THE EFFECTS OF METHOTREXATE ON HUMAN BONE CELL RESPONSES TO MECHANICAL STIMULATION

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For my Mum and Dad

Thank You For Everything

xxx

DECLARATION

I hereby declare that this thesis has been composed by myself and has neither been presented nor accepted in any previous application for a degree. All work presented in this thesis was, unless acknowledged, carried out by myself. All sources of information have been acknowledged by reference.

Kerry Jane Elliot.

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ABBREVIATIONS USED IN THIS THESIS

AP	alkaline phosphatase
ABC-HRP	avidin biotin complex – horseradish peroxidase
ADA	adenosine deaminase
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
ALL	acute lymphoblastic leukaemia
AMP	adenosine monophosphate
AMPDA	adenosine monophosphate deaminase
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CH_3	methyl
CM	conditioned medium
COX-2	cyclooxygenase-2
DAB	diaminobenzidine
DAG	diacylglycerol
DHFR	dihydrofolate reductase
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2' deoxynucleoside 5'-triphosphate
dTMP	dioxythymidylate
DTT	dithiothreitol
dUMP	dioxyuridylate
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid

ERK	extracellular-signal related kinase
FAICAR	formyl 5-aminoimidazole-4-carboxamide ribonucleotide
FCS	foetal calf serum
FH_2	dihydrofolate
FH_4	tetrahydrofolate
GAPDH	glyceraldehyde phosphate dehydrogenase
G-protein	guanosine trisphosphate-binding protein
GH	growth hormone
Glu	glutamyl
GRGDSP	glycine-arginine-glycine-aspartic acid-serine-proline
GTP	guanosine trisphosphate
HBC	human bone cell
HCl	hydrochloric acid
Hz	hertz
ICTP	C-terminal telopeptide of type 1 collagen
IGF	insulin growth factor
IL	interleukin
IL1R	interleukin-1 receptor
IP_3	inositol trisphosphate
kD	kilo dalton
KCL	potassium chloride
LDH	lactate dehydrogenase
mAb	monoclonal antibody
MEM	minemal essential medium
mRNA	messenger ribonucleic acid
MS	mechanical stimulation
MSCs	mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide
MTX	methotrexate

MTX-glu	methotrextae polyglutamates
NBT/BCIP	nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl phosphate
NCP	non-collagenous protein
NH_2	amino
NO	nitric oxide
Pa	pascal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PICP	C-terminal propeptide of type 1 collagen
PKC	protein kinase C
PLC	phopholipase C
PTH	parathyroid hormone
RA	rheumatoid arthritis
RNA	ribonucleic acid
RGD	arginine-glycine-aspartic acid
RT	reverse transcription
RT-PCR	reverse transcriptase-polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
SK	small conductance calcium activated potassium ion channels
STWS	scott's tap water substitute
TBS	tris buffered saline
TGF-β	transforming growth factor-β
TNF-α	tumour necrosis factor-α
TRAP	tartrate-resistant acid phosphatase
TRITC	tetramethyl rhodamine isothiocyanate
TS	thymidylate synthetase
VCAM	vascular cell adhesion molecule

PUBLICATIONS ASSOCIATED WITH THE WORK IN THIS THESIS

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1. **Elliot, K.J., Millward-Sadler, S.J., Wright, M.O., Robb, J.E., Wallace, W.H.B. and Salter, D.M.** Effects of Methotrextae on Human Bone Cell Responses to Mechanical Stimulation [*submitted to Br. J. Rheumatol*].

Abstracts:

1. **Elliot, K.J., Millward-Sadler, S.J., Wright, M.O., Robb, J.E., Wallace, H. and Salter, D.M.** Methotrextae Inhibits Normal Bone Mechanotransduction: Is Adenosine Signalling Involved? Int. J. Exp. Pathol. [*In Press*].

2. **Elliot, K.J., Millward-Sadler, S.J., Wright, M.O., Robb, J.E., Wallace, H. and Salter, D.M.** 2001. Methotrexate Influences Human Bone Cell Responses to Mechanical Stimulation. Int. J. Exp. Pathol. **82**:A9.

ABSTRACT

Methotrexate (MTX) is prescribed widely, both in the treatment of malignancy and autoimmune disease. MTX has detrimental effects on a number of organ systems including bone, and children who receive MTX therapy in the treatment of childhood malignancies often show growth retardation throughout the course of the treatment. On a longer time scale, this may result in a predisposition to osteoporosis. The mechanism of action of MTX on bone at the cellular level is not clear, but may involve interaction with the function of a number of bone cells and their precursors.

Mechanical forces influence human bone cell (HBC) metabolism and function. It has been shown that 0.33 Hz cyclical mechanical stimulation results in membrane hyperpolarisation of HBC. Membrane hyperpolarisation follows activation of a mechanotransduction pathway which involves the $\alpha_5\beta_1$ integrin, tyrosine kinase activity, the actin cytoskeleton and interleukin-1β (IL-1β) secretion. IL-1β binds to IL-1 receptors and stimulates a signal cascade involving phospholipase C (PLC) and protein kinase C (PKC), leading to the activation of small conductance Ca^{2+} activated K^+ (SK) channels.

Experiments have been performed to further elucidate the mechanotransduction pathways activated following 0.33 Hz cyclical mechanical stimulation. It has been demonstrated that type 1 collagen mRNA levels are increased following 0.33 Hz cyclical mechanical stimulation, whereas bone morphogenetic protein-4 (BMP-4) mRNA levels are decreased following 0.33 Hz cyclical mechanical stimulation.

The effect of MTX on HBC responses to 0.33Hz cyclical mechanical stimulation was investigated. MTX dose-dependently inhibits the hyperpolarisation response following mechanical stimulation. MTX appears to block mechanically induced IL1-β release, rather than interfering with downstream IL1-β responses. Methylene blue cell adhesion assays have provided evidence to indicate that MTX may have effects on integrin receptors (in particular the β_1-integrin subunit), although at present, the exact mechanisms involved are unknown. MTX does not affect mechanically induced changes in type 1 collagen and BMP-4 mRNA levels, which would indicate that the mechanotransduction pathways by which type 1 collagen and BMP-4 gene expression are regulated are not reliant on IL1-β function.

MTX is known to affect purine biosynthesis resulting in an overproduction of adenosine. Studies have been performed to investigate the effects of adenosine on HBC responses to 0.33 Hz cyclical mechanical stimulation. It has been demonstrated that adenosine inhibits HBC hyperpolarisation following 0.33 Hz cyclical mechanical stimulation, and that the inhibitory effects of MTX on HBC responses to mechanical stimulation can be prevented by adenosine deaminase (ADA). These results suggest that adenosine signalling may be involved in MTX mediated inhibition of HBC responses to cyclical mechanical stimulation, although the specific subset(s) of adenosine receptors involved could not be determined.

CHAPTER 1.

INTRODUCTION.

The following section will give an introduction and background to the work detailed in this thesis. A substantial part of this work has involved investigating the responses of human bone cells (HBC) to regimes of cyclical mechanical stimulation (in the presence and absence of methotrexate (MTX)), and therefore, particular attention is given to the structure and biology of bone and also to bone mechanotransduction. Major findings in the mechanotransduction field are discussed, and the relative roles of different types of bone cells and the extracellular matrix in recognition and conversion of mechanical signals to biochemical events are described. The structure and mechanism of action of MTX is also discussed, as are its effects on bone, with particular attention to its effects on osteoblasts.

This study is the first time that the effects of MTX on HBC responses to mechanical stimulation have been investigated.

1.1 Bone Structure and Biology.

Bone is a specialised connective tissue that provides many essential functions of the body. These functions include (1) the protection of vital organs, such as the brain, spinal cord and heart, (2) providing a rigid framework to support the body, (3) providing sites for muscle attachment to allow mobility, (4) generation of red and white blood cells for immunoprotection and oxygenation of other tissues and (5) retaining reserve stores of calcium, phosphate and magnesium (Yaszemski et al, 1996).

1.1.1 Gross Structure.

The skeleton provides the framework of the body and is divided into (a) the axial skeleton, which includes the vertebrae, skull, ribs, sternum and hyoid bone and (b) the appendicular skeleton, which includes the bones of the upper and lower limbs and the pelvis (Van Wynsberghe et al, 1995).

The long bones of the body (e.g. humerous, tibia and femur) provide probably the easiest descriptive model for the gross anatomy of a typical bone (Van Wynsberghe et al, 1995). Most adult long bones have a tubular shaft called the diaphysis, which is essentially a hollow cylinder of compact (dense) bone tissue. In the centre of the cylinder is the medullary cavity, which is filled with marrow and trabecular (spongy, cancellous) bone tissue (Buckwalter et al, 1996, Buckwalter et al, 1997). At each end of the bone is a roughly spherical epiphysis of trabecular bone tissue. The epiphysis is usually wider than the shaft of the bone. The flat bones and irregular bones of the trunk and limbs have many epiphyses, whereas the long bones and the fingers and toes have only two epiphyses.

Separating the diaphysis from the epiphyses at either end of the bone is the metaphysis. It comprises the epiphyseal (growth) plate and the adjacent bony trabeculae of spongy bone tissue on the diaphyseal side of the long bone. The epiphyseal plate is a thick plate of hyaline cartilage that provides the framework for the synthesis of the cancellous bone

tissue within the metaphysis. The epiphyseal plates are the only place where long bones continue to grow in length after birth (Van Wynsberghe et al, 1995).

An outer bone sheath called the periosteum, covers both the cortical and the cancellous bone. The periosteum is a fibrous membrane that has the potential to form bone during growth periods and in fracture healing and is often attached to the underlying bone by collagenous fibres called Sharpey's fibres. The periosteum contains nerves, lymphatic vessels, and many capillaries, which provide nutrients to the bone and is composed of two distinct layers. The outer layer is fibrous and is used to connect bones at the joint, whereas the inner layer is more vascularised and contains cells that are capable of becoming osteoblasts. The periosteum is continuous, except over articular surfaces, which are covered by cartilage. As well as playing an important role in endochondral bone formation, the periosteum provides a large proportion of the blood supply to the bones throughout life (Buckwalter et al, 1996).

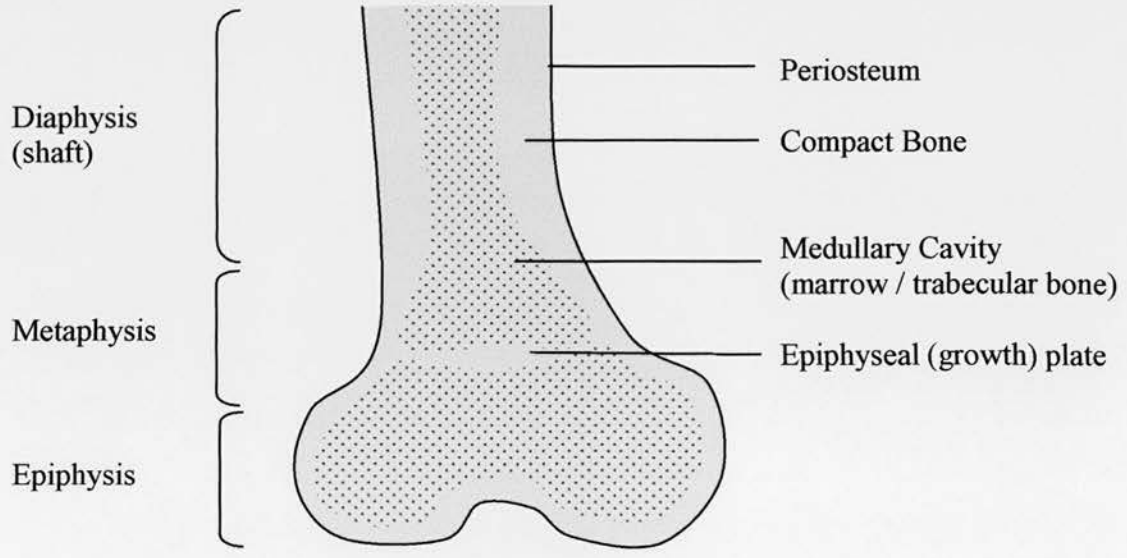


Figure 1.1 – Gross anatomy of a typical long bone.

1.1.2 Macroscopic Physiology.

1.1.2.1 Compact Bone Tissue.

Compact bone tissue is very hard and dense and appears to the naked eye to be solid although it is not. The basic unit of compact bone is the Haversian system (osteon). The Haversian system is a hollow, laminated cylinder of collagen and calcium phosphate. The cylinders are made of concentric layers (lamellae) of bone. These lamellae are arranged like wider and wider drinking straws; each one nestled inside the next wider one (Van Wynsberghe et al, 1995; Buckwalter et al, 1996). This structure provides the great strength needed to resist typical, everyday compressive and bending forces on long bones **(Figure 1.2)**.

In the centre of osteons are central canals (Haversian canals). The Haversian canals are longitudinal channels that contain blood vessels, nerves and lymphatic vessels **(Figure 1.2)**. Central canals usually have branches called perforating canals (Volkmans canals) which run at right angles to the central canals and therefore extend the system of nerves and vessels outward to the periosteum, and inward to the endosteum (inner lining) of the bone marrow cavity (Sikavitsas et al, 2001).

Lamellae contain lacunae, which contain osteocytes. Canaliculi radiate from each lacuna, and contain the cell processes of the osteocytes. Nutrients and waste products can pass to and from the blood vessels in the central canals (1) by normal processes of intracellular transport within each osteocyte, (2) over gap junctions from one osteocyte to another, or (3) possibly via tissue fluid in the tiny spaces between the osteocytes and their surrounding lacunae (Van Wynsberghe et al, 1995) **(Figure 1.2)**.

(a) SINGLE OSTEON

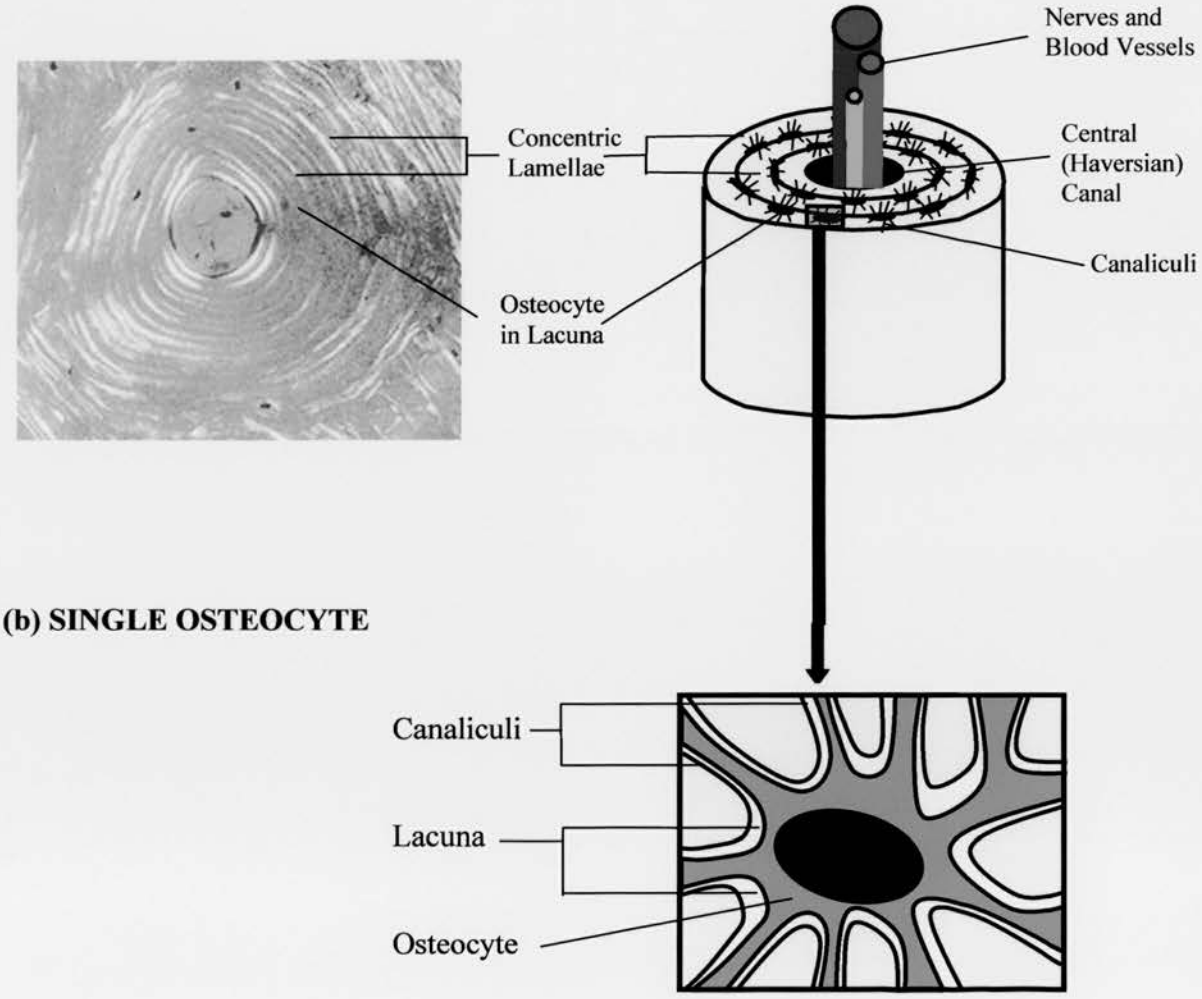


Figure 1.2 – Compact bone tissue. (a) an enlargement of a single osteon with lacunae, canaliculi and a central (Haversian) canal visible. Photograph taken from Athansou, 1999. (b) An enlarged osteocyte, housed within a lacuna.

1.1.2.2 Trabecular (cancellous, spongy) Bone Tissue.

Trabecular bone tissue is in the form of an open interlaced pattern that withstands maximum stress and supports shifts in weight distribution. The mesh like structure is designed for strength in a similar manner to steel rods within a concrete structure. Prominent in the interior structure of cancellous bone tissue are trabeculae, tiny spikes of bone tissue surrounded by bone matrix that has calcified, or become hardened by the deposition of calcium salts. Trabeculae are relatively thin and do not usually contain Haversian systems. They are nourished by surrounding vessels within haematopoietic or fatty marrow. Bone trabeculae are lined by a continuous layer of flattened bone lining cells or osteoblasts and are separated by haematopoietic or fatty marrow. The trabeculae form along the lines of greatest pressure or stress. This arrangement provides the greatest strength with the least weight. Trabecular bone tissue is found within most bones (Van Wynsberghe et al, 1995; Buckwalter et al, 1996).

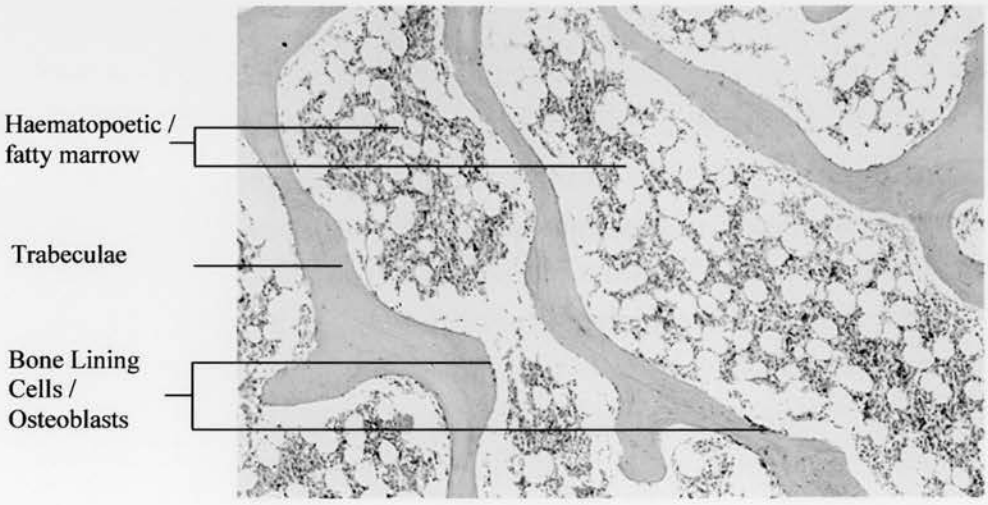


Figure 1.3 – Trabecular (cancellous, spongy) bone tissue. Photograph taken from Athansou, 1999.

1.1.3 Bone Matrix.

1.1.3.1 Organic Components.

Bone is a specialised connective tissue in which bone cells lie within a connective matrix that has organic and inorganic components (Fujisawa et al, 1998). The organic component, which comprises approximately 35% of the bone matrix, is composed predominantly of type 1 collagen fibres (95%), although trace amounts of other collagen types, such as collagen III, V, XI and XIII, have also been found (Robey et al, 1993; Athanasou, 1999). The collagen fibres in the matrix are responsible for giving bone its tensile strength and flexibility and although the hardness of bone comes from the organic salts, its structure depends equally on the collagen framework (Van Wynesberghe et al, 1995).

All collagens share the same basic structure, which is a long and thin diameter rod-like protein (Gross et al, 1974; Ottani et al, 2002). Type 1 collagen is 300nm long, 1.5nm in diameter and consists of 3 coiled subunits composed of two α_1 (I) chains and one α_2 (I) chain (Sandberg et al, 1987). Each chain consists of 1050 amino acids wound around each other in a right-handed triple helix. Lateral interactions of triple helices of collagens result in the formation of collagen fibrils roughly 50nm in diameter. The packing of collagen is such that adjacent molecules are displaced approximately ¼ of their length (67nm). This staggered array produces a striated effect that can be seen under the electron microscope.

Collagenous fibres are whitish in colour and are found in all kinds of connective tissue. They are composed of bundles of parallel collagen fibrils. Collagenous fibres are sturdy, flexible, insoluble and virtually unstretchable. The arrangement of collagenous fibres varies from loose and pliable as in the loosely woven connective tissue that supports most of the vital organs, to tightly packed and stretch-resistant as in tendon and bone. In bone, osteoblasts are responsible for the synthesis and deposition of type 1 collagen. The type 1 collagen formed by osteoblasts is typically deposited in parallel bundles or concentric layers.

The remainder of the organic bone matrix is composed of non-collagenous proteins (NCP) of bone (Heinegard et al, 1989; Young et al, 1992). NCP's can be classified according to their structure into three main groups:

1. **Proteoglycans** are macromolecules, which consist of a central core protein and covalently attached polysaccharide chains. On the basis of side chains, three subgroups of proteoglycans can be found in bone: a.) chondroitin sulfate side chains are found in biglycan, decorin and in a protein which resembles versican (Krusius et al, 1987), b.) Heparan sulphate side chains are found in association to membrane proteoglycans, e.g. receptors for transforming growth factor-β (TGF-β). c.) keratan-sulfate side chains are associated with leucine rich proteins and include osteoglycin and osteoadherin.
2. Osteocalcin and vitronectin are examples of **g-carboxylated (gla)-proteins**, (Ruoslahti, 1988; Hauschka, 1986). Osteocalcin is a valuable biochemical indicator of osteoblastic activity and metabolic turnover in bone (Neradilova et al, 1990).
3. Osteonectin is an example of a **glycoprotein** found in bone matrix. Others include thrombospondin, fibronectin (Hauschka, 1986), tetranectin and osteopontin (Gehron Robey, 1989; McKee et al, 1996).

