] and suppresses the synthesis of inflammatory mediators (e.g. prostanoids), and hydrocortisone stimulates the synthesis of anti-inflammatory mediators (e.g. lipocortins) [106]. Glucocorticoids, but not mineralocorticoids, can also promote the phagocytosis of apoptotic leukocytes [107], and so contribute to the resolution of inflammation. The expression of 11HSD1 in VSMCs [50] and in activated macrophages [108] suggests that generation of glucocorticoid within these cells may contribute to regulation of inflammation.

Influence of 11HSDs on vascular inflammation

The demonstration that pro-inflammatory cytokines selectively upregulate 11HSD1 activity in human VSMCs suggests that glucocorticoid generation within the vascular wall provides a mechanism for local feedback regulation of inflammation [50]. However, this has not been examined *in vivo*. The ability of inflammatory mediators to regulate 11HSD activity in VSMCs may be dependent upon the phenotypic state of the cells (with enzyme ac-

tivity upregulated in actively proliferating, but not in quiescent, cells), the anatomical origin of a particular vessel, the prevailing local glucocorticoid concentrations and the modulation of the inflammatory response by neighbouring tissues [A. R. Dover et al., unpublished data]. Further work is required to clarify the significance of cytokine-mediated regulation of 11HSD1 in arterial cells, particularly given the importance of inflammation in the vascular response to injury [109].

Alternatively, 11HSD1 may regulate inflammation by controlling generation of glucocorticoids within the inflammatory cells. Certainly, the ability of inflammatory cytokines to upregulate 11HSD1 activity in activated human macrophages [108] suggests, as in human VSMC [50], a means of feedback regulation of inflammation within these cells.

Glucocorticoids and vascular remodelling

The term 'vascular remodelling' has been used to cover a range of structural changes in the arterial wall, and its correct definition is the subject of debate (for review see Bund and Lee [110]). In this review, the term 'vascular remodelling' encompasses medial hypertrophy (thickening of the vessel wall caused by increase in cell size) and hyperplasia (thickening of the cell wall caused by an increase in cell number), as well as the intimal remodelling seen in neointimal proliferation and the development of atherosclerotic lesions. It has also been extended to include angiogenic growth of new blood vessels.

The relationship between glucocorticoids and arterial remodelling is well-established; for example, one year following remission, patients with Cushing's syndrome show reduced intimal/medial thickness and increased lumen diameter in the carotid artery [111]. It should be noted, however, that remodelling may be the indirect result of systemic changes (e.g. increased blood pressure) rather than direct interactions of glucocorticoids with the vessel wall. Glucocorticoids may induce vascular remodelling by altering expression of genes for relevant growth factors or by inhibiting processes that modulate growth factor activity. For example, dexamethasone induces a GR-dependent upregulation of endothelin-1 expression [76], and cortisol attenuates the activity of nitric oxide [77] (itself a potent inhibitor of cell growth). Similarly, dexamethasone- and hydrocortisone-mediated increases in ACE activity in VSMCs [112] and ECs [75] may enhance local generation of angiotensin II (a stimulant of VSMC growth both *in vitro* [113] and *in vivo* [114]).

Hypertrophy

Vascular hypertrophy in rats exposed to mineralocorticoids, predominantly deoxycorticosterone acetate, and salt [115] has been attributed to upregulation of the endothelin-1 gene [116]. Similarly, glucocorticoids (dexamethasone, hydrocortisone) have the ability to induce vascular hypertrophy by augmenting the production of [112, 117], and hypertrophic response to [118, 119], angiotensin II. The significance of this hypertrophy is unclear, however, as many investigations that demonstrate enhanced vascular contractility in response to corticosterone involved a duration of exposure that would be insufficient for vascular hypertrophy to occur [120]. Furthermore, glucocorticoid-mediated stimulation of growth in the vascular wall is counterintuitive given that dexamethasone inhibits VSMC growth in culture [121–123] and glucocorticoids prevent neointimal hyperplasia (see below). Thus, the direct influence of glucocorticoids on vascular hypertrophy/hyperplasia is unclear, and any role of local glucocorticoid metabolism by 11HSDs in the process has yet to be investigated.