In addition to these three groups, several other NCP's can be found in variable amounts. These include growth factors known to be involved in bone formation, and include bone morphogenetic proteins (BMP's), which are members of TGF superfamily (Ripamonti et al, 1992) and are important in bone maturation. Bone NCP's can also be classified according to whether they possess an RGD (arg-gly-asp) sequence or not. This is especially important in cell-matrix interactions, where RGD–sequences are known to play a role in attachment of cells to the ECM. RGD-sequences are found in bone sialoprotein (osteopontin), thrombospondins, vitronectin, osteoglycin and osteoadherin.

1.1.3.2 Inorganic Components.

Bone is a major store of minerals, containing 99% of body calcium and 88% of body phosphate. Inorganic bone matrix (65%) is composed of crystalline salts, mainly calcium and phosphate, in the form of calcium hydroxyapatite [$(Ca)_{10}(PO_4)_6(OH)_2$].

Hydroxyapatite crystals are spindle or platelet-shaped, measuring up to 200 nm in length. This offers a large surface area available for mineral exchange, about 10 m^2 per 1g of bone. Other components include calcium carbonate, calcium fluoride, and magnesium fluoride (Athanasou, 1999).

1.1.4 Bone Cells.

Bone contains five different cell types, which are capable of changing their roles as the needs of the body changes in the growing and adult skeletons.

1. **Osteogenic (osteoprogenitor)** cells are small, spindle-shaped, mesenchymal stem cells. They are found mostly in the deepest layer of the periosteum and in the endosteum. These cells have a high mitotic potential and can be transformed into bone forming cells (osteoblasts) during growth, remodelling and healing (Van Wynsberghe et al, 1995).
2. **Osteoblasts** are located on the surface of bone tissue and are usually found in growing portions of bone, including the periosteum. Osteoblasts are derived from mesenchymal stem cells of the bone marrow stroma and possess a single nucleus. They have a shape that varies from flat to plump, reflecting their level of cellular activity. When active, they have a plump, polygonal appearance and when inactive, have a flattened morphology. Osteoblasts are responsible for the synthesis and secretion of an unmineralized ground substance called osteoid, which consists of collagen and NCP's. Mature osteoblasts are also rich in alkaline phosphatase, an organic phosphate-splitting enzyme (Robey, 1989). Hormones, growth factors, physical activity and other stimuli act mainly through osteoblasts to bring about their effects on bone. Osteoblasts are known to express receptors for estrogen, testosterone, parathyroid hormone (PTH) (Buckwalter et al, 1996), transforming growth factor-β (TGF–β), tumour necrosis factor-α (TNF–α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-11 (IL-11). Osteoblasts also express receptors for vitamin D_3, prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2). Osteoblasts also act as a pump to move calcium and phosphate into and out of bone tissue, thereby respectively calcifying or decalcifying it (Van Wynsberghe et al, 1995, Buckwalter et al, 1996, Athanasou et al, 1999).

3. **Osteocytes** are the most numerous cells of fully developed bone. Osteocytes are derived from osteoblasts which have secreted bone matrix around themselves. Each osteocyte has a cell body that occupies a lacuna within the bone matrix and long slender cell processes that extend through the matrix via canaliculi. These processes interconnect to form gap junctions between neighbouring osteocytes. They, along with osteoclasts, play an active role in homeostasis by helping to release calcium from bone interstitial fluid into the blood, thereby regulating calcium concentration in the body fluids. Osteocytes also keep the matrix in a stable and healthy state by secreting enzymes and maintaining the mineral content of the matrix (Van Wynsberghe et al, 1995).

4. **Osteoclasts** are multinuclear giant cells with a size up to 100 μM. They are derived from hematopoietic stem cells that also give rise to monocytes and macrophages. Osteoblasts are found on the surface of the bone resorbing bone from sites where it is either deteriorating or no longer needed, and are normally found where bone is resorbed during its normal growth. Osteoclasts appear to be activated by "signals" from osteoblasts. For example, osteoblasts have receptors for PTH whereas osteoclasts do not, and PTH-induced osteoclastic bone resorption does not occur in the absence of osteoblasts.

5. **Bone-Lining Cells** are found on the surface of most bones. They are thought to be derived from osteoblasts that are no longer active and that have flattened out on the bone surface. Such cells may have several functions. They may serve as osteogenic cells that can divide and differentiate into osteoblasts, or may serve as an ion barrier around bone tissue (Van Wynsberghe et al, 1995).

1.1.5 Bone Formation and Development.

1.1.5.1 Bone Formation.

Bones develop through a process called ossification (osteogenesis). The primitive skeleton of the human embryo is composed of either hyaline cartilage or fibrous membrane and bones can develop by two ways: intramembranous or endochondral ossification. Intramembranous ossification occurs when bone develops directly from

mesenchymal tissue. Bones that develop by intramembranous ossification include the frontal and parietal bones and the bones of the face (Van Wynsberghe et al, 1995).

The process of endochondral bone ossification begins when mesenchymal stem cells (MSCs), progenitor cells which can differentiate into bone or cartilage-forming cells start to differentiate into chondrocytes and secrete a cartilaginous matrix. The cells then pass through various lineage states and eventually lose the capability to proliferate (Caplan et al, 1994; Caplan et al, 1997). At this time a periosteal layer appears around the cartilage model (the periosteal layer is comprised of MSC's which are predisposed to form bone rather than cartilage). Shortly after this time, the chondrocytes enlarge and produce alkaline phosphatase, which triggers a chemical reaction in the matrix that causes ossification. The calcified matrix blocks the diffusion of nutrients to the chondrocytes, which die and are eventually resorbed, leaving irregular cavities in the ossifying matrix. Once the bone is formed, these cavities contain the bone marrow. Pluripotent cells lining the cavities begin to differentiate into osteoblasts and osteocytes. The osteoblasts deposit osteoid on the mineralized cartilage cores forming a thin layer of spongy bone tissue (Buckwalter et al, 1996).

1.1.5.2 Determinants of Peak Bone Mass.

There are many factors which have a bearing on the magnitude of peak bone mass, and these will be described in the following sections;

1. It is known that there is a **genetic component**, which accounts for >70% of peak bone mass. Strong genetic influences affect body size and bone geometry (Marcus, 1996), which can be demonstrated in the variation in skeletal size and density between various ethnic groups – with Caucasians having higher bone masses on average than Asians (Pollitzer et al, 1989).

2. Mechanical stresses and **strains** are known to be important, both by way of gravitational stress related to the weight of the individual and also to physical exercise. For example, in individuals who greatly increase the size of their muscles, bone mass

may also increase at the same time. If the bones do not increase in mass they may fracture because they are unable to cope with the increased pull of the stronger muscles.

3. **Dietary calcium intake** plays a substantial role in determining peak bone mass. The skeleton is responsible for 99% of body calcium stores (in addition to 88% of phosphate stores and 54% of magnesium stores) and constitutes a reservoir of mineral that can support plasma calcium levels in times of need. Without calcium, some enzymes would be unable to function, the permeability of cell membranes would be affected, muscles (including the heart) could not contract, nerve function would be impaired and blood could not clot. Clinical trials have shown that calcium intake is likely to be particularly important to bone mass during the first three decades of life (Recker et al, 1992), and that calcium supplementation of adolescent boys and girls, leads to increased bone acquisition (Johnston et al, 1992). In contrast, through the third to fifth decades, growth has stopped and calcium nutritional state does not appear to be a major influence on maintenance of bone mass during this period (Elders et al, 1991; Riis et al, 1987). General nutrition, in addition to calcium intake, is also important and girls with anorexia nervosa have been reported to have as much as 25% reduction in the trabecular bone density (Mazess et al, 1990).

4. **Growth Hormones** also play an important role in the acquisition and maintenance of bone mass. Patients with deficient growth hormone (GH) secretory function show low bone mass and it has been demonstrated that GH replacement of such patients promotes significant increases in bone mineral density.

5. There is a great deal of evidence to suggest a role for **reproductive hormones** in the acquisition and maintenance of bone mass. Hypogonadal boys and girls show deficits in both cortical and trabecular bone mineral and loss of endogenous sex steroids during adult life regualrly leads to loss of bone mineral. 17β-estradiol is of particular importance for bone acquisition and mutations in the estradiol receptor in the male can lead to bone mineral densities which are several standard deviations below predicted values (Smith et al, 1994). In women, loss of estrogen has dual effects. Decreased efficiency of intestinal and renal calcium handling increases the level of calcium necessary to maintain neutral calcium balance (Heany et al, 1977). In addition, estrogen

directly affects bone cell function. In animal models, strong evidence indicates that estrogen directly regulates osteoblast production of IL-6, a potent regulator of osteoclast recruitment (Girasole et al, 1992). The precise details of estrogen regulation of skeletally active cytokines in humans are not clear but such interactions are thought to underlie the accelerated bone loss of early estrogen deficiency. Estrogen deficiency permits osteoclasts to resorb bone with greater efficiency, which may eventually lead to elimination of entire trabecular struts so that no scaffold remains for the initiation of bone formation.

1.2 Bone Mechanotransduction.

Terms Used in Mechanical Studies (Armstrong et al, 1992; Woo et al, 1992).

Strain is a measure of force per unit length of a body. It is a ratio of deformation / initial length.

Tensile strain is an increase in length per unit length of material along a given line, while **compressive strain** is the decrease in length per unit length.

Shear strain is the change in angle in the deformed state between two lines that were vertical in the undeformed state.

1.2.1 Introduction to Mechanotransduction.

Mechanotransduction is the process by which cells within a living tissue perceive physical stimuli and respond with biochemical signals. Mechanotransduction is integral to the successful function of organ systems (Ingber, 1991; Ingber, 1997; Davies et al, 1997; Chicurel et al, 1998), whole organisms and plants (Johnson et al, 1998). Studies with cultured cells confirm that mechanical stresses can directly alter many cellular processes, including signal transduction, gene expression, growth, differentiation and survival (Chen and Ingber, 1999). In principal, all eukaryotic cells are mechanosensitive and physical forces, including gravity, tension, compression, and shear, influence growth and remodelling in all living tissues at the cellular level (Ingber, 1997).

1.2.2 Mechanotransduction and Bone.

It has been known for many decades, that the shape, mass and general morphology of bones are moulded, to a certain degree, by the strains to which they are exposed in their daily environment (Turner et al, 1998; Sakai et al, 2002). Therefore, bone develops not only as a structure designed specifically for mechanical tasks, but is able to adapt during the life of an individual towards a more effective mechanical performance. This has been accepted for a great number of years, emerging initially in the ideas of Roux (Roux

et al, 1905), Meyer (Meyer, 1867), and most significantly Julius Wolff (Wolff et al, 1892). Wolff's mathematical law of bone transformation, published in 1892 states that the form of bone is related to mechanical stresses to which it is exposed. This law is still generally accepted, although many of its components have since been disproved.

Evidence for a relationship between mechanical forces and skeletal form is abundant. Prolonged skeletal disuse in humans, whether due to spaceflight (Mack et al, 1967; Morey et al, 1978, Vico et al, 1998), bed rest (Leblanc et al, 1990), or immobilization (Prince et al, 1988), results in osteopenia due to a decrease in bone formation while bone resorption remains unchanged or is transiently increased (Cann et al, 1983; Turner et al, 1985). Conversely, increased skeletal loading can increase bone formation and decrease bone resorption (Rubin et al, 1984; Sessions et al, 1989). Although the relationship between bone tissue formation and mechanical strain has been known for a number of years, until fairly recently the mechanisms involved have remained poorly understood.

Frosts mechanostat theory provides a hypothesis of how bone might adapt to strain (Frost, 1987; Frost 1998). The mechanostat theory hypothesises that when bone cells are in equilibrium with the surrounding environment, a basal / physiological level of stress (200 – 2500 μstrain) exists. At this level of stress there is assumed to be no net formation of bone, with an equilibrium existing between bone formation by osteoblasts and bone resorption by osteoclasts. At the upper end of the physiological scale of strain, there exists a maximum effective strain (2500 μstrain), which results in osteoblast recruitment and matrix deposition. Similarly, at the lower end of the physiological scale of strain, a mimimum level of strain exists (200 μstrain) below which there is net bone loss. Although the mechanisms involved in the events described by the mechanostat theory are unknown, the theory assumes that biophysical forces are detected by the cell and converted into a biochemical response, thereby allowing the organism to respond and adapt accordingly **(Figure 1.4).**

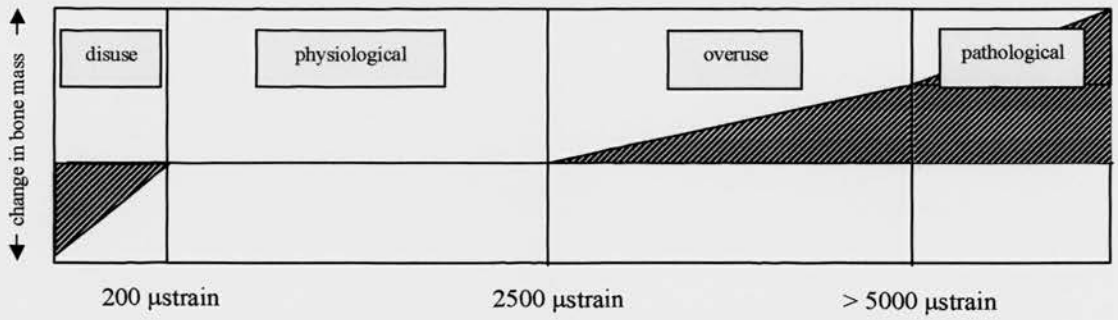


Figure 1.4 – Frosts mechanostat theory (adapted from Frost, 1987). The theory states that, under physiologically normal loading, bone formation and resorption are in equilibrium. Low strains experienced during disuse result in bone resorption, while overuse results in bone formation.

It is expected that the detection of the applied mechanical forces is performed either by each individual cell, and the sensation is restricted to the cellular level, or by certain sensor cells which generate biochemical signals, which can activate various cell signalling pathways and cascades which modulate bone formation and resorption and maintain bone homeostasis (Sikavitsas et al, 2001). In the latter case, the sensation is at the tissue level.

Osteocytes and osteoblasts are the two most likely cell types involved in mechanotransduction in bone (Bloomfield, 2001). A number of studies have proposed roles for both of these cell types. The techniques employed and the major findings of these studies will now be described.

1.2.3 Techniques employed in *in-vitro* Mechanical Stimulation of Bone Cells.

Because of the complexity of the *in-vivo* environment, study of cellular response to mechanical stimulation has relied heavily on the development and use of *in-vitro* systems designed to mimic the *in-vivo* situation. It is known that normal, physiological loading of bone cells results in bending forces resulting in mechanical stretch and pressure gradients within the lacuno-canalicular porosity leading to stress on the membranes of osteocytes, osteoblasts and bone lining cells (and fibroblasts).

There are four main classes of *in-vitro* model systems commonly used in the investigation of mechanical loading of bone. These systems generate fluid shear, hydrostatic compression, biaxial stretch, uniaxial stretch, or a combination of two or more of these forces **(Figure 1.4)**. The relevance of each of these approaches will be discussed.

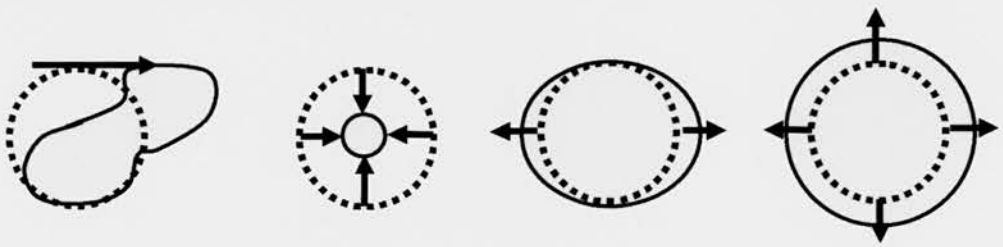


Figure 1.5 – Cell deformation in response to *in-vitro* loading. The four main types of mechanical loading systems generate fluid shear, hydrostatic compression, uniaxial stretch or biaxial stretch on cultured bone cells. Figure adapted from Duncan and Turner, 1995.

1.2.3.1 Fluid Shear Systems.

A wide range of cellular phenomena are recognized as being influenced by fluid shear, including both mechanoreception (e.g. by membrane bound receptors, ion channels, and integrins) and cell response (e.g. changes intracellular calcium and nitric oxide (NO)) (Davies et al, 1995). To date, there have been two devices used to study fluid shear alone. The first is the cone and plate system (Dewey et al, 1984; Mohtai et al, 1996, Hermann et al, 1997), which generates continuous laminar shear by stirring medium in a 100 mm-diameter dish. This system achieves spatially homogeneous fluid shear stresses on both of the respective surfaces. It has been used to investigate the effect of fluid shear on the release of TGF-β, insulin growth factors-1 and 2 (IGF-1 and 2) and IL-6 in osteosarcoma cell lines. It was found that TGF-β mRNA was increased following 3 hours of continous fluid flow whereas mRNA levels for IGF-1, IGF-2 and IL-6 were undetectable.

The second fluid shear system is the parallel plate flow chamber, which produces homogeneous shear between two openings at either end of the chamber (Levesque et al, 1985; Frangos et al, 1988; Hung et al, 1995; Tseng et al, 1995; Chun et al, 1997). The parallel plate flow chamber has many advantages, including the homogeneity of the stress stimulus, simplicity of the equipment, ability to microscopically monitor changes in the culture and ease of medium sampling and exchange. This system has been used to demonstrate increases in NO and prostaglandin E_2 (PGE_2) production in mouse MC3T3-E1 cells, UMR-106-01 cells, ROS17/2.8 cells and rat calvarial cells in response to fluid shear (Smalt et al, 1997, Tjandrawinata et al, 1997; McAllister and Frangos, 1999). In addition to being used to monitor the release of factors into the cell culture media following periods of fluid shear, the parallel flow chamber has also been used to study the effects of fluid shear on the conformation and appearance of cytoskeletal components. It has been demonstrated that MC3T3-E1 cell respond to a shear force of 12 dyn/cm^2 for 60 minutes with recruitment of α-actinin and β_1-integrin receptor subunit to the focal adhesion sites, which is likely to promote intracellular signalling.

Expression of the early response gene c-fos was also increased following fluid shear (Pavalko et al, 1998).

1.2.3.2 Hydrostatic Compression.

The hypothesis that cytoskeletal elements, in particular focal adhesions, are important in the process of mechanotransduction and signal transduction has led to the development of hydrostatic devices. The effect of hydrostatic compression cannot be used to study free-floating cells because of the high water content of the cytoplasm. However, in monolayer culture, cells attach to the culture substratum via integrin receptors located at focal adhesions and perturbation of the attached cells by hydrostatic forces may result in the generation of shear stresses along this interface although this is a focus of debate as there is no evidence of fluid shear as a result of hydrostatic compression of cells in culture. The hydrostatic system described commonly in the literature is capable of delivering both intermittent / pulsatile and continuous compressive forces to the cells (Klein-Nulend et al, 1986). This system has been used successfully to examine articular cartilage development during joint loading (Klein-Nulend et al, 1986). It has also been used to study osteoblastic function, expression of osteoblast markers and production of second messenger signalling molecules such as PGE_2 in osteoprogenitor-like and osteoblast-like cells (Roelofsen et al, 1995; Glantschnig et al, 1996, Klein-Nulend, 1997; Macdonald et al, 1999).

1.2.3.3 Uniaxial Stretch Systems.

Loading systems have been used widely, which utilise controlled uniaxial stretching of a deformable substrate upon which cells have been cultured. Such systems are generally considered acceptable as stretching in one direction is accompanied by compression of the membrane in the perpendicular plane, which resembles the *in-vivo* environment (Murray et al, 1990; Smalt et al, 1997).

1.2.3.4 Biaxial Stretch Systems.

Another model used to investigate the effects of mechanical forces on bone cells *in-vitro* involves systems, which produce biaxial cell stretch. The term biaxial stretch is used to describe the stretching of a deformable membrane (usually circular) and hence the adherent cells, in all directions. The first system of this type was reported in 1985 by Hasagawa et al (1985), and consisted of a plastic culture dish whose base was pressed upon a convex template by means of a static weight. Anderson and Norton, (1991), employed a similar system and various modifications of this system have since been designed. Vandenburgh et al, (1988), used pulsating prongs to create an upward tent in a deformable membrane from below, while Soma et al, (1997), used a similar mechanism to tent deformable membranes in a downward direction, from above. Banes et al, (1985), introduced a system which utilised specially designed flexible-bottomed culture dishes whose bases were made from a deformable material. These plates were used in conjunction with a vacuum system controlled by a programmable device and when the vacuum was applied, the base of the culture dish was stretched downwards, thus imparting strain to the culture layer. This system had the advantage that it was easily programmable and relatively easy to set up and operate. Because of these factors, this system has been extensively redesigned and was commercially manufactured in 1987, and known as the Flexercell® system. This system has been used extensively to investigate the effects of cyclic biaxial stretch on a variety of cell types including osteoblasts (Buckley et al, 1990; Stanford et al, 1995).

A modification of the Flexercell® system was introduced in 1989 (Winston et al, 1989), which used positive pressure as opposed to negative, vacuum pressure. The system used circular membranes, which were clamped peripherally by 'O'-rings, and caused to bulge upward by increased air pressure from below, at a controlled frequency.

The main disadvantage of any biaxial strain system presently available is heterogeneity of strain. Strain varies across the diameter of the dish falling to zero in the centre of the dish and therefore, cells at different locations of the dish are subject to different amounts

of strain (Gilbert et al, 1990). It is also difficult to assess the actual amount of strain that individual cells are subjected to. Another potential complication of the biaxial stretch system is the influence of fluid shear, as it is likely that some of the effects of cyclic stretch in such systems may be as a result of agitation of the culture medium and therefore production of fluid shear.

1.2.4 Osteocytes and Bone Mechanotransduction.

Unlike osteoblasts, osteocytes are literally entombed in bone matrix. They are derived from differentiated osteoblasts, and form contacts with the osteoblastic cells on the cell surface and with other osteocytes via an intricate network of slender cell processes and gap junctions (Burger et al, 1999). This three dimensional network presents a very attractive system for the possible detection of local mechanical stimuli (Sikavitsas et al, 2001). Osteocytes have proved very difficult to study, owing to the fact that they are post-mitotic (Smith et al, 1997), and their location deep within the bone matrix makes them extremely difficult to extract and purify. They also appear to be inactive in respect of protein synthesis.

It is known that *in-vivo*, osteocytes express mRNA for β-actin, osteocalcin, connexin-43, IGF-1, c-fos and c-jun, but not tumor necrosis factor-α (TNF-α) or tartrate-resistant acid phosphatase (TRAP) (Mason et al, 1996; Moalli et al, 2000). *In-vitro* it has been shown that they establish a network allowing communication with other osteocytes via their cell processes and gap junctions (Nijweide et al, 1986; Van der Plas et al, 1992; Van der Plas et al, 1994; Tanaka et al, 1995). Osteocytes also produce small amounts of fibronectin and type 1 collagen (although in lesser amounts than osteoblasts) but produce greater quantities of the bone matrix proteins osteocalcin, osteonectin and osteopontin than osteoblasts (Aarden et al, 1996).

Evidence for the involvement of osteocytes in bone cell mechanotransduction has been accumulating over the last decade. Early strain related changes in glucose-6-phosphate dehydrogenase were found in osteocytes following bone-loading *in-vivo* (Skerry et al, 1989). These results have been confirmed in *in-vitro* studies (Dallas et al, 1993; Van der Plas et al, 1994). Expression of c-fos mRNA was transiently induced in cortical bone osteocytes of rat tail vertebrae, *in-vivo*, one hour after a 5 minute period of loading (Inaoka et al, 1995), whereas in the same study, no effect was observed in the mRNA levels of IGF-1. In contrast, a separate study demonstrated that rat caudal vertebrae osteocytes increased IGF-1 expression after mechanical stimulation (Lean et al, 1995). IGF-1 is known to promote bone formation (Conover, 1996) and stimulate the differentiation of osteocytes from osteoblasts (Gohel et al, 1995). Hypoxia was observed in osteocytes from avian ulnae that were subjected to unloading for 24 hours. A brief period of loading restored oxygenation to these cells, which may suggest that mechanotransduction plays a role in oxygenation of osteocytes (Dodd et al, 1999). Osteopontin is a member of a family of phosphorylated sialoproteins found in mineralized bone matrix and is believed to have a general role in cell attachment (Miyauchi et al, 1991). The expression of osteopontin is known to be mechanically regulated in bone and it has been shown that mechanical loading increases osteopontin mRNA expression *in-vitro* in osteocytes (Toma et al, 1997; Klein-Nulend et al, 1997). Similar results have also been demonstrated in osteocytes *in-vivo* (Miles et al, 1998).

It is clear that mechanical loading at physiologically appropriate magnitudes results in the activation of several metabolic processes in osteocytes including energy metabolism, growth factor production, and alterations in gene expression and matrix synthesis. Such findings provide evidence for a role for osteocytes in bone mechanotransduction (Mikuni-Takagaki et al, 1999). At the present time, however, it is not clear how osteocytes sense mechanical loading and transduce it into a cellular response. A number of investigations have provided evidence to suggest that the flow of interstitial fluid is

the most likely mechanism of delivering information to osteocytes about the degree of mechanical loading (Kufahl et al, 1990; Weinbaum et al, 1994; Cowin et al, 1995; Klein-Nulend et al, 1995, Knothe-Tate et al, 1998). This will be discussed in the following section.

1.2.4.1 Effect of Interstitial Fluid Flow on Osteocytes *in-vitro*.

Strain-derived flow of interstitial fluid through the lacuno-canalicular pores is believed to mechanically activate osteocytes as well as ensuring transport of cell signalling molecules, nutrients and waste products. Flow of interstitial fluid occurs because the application of mechanical strain causes the volume of some pores to decrease slightly and the volumes of other pores to increase slightly, creating differences in bone fluid pressure, resulting in fluid flow. Although fluid flow is important in the lacuno-canalicular porosity, it is negligible in the Haversian canals and Volkmans canals, because these are 1000 times larger and their pressure is more uniform being almost equal to blood pressure (Cowin et al, 1998).

It has been shown that osteocytes are extremely responsive to fluid flow and hydrostatic compression (Klein-Nulend et al, 1996; Ajubi et al, 1996). Pulsating fluid flow with a mean stress of 0.5 Pa and 5 Hz pulses of 0.02 Pa had an immediate effect on osteocytes; release of PGE_2 and PGI_2 increased fivefold and their NO release increased twofold after the application of flow (Klein-Nulend et al, 1995). Prostaglandins are essential agents in the transduction of mechanical stimuli into bone formation. PGE_2, in particular, stimulates osteoblastic cell proliferation and bone formation. NO is a highly diffusible free radical implicated in neurotransmission and vasodilation and has recently been shown to inhibit osteoclastic activity and stimulate osteoblastic proliferation

Flow of fluid over the cell surface subjects the cell to two types of stimuli, fluid shear stresses (or fluid-induced drag forces) and streaming electrical potentials (Salzstein et al,

1987). Although many studies have provided evidence to suggest that streaming electrical potentials are primarily responsible for initiating cellular responses in bone (Weinbaum et al, 1994; Cowin et al, 1995), other studies have produced results which argue that fluid shear stresses and direct perturbance of the cell membrane is the stimulus that conveys the mechanical message to bone cells (Hung et al, 1996).

It appears that the lacuno-canalicular network, with its cellular part (osteocytes and gap junctions) and its porosity is very important for mechanotransduction in bone. It is known that mechanical stimulation of bone is required to keep osteocytes viable, by enhanced displacement of nutrients and waste but also to provide them with a basal level of mechanical stimulation by fluid shear stress. Under these conditions, no osteoblasts or osteoclasts are recruited. However, overstimulation of the osteocytes by abnormally high fluid shear stresses will result in recruitment of osteoblasts to the bone surface. The extra bone produced by the osteoblasts restores the dynamic equilibrium in bone and bone growth stops. Disuse, in contrast, reduces osteocyte shear stress stimulation as well as reducing transport of nutrients and waste products. The latter will reduce osteocyte viability and may even lead to osteocyte apoptosis (Burger et al, 1999).

1.2.5 Osteoblasts and Bone Mechanotransduction.

A number of studies have demonstrated that in addition to osteocytes, osteoblasts are also involved in the recognition and conversion of mechanical signals into a cellular response.

It has been shown that osteoblasts respond to mechanical stimulation *in-vitro* (Somjen et al, 1980, Buckley et al, 1988; Murray et al, 1990; Harter et al, 1995) although opinion is divided upon whether osteoblasts are more sensitive to mechanical strain or fluid flow. Results obtained to date suggest that the response of the osteoblast to mechanical stimuli is dependent on both the magnitude and the type of stimulus applied. Osteoblasts

exposed to high levels of mechanical strain (≥10,000 μstrain) respond with increases in cell proliferation, DNA synthesis, and PGE_2 production and secretion. In addition, production of osteoblastic phenotypic markers, such as alkaline phosphatase are decreased. At more physiological levels of strain (2000 – 4000 μstrain), the response of the osteoblast indicates a more differentiated state, with an increase in production of type 1 collagen, osteopontin, osteocalcin (Harter et al, 1995), and alkaline phosphatase (Buckley et al, 1990), while osteoblast proliferation and matrix mineralization are also increased (Murray et al, 1990; Chambers et al, 1993; Turner et al, 1996).

Experiments have also been performed which provide evidence that osteoblasts are responsive to fluid shear stresses and that fluid shear stresses may act as mechanical stimuli in osteoblasts (Owan et al, 1997; Kapur et al, 2003). Continuous fluid shear rapidly increases NO release (Johnson et al, 1996; Bakker et al, 2001), cyclic adenosine monophosphate (cAMP) production (Reich et al, 1990), inositol trisphosphate (IP_3) and PGE_2 release (Reich and Frangos, 1991; Reich et al, 1997; Bakker et al, 2001) and intracellular calcium levels in osteoblast cultures. It is known that in marrow stromal cells, prostaglandins induce the production of cAMP so therefore, it is possible that the increased level of cAMP in osteoblasts may be induced by shear stress indirectly via an autocrine mechanism involving increased prostaglandin secretion. It has been demonstrated that PGE_2 release is stimulated by shear stress in a dose dependent manner, together with increased levels of IP_3 (Reich et al, 1991). NO release is rapidly increased in rat calvarial osteoblasts in response to fluid shear stresses of 6 dyn/cm^2 and is sustained for up to 12 hours post stimulation (Jonhson et al, 1996). The addition of an NO synthase inhibitor blocked NO production in response to fluid flow. Similar results have been obtained using human primary osteoblast like cells (Klein-Nulend et al, 1998) and it has been proposed that fluid flow stimulated NO release in osteoblastic cells is biphasc and follows two distinct signalling pathways, a G-protein calcium-dependent pathway and a G-protein calcium-independent pathway (Klein-Nulend et al, 1998).

Fluid flow has also been shown to up-regulate the mRNA level of the early response gene egr-1, by a tyrosine kinase-mediated mechanism (Fitzgerald et al, 1999). Fluid flow enhances the tyrosine phosphorylation of many proteins including the adaptor protein Shc (Ogata et al, 2000) and the extracellular-signal regulated kinases-1 and 2 (ERK-1 and 2) (Jessop et al, 2002; Kapur et al, 2003). ERK-1 and ERK-2 are members of the mitogen activated protein kinase (MAPK) family and both ERK-1 and ERK-2 are essential mediators of growth factor induced cell proliferation and differentiation in various cell types including osteoblasts. The activation of ERK-1/2 is involved in various cellular responses induced by mechanical stimuli, such as collagen synthesis (Gebken et al, 1999), cyclo-oxygenase (COX)-2 expression (Wadhwa et al, 2002), and osteopontin production (You et al, 2001).

In an attempt to clarify the nature of the actual sensing mechanism of mechanotransduction in osteoblasts, several studies have compared the mechanosensing in osteoblasts exposed to shear stress to mechanosensing in osteoblasts exposed to mechanical strain. As it is known that fluid shear stresses induce PGE_2 and NO production, these two substances have been monitored when rat calvarial osteoblasts, rat long bone cells, MC3T3-E1 cells, ROS 17/2.8 cells and UMR-106-01 cells have been subjected to shear stress and linear mechanical strain whilst attached to tissue-culture treated polystyrene film. The results have shown that production of PGE_2 and NO was rapidly increased in the osteoblast and osteoblast-like cells when exposed to wall shear stresses of 148 dyn/cm^2. When the osteoblasts were subjected to unidirectional linear strains in the range of 500-5000 μstrain no apparent increase in NO and PGE_2 production has been observed (Smalt et al, 1997), suggesting that osteoblasts are more responsive to fluid flow than mechanical strain or that different mechanotransduction pathways are activated by the different types of mechanical stimuli.

1.2.6 The Cellular Cytoskeleton and Mechanotransduction.

Another mechanism that bone cells may use to sense mechanical forces is cytoskeletal – integrin interactions. The integrins are a heterodimeric family of transmembrane adhesion molecules composed of an α - and a β-subunit. Sixteen α and eight β integrin subunits have been cloned. These subunits combine to from the 22 described integrin heterodimers. These subunits are the products of two different genes and the expression of both subunits is required for cell surface expression (O'Toole et al, 1989). Integrins physically bridge the extracellular matrix proteins outside the cell with the cytoskeletal proteins within the cell (Hynes, 1992). In addition to their structural function of anchoring cells to the extracellular matrix, the integrins are also known to be intimately involved in the generation and propagation of intracellular signalling (Parsons, 1996; Longhurst et al, 1998; Vuori, 1998). Such conclusions are supported by studies which have demonstrated that ligation of integrins with either inhibitory antibodies or by binding to their ligands leads to changes in the intracellular pH, intracellular calcium concentration, and the tyrosine phosphorylation of several proteins (Juliano et al, 1993; Clark et al, 1995). Clustering of integrins due to ligand binding causes the attachment of stress fibres to integrin receptors at specialised sites called focal adhesions. Linker proteins, including α-actinin, vinculin and talin, anchor actin filaments to the integrins (Dedhar et al, 1996; Hemler et al, 1998). There is substantial evidence to suggest that the development of internal tension by actin and myosin plays a central role in signal transduction from the extracellular matrix to the nucleus to regulate gene expression (Ingber et al, 1999).

Experiments *in-vitro* have suggested a role for mechanosensitive ion channels and integrins as mechanotransducers. Salter et al, (1997), have shown that the hyperpolarisation response associated with cyclic mechanical stimulation of human bone cells is associated with the activation of small conductance calcium activated potassium channels. The transduction pathways involved in the hyperpolarisation response to

cyclical mechanical stimulation also involve the $\alpha_5\beta_1$ integrin (Salter et al, 1997). The downstream intracellular signalling pathways are inhibited by anti-integrin α_5 and β_1 antibodies. These results suggest that the $\alpha_5\beta_1$ integrin in an important human bone cell mechanotransducer and a potential regulator of human bone cell function.

Application of fluid shear stress causes an increase in the early response gene product c-fos and cyclooxygenase-2 (COX-2), the reorganization of actin filaments into contractile stress fibres and involves the recruitment of β_1-integrins and α-actinin to focal adhesions (Pavalko et al, 1998). Treatment of cells with cytochalasin D, which disrupts filamentous actin, blocks the upregulation of COX-2. Similarly, microinjection of a proteolytic fragment of α-actinin, which competes with the endogenous protein for binding with the integrin receptor, blocked both COX-2 and c-fos upregulation. Finally, cells transfected with a dominant negative mutant of the GTPase, Rho, which blocks stress fibre and focal adhesion formation, also inhibited c-fos and COX-2 upregulation, suggesting that stress fibres and their anchorage to the membrane at focal adhesion points in response to fluid flow may facilitate the transduction of applied forces into intracellular signals (Pavalko et al, 1998).

Osteopontin possesses an RGD (Arg-Gly-Asp) sequence that binds to certain integrins leading to alterations in intracellular calcium levels (Miyauchi et al, 1991). Several studies have demonstrated that osteopontin levels are increased in cultured osteoblasts in response to mechanical stimulation (Harter et al, 1995; Kubota et al, 1993; Owan et al, 1997). One such study investigated the expression of osteopontin mRNA in embryonic chick osteoblasts following exposure to a dynamic spatially uniform biaxial strain at 0.25 Hz for 2 hours (Toma et al, 1997). It was found that osteopontin mRNA expression increased fourfold, 9 hours after the embryonic chick osteoblasts had been exposed to the stimulation and that cytochalasin-D blocked the upregulation of the osteopontin gene, while colchicine (which inhibits microtubule assembly and disassembly) did not

block osteopontin expression. These results offer additional evidence in the involvement of the actin cytoskeleton in osteoblast mechanotransduction.

A separate study demonstrated that osteopontin mRNA expression can also be induced in the absence of shear following plating of chicken calvaria osteoblasts on fibronectin coated surfaces (Carvalho et al, 1998). This induction disappeared when the cells were seeded on uncoated plastic. The observed osteopontin induction was not affected by cytochalasin-D, which is known to block the mechanically stimulated up-regulation of the osteopontin gene, while the addition of cyclohexamide, which inhibits protein synthesis, blocked the induction of the osteopontin mRNA expression following adhesion to fibronectin but left unchanged the up-regulation of the osteopontin gene by mechanical forces. These results imply that fibronectin itself may induce osteopontin expression via integrin receptors but by a different mechanism than that following mechanical stimulation (Carvalho et al, 1998).

1.3 The Human Bone Cell (HBC) Mechanotransduction Pathway Studied by the Use of Electrophysiological Techniques.

1.3.1 Responses of the Plasma Membrane to Cyclic Mechanical Stimulation *in-vitro*: Evidence for the Presence of Stretch Activated Ion Channels.

Salter et al (1997) investigated the effects of cyclic mechanical stimulation on the membrane potentials of cultured human bone cells (HBC). The cells were mechanically stimulated by culturing HBC on plastic culture dishes, which were placed in a sealed polypropylene chamber with gas inlet and outlet ports. Cyclical pressurisation (using nitrogen or helium gas) was applied to the pressure chamber, and caused deformation of the base of the culture dish to which the HBC were adherent and therefore deformation (strain) was applied to the HBC. Hyperpolarisation of the HBC plasma membrane was induced by cyclic mechanical stimulation at 0.33Hz, 120 mmHg for 20 minutes, whereas depolarisation of the plasma membrane was induced by cyclic mechanical stimulation at 0.104 Hz, 120 mmHg for 20 minutes. It has been demonstrated in similar experiments using human articular chondrocytes, that the amplitude of the hyperpolarisation response is proportional to the strain (measured in μstrain) to which the cells were subjected (Wright et al, 1992; Wright et al, 1996). It has also been demonstrated that the hyperpolarisation response did not occur when chondrocytes were subjected to the same regime of cyclic mechanical stimulation when plated on rigid glass culture dishes or when the plastic culture dishes were placed in the pressurisation chamber in such a way as to avoid deformation of the base of the dish (Wright et al, 1996).

To investigate possible ion channels involved in the electrophysiological response of HBC to cyclical mechanical stimulation, the effects of a panel of pharmacological

inhibitors of ion channel activity were investigated (Salter et al, 1997). Inhibitors of ion channel activity included, tetrodotoxin, which blocks Na^{2+} channels (Chesnoy-Marchais et al, 1988), apamin and quinidine which blocks Ca^{2+} activated K^{+} channels (Blatz et al, 1986; Lew et al, 1976) and gadolinium, which blocks stretch activated ion channels (Yang et al, 1989).

None of the ion channel inhibitors studied had an effect on the resting membrane potential of the HBC. Gadolinium abolished the hyperpolarisation response to 0.33 Hz cyclical mechanical stimulation, but had no effect on the depolarisation response to 0.104 Hz cyclical mechanical stimulation. Apamin and quinidine also inhibited the hyperpolarisation response to 0.33 Hz cyclical mechanical stimulation. The effect of apamin and quinidine on 0.104 Hz cylical mechanical stimulation was not studied. In contrast, tetrodotoxin inhibited the membrane depolarisation following 0.104 Hz cyclical mechanical stimulation, suggesting a role for Na^{+} channels in this response, but had no effect on membrane hyperpolarisation at 0.33 Hz stimulation (Salter et al, 1997).

Osteoblast like cells are known to possess a number of mechanosensitive ion channels (el Haj et al, 1999; Walker et al, 2000), nonselective cation and K^{+}- selective channels, which may cause alterations in membrane potential following mechanical stimulation (Dixon et al, 1984; Ypey et al, 1988; Davidson et al, 1990). Studies from our group have shown that human chondrocytes and fibroblasts also show changes in membrane potential in response to cyclical mechanical stimulation. Fibroblasts however, unlike HBC and chondrocytes, show membrane depolarisation following stimulation at 0.33 Hz, suggesting cell type-specific responses to mechanical stimulation. The hyperpolarisation response is a result of activation of small conductance Ca^{2+} activated K^{+} (SK) channels, resulting in an efflux of K^{+} ions from the cell and cell hyperpolarisation. Bone cells also possess a gadolinium sensitive stretch activated ion channel that may be modulated by parathyroid hormone and chronic, intermittent load-

bearing. The results obtained by Salter et al, (1997) are consistent with a role for gadolinium sensitive ion channels in HBC responses to cyclical mechanical stimulation.

1.3.2 Evidence for a Role for $\alpha_5\beta_1$ Integrin Receptor as a HBC Mechanoreceptor.

The integrin family of transmembrane proteins, are known to be involved in the transduction of mechanical forces to cells via the ECM. The integrins are a family of heterodimeric (α and β chain) transmembrane glycoproteins, which form specific receptors for a variety of ECM proteins (Hynes et al, 1992). Integrin receptors are expressed by cells in all tissues including bone. All bone types express α_5 and β_1 integrins while α_4 and α_V integrins are heterogeneously expressed by cells of the osteoblast lineage and α_2 and the $\alpha_V\beta_3$ complex are expressed exclusively by osteoclasts (Clover et al, 1992; Hughes et al, 1993; Grzesik et al, 1994). As well as acting as adhesion molecules, integrins have been shown to act as signalling molecules (Clark et al, 1995; Schoenwaelder et al, 1999), and are known to act as mechanotransducers in a variety of cell types including bone cells. A series of experiments were undertaken to investigate a possible role for integrin molecules and integrin-associated signalling pathways in mediating the hyperpolarisation response in cultured HBC following cyclic mechanical stimulation (Salter et al, 1997).

Anti-integrin antibodies, including those against α_1, α_2, α_3, α_5, α_V, β_1, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ were used to test for a role for the specific integrin subunits in the mechanotransduction process. None of the antibodies tested had any effect on resting membrane potential but showed variable effects on HBC responses to cyclic mechanical stimulation. The anti-β_1 antibody inhibited both the depolarisation response to 0.104 Hz stimulation and the hyperpolarisation response to 0.33 Hz stimulation. The $\alpha_V\beta_5$ antibody reduced the depolarisation response to 0.104 Hz but had no effect on the hyperpolarisation response

to 0.33 Hz, whereas the $\alpha_V\beta_3$ antibody had no effect on the membrane potential response at either frequency of stimulation. Of the antibodies used against the α-integrin subunits expressed by bone cells, only two showed significant effects. The anti-α_V antibody inhibited the response to 0.104 Hz stimulation but had no effect on the 0.33 Hz stimulation response. In contrast, the anti-α_5 antibody inhibited the 0.33 Hz hyperpolarisation response but had little effect on the 0.104 Hz depolarisation response. Antibodies to α_1, α_2, and α_3 integrins did not inhibit the membrane potential responses of bone cells to either frequency of cyclical mechanical stimulation. The results suggest that changes in membrane potential as a result of stimulation by different frequencies involve different integrins. The results suggest that α_5 and β_1 integrins are involved in the transduction of strain at 0.33 Hz, resulting in the hyperpolarisation response via a Ca^{2+} activated K^+ channel, whereas, the depolarisation response mediated by a tetrodotoxin sensitive Na^+ channel seen at 0.104 Hz involves α_V, β_1 and $\alpha_V\beta_5$ integrins (Salter et al, 1997).

A number of integrins recognise and interact with ECM proteins via RGD (arg-gly-asp) sequences. RGD dependent integrin binding has been shown for a number of ECM proteins, such as fibronectin, osteopontin, thrombospondin, type 1 collagen and vitronectin (Hynes et al, 1992; Grzesik et al, 1994; Sommermann et al, 1988; Davies et al, 1989) and therefore experiments have been carried out to see whether RGD-containing oligopeptides known to interfere with integrin-mediated cell binding influenced HBC responses to 0.33 Hz and 0.104 Hz cyclical mechanical stimulation. The exposure of HBC to GRGDSP peptide, which competitively inhibits integrin binding to RGD-containing substrates, resulted in the inhibition of both the hyperpolarisation and depolarisation responses, whereas the control GRADSP peptides had no effect on either of these responses. These results suggest that RGD-mediated interactions are involved in the transduction of strain to HBC at both 0.33 Hz and 0.104 Hz (Salter et al, 2000).

Using inhibitors to the intracellular second messenger signalling molecules activated by integrin signalling, the hyperpolarisation response induced by 0.33 Hz cyclical mechanical stimulation was investigated (Salter et al, 1997; Salter et al, 2000). It was found that the hyperpolarisation response was inhibited by cytochalasin D (which disrupts the actin cytoskeleton) (Casella et al, 1981; Wang et al, 1994), genistein (which inhibits tyrosine kinase activity) (Akiyama et al, 1987; O'Dell et al, 1991), neomycin and W7 (inhibitors of phospholipase C / IP_3 pathway) (Hidaki et al, 1981; Liscovitch et al, 1991), indomethacin (inhibitor of arachadonic acid metabolism) and calphostin C (inhibitor of protein kinase C activity). However, caffeic acid (inhibitor of lipo-oxygenase) (Cho et al, 1991; Sudina et al, 1993) had no effect on the membrane potential response to 0.33 Hz cyclical mechanical stimulation, suggesting that the lipo-oxygenase pathway is not involved in the hyperpolarisation response of HBC to 0.33 Hz cyclic mechanical stimulation.