Neointimal proliferation

The development of neointimal lesions (e.g. in atherosclerosis and in restenosis following revascularisation) is a consequence of an excessive wound healing response in the vessel wall [124, 125]. Vascular injury results in infiltration by inflammatory cells and subsequent migration and proliferation of VSMCs [109]. Consequently, inhibition of either the inflammatory response [126] or VSMC proliferation/migration [127] inhibits lesion development in a variety of models. Since glucocorticoids (dexamethasone) can inhibit inflammation and VSMC proliferation [121–123] and migration [128], it is not surprising that their potential as anti-atherosclerotic [129] and anti-restenotic agents [130] has been investigated [6]. It is also possible, however, that the action of glucocorticoids on the vessel wall is deleterious in patients with vascular disease. For example, given that ACE inhibition limits neointima formation following balloon injury [131, 132], stimulation of ACE activity by dexamethasone [75, 112] could exacerbate lesion development. Also, inhibition of endothelium-derived nitric oxide activity by glucocorticoids could increase both VSMC proliferation and vascular contraction. Further, the systemic effects of glucocorticoids on cardiovascular risk factors (glucose, insulin, lipids and blood pressure) may offset beneficial effects within the vessel wall.

Dexamethasone reduces cholesterol ester accumulation in the aorta [133], and glucocorticoids (dexamethasone, hydrocortisone) inhibit neointimal lesion formation in rats [134, 135], rabbits [136–138] and dogs [139] (with a few contradictory reports [140, 141]). Clinical trials in humans, by contrast, have proved disappointing (with notable exceptions [130]): methylprednisolone did not inhibit restenosis after coronary angioplasty [142] or stent implantation [143], whilst the combination of a glucocor-

ticoid with colchicine increased the risk of coronary aneurysm following stent placement [144]. Discrepancies between clinical studies and animal models could be attributed to species differences or, more probably, to methodological variation (e.g. small sample size; inappropriate patient selection, dose, duration of treatment, route of administration). Exacerbation of lesion development by glucocorticoids could be explained by systemic effects (e.g. weight gain with elevated blood pressure and plasma lipids, which may be more prominent in humans than in other species) or by a net stimulation of vascular cell proliferation. Alternatively, changes in plasma lipids could influence the ability of glucocorticoids to interact with vascular cells. Lipoprotein(a) can downregulate GR gene expression in human VSMCs, thus inhibiting any protective actions of glucocorticoids and, possibly, representing a novel atherogenic mechanism [145].

Angiogenesis

Angiogenesis, in which new blood vessels are formed from an existing vascular network, is a complex process regulated by a balance between counteracting endogenous activators and inhibitors [146]. Physiological angiogenesis is an essential component of reproduction and embryonic development. In postnatal and adult life, it is a discrete process (e.g. in the reproductive tract, wound healing and exercised skeletal muscle) of relatively short duration [147]. In contrast, pathological angiogenesis is usually persistent and unabated and often continues for months or years [147]. Numerous disorders are characterised by excessive angiogenesis, including neoplasia, rheumatoid arthritis and diabetic retinopathy [148]. Consequently, modulation of angiogenesis is regarded as an attractive therapeutic goal in a variety of conditions.

A comprehensive review of the mechanisms of angiogenesis is beyond the scope of this article (for recent reviews see [146, 149]). For the present purposes it is useful to consider angiogenesis to be a stepwise process comprising four distinct phases: (i) basement membrane disintegration, (ii) endothelial cell migration, (iii) channel formation and (iv) maturation. Of the numerous factors that control this process, vascular endothelial cell growth factor (VEGF) is widely considered to be of central importance, since it is crucial for vascular development both in the embryo and in adult tissues and it is EC specific.

Since its first demonstration by Folkman and colleagues, over 20 years ago [150], the ability of glucocorticoids to inhibit angiogenesis has been confirmed *in vitro, in vivo* and in tumour-bearing animals [150]. It was suggested that inhibition of angiogenesis in the rabbit cornea was independent of classical GR and MR activity [151]. For example, 17α-hydroxyprogesterone and tetrahydro-S, which have no glucocorticoid or mineralocorticoid activity, retained an anti-angiogenic capability equivalent to, or greater than, that of hydrocortisone. Taken together, these studies demonstrated a class of steroids for which inhibition of angiogenesis appears to be the principal function and hence were named 'angiostatic steroids.' [151].