Therefore, the above studies suggest that the mechanotransduction pathway activated by 0.33 Hz cyclical mechanical stimulation involves the $\alpha_5\beta_1$ integrin receptor, the actin cytoskeleton, tyrosine kinase activity, the phospholiase C (PLC) / IP_3 pathway and protein kinase C (PKC) activity.

1.3.3 Interleukin-1β in HBC Mechanotransduction.

A number of studies have reported the production of a number of soluble factors such as prostaglandins (Somjen et al, 1980; Ayajiki et al, 1996; Sterck et al, 1998) and NO (Pitsillides et al, 1995; Klein-Nulend et al, 1998), by bone cells and endothelial cells in response to mechanical stimulation. These factors could potentially act in an intermediate, autocrine or paracrine step to regulate cell function in the response of cells to mechanical stimulation.

Studies have been undertaken to investigate whether soluble mediators are involved in the hyperpolarisation response of HBC to 0.33 Hz cyclical mechanical stimulation. Conditioned medium from mechanically stimulated HBC, when transferred to unstimulated cells, caused membrane hyperpolarisation of these cells of a similar magnitude to that of the directly mechanically stimulated cells, suggesting the presence of a soluble transferable factor secreted by the mechanically stimulated HBC. These conditioned medium experiments were repeated in the presence of anti-integrin antibodies. Medium from cells which had been incubated with anti-β_1 antibody inhibited the direct hyperpolarisation response of HBC to 0.33 Hz cyclical mechanical stimulation. Similarly, medium from HBC which had been mechanically stimulated in the presence of anti-β_1 antibody, when transferred to unstimulated cells did not alter the membrane potential of these cells, suggesting that the anti-β_1 antibody inhibited the production of the transferable factor. In contrast, an anti-$\alpha_V\beta_5$ integrin antibody did not inhibit 0.33 Hz induced hyperpolarisation nor did it inhibit the production of a transferable factor which could induce membrane hyperpolarisation of unstimulated HBC. These results suggest that the secretion of the transferable factor is β_1, and not $\alpha_V\beta_5$ integrin mediated (Salter et al, 2000).

As HBC are known to secrete and respond to a number of chemical mediators, including prostaglandins and cytokines, a series of experiments were undertaken to investigate whether such agents might be involved in the hyperpolarisation response of HBC to cyclic mechanical stimulation. HBC were incubated with a panel of cytokines, including IL-1α, IL-1β, IL-4, IL-6 and TGF-β1 and the eicosanoids PGE_2 and $PGF_{2\alpha}$. IL-1α, IL-1β and PGE_2 each induced a hyperpolarisation response that was similar in extent to that seen following 0.33 Hz cyclical mechanical stimulation, whereas the other cytokines studied and $PGF_{2\alpha}$ induced membrane depolarisation. Dose response studies showed that IL-1β could induce significant membrane hyperpolarisation at levels as low

as 1 pg/ml. Neutralizing antibodies to IL-1β abolished the hyperpolarisation response to cyclical mechanical stimulation, whereas antibodies to IL-1α, IL-6 and TGF-β1 had no effect. Similarly, anti IL-1β antibodies, added to the conditioned medium after mechanical stimulation but before transfer of that medium to unstimulated cells, prevented the subsequent hyperpolarisation. These results demonstrate the IL-1β is necessary for the membrane hyperpolarisation response to mechanical stimulation (Salter et al, 2000).

The hyperpolarisation response of HBC to recombinant IL-1β and PGE_2 was unaffected by gadolinium (an inhibitor of stretch activated ion channels), genestein (a tyrosine kinase inhibitor) and cytochalasin D (which disrupts the actin cytoskeleton), which have previously been shown to inhibit the HBC hyperpolarisation response to 0.33 Hz cyclical mechanical stimulation (Salter et al, 1997). These results indicated that mechanically-induced secretion of IL-1β in the mechanotransduction pathway is downstream of the activation of stretch-activated ion channels, tyrosine kinases and the actin cytoskeleton. However, indomethacin (inhibitor of arachadonic acid metabolism), neomycin, flunarizine, W7 (inhibitors of the PLC / IP_3 pathway), calphostin C (inhibitor of PKC) and apamin (SK channel blocker) each inhibited the HBC hyperpolarisation response to recombinant IL-1β. These results indicate that IL-1β secretion is upstream of activation of PLC and IP_3 – mediated Ca^{2+} release and the resultant opening of SK channels (Salter et al, 2000).

Similarly, apamin and calphostin C inhibit PGE_2-induced cell membrane hyperpolarisation but neomycin, flunarizine and W7 did not. This would suggest that PGE_2 production lies downstream of the PLC / IP_3 signalling, and that PKC may be involved in PGE_2 production by IL-1β and / or the activation of the SK Ca^{2+} activated K^+ channels.

1.3.4 Summary.

When HBC are mechanically stimulated at 0.33 Hz, they undergo hyperpolarisation of the plasma membrane via the activation of Ca^{2+} activated K^+ channels. The mechanotransduction pathway involved includes the $\alpha_5\beta_1$ integrin and stretch activated ion channels. Downstream intracellular signalling molecules include the actin cytoskeleton, tyrosine kinases and the production and secretion of IL-1β. IL-1β acts in an autocrine / paracrine way to activate downstream signalling, components of which include the PLC and IP_3 pathways. The proposed mechanotransduction pathway is summarised in **Figure 1.6**.

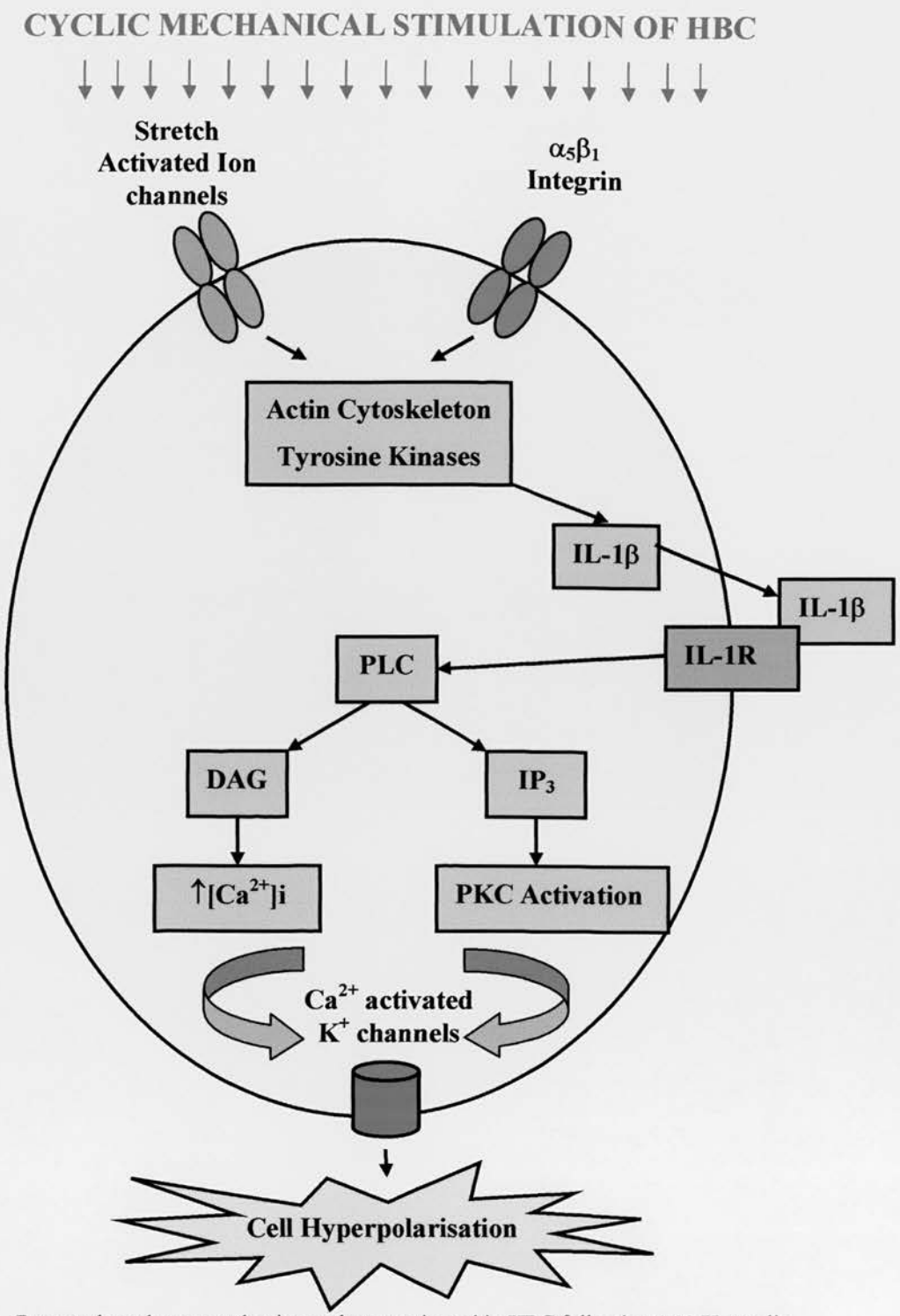


Figure 1.6 - Proposed mechanotransduction pathway activated in HBC following 0.33 Hz cyclic mechancial stimulation. IL-1β = interleukin-1β, IL-1R = interleukin-1 receptor, PLC = phopholipase C, DAG = diacylglycerol, IP_3 = inositol trisphosphate, PKC = protein kinase C.

1.4 Methotrexate (MTX).

Since 1948, when Farber *et al*, showed that the antifolate aminopterin could induce remissions in children with acute leukaemia, folate dependent enzymes have become a major target of chemotherapy, because they are known to play an essential role in the synthesis of DNA precursors (Heunnekens et al, 1994; Chu et al, 1996). Methotrexate (MTX), the most widely used anti-folate in cancer chemotherapy, has an essential role in the treatment of such diverse diseases as acute lymphocytic leukaemia, non-Hodgkin's lymphoma, osteosarcoma, head and neck cancer and breast cancer (Chabner, 1982). It has also become an important therapeutic treatment for various autoimmune diseases such as rheumatoid arthritis and psoriasis (Weinstein, 1977) and for the prevention of graft versus host disease after transplantation (Blume et al, 1980; Gorlick et al, 1996).

1.4.1 Cellular Effects of MTX.

1.4.1.1 MTX Disrupts DNA Synthesis Via Dihydrofolate Reductase (DHFR) Inhibition.

The folate vitamins are a class of essential cofactors that carry single carbon groups. Reduced folate (tetrahydrofolate) is the proximal single carbon donor in several reactions involved in the synthetic pathways for purine and pyrimidine precursors of DNA and RNA required for cell proliferation. Also, tetrahydrofolate plays a part in a second important biochemical step: the methionine homocysteine cycle, which is necessary to provide a methyl group for several downstream reactions such as methylation of DNA, RNA proteins and others (Seeger et al, 1949; Huennekens et al, 1994; Chu et al, 1996). Thus, in the design of inhibitors of DNA synthesis, folic acid and its derivatives are a logical target.

The physiological folate cofactors all share the common structural features, as shown in **Figure 1.7**. The structure consists of three main elements: a multi-ring pteridine group linked to para-aminobenzoic acid, which in turn connects with a terminal glutamic acid residue. Although folates found in the blood have a single terminal glutamate, most

intracellular folates are converted to polyglutamates, which contain multiple glutamate groups linked by gamma peptide bonds (McGuire et al, 1980; McGuire et al, 1981). The polyglutamate forms of folic acid have unique properties. They are preferentially retained within cells and are usually more efficient cofactors than the monoglutamated compounds. A second, important feature of folate biochemistry is that the cofactors must be reduced to their tetrahydo form, with hydrogen atoms in positions 5, 6, 7 and 8 of the pteridine ring (tetrahydrofolates [FH_4]), in order to be active in enzymatic reactions **(Figure 1.7a)**. The enzyme dihydrofolate reductase (DHFR), which is responsible for the conversion of oxidized folates to their active reduced form, is potently inhibited by MTX.

MTX is a folate analogue with an amino group (NH_2), methyl group (CH_3), and a fully oxidised pteridine ring, rendering the molecule inactive as a cofactor **(Figure 1.7b)** (Seegar et al, 1949). Once administered, MTX is delivered to cells via the same active transport system used by the parenteral folates. After entering the cell, MTX quickly binds to and inactivates DHFR. As DHFR has an essential role in the maintenance of intracellular FH_4 levels by reducing dihydrofolate (FH_2), the critical result produced by inhibition of DHFR is depletion of intracellular pools of reduced folate. The reaction most sensitive to folate depletion is thymidylate synthesis, which requires N^{5-10}methylene-FH_4. This reaction ceases at concentrations of $1x10^{-8}$ M of MTX (Chabner et al, 1973). N^{10}-formyl-FH_4, the folate involved in purine synthesis (Smith et al, 1981) is also depleted, leading to the cessation of purine synthesis at slightly higher concentrations (approximately $1 x10^{-7}$ M) of MTX (Zaharko et al, 1977). The lack of either thymidylate or purines block synthesis of DNA **(Figure 1.8)**.

Like the pareteral folates, MTX is metabolised extensively intracellularly to yield polyglutamate derivatives (MTX-glu) (Whitehead et al, 1975; Rosenblatt et al, 1978; Witte et al, 1980; Gewirtz et al, 1980). Upon administration, 3-12% of MTX is hydroxylated in the liver and circulates as 7-hydroxy-MTX (7-OH -MTX) (Baugh et al, 1973; Jacobs et al, 1977). MTX-glu synthesis increases in respect to drug concentration

and duration of exposure. MTX-glu generally represent long-lived MTX derivatives (Rosenblatt et al, 1978; Jolivet et al, 1982; Jolivet et al, 1983), and may be detected for as long as two weeks after a single administration of MTX. Because there is a latent period of several weeks before the effects of MTX are observed in patients receiving treatment, it may be the intracellular MTX-glu derivatives which are the true therapeutic agents (Cutolo et al, 2001; Ramanan et al, 2003).

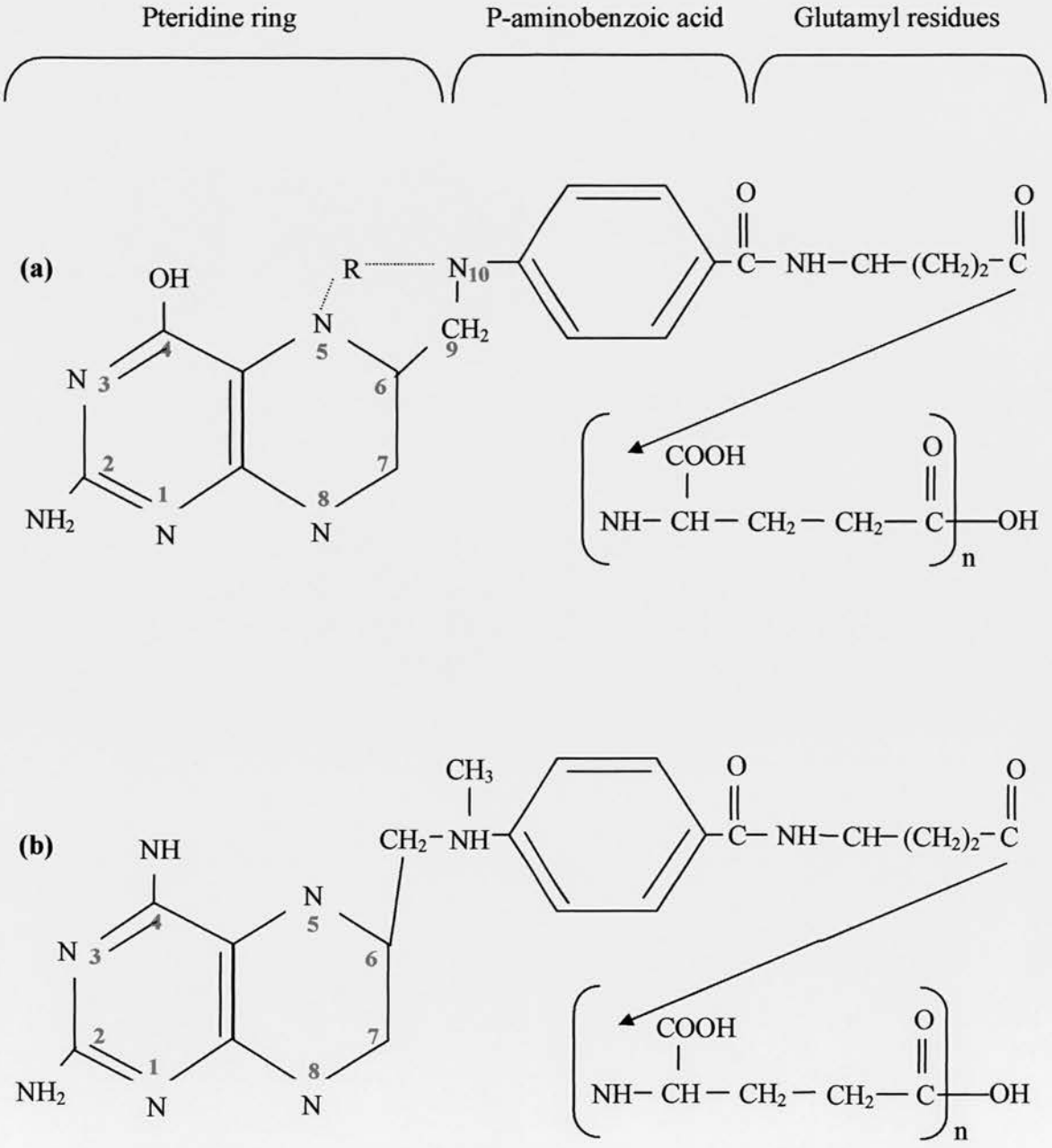


Figure 1.7 - Structure of tetrahydrofolate **(a)** and methotrexate **(b)** polyglutamates. In **(a)** one–carbon groups (R) are transported on nitrogen 5 or 10 or both. Figure adapted from Jolivet et al, 1983.

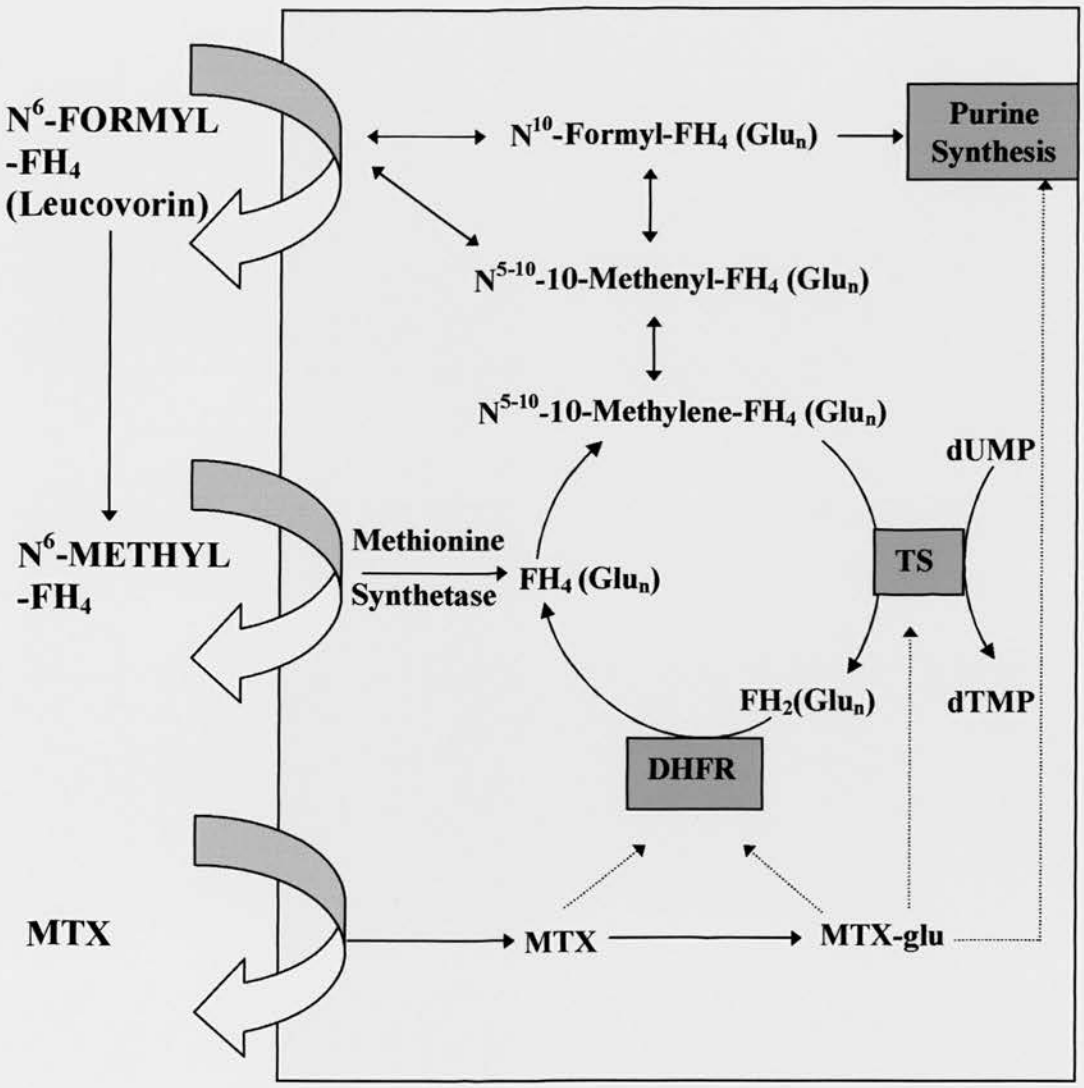


Figure 1.8 - The mechanism of action of methotrexate via dihydrofolate reductase inhibition.

MTX = methotrexate, DHFR = dihydrofolate reductase, TS = thymidylate synthetase, FH_4 = tetrahydrofolate, FH_2 = dihydrofolate, Glu = glutamyl, dTMP = dioxythymidylate and dUMP = dioxyuridylate.

Broken lines indicate enzyme inhibition. Figure adapted from Jolivet et al, 1983.

1.4.1.2 MTX Effects Via Adenosine Induced Immunosuppression.

In addition to inhibiting DHFR, MTX may also interfere with de novo purine biosynthesis by inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, an enzyme involved in the purine biosynthetic pathway (Baggot et al, 1986). This has several consequences, one of which is an overproduction of adenosine (Cronstein et al, 1991; Cronstein et al, 1992; Cronstein et al, 1993; Kremer et al, 1995; Montesinos et al 2000; Cutolo et al, 2001). It is thought that a number of anti-inflammatory effects exerted by MTX may be related to an increase in the extracellular concentration of adenosine and its interaction with specific cell membrane receptors.

It is known that MTX inhibits the enzyme AICAR transformylase, which results in a relative increase in AICAR levels **(Figure 1.9)**. AICAR inhibits important steps of degradation of adenosine monophosphate (AMP) and adenosine by AMP deaminase (AMPDA) and adenosine deaminase (ADA) respectively. Inhibition of degradation of AMPDA and ADA leads to increased intracellular and extracellular AMP and adenosine. Extracellular adenosine can bind to adenosine receptors, which are seven transmembrane spanning in nature. To date, four sub-types of adenosine receptor have been characterised, namely A_1, A_{2A}, A_{2B} and A_3 (Collis et al, 1993; Palmer et al, 1995; Klotz, 2000). The rank order of affinity of adenosine binding to these receptor subtypes is $A_1 > A_{2A} > A_{2B}$ (Mazzoni et al, 1993). The A_1 and A_3 receptors are coupled to inhibitory G-proteins ($G\alpha_{i/o}$), whereas the A_{2A} and A_{2B} receptors are coupled to stimulatory G-proteins ($G\alpha_S$). Therefore, binding to the A_1 or A_3 receptor subtypes decreases intracellular cAMP, whereas binding of adenosine to either of the two A_2 receptors increases intracellular cAMP. If the pathways through the two different receptor subtypes (A_1 or $A_{2A/2B}$) were functionally intact one would expect a prominance of the A_1 pathway owing to the higher affinity of adenosine to the A_1 receptor subtype, resulting in a decrease of cAMP. Evidence to date, however, suggests that low dose MTX exerts its anti-inflammatory effects by inducing extracellular adenosine which acts predominantly via A_{2A} receptors (Cronstein et al, 1993; Cronstein et al, 1997; Morabito

et al, 1998; Montesinos et al, 2000), and it would appear that A_1 receptor signalling is in someway 'switched off' (Straub et al, 1999) **(Figure 1.9)**.

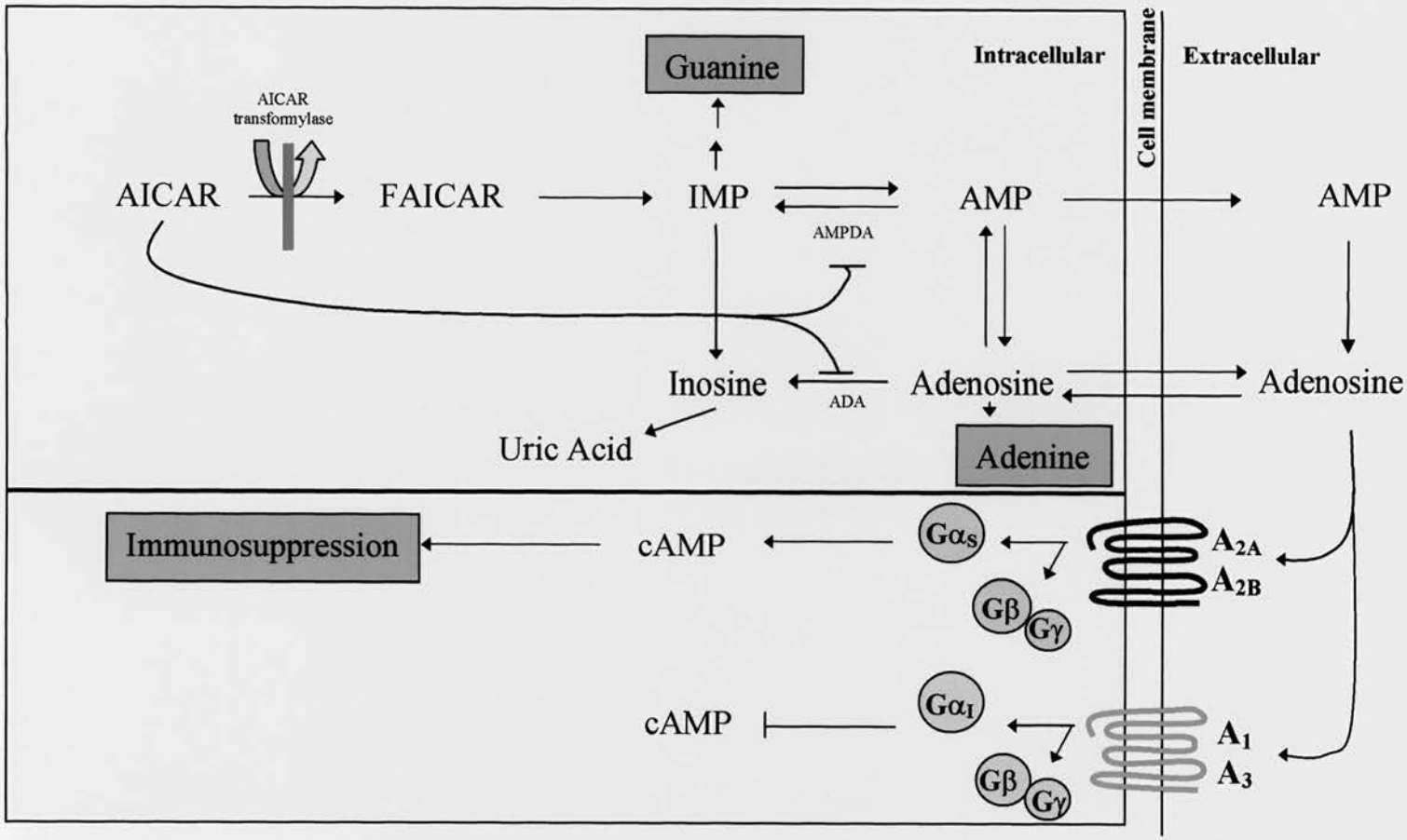


Figure 1.9 - Adenosine increase by MTX and subsequent immunosuppression through G-coupled adenosine receptors. MTX inhibits the conversion of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) to formyl-AICAR (FAICAR) resulting in an accumulation of AICAR. Accumulated AICAR inhibits adenosine monophosphate deaminase (AMPDA) deaminase and adenosine deaminase (ADA), which increases AMP and adenosine release into the extracellular space, where AMP is converted to adenosine. Adenosine binds membrane bound adenosine receptors (A_1, A_{2A}, A_{2B} and A_3). It is generally believed that there is a preponderance of the A_2 receptor pathway activation leading to increased cAMP and immunosuppression. Figure adapted from Cutolo et al, 2001.

1.5 Methotrexate and Bone.

1.5.1 Effects of Intensive Chemotherapy Regimes on Bone Turnover and Growth.

Although extremely efficacious in the treatment of both malignant disease and autoimmune disease, MTX has a number of side effects in a variety of organ systems. High dose regimes may induce myelotoxicity, nephrotoxicity (Abelson et al, 1983), mucositis, ocular irritation (Doroshow et al, 1981), reversible elevation of hepatic enzymes and nausea (Frei et al, 1980). MTX also has detrimental effects on bone formation, and there have been reports of poor growth in children receiving MTX treatment, especially during periods of intensive chemotherapy (Clayton et al, 1988; Möell et al, 1988; Thun-Hohenstein et al, 1992; Hokken-Koelega et al, 1993; Wallace et al, 1996). Growth rate may return to normal during less-intensive periods of treatment and may show evidence of further catch-up upon completion of chemotherapy (Caruso-Nicoletti et al, 1993; Holm et al, 1994; Ahmed et al, 1997). Also of great concern are reports of reduced bone mineral content and bone mineral density at various sites which are associated with increased fracture risk, reduced peak bone mass, and a theoretical risk of osteoporosis in later life, a condition known as MTX osteopathy (Ragab et al, 1969; O'Regan et al, 1973; Stanisavljevic et al, 1976; Stevens et al, 2001; Wijnands et al, 2001). In addition to patients receiving intensive chemotherapy regimes, MTX may also have detrimental effects on bone in patients receiving long term, low dose MTX treatment for rheumatoid arthritis (Preston et al, 1993).

A number of bone markers exist that can be used as indicators of the dynamic process of bone turnover and growth. Using these markers, it has been demonstrated that at diagnosis, children with acute lymphoblastic leukaemia (ALL) have reduced bone alkaline phosphatase (a marker of the differentiated osteoblast), reduced circulating C-terminal propeptide of type 1 procollagen (which reflects type 1 collagen synthesis and is a marker of the osteoblast in its early proliferative stage), reduced C-terminal telopeptide of type 1 collagen (which reflects the breakdown of type 1 collagen), and reduced insulin growth factor-1 and insulin growth factor-1 binding protein-3 (which are important regulators of osteoblast activity and bone

turnover). During the first year of treatment, periods of intensive chemotherapy were associated with further suppression of markers of collagen turnover, with marked increases in the levels of these markers during periods of less-intensive treatment (Crofton et al, 1998; Crofton et al, 1999; Crofton et al, 2000). Children are, however, given combinations of chemotherapeutic agents during treatment, and it is therefore impossible to determine from clinical studies the relative contribution of each individual agent administered to the resulting osteopenia. There are surprisingly few *in-vitro* investigations examining the local effects of individual chemotherapeutic agents on bone cells and the majority of *in-vitro* studies of the effects of chemotherapeutic agent on bone cells have been with MTX, dexamethasone or prednisolone (Scheven et al, 1995; Cheng et al, 1994).

1.5.2 Effects of MTX on Osteoblasts.

The mechanism of action of MTX on bone at the cellular level is unclear but may involve interaction with the function of osteoblasts and their precursors. Several studies have reported that MTX affects osteoblasts *in-vitro* and *in-vivo*, including adverse influences on recruitment and differentiation of mesenchymal precursors (Minaur et al, 2002), and inhibition of osteoblast function including bone formation (Uehara et al, 2001). The mechanisms by which MTX has these effects have yet to be defined and, although inhibition of folate metabolism is likely to be important in prevention of proliferation-associated events, it is also likely that MTX influences the ability of osteoblasts to respond to environmental cues such as growth factors (Beresford et al, 1984), cytokines or mechanical stimulation (Buckley et al, 1990, Lanyon et al, 1992; Harter et al, 1995; Owan et al, 1997), which are important in maintaining bone homeostasis.

The effect of both high and low dose MTX regimes has been the focus of many laboratory based and clinical studies. The major findings of these studies will be described in the following sections.

1.5.2.1 *In - Vivo* Investigations.

The majority of *in-vivo* laboratory studies have been conducted using a rat model, with the aim to give a better understanding of the effects of MTX on the normal physiological remodelling of the intact skeleton. Tross et al, (1980), demonstrated, using the rat, that MTX decreased bone turnover and strength, 14 days after administration of a single dose, whereas May et al, (1994), provided evidence to show that prolonged administration of low-dose MTX in normal rats causes significant osteopenia via suppression of osteoblast activity and stimulation of osteoclast recruitment, resulting in increased bone resorption. The animals in this study received weekly injections of 3 mg/kg of MTX for 16 weeks before being sacrificed at the end of week 16. The results of this study demonstrated that prolonged administration of weekly low-dose MTX caused a reduction serum alkaline phosphatase and osteocalcin levels and mineral apposition rates, although osteoblast numbers were not affected. Thus, the decrease in bone formation is likely to be a consequence of reduced osteoblastic activity rather than depletion of osteoblast numbers. Another study (Freidlander et al, 1984), also using a rat model, noted a significant reduction in cancellous bone volume, osteoid surface and osteoblast number 14 days after treatment with a 5-day course of MTX (0.75mg/kg/day), although no changes were found in the torsional strength, stiffness, energy absorbed at failure, osteoclast surface or mean cortical thickness over this 14-day period. These findings further support the hypothesis that, in rats, the mechanism of MTX-induced osteopenia is a result of decreased osteoblast activity.

Wheeler et al, (1995) investigated the effect of long-term MTX administration on the rat skeleton. MTX decreased cancellous bone volume, cancellous bone formation and increased cancellous bone resorption. Osteoblast (and osteoclast) recovery was not observed at the conclusion of the study, which was 170-days after MTX administration, and therefore as a result, cancellous bone mass remained at a reduced level in the MTX treated animals.

Studies have also been performed to investigate the effect of low dose MTX therapy on bone mass in adjuvant-induced arthritic rats. Segawa et al (1997) showed that

MTX decreases bone formation in both adjuvant-induced arthritic rats, and control (no-arthritis) rats but that the effect of MTX on osteoclast activity differed between arthritic rats and control rats. While MTX appears to increase osteoclast activity in the control rats, it prevents the increase in trabecular osteoclast number in arthritic rats. As a result, normal bone mass can be maintained (Segawa et al, 1997).

1.5.2.2 *In – vitro* Investigations.

Several *in-vitro* studies have investigated the effects of MTX on bone cells in culture (Preston et al, 1993; Scheven et al, 1995; May et al, 1996; Van der Veen 1996; Uehara et al, 2001; Davies, 2002). May et al (1996) investigated the effects of different concentrations of MTX (0.5 nM – 0.6 μM) on mouse bone cells in culture 7-days after addition to the culture. The lowest concentration used (0.5 nM) is comparable to that found in human synovium and bone (0.28 nM and 0.29 nM, respectively) following administration of intramuscular MTX (Preston et al, 1993), and the highest concentration (0.6 μM) is similar to that found in serum during standard dosing (Kremer et al, 1986). The results of this study provide evidence to show that MTX dose dependently inhibits osteoblastic cell function, as indicated by reduced osteocalcin levels and decreased matrix calcification. The authors concluded that the diminished matrix calcification was likely to be related to the reduced osteocalcin production, although no alteration in osteoblast proliferation was noted following MTX exposure.

Three further studies (Scheven et al, 1995; Van der Veen et al, 1996), have also investigated the *in-vitro* effects of MTX on human osteoblasts. These studies conclude that MTX decreases osteoblast proliferation by as much as 30% after 4 days of exposure. These results contradict those obtained by May et al (1996). This may be due to differences in MTX sensitivity between mouse and human osteoblasts, or because the response to MTX was measured in the human osteoblasts after just 4-days as opposed to 7-days in the mouse osteoblasts. MTX had no effect on alkaline phosphatase expression in either study. It is somewhat surprising that alkaline phosphatase levels, which are representative of cellular differentiation, remain

unaffected following incubation with MTX while cellular proliferation is diminished. It has been suggested (May et al 1996; Van der Veen et al, 1996), that MTX may be able to selectively 'target' and eliminate immature osteoblasts without affecting fully differentiated cells. Consistent with the above studies, Uehara et al (2001) have provided further evidence to support the hypothesis that MTX can inhibit the differentiation of immature osteoblasts. This study investigated the inhibitory effects of MTX on the differentiation of MC3T3-E1 cells, which have the ability to differentiate into mature osteoblasts or osteocytes. The results show that the inhibitory effects of MTX on alkaline phosphatase activity decrease according to the length of time the cells have been in culture before the addition of MTX. Therefore, these results suggest that MTX predominantly affects early osteoblastic cells that do not express alkaline phosphatase, and inhibits their differentiation into mature osteoblasts, but has little influence on either the function or proliferation of more mature osteoblasts.

Although the adverse effects of chemotherapy on bone have always largely been attributed to steroid therapy and the administration of MTX, Davies et al, 2002, have provided evidence to suggest that more chemotherapeutic agents than previously appreciated may have deleterious effects on bone metabolism. The relative chemosensitivity of osteoblast-like cells representing different stages of cell differentiation were evaluated. The chemosensitivity of an established human osteoblast-like cell line (MG63) was compared to that of a human osteoprogenitor cell line (HCC1) and primary HBC. Cell numbers were reduced by MTX, mercaptopurine, thioguanine, cytarabine, vincristine, etoposide, daunorubicin, asparaginase, prednisolone and dexamethasone in all cell types although there was a varied response between agents at equimolar concentrations. The less differentiated osteoblast phenotypes were significantly more chemosensitive than the more differentiated phenotypes. These results suggest that a variety of chemotherapeutic agents may contribute to osteopenia in childhood malignancy by direct effects on osteoblast numbers (Davies et al, 2002).

1.6 Hypothesis and Aims.

The work described in this thesis was undertaken with the goal of providing new insights into the mechanisms involved in the responses of HBC to mechanical stimulation, and to investigate the effects of MTX on these responses. As such, these experiments have involved the characterisation of HBC grown from trabecular bone fragments, and investigation of the signalling pathways activated following mechanical stimulation in control and MTX treated cells.

Hypothesis.

The hypothesis of this project was that detrimental effects of MTX on bone growth and resultant osteopenia, can be attributed to inhibitory effects of MTX on bone cell responses to mechanical stimulation, which are known to increase bone mass.

Scientific Questions

Chapter 3 – Do HBC obtained by outgrowth from trabecular bone fragments exhibit characteristics typical of the osteoblastic phenotype?

Chapter 4 – What is the electrophysiological response of cultured HBC to cyclical mechanical stimulation? What are the signalling mechanisms involved? Does cyclical mechanical stimulation result in alterations in gene expression?

Chapter 5 – Does MTX affect HBC responses to cyclical mechanical stimulation? How does MTX affect HBC viability? What are the mechanisms involved in MTX inhibition of HBC responses to cyclical mechanical stimulation?

Chapter 6 – Is adenosine signalling involved in MTX inhibition of HBC responses to mechanical stimulation? Does adenosine inhibit HBC responses to mechanical stimulation? Which specific subset (s) of adenosine receptor are involved?

CHAPTER 2.

MATERIALS AND METHODS.

All human bone tissue was handled in a BioMAT Class 1 Microbial Safety Cabinet.

2.1 Human Bone Cell (HBC) Culture.

2.1.1 Isolation of HBC.

HBC cultures were established from trabecular bone explants as described previously (Beresford et al, 1984). Bone was collected at surgery with informed consent from children undergoing corrective osteotomy, or from adults undergoing above or below knee amputation as a result of diseases unrelated to the osteoarticular system. The inner, soft trabecular bone was removed from the hard outer cortical bone using both sterile forceps and a scalpel and was retained. The cortical bone was discarded. The trabecular bone was chopped into 0.5 cm fragments using a sterile scalpel and then placed in a sterile universal container. The fragments were washed with sterile phosphate buffered saline (PBS), by shaking vigorously so that any non-adherent marrow associated cells were removed. This washing step was repeated three times. The trabecular fragments were then transferred to a sterile tissue culture petri dish (Nunc) containing 20ml complete medium consisting of, Minimal Essential Medium (MEM) (Sigma, Poole, UK) supplemented with 10% foetal calf serum (FCS) (Sigma), 10 I.U/ml penicillin, 10 μg/ml streptomycin (Gibco, Paisley, UK) and 2 mM L-glutamine (Gibco) (complete medium). The petri dish was placed overnight in an incubator at 37°C in an atmosphere of 95% O_2, 5% CO_2. The following day the fragments were examined microscopically for any signs of microbial infection and if none was present the fragments were transferred to a fresh sterile tissue culture petri dish (Nunc) containing 20 ml of the complete medium and returned to the incubator in the same culture conditions as above. Bone cell outgrowth from the trabecular bone fragments was usually apparent after 1-2 weeks, and the dish was confluent with HBC after 4-6 weeks.

2.1.2 Passaging Adherent Human Bone Cells

Once the petri dish was confluent with HBC, the trabecular bone fragments were removed to a fresh sterile tissue culture grade petri dish (Nunc) using sterile forceps. Complete media (20 ml) was added to the petri dish and the fragments were cultured at 37°C, 95% O_2, 5% CO_2 as described previously, until the dish was confluent with HBC. Bone fragments were recultured in this manner a maximum of three times.

After the bone fragments had been removed from the culture dish, the confluent cells were washed by the addition of 10 ml sterile PBS. The dish was gently agitated for 5-10 seconds and the PBS removed using a sterile pipette. 10 ml of 0.02% EDTA (Sigma) was added to the confluent HBC and the dish incubated at 37°C, 95% O_2, 5% CO_2 for 5 minutes. EDTA chelates divalent cations and metal ions and thus promotes loss of cell adhesion. Remaining adherent cells were then scraped from the dish using a sterile cell scraper (Greiner, Shroudwater Business Park, Gloucestershire) and the cell suspension was pipetted into a sterile universal container. The universal container was then filled to the 25 ml mark with sterile PBS and centrifuged at 300g (1000 rpm) for 10 minutes. The EDTA/PBS solution was discarded and the cell pellet was resuspended in 25 ml sterile PBS before being centrifuged again at 300g (1000 rpm) for a further 10 minutes. The cell pellet was resuspended in 5 ml complete medium, which was then transferred to a sterile tissue culture flask (Nunc) containing 75 ml complete medium. The cells were passaged as required. Cells were not used beyond passage 5.

2.1.3 Plating of Cells for Experiments

HBC were grown in sterile tissue culture flasks until the cells covered approximately 75% of the base of the tissue culture flask. To remove the cells, 10 ml EDTA (0.02%) was added to the flask and incubated at 37°C, 95% O_2, 95% CO_2 for 5 minutes. After the incubation period, the flask was tapped gently to dislodge any cells still adhering to the plastic. The cells were then removed from the flask using a sterile pipette, transferred to a sterile universal container, which was filled to the 25 ml mark with sterile PBS and were centrifuged at 300g (1000 rpm) for 10 minutes.

The EDTA/PBS solution was discarded and the cells were resuspended in 25 ml fresh PBS before being centrifuged again at 300 g (1000rpm) for a further 10 minutes. The cell pellet was then resuspended in 5 ml of complete medium and then filtered through a 100-micron cell strainer (Falcon, Becton Dickinson, UK) to remove any large cell clumps or pieces of debris. The cell number in the suspension was then assessed using a Neubauer haemocytometer. With a coverslip in place, 10 µl cell suspension was added to one chamber by touching the tip of the pipette to the edge of the coverslip and allowing the chamber to fill by capillary action. The number of cells was assessed by counting all the cells in the 1 mm centre square and two of the 1 mm corner squares **(Figure 2.1)**. Each 1mm square represents a total volume of 10^{-4} cm^3, and since 1 cm^3 is equal to 1 ml, the number of cells per ml is equal to the average count per 1 mm square x 10^4. The total number of cells is equal to the number of cells per ml x 5.

Cells were seeded in complete medium in tissue culture grade dishes and multiwell plates (Nunc) at differing densities, depending on the experiment to be performed **(Table 2.1)**. After seeding, all dishes/plates were incubated at 37°C for 48 hours to allow cells to adhere to the base of the dish/well. For all experiments, complete media was removed and replaced with MEM containing 10 I.U/ml penicillin, 10 µg/ml streptomycin and 2 mM L-glutamine (serum free medium) 30 minutes before commencing the experiment.