Despite considerable research, the mechanisms through which glucocorticoids inhibit angiogenesis have not been identified. Indeed, the role of GR is still controversial, as some of the 'angiostatic steroids' may actually have the ability to stimulate these receptors. For example, we have recently shown that inhibition of angiogenesis by tetrahydrocorticosterone, one of the original angiostatic steroids, is dependent upon GR activation in mouse aortic ring explants [G. R. Small et al., unpublished]. Some indication of mechanism was provided by early studies which demonstrated, using nude mice or the non-anticoagulant hexasaccharide fragment of heparin, that the combination of glucocorticoid and heparin was independent of an immune response and anti-coagulant function, respectively [150]. At present, however, there are still several possible pathways through which glucocorticoids may inhibit angiogenesis: (i) Degradation of extracellular matrix, (ii) modification of cytokine production, (iii) inhibition of protease activity, (iv) impairment of vessel maturation and stabilisation, (v) inhibition of growth factor activity, (vi) inhibition of the arachidonic acid cascade, (vii) inhibition of EC-leukocyte interactions and (viii) non-transcriptional effects. The relative significance of these pathways has not been established.

11HSDs and vascular remodelling

Although the initial focus was on 11HSD2 and vascular function, the most recent work in the field of intra-vascular glucocorticoid metabolism has highlighted novel roles for 11HSD1 in influencing vascular structure and remodelling.

Neointimal remodelling and atherogenesis

The potential link between atherosclerosis and tissue-specific generation of glucocorticoids by 11HSDs has been underlined by recent demonstrations that selective upregulation of 11HSD1 in the adipose produces features of the metabolic syndrome, including central obesity, hypertension and hypertriglyceridaemia [10, 152]. This supports the concept that similarities between the metabolic syndrome and Cushing's syndrome are explained by tissue-specific increases in 11HSD1 activity resulting in tissue-specific elevation of glucocorticoid generation [153]. This link between 11HSD1 activity in glucocorticoid-target tissues and atherosclerotic risk factors is not limited to the adipose, as hepatic overexpression of 11HSD1 also results in elevated blood pressure and dyslipidaemia

[154]. It has been proposed, therefore, that 11HSD1 inhibition may reduce atherogenesis. Very recently, systemic administration of a selective 11HSD1 inhibitor was reported to virtually abolish lipid accumulation in the aorta of atherosclerotic (apolipoprotein $E^{-/-}$) mice. However, inhibition of 11HSD1 in $ApoE^{-/-}$ mice produced only a relatively modest reduction in serum triglycerides and cholesterol [155], suggesting that mechanisms over and above amelioration of systemic cardiovascular risk factors may be responsible. It may be that inhibition of 11HSD1 within the vessel wall or within invading macrophages [156] is crucial, but these mechanisms require further clarification.

11HSD2-dependent protection of MR from inappropriate occupation by glucocorticoids may also influence atherogenesis. The role of MR activation in the pathogenesis of atherosclerosis [157] is demonstrated by aldosterone-induced enhancement of lesion development in atherosclerotic (apolipoprotein $E^{-/-}$) mice (probably by increasing oxidative stress in macrophages and cells of the vascular wall [157, 158]). This potentiation of lesion development by aldosterone, which is largely independent of blood pressure, is attenuated by MR antagonists [157], as is constrictive remodelling following angioplasty [159].

The potential importance of 11HSD activity to the development of atherosclerotic lesions was recently extended by the demonstration that, in addition to glucocorticoid metabolism, 11HSDs catalyse the conversion of the atherogenic oxysterol 7-ketocholesterol to 7β-hydroxycholesterol [160]. 7-Ketocholesterol is present in micromolar concentrations in human atherosclerotic lesions and in nanomolar concentrations in the plasma [161]. Its