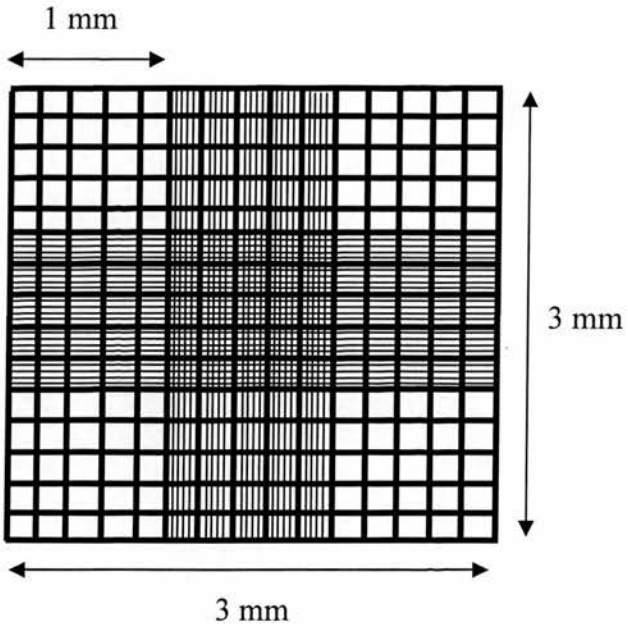


Figure 2.1 – Diagrammatic representation of the Neubauer haemocytometer used to assess cell number.

Experiment	Cell Density (cells/ml)	Seeding Vessel	Total Volume (per dish/well)
Electrophysiology	5×10^4	58 mm petri dish	5 mls
Acridine Orange Staining	5×10^4	6-well plate	2 mls
MTT Assays	5×10^4	96-well plate	100 µl
Immunofluorescence	5×10^4	58 mm petri dish	5 mls
Immunocytochemistry	5×10^4	58 mm petri dish	5 mls
RNA Extraction	5×10^4	58 mm petri dish	5 mls
Lactate Dehydrogenase Assays	1×10^5	12-well plate	1 ml

Table 2.1 – Different seeding conditions for the various experimental techniques performed throughout this study.

2.2 Induction of Mechanical Stimulation

The system used to mechanically stimulate the cells **(Figure 2.2)** is a modification of that described previously (Wright et al 1992, 1996). Flexible 58 mm tissue culture dishes (Nunc) were placed in a sealed polypropylene stimulation chambers with inlet and outlet ports **(Figure 2.3)**. The culture dish was supported by a rubber 'O'-ring in the central well of the stimulation chamber, below which there is a space through which nitrogen gas could circulate via inlet and outlet ports. The lid was screwed down and held the dish in position. The lid and the 'O'-ring ensured an airtight seal between the chambers below and above the dish **(Figures 2.3 and 2.4)**.

Nitrogen gas enters the space below the culture vessel via the inlet port, the frequency being dictated by an electronic timer, which controls both the inlet and outlet valves. When nitrogen gas enters the lower chamber, there is an increase in the pressure in the lower chamber, which causes the base of the dish to balloon upwards – therefore the base of the dish and its adherent HBC are exposed to strain.

In these studies, a gas pressure of 30 mmHg (0.025 atmospheres) was used which results in ~ 4000 microstrain on the base of the dish **(Figure 2.5)**. The system had been extensively calibrated by Dr Malcolm Wright and Mr Paul Parker using strain gauges which were glued to the base of the culture dish to measure the strain induced by various pressures. The standard stimulation regime used was a frequency of 0.33 Hz (2 seconds pressure on, 1 second pressure off) at 37°C. Each experiment was carried out using HBC from at least 3 different donors.

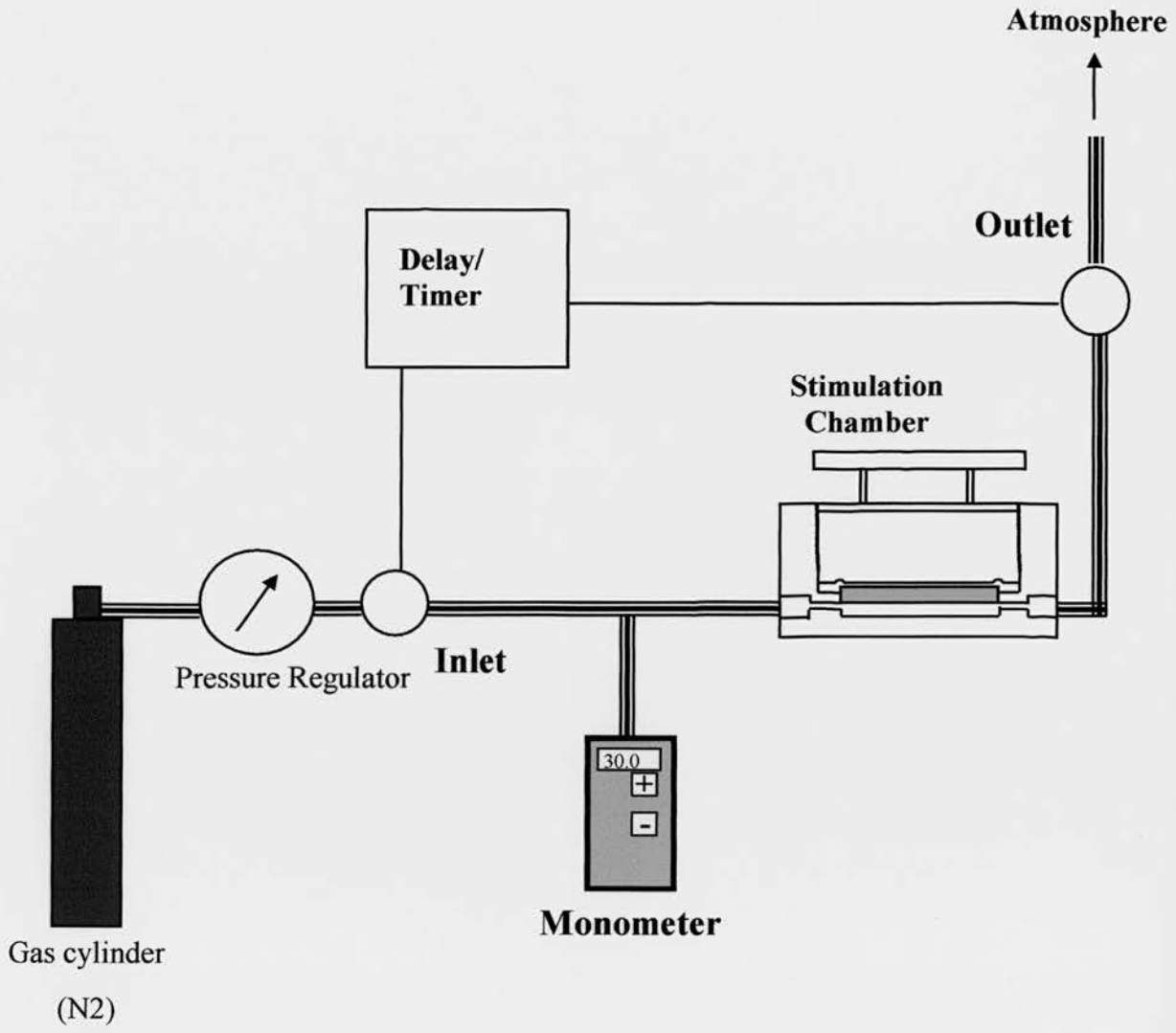


Figure 2.2 – Apparatus for the induction of cyclic mechanical stimulation. Nitrogen gas entered the system from a nitrogen cylinder, via a pressure regulator, which controlled the inlet and outlet valves. A delay/timer allowed variation in duration of pressure pulse and time between pulses. The pressure in the system was monitored by a digital monometer.

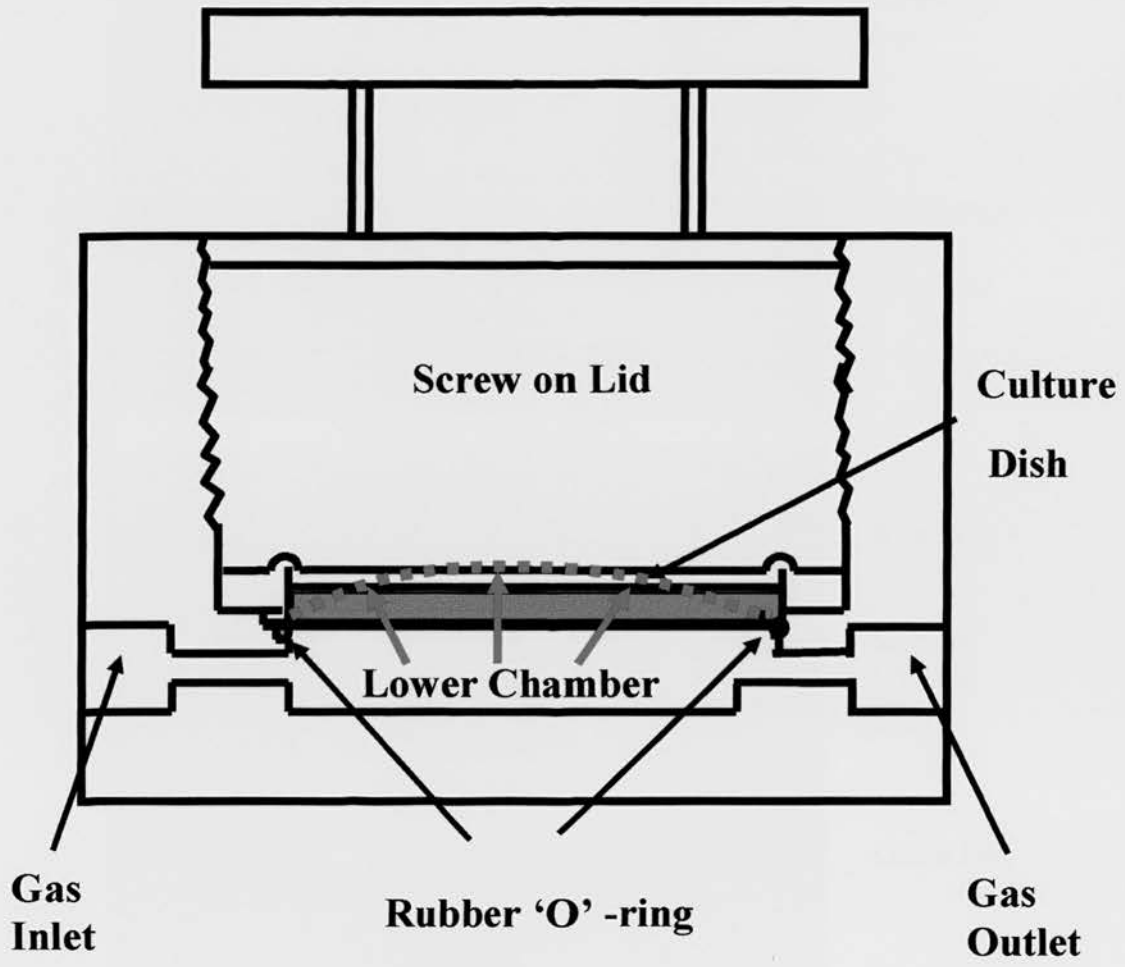


Figure 2.3 – Details of the mechanical stimulation chamber. Nitrogen gas enters the lower chamber via the gas inlet port resulting in an increase in the pressure in the lower chamber, which causes the base of the dish to balloon upwards (represented by the dotted line).

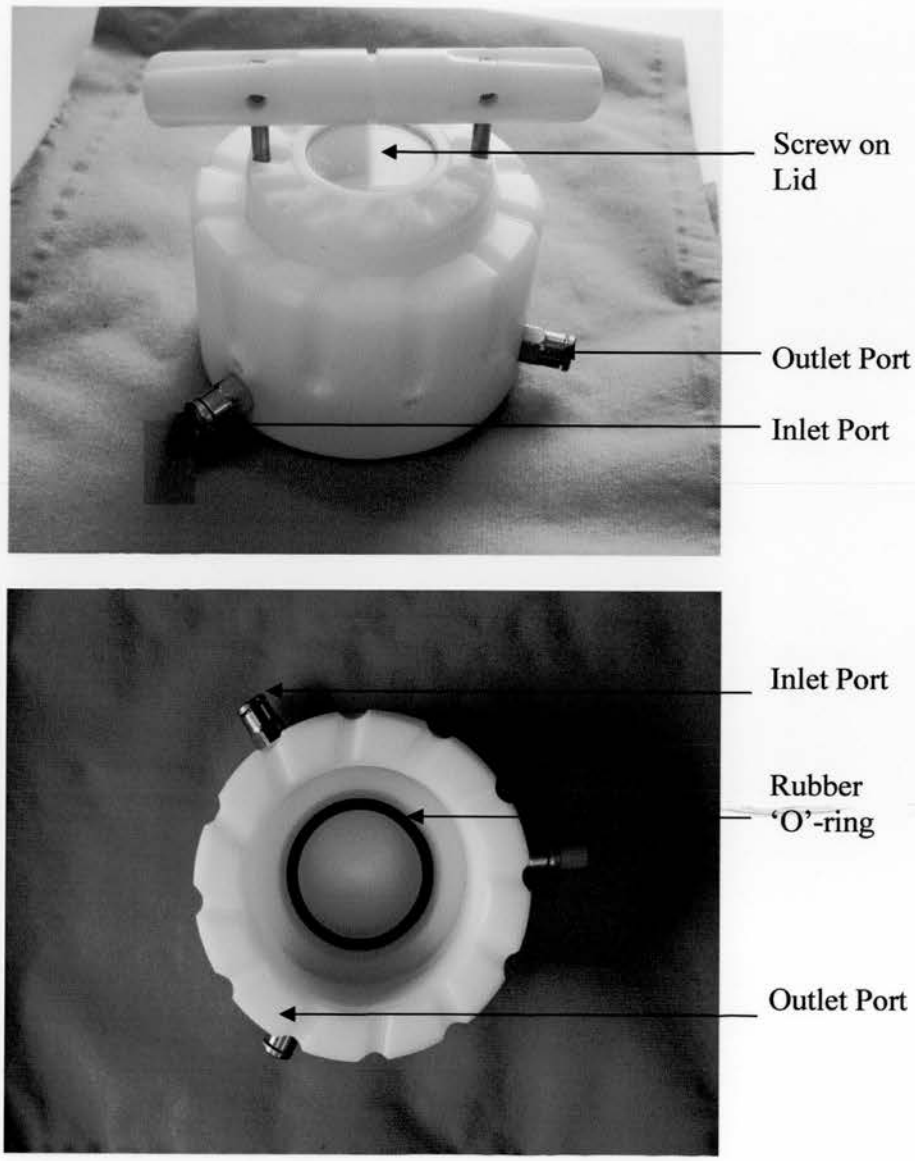


Figure 2.4 – Photograph of the mechanical stimulation chamber.

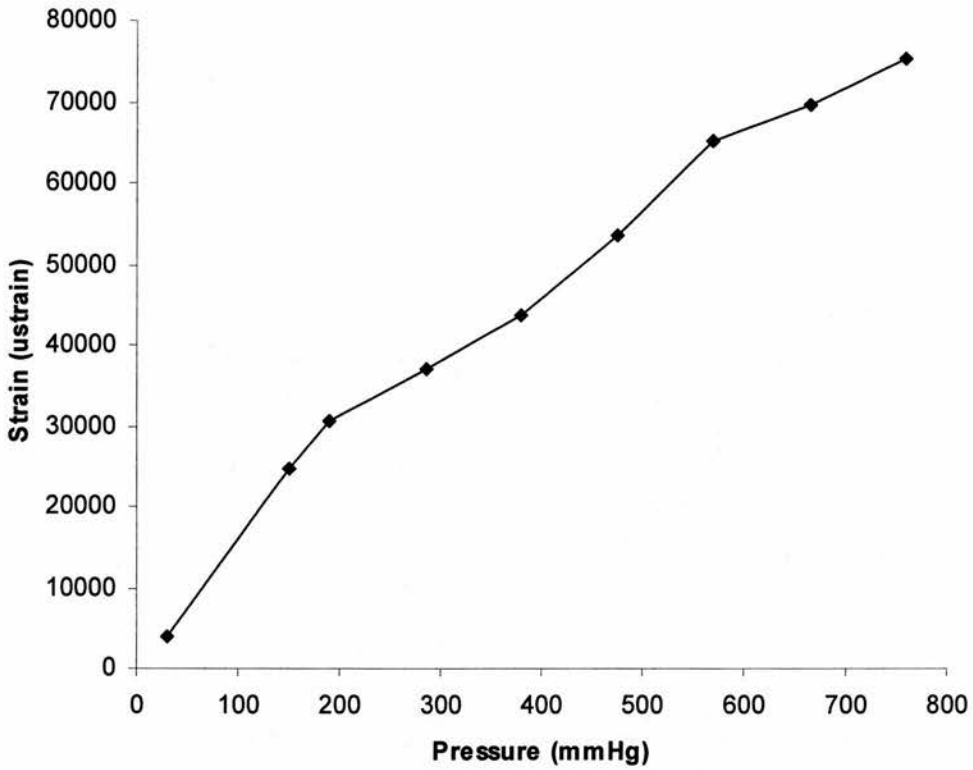


Figure 2.5 – Calibration graph for mechanical stimulation system. In the following studies a gas pressure of 30mm Hg (0.025 atmospheres) was used which results in ~ 4000 microstrain on the base of the dish.

2.3 Electrophysiology

2.3.1 Preparation of microelectrodes

A batch of microelectrodes was manufactured prior to each experiment from borosilicate glass capillaries, 1.5mm inner diameter and 1.86 outer diameter with inner filament (Harvard Apparatus, Edenbridge, Kent, England). The electrodes were made using a Narshege microelectrode puller (Narshege, Japan) and were stored in a clean container with their tips uppermost.

2.3.2 Filling of electrodes

Prior to use, the electrodes were filled with 3M potassium chloride solution (3M KCl). A glass tissue culture beaker was filled with 3M KCl and plasticine was pressed firmly around the rim of the beaker. Electrodes were then pressed onto the plasticine so that their tips were submerged in the KCl. The tips filled by capillarity and the shafts of the electrodes were filled using a syringe and lumbar puncture needle. After filling the electrodes were left for at least 3 minutes to allow the tips to fill by capillary action. Electrodes were used when there were no air bubbles visible in either the tip or the shaft **(Figure 2.6)**.

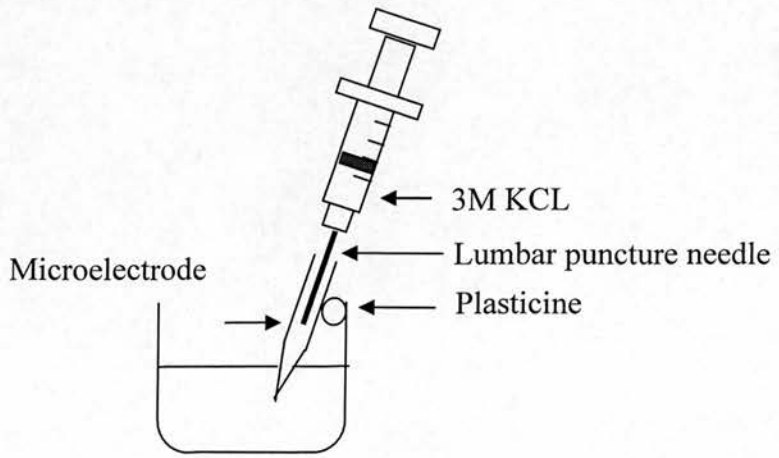


Figure 2.6 - Filling of Microelectrodes with 3M KCl.

2.3.3 Electrophysiological Recording

Electrophysiological studies were carried out at room temperature (~ 20°C). Dishes of cells were placed on the stage of a Wild M40 inverted microscope **(Figure 2.7)** and were observed under x200 magnification. A silver/silver chloride (Ag/AgCl) earth wire was placed in the periphery of the cell culture dish making contact with the culture medium. The other end of this wire was connected to the ground terminal of the input headstage of the Axoclamp Amplifier. The microelectrode was connected to the headstage via a microelectrode holder, which housed an Ag/AgCl wire. A pre-filled micreoelectrode was connected to the microelectrode holder so that the Ag/AgCl wire of the holder passed down the shaft of the microelectrode making contact with the 3M KCl. The microelectrode was secured firmly by the pipette seat of the holder **(Figure 2.8)**. The microelectrode holder was connected to the input of the headstage of the Axoclamp Amplifier. Using a Zeiss micromanipulator **(Figure 2.7)**, the microelectrode was manoeuvred until its tip was just below the surface of the culture medium. The tip of the microelectrode was then located in the field of view using the x200 magnification. Once the tip of the microelectrode had been located and focussed, the experiment was ready to commence.

Power to the Axoclamp-2B (Microelectrode Clamp, Axon Instruments) was switched on **(Figure 2.9)**. Any voltage observed at this time was offset to the zero baseline on the oscilloscope (Gould Advance type OS 4000/4001), using the input offset potentiometer of the Axoclamp-2B.

A Wheatstone bridge circuit incorporated into the circuits of the Axoclamp-2B allowed the determination of microelectrode resistance whilst the experiment was in progress. A command current of 5 nA was set with the step command thumbwheel switch. A voltage pulse was observed on the oscilloscope. The bridge balance control was advanced until this voltage step was eliminated. The bridge was then in balance. The microelectrode tip resistance was read from the bridge balance dial and was usually in the range of 30-50 MΩ, which was acceptable for experimentation. Lower tip resistances indicated that there was some damage to the tip or that the

electrodes had a large tip diameter. High tip resistances (> 100 MΩ) indicated the probability of air bubbles in the electrode tip, due to inadequate filling by the capillary. Therefore, electrodes with tip resistances <30 MΩ or >100 MΩ were discarded and not used for experimentation.

2.3.4 Electrophysiological Procedure

A suitable HBC was selected for impalement. As the culture was mixed and contained fibroblasts as well as HBC cells, for cells to be chosen for impalement they had to exhibit several criteria.

- Polygonal morphology (opposed to the spindle morphology of fibroblasts).
- Isolated, with no visible contact with neighbouring cells as it has been previously shown that resting membrane potentials are higher in cells which are in contact with one or more neighbouring cells (Bard, 1974).

The voltage dependent oscillator was switched on. This produces a tone, which is dependent on voltage, and changes in accordance with changes in membrane potential **(Figure 2.10)**. This piece of equipment provides the advantage that the experimenter can observe the microelectrode on cell impalement and not have to continually check the oscilloscope monitor, as a change of note alerts the experimenter that the electrode has entered the cell. The microelectrode tip was positioned over the cell using the micromanipulator. The electrode tip was then lowered whilst listening to the note of the voltage dependent oscillator. When the electrode tip entered the cell, the note changed suddenly from high to a lower frequency. The digital readout of membrane potential was then observed on the Axoclamp-2B and this was noted. The electrode tip was then removed from the cell using the micromanipulator.

The results from individual cells were accepted, if, upon impalement there was a rapid change in voltage which remained constant for at least 20 seconds. A microelectrode was changed if there was a dramatic increase or decrease in electrode

resistance as this usually indicated that the electrode had become blocked with debris, or cellular material or that the electrode had become damaged during cell impalement.

Membrane potentials of a minimum of 5 cells were measured before and after addition of each reagent to be tested and again following mechanical stimulation.

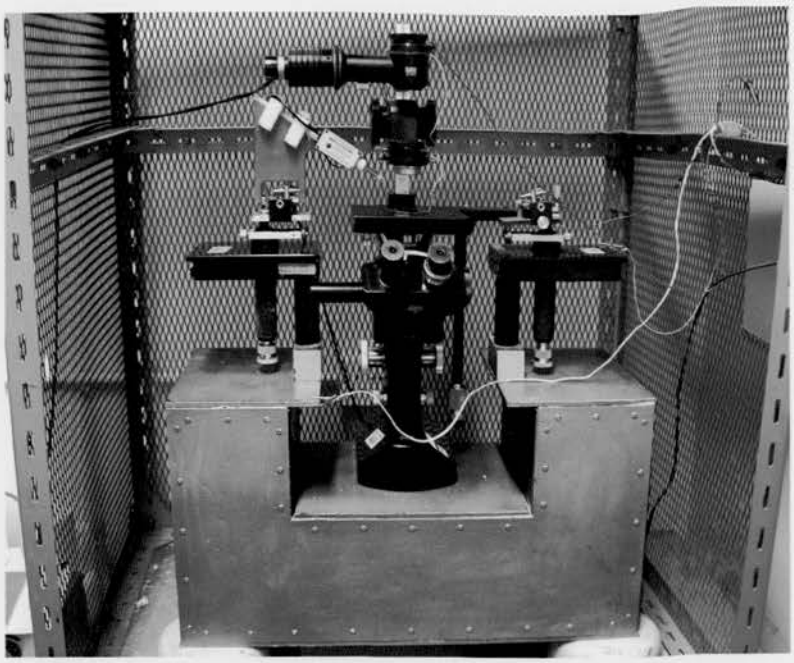


Figure 2.7 – Photograph of Wild M40 inverted microscope and micromanipulation system.

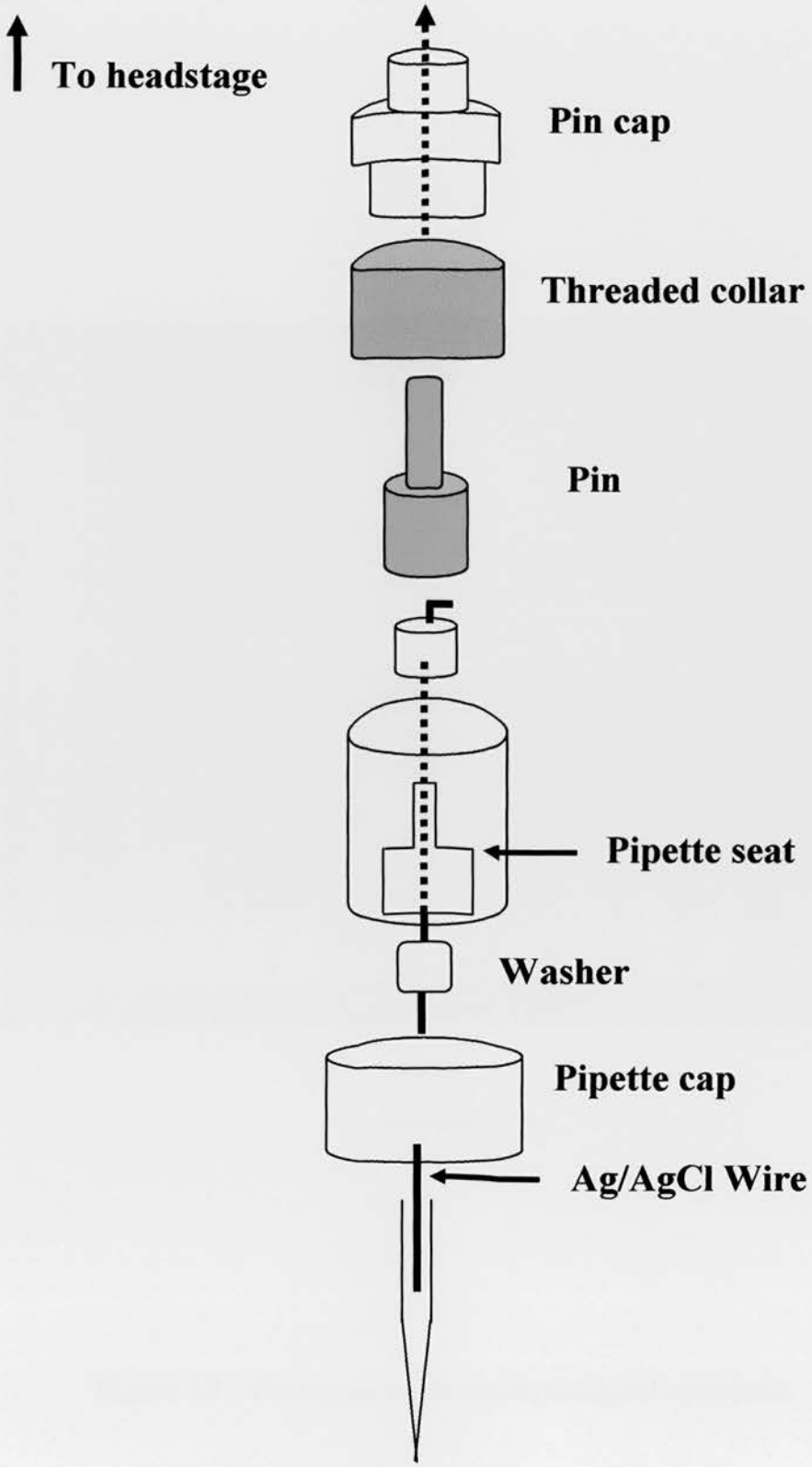
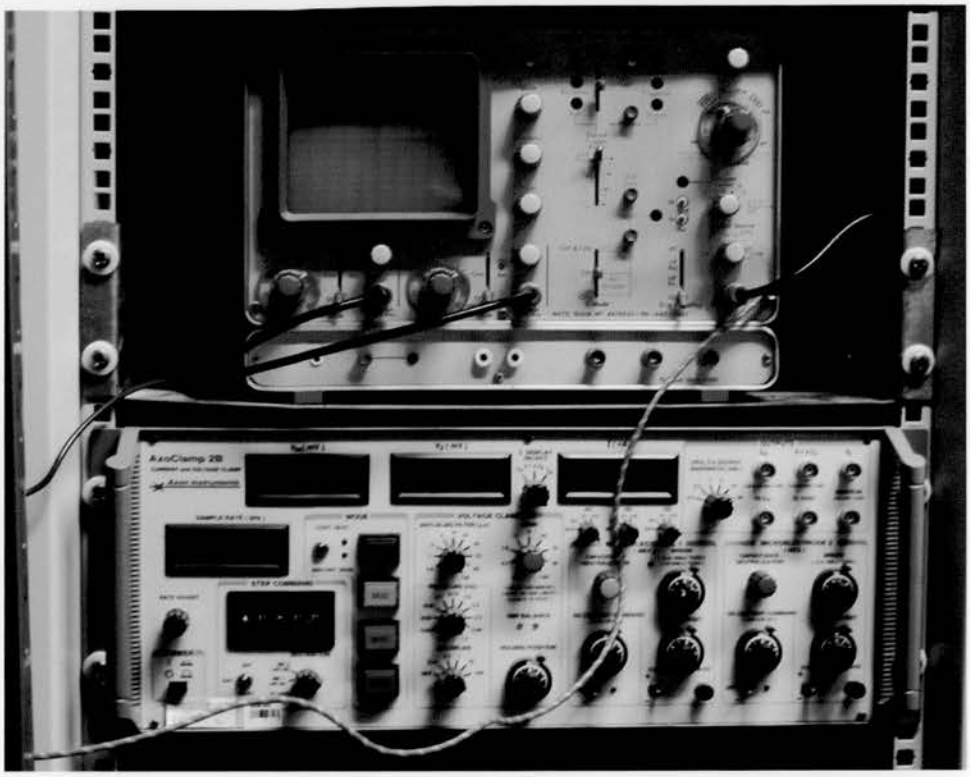


Figure 2.8 - Details of the microelectrode holder.

Oscilloscope



Axoclamp-2B

Figure 2.9 – Photograph of electrophysiological equipment.

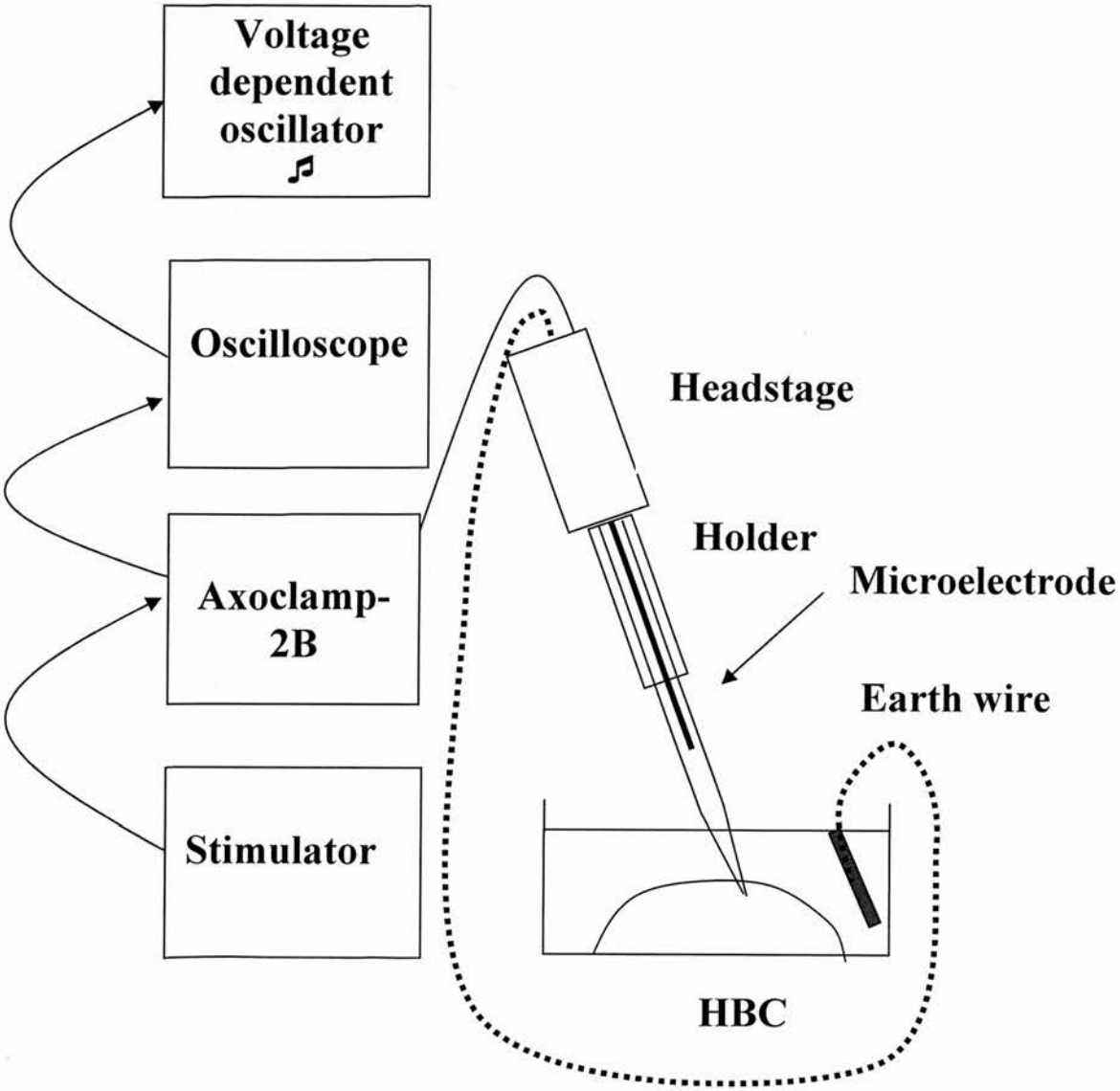


Figure 2.10 – Diagram of Axoclamp-2B system.

2.4 Chemical Reagents

To investigate the mechanotransduction pathway induced by mechanical stimulation and the effect of methotrexate (MTX), a variety of reagents including cytokines and receptor antagonists were added to cell cultures separately, or in conjunction with MTX. Chemical reagents were still in contact with HBC during the standardised stimulation procedure and also when the post-stimulation measurements were being made. These chemical reagents and the concentrations utilised are detailed in **Table 2.2**.

Reagent	Source	Target / Effect	Conc Utilised	Reference
Methotrexate	Sigma	Dihydrofolate reductase inhibitor.	1μM, 50 nM, 1 nM	May et al; 1996.
1-25-dihydroxy-vitamin D_3	Sigma	Induces osteoblast differentiation	10 nM	Scheven et al, 1995.
Adenosine	Sigma	Binds G-protein coupled adenosine receptors to positively or negatively regulate adenylate cyclase.	100 μM	Shimegi et al, 1996.
Adenosine Deaminase	Sigma RBI	Degrades adenosine to inosine.	0.125U/ml	Shimegi et al; 1996.
8-Cyclopentyl –1, 3-dipropylxanthine	Sigma RBI	A_1 receptor antagonist	1 μM	Ruiz et al; 2000.
8-(3-chlorostyryl) caffeine	Sigma RBI	A_{2A} receptor antagonist	10 nM	Jacobson et al; 1993.
Alloxazine	Sigma RBI	A_{2B} receptor antagonist	1 μM	Shin et al; 2000.
MRS-1191	Sigma RBI	A_3 receptor antagonist	100 nM	Mitchell et al; 1999.
IL-1β	R&D*	Recombinant cytokine	10 ng/ml	Salter et al; 2000.

Table 2.2 – Chemical Reagents used throughout these studies. *Abingdon, England.

2.5 Methylene Blue Cell Adhesion Assays

Flat bottomed, 96 well tissue culture plates (Nunc) were coated with 10 μg/ml of the extracellular matrix (ECM) proteins fibronectin (FN) (Sigma) or type 1 collagen (Coll 1) (Gibco) in 50 μl PBS overnight at 4°C. The following day, the ECM proteins were removed using a sterile pipette and non-specific binding was blocked with 100 μl filter sterilised bovine serum albumin (BSA) (2 mg/ml) for 1 hour at 37°C, 95% O_2, 5% CO_2. HBC were removed from tissue culture flasks using EDTA and washed with PBS as described in section 2.1.2. Following the PBS washes, cells were resuspended in serum free medium. The cell suspension was filtered through a 100-micron cell strainer (Falcon) and the cell number was assessed using a haemocytometer as described in section 2.1.3. The cell suspension was made up to a final concentration of $3.0x10^5$ cells per ml in serum free medium. 100μl of cell suspension was transferred to respective wells in the test plate and the plate was incubated at 37°C, 95% O_2, 5% CO_2, for 3 hours. After the incubation period, wells were washed gently with 100 μl PBS, taking especial care not to touch the bottoms of the wells with the pipette tip as so not to dislodge any adherent cells and 100 μl of 4% formalin was added to each well. The cells were fixed at room temperature for 1 hour. The plate was centrifuged at 1000 rpm for 5 minutes and the formalin removed using a pipette. Pre-filtered methylene blue (100 μl, 1% in dH_2O) was added to each well and the plate was incubated at room temperature for 30 minutes. The methylene blue was removed and the wells were washed several times in distilled water until the washes ran clear. HCl (100 μl, 0.1N) was added to each well for 5 minutes and mixed thoroughly to ensure the dye was properly resuspended. The plate was read at 630 nm. The number of cells present is proportional to the absorbance at 630 nm **(Figure 2.11)**. All experiments were performed in triplicate.

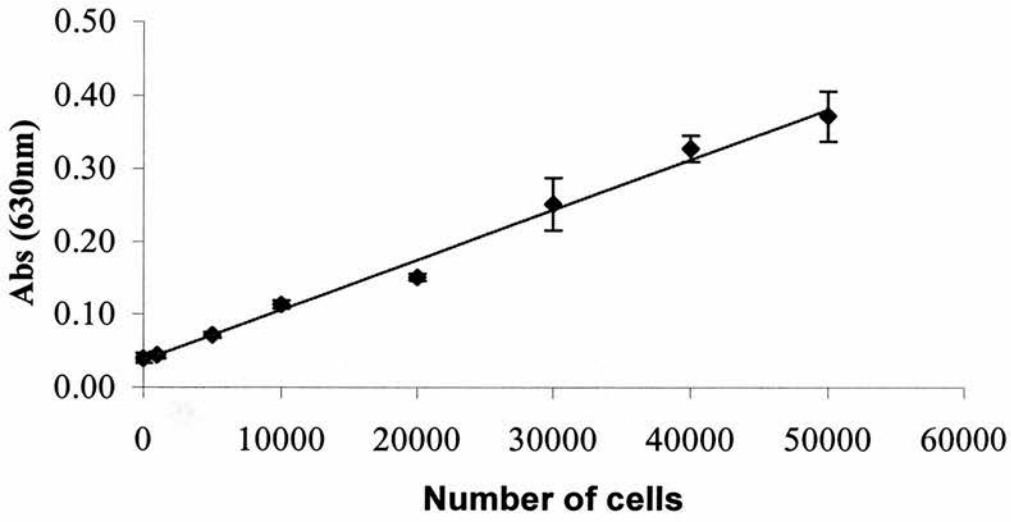


Figure 2.11 - Methylene blue cell adhesion assay standard curve. The absorbance at 630 nm is proportional to the amount of methylene blue bound, which is proportional to the number of adherent cells. Values are representative of three experiments carried out in triplicate ± SEM.

2.6 Cell Viability Assays.

2.6.1 Lactate dehydrogenase (LDH) assays.

To examine the effects of MTX on cell toxicity LDH assays were performed using a commercially available kit (Sigma), which is based upon the technique developed by Cabaud and Wroblewski (Cabaud and Wroblewski, 1960). This colorimetric assay kit was originally developed to allow determination of LDH concentrations in serum and the protocol was modified accordingly to allow quantification of LDH levels in HBC supernatants. LDH is a stable cytosolic enzyme, which is released from the cell upon its death and therefore the presence of LDH is an indication of cell necrosis.

HBC, cultured in 12-well plates **(Table 2.1)** were incubated with 1 μM, 50 nM or 1 nM MTX or sterile PBS for 90 minutes at 37°C. Triton-X 100 (0.2%) was used as a positive control. Following the incubation period, the culture medium (sample supernatant) was removed and stored at room temperature. Sigma pyruvate solution (1 ml) was added to a Sigma NADH vial and heated in a 37°C H_2O bath for 5 minutes. Aliquots (100 μl) of the pyruvate / NADH mixture were pipetted into microfuge tubes and 10 μl of sample supernatant added to appropriate tubes. The tubes were vortexed to mix and placed in a 37°C water bath for 30 minutes. The tubes were removed from the water bath and Sigma colour reagent (100 μl) was added to each tube and incubated at room temperature for 20 minutes. NaOH (1 ml of 0.4N in distilled water) was added to each tube and vortexed to mix. Samples were transferred to a sterile flat-bottomed 96 well plate (200 μl per well). After at least 5 minutes, but not more than 30 minutes absorbance was read at 450 nm versus distilled water as a reference. The absorbance at 450 nm is inversely proportional to the LDH concentration.

2.6.2 Acridine orange staining.

To check for altered levels of apoptosis following incubation with MTX, HBC were stained with acridine orange (Sigma), a fluorescent nucleic acid stain (Carmichael et al, 1980). HBC, cultured in 6-well plates **(Table 2.1)** were incubated with 50 nM

MTX or PBS control at 37°C for 90 minutes. Following the incubation period, the medium was removed from the wells and the cells were washed gently with 2 ml sterile PBS using a pipette. 1 ml acridine orange (100 μg/ml in PBS) was added to each well, the wells were coverslipped using alcohol cleaned coverslips and the cells were examined immediately under a Zeiss axiphot fluorescence microscope for the presence of apoptotic nuclei.

2.6.3 MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide] assays

The MTT assay is a simple and reliable colorimetric assay to assess cell number in drug toxicology studies. It is based on the ability of mitochondrial succinate dehydrogenase to catalyse conversion of MTT to a blue formazan product. The cells must be viable for the reaction to occur. HBC, cultured in 96-well plates **(Table 2.1)** were incubated with 50 nM MTX (or PBS control) at 37°C, 95% O_2, 5% CO_2, for various lengths of time before the assay was performed (24h, 6h, 3h or 1h). Triton-X 100 (0.2%) was included as a positive control. After the incubation period, the culture supernatant was removed and the cells were washed gently with PBS. MTT working solution was made up by adding 1.6 ml of MTT stock (10 mg/ml in PBS) (Sigma) to 5.4 ml of serum-free MEM. 100 μl of working solution was added to each well and the cells were incubated at three hours in the dark at 37°C, 95% O_2, 5% CO_2. Following incubation, the MTT solution was removed and the cells were gently washed with PBS. 100 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan salt and the plate agitated gently for a few seconds. The plate was then read using a spectrophotometer at a wavelength of 630 nm within 30 minutes of the addition of the DMSO. The absorbance is proportional to the number of viable cells.

2.7 Immunofluorescent staining of cultured HBC using rhodamine (TRITC) labelled phalloidin.

Effects of MTX on the integrity of the actin cytoskeleton were investigated using rhodamine (TRITC) labelled phalloidin, a fluorescent phallotoxin that can be used to identify filamentous actin (Faulstich et al, 1983). HBC, cultured in 58 mm culture dishes were incubated with 50 nM MTX or sterile Tris buffered saline (TBS) for 30 mins at 37°C, before being mechanically stimulated as described previously in section 2.2. Following mechanical stimulation, cells were fixed using 4% buffered formalin at room temperature, for 5 minutes. The formalin was removed using a sterile pipette and the cells were permeabilised using 1 ml 1% Triton-X 100 for 5 minutes at room temperature. Triton-X 100 was removed using a pipette and the cells were washed using 2 ml TBS accompanied by gentle agitation at room temperature for 5 minutes. This wash step was repeated a further two times. The TBS was removed using a pipette and the cells were treated with 1 ml rhodamine conjugated phalloidin (Sigma) (1:100 in 1 ml TBS) for 30 minutes. As rhodamine conjugated phalloidin is light sensitive, the plate was wrapped in aluminium foil during this and all subsequent steps. The phalloidin was removed and the cells were washed using 2 ml TBS with gentle agitation at room temperature for 5 minutes. This wash step was repeated a further two times. The cells were then mounted in alcohol-cleaned coverslips using Vectashield (Vector Laboratories, Burlingame), to help prevent fluorescence quenching. The edges of the coverslip were sealed with clear nail varnish. The cells were visualised using a Zeiss axiphot fluorescence microscope. The actin filaments appeared red, stained by the TRITC-labelled phalloidin.