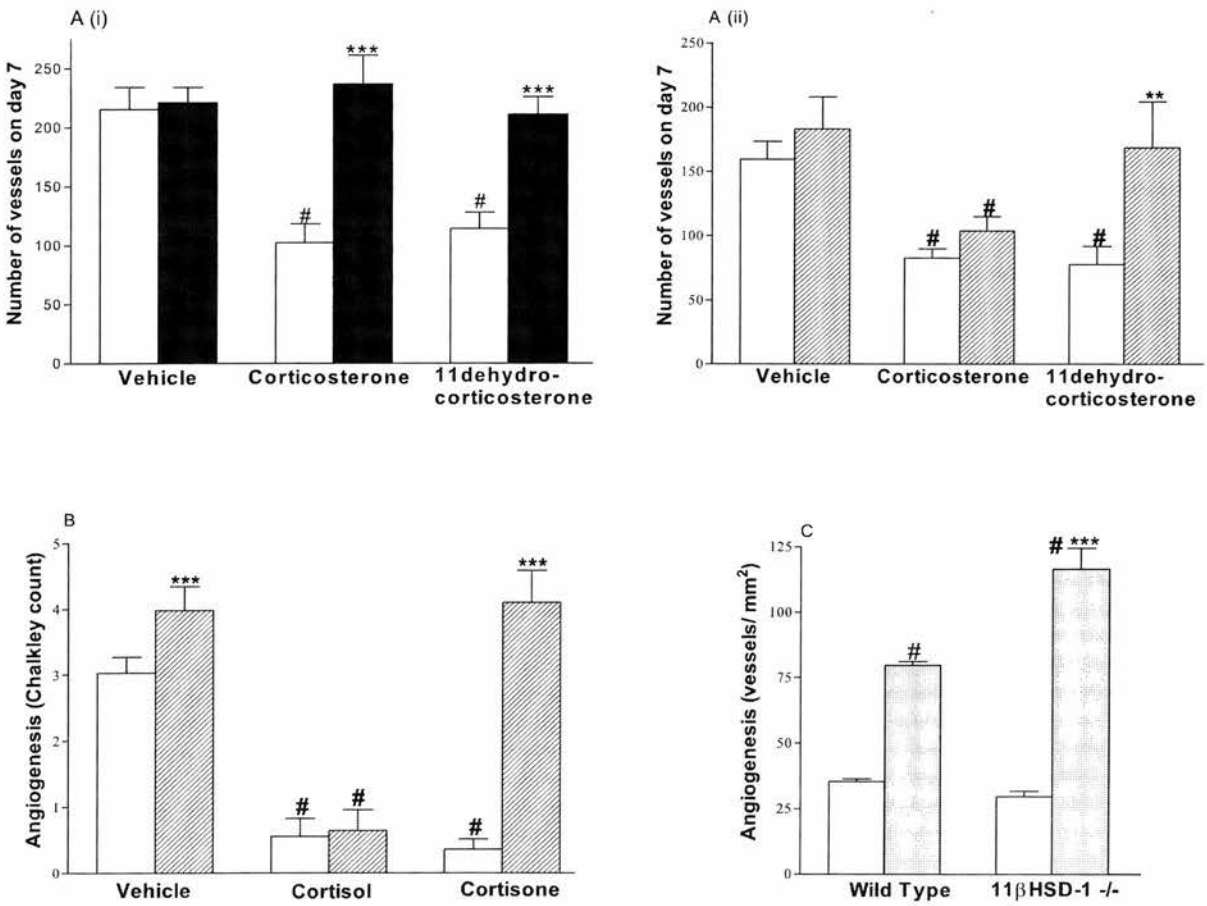

Figure 4. The influence of 11HSD1 on glucocorticoid-mediated angiogenesis. (*A*) In mouse aortic rings cultured in Matrigel, corticosterone and its inactive metabolite 11-dehydrocorticosterone attenuated new vessel growth. Glucocorticoid receptor antagonism (with RU38486, filled columns) abolished the angiostatic response to both compounds (*Ai*), but 11HSD1 deletion (hatched columns) selectively prevented 11-dehydrocorticosterone-mediated angiostasis (*Aii*). Similar results were obtained *in vivo*, using subcutaneous sponge implants (*B*), with 11HSD1 deletion (hatched columns) increasing angiogenesis under basal conditions and abolishing cortisone-, but not cortisol-, mediated inhibition of vessel growth. This mechanism contributed to regulation of myocardial angiogenesis following coronary artery ligation (*C*) with increased vessel growth in $11HSD1^{-/-}$ mice (coronary artery ligation, shaded bars; sham, open bars). #$p < 0.05$ compared with relevant vehicle-treated control; **$p < 0.01$ compared with wild-type mice; ***$p < 0.001$ compared with relevant wild-type control or sham-operated mouse. Reproduced with permission from [168].

association with atherosclerosis is demonstrated in the condition cerebrotendinous xanthomatosis, in which patients who have normal circulating cholesterol levels but increased 7-ketocholesterol develop atherosclerosis prematurely [162]. Conversion of 7-ketocholesterol to 7β-hydroxycholesterol by 11β-HSD1 may represent the rate-limiting step in a clearance pathway: *in vivo* inhibition of 11HSD1 in rats resulted in an accumulation of 7-ketocholesterol in the liver and increased concentrations in the plasma [163]. In addition to these hepatic effects, reduction of 7-ketocholesterol by 11HSD1 within the vascular wall may also be important. 7-Ketocholesterol and 7β-hydroxycholetserol are both toxic to cells of the vascular wall [164] and are potent inhibitors of endothelium-dependent relaxation [165–167]. Consequently, reduction of 7-ketocholesterol, and subsequent clearance of 7β-hydrocholesterol, by protecting the vascular wall from damage may have a role in preventing lesion development.

The influence of 11-HSD activity on angiogenesis

Since inflammatory cytokines can promote angiogenesis, we hypothesised that 11β-HSD1 in the vessel wall may regulate new vessel formation by controlling the local regeneration of active glucocorticoids. This possibly was addressed using a combination of *in vitro*, *in vivo* and pathological models of angiogenesis [168]. Using a model of tube formation from mouse aortic rings cultured in Matrigel [169], we demonstrated (fig. 4a) that angiogenesis was inhibited by physiological concentrations of active glucocorticoid (corticosterone) but also by its inactive metabolite (11-dehydrocorticosterone). Both these responses were blocked by RU38486, but not by spironolactone, indicating GR dependence. However, whereas 11HSD1-inhibition (with carbenoxolone) or deletion (aortic rings from 11HSD1$^{-/-}$ mice) had no effect on the response to corticosterone, they abolished the ability of 11-dehydrocorticosterone to inhibit angiogenesis (fig. 4b). This indicated that 11HSD1-dependent regeneration of active glucocorticoid within the vascular wall regulates new vessel growth. Application of a model of angiogenesis in sub-cutaneous sponge implants, confirmed this role for 11HSD1 *in vivo*, showing that 11HSD1 deletion produced increased angiogenesis in un-treated sponges and blocked the ability of cortisone (but not cortisol) to inhibit new vessel formation. The pathophysiological significance of these observations was emphasised in healing cutaneous wounds and in the myocardial response to coronary artery ligation (fig. 4C). In both cases, 11HSD1 deletion resulted in increased angiogenesis, demonstrating that 11HSD1 regulates the growth of new blood vessels in healing tissues.

Altered angiogenesis in 11HSD1$^{-/-}$ [168] mice could, conceivably, be the result of changes in macrophage activity. Given that 11HSD1 is expressed in macrophages [170], and regeneration of glucocorticoids enhances phagocytosis of apoptotic neutrophils [107], absence of 11HSD1 may confer a prolonged and enhanced acute inflammatory response, thus stimulating angiogenesis. The use of *ex vivo* models such as isolated aortic rings cultured in extracellular matrices [171] has made it possible to differentiate between these two intimately related pathways, angiogenesis and inflammation, and specifically address the effects of glucocorticoids on angiogenesis in the absence of a systemic response. This has produced evidence that glucocorticoids regulate angiogenesis by direct interaction with the vessel wall [168].

Conclusions

It is apparent that glucocorticoids have the ability to regulate both the structure and the function of the artery wall, with significant implications for vascular physiology and pathophysiology. Emerging evidence suggests that pre-receptor metabolism of glucocorticoids within vascular ECs and VSMCs provides a mechanism for regulating these interactions. Relatively few studies have addressed the role of intravascular 11HSD activity, and most of those available have focussed on vascular function. There is, however, a growing body of evidence to suggest that 11HSD isozymes within the arterial wall modulate vascular contractility, the angiogenic growth of new blood vessels, and the atherosclerotic process. Whether these isozymes also influence the inflammatory response to vascular injury and the inter-conversion of atherogenic oxysterols in vascular smooth muscle has still to be determined. Further clarification of the role of 11HSDs in vascular cells is likely to increase our understanding of the link between glucocorticoids and a variety of vascular diseases, and to demonstrate their potential as therapeutic targets for treatment of these conditions.

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