2.8 Immunocytochemistry.

For immunocytochemistry, HBC cultured in 58 mm culture dishes were fixed using 2 ml methanol/acetone (1:1) at -20°C for 20 minutes. The methanol/acetone was removed using a pipette and the plates were air dried and then washed with 2ml PBS. The PBS was removed using a pipette and the cells were permeabilised with 0.1% Triton -X 100 for 5 minutes at room temperature with agitation. Triton-X 100 was removed using a pipette and the cells were washed using 2 ml PBS at room temperature, for 5 minutes with agitation. This wash step was repeated a further two times. Non-specific binding by the cells was blocked by incubating them with normal rabbit serum (diluted 1:5 with PBS) for 10 minutes at room temperature with agitation. The serum was discarded and cells were incubated with 1 ml of the appropriate primary antibody for 1 hour at room temperature with agitation. Antibodies were diluted optimally in PBS **(Table 2.3)**. The antibody was discarded and cells were washed using 2 ml PBS at room temperature, for 5 minutes with agitation. This wash step was repeated a further two times. Non-specific binding was blocked using normal rabbit serum (diluted 1:5 in PBS) for 10 minutes at room temperature with agitation. Cells were incubated with biotinylated rabbit-anti-mouse secondary antibody (diluted 1:300 in 20% normal rabbit serum). Cells were washed using 2 ml PBS at room temperature, for 5 minutes with agitation. This wash step was repeated a further two times. Cells were incubated in Avidin-biotin complex – horseradish peroxidase (ABC-HRP) complex for 30 minutes at room temperature with agitation. ABC-HRP was removed and cells were washed using 2 ml PBS at room temperature before being visualised using diaminobenzidine (DAB) substrate for 3 minutes. DAB substrate was removed and discarded and cells were washed gently in running tap water and lightly counterstained in haematoxylin. Cells were 'blued up' using Scotts Tap Water Substitute (STWS) and mounted using glycerol:PBS (9:1). The edges of the coverslip were sealed using nail varnish. The cells were visualised using an Olympus CX2 microscope.

Antibody	Clone	Source	Dilution in PBS
Mouse Anti-Human IL-1β	2805.31	R&D Systems	1:10
Mouse Anti-Human IL-1 RI	35730.111	R&D Systems	1:20
Mouse Anti-Human IL-1 RII	32437.111	R&D Systems	1:20

Table 2.3 – Monoclonal primary antibodies used to perform immunocytochemistry.

2.9 Staining for Alkaline Phosphatase.

HBC, cultured in 6-well plates were stained for alkaline phosphatase, which is a useful indicator of the differentiated osteoblast phenotype. Cell culture medium was removed and the cells were washed using 2 ml sterile PBS. Nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) stock solution was diluted 1:50 in buffer (0.1 M Tris-buffer, pH 9.5, 0.05 M $MgCl_2$, 0.1 M NaCl) and 2ml of this solution was added to each well. The plates were covered in aluminium foil and incubated at room temperature for 2 hours. During the incubation period, the plates were placed upon a rocking platform. Following the incubation period, HBC were washed two times using sterile PBS and the colour was allowed to develop on the bench for 2-3 minutes. The cells were then fixed in 2 ml 70% (v/v) ethanol at 4°C for 5 minutes. The ethanol was removed, and the cells were counterstained with neutral red for 1 minute at room temperature. Neutral red was removed by rinsing with tap water, the cells were cover slipped using an aqueous mount, and the edges were sealed with nail varnish. The cells were visualised using an Olympus CX2 microscope.

2.10 RNA Purification

2.10.1 RNA extraction

HBC, cultured on 58 mm culture dishes, were incubated for 30 minutes with 50 nM MTX or PBS control for 30 minutes before induction of 20 minutes mechanical stimulation. Following mechanical stimulation, RNA was extracted immediately or the dishes were incubated at 37°C, 95% O_2, 5% CO_2 for 1, 3, 6 or 24 hours before RNA extraction.

To extract the RNA, the culture medium was poured from the dishes and the cells washed gently with 5 ml sterile PBS. 750 µl RNA extraction medium (4 M guanidine isothiocyanate, 0.75 M sodium citrate, 10% (wt/vol) lauryl sarcosine and 7.2 µl/ml β-mercaptoethanol) was added directly to the cells. The plates were swirled gently for 1 minute at room temperature to ensure all cells were lysed. Plates were sealed with parafilm and stored at -20°C until required.

The plates were thawed at an angle to pool the solution and as much of the solution as possible was transferred to a DNase / RNase – free microfuge tube. To each tube, 75 µl of 2 M sodium acetate (pH 4.5) was added and the tubes were inverted several times to mix the solution. To each tube, 750 µl of a phenol (pH 4.5)/chloroform mix (1:1 mix) was added and the tubes were vortexed to mix and incubated on ice for 15 minutes. Following the incubation period, tubes were centrifuged at 13,000 g for 15 minutes at 4°C. Following centrifugation, as much of the clear aqueous phase as possible was transferred to a fresh RNase / DNase-free microfuge tube. The tubes were centrifuged at 13,000 g for 10 minutes at room temperature to pellet RNA. The RNA pellet was washed in 500 µl ethanol and air-dried at 37°C. Once dry, the pellet was resuspended in 30 µl sterile RNase-free water and stored at -80°C until required.

2.10.2 RNA Quantification

RNA was quantified using a Biomate II spectrophotometer and 600 µl quartz cuvettes. The absorbance of 6 µl RNA in a total of 600 µl was measured using RNase-free water. RNase-free water was used to zero the instrument. Quantified RNA was stored at -80°C until required for reverse transcription reactions.

2.11 Polymerase Chain Reaction (PCR) Methods.

2.11.1 Primer design

The sequences used for PCR primers came from publications or were designed in-house using mRNA sequences obtained from the GenBank database held at the National Centre for Biotechnology Information web site **(Table 2.4)**. Wherever possible primers were designed which were intron spanning to minimise the chances of genomic DNA contamination. All primers were synthesised by Life Technologies Ltd.

Target	GenBank Access No	Sequence 5'⇒ 3' (S=sense, AS=anti-sense)	Product Size (bp)	Reference
GAPDH	J0438	**S** = CCA CCC ATG GCA AAT TCC ATG GCA **AS** = TCT AGA CGG CAG GTC AGG TCC ACC	600	Designed in-house.
Collagen 1	AF017178	**S** = AAG ATG GAC TCA ACG GTC TC **AS** = AAC CAG ACA TGC CTC TTG TC	523	Designed in-house.
Osteopontin	NM_000582	**S** = ATG AGA ATT GCA GTG ATT TG **AS** = TTT TGA CCT CAG TCC ATA AA	468	Designed in-house.
BMP-4	X56848	**S** = CAG CGG TCC AGG AAG AAG AAT AAG **AS** = TCT GCA CAA TGG CAT GGT TG	188	Ikegame et al, 2001.
Osteocalcin	NM_000711	**S** = ATG AGA GCC CTC ACA CTC CT **AS** = GTCAGC CAA CTC GTC ACA GTC C	255	Kuliwaba et al, 2000

Table 2.4 – Details of PCR primers used throughout these studies.

2.11.2 Reverse Transcription (RT) Reactions.

RT reactions were performed using sterile 0.5 ml RNAse / DNAse-free tubes and each RT reaction contained 0.5 μg RNA, 0.25 ng Oligo dT and 38.2U RNAse inhibitor. The reaction volume was made up to 11 μl using RNAse free water.

Tube contents were mixed thoroughly and then pulsed in a micro-centrifuge to ensure contents were gathered at the bottom of the tube. Contents were then denatured by heating for 10 min at 70°C on an Omn-E™ thermal cycler (Hybaid, Teddington, Middlesex, UK). Following this 10-minute denaturation, 0.01M Dithiothreitol (DTT), 0.1mM of each 2' Deoxynucleoside 5'-Triphosphates (dNTPs), 1x RT reaction buffer and 200U superscript II was added to each tube (Note - The above volumes are those added for 0.5 μg RNA. For larger quantities all reagents are scaled up accordingly except RNAse inhibitor – 38.2U is sufficient for up to 60 μl final volume).

The tubes were then placed on the Omn-E™ thermal cycler for 1 hour at 42°C and 70°C for 10 minutes to allow the RT reaction to proceed. Once the RT reactions were complete, synthesised cDNA was stored at -20°C until required for PCR.

2.11.3 PCR Protocol.

PCR reactions were performed using sterile 0.5 ml RNAse / DNAse-free tubes and each PCR reaction was made up to a final volume of 20 μl. A typical 20 μl PCR reaction contained 16 mM ammonium sulphate, 67 mM Tris/HCl, pH 8.8, 0.01% (vol/vol) Tween 20, 1 μM of each primer, 2 μl cDNA and 0.25 U Taq polymerase. The magnesium chloride ($MgCl_2$), dNTP and BSA concentrations varied according to the primer pair and are summarised in **Table 2.5**.

Primer Pair	$MgCl_2$ (mM)	dNTP's (μM)	BSA (wt/vol)
GAPDH	2.5	100	0.1%
Collagen 1	2	50	0.1%
Osteopontin	2	100	0.1%
BMP-4	1	200	nil
Osteocalcin	1.5	200	0.1%

Table 2.5 – Primer specific conditions required for PCR reactions.

2.11.4 PCR Programs.

The following programs were run on an Omn-E™ thermal cycler with tube temperature control (Hybaid) **(Table 2.6)**. Once the appropriate number of cycles had been completed or the program had finished, PCR samples were stored at -20°C until required for agarose gel electrophoresis.

Primer Pair	Temp (°C)	Time	Number of Cycles
GAPDH	95	3 min	1
	95, 60, 72	1min, 1min, 1 min 30 sec	36
	72	10 min	1
Collagen 1	95	3 min	1
	95, 60, 72	1 min, 1 min,1 min 30 sec	36
	72	10 min	1
BMP-4	94	2 min	1
	94, 57, 72	30 sec, 35 sec, 1 min	36
	72	7 min	1
Osteocalcin	95	9 min	1
	95, 62, 72	1 min, 1 min, 1 min	36
	72	5 min	1

Table 2.6 – Conditions used for PCR reactions.

2.11.5 Agarose gel electrophoresis

PCR samples were run on 1% agarose (Sigma) gels. Agarose gels were manufactured by mixing 1.5 g agarose with 150 mls 1x Tris/acetic acid/EDTA (TAE) buffer in a glass conical flask and heating in a microwave until all the agarose had dissolved. After heating the agarose solution was allowed to cool at room temperature. Once the agarose had cooled enough to comfortably hand hold, but was still liquid, it was poured into the a gel tray and the relevant number of wells were created by inserting combs, ensuring no air-bubbles were present. Once solid, the agarose gel (and tray) were transferred into the gel tank which was filled with 1x TAE so that the gel was completely submerged in the buffer.

PCR samples were allowed to defrost at room temperature. Once liquid, 20 µl of 2x Orange-G gel loadng dye was added to each sample and mixed thoroughly. The samples were then pulsed in a micro centrifuge to ensure the contents were gathered at the bottom of the tube before loading the sample in to the appropriate wells in the agarose gel using a pipette. Gel electrophoresis was run at 75V for 30-60 min depending on how far it was necessary to run the samples. Once the gel had run far enough, the power was disconnected and the gel was removed from the gel tank and gel tray and transferred to a box specifically used for ethidium bromide staining. The gel was completely submerged in ethidium bromide (2 µg/ml) and left to soak for 15-30 min. Once the gel was stained the ethidium bromide was poured back into the bottle for re-use and the gel was visualised.

2.11.6 Gel Visualisation

Gels were visualised by UV trans-illumination. Images were saved to floppy disk and were printed using the Enhanced Analysis System (EASY, Scotlab, Coatbridge, Lanarkshire, Scotland). This program allows semi-quantitative analysis of PCR products. It designates the brightest band on the gel a value of 1000 (background = 0) and thus can designate all the other bands a value between 0 and 1000 depending on their intensity. Therefore this program allows the intensity of PCR bands (which is proportional to the amount of PCR product) to be semi-quantitatively assessed following a range of different treatments and conditions.

2.12 Statistics

- The following generalised linear model was fitted to the data obtained from adult and juvenile bone cells before / after mechanical stimulation.

$$\mu_{ij} = \mathbf{k} + \alpha_i + \beta_j$$

where k, α and β are parameters to be estimated from the data for **i** = c (child) or a (adult) and **j** = b (before MS) or af (after MS). We set $\alpha_c = -\alpha_a$ and $\beta_b = -\beta_{af}$.

This model was used to investigate two hypotheses;

1. Mechanical stimulation has no effect on membrane potential.
2. The membrane potentials of adult and juvenile bone cells are not significantly different.

- For all other experiments, the mean, standard deviation (SD) and standard error of the mean (SEM) were determined in each experiment. For statistical comparisons, when the F-ratio of the two variances reached significance, the non-parametric Mann-Whitney test was used. When the ratio did not reach significance, the Student's *t* test was used.

CHAPTER 3.

THE PHENOTYPE OF HUMAN BONE CELLS (HBC) IN CULTURE.

3.1 The Morphology of Cultured HBC.

HBC were obtained by outgrowth from trabecular bone fragments obtained from patients undergoing orthopaedic surgery **(Figure 3.1)**. Morphologically, the cultures obtained were heterogeneous, containing approximately equal proportions of spindle shaped fibroblastic cells and polygonal osteoblast-like cells.

Osteoblasts exhibit several characteristics useful in their identification, including alkaline phosphatase activity (Doty et al, 1984), the ability to form a mineralised extracellular matrix comprised of type 1 collagen, and certain bone specific NCP's such as osteocalcin and osteopontin (Gehron Robey et al, 1989). To identify whether the osteoblast-like cells obtained *in-vitro* expressed similar markers to those of osteoblasts *in-vivo*, these cells were assessed for expression of alkaline phosphatase and mRNA for the bone cell markers type 1 collagen, osteocalcin, osteopontin and bone morphogenetic protein 4 (BMP-4).

Alkaline phosphatase staining was performed both on resting HBC and HBC which had been incubated for various lengths of time (0 – 4 days) with 1-25-dihydroxyvitamin D_3 (Vitamin D_3). Vitamin D_3 has been shown to be a potent inducer of osteoblast differentiation (Scheven et al 1995). Alkaline phosphatase is secreted by osteoblasts (Gehron Robey et el, 1989) and is commonly used as a biochemical (Osdoby et al, 1981; Beresford et al, 1986) and histochemical (Osodby et al, 1981; Thavarajah et al, 1993) marker to identify osteoblasts. Osteoblasts stain positively for alkaline phosphatase whereas fibroblasts are negative for alkaline phosphatase staining. The results demonstrated considerable alkaline phosphatase staining in the HBC cultures **(Figure 3.2a)** and this staining was increased in a time dependent manner following exposure to Vitamin D_3 in culture (10 nM) for 0-4 days **(Figure 3.2b)**.

Further characterisation of the HBC was achieved by the use of RT-PCR. RT-PCR was performed using primers specially designed to investigate expression of collagen type 1, osteocalcin (Kuliwaba et al, 2000), osteopontin and BMP-4 (Ikegame et al, 2001). Collagen type 1 fibres comprise 95% of the organic part of bone (Athanasou, 1999), the remainder consisting of non-collagenous proteins of bone, which include osteocalcin and osteopontin (Athanasou, 1999). Osteocalcin, also known as bone-gla protein, is one of the most abundant non-collagenous proteins of bone. The exact role of osteocalcin in bone metabolism is still not known but may involve bone resorption (Gehron Robey, 1989). The presence of osteocalcin appears to be unique to bone (Nishimoto et al, 1979) and dentine (Linde et al, 1980). Osteopontin is an acidic sialoprotein, which is implicated in general cell attachment (Robey, 1989). BMP-4 is a member of a cytokine family that stimulates proliferation of both osteoblasts and chondrocytes, and causes an increase in matrix production (Buckwalter et al, 1996; Rosen et al, 1992). The results obtained using RT-PCR in this study demonstrate that, *in-vitro*, HBC produce collagen type 1, osteocalcin, osteopontin and BMP 4 mRNA **(Figure 3.3)**.

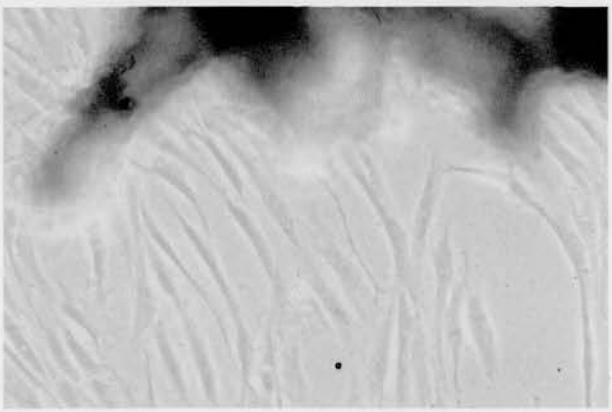
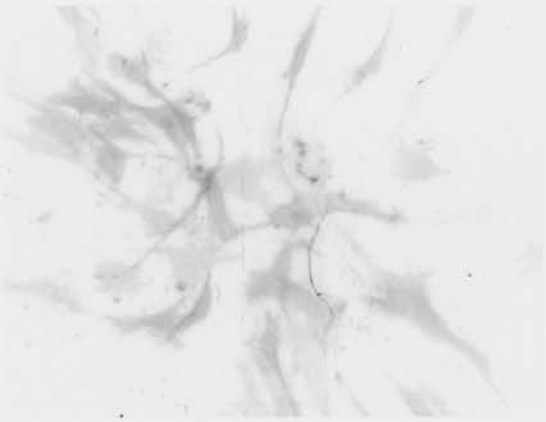


Figure 3.1 – The appearance of HBC in culture.

HBC are obtained by outgrowth from trabecular bone fragments obtained from orthopaedic surgery. The culture is heterogeneous, consisting of both fibroblasts and osteoblast-like cells (original magnification x 100).

(a)



(b)

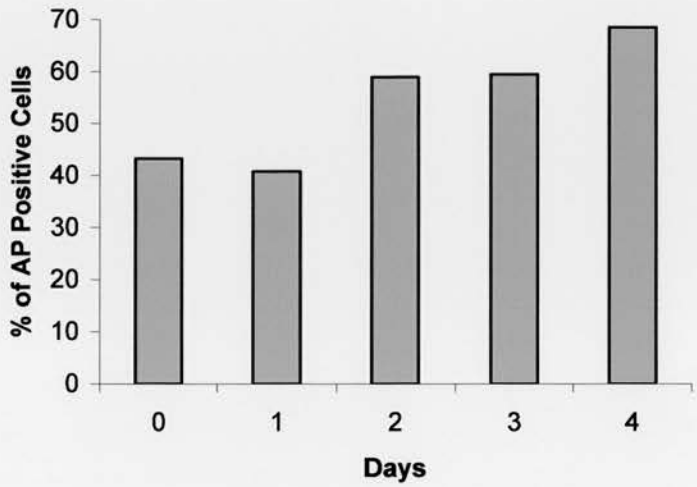


Figure 3.2 – Expression of alkaline phosphatase by HBC in culture.

(a) Alkaline phosphatase expression by unstimulated HBC (purple staining). Cells are counterstained with neutral red (original magnification x 200).

(b) Alkaline phosphatase expression following incubation over a 4-day period with 10 nM Vitamin D_3. Results are expressed as % of positively stained cells, n = 2 (200 cells were counted for each time point).

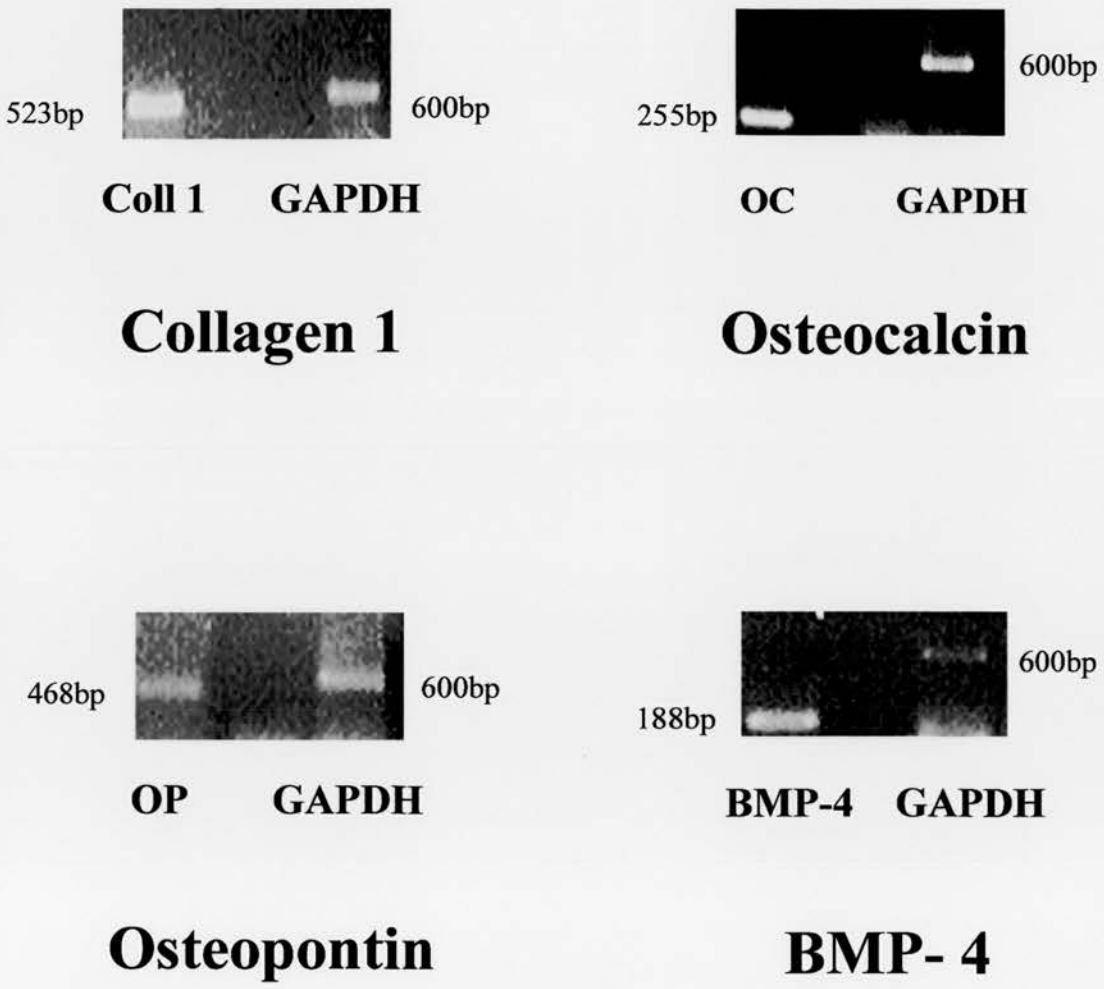


Figure 3.3 – RT-PCR shows expression of collagen 1, osteocalcin (OC), osteopontin (OP) and bone morphogenetic protein-4 (BMP-4) mRNA.

3.2 HBC in Culture Express Interleukin 1β (IL-1β) and Interleukin 1β Receptors Type I and II (IL-1β RI and II).

It has been shown that following mechanical stimulation, HBC in culture exhibit considerable cell membrane hyperpolarisation, and that this occurs as a result of the activation of a novel signalling pathway which involves an Interleukin-1β (IL-1β) autocrine/paracrine loop (Salter et al 1997; Salter et al 2000).

Immunocytochemistry was performed to identify whether the HBC in culture express IL-1β and its receptors IL-1β RI and II. The cells were subjected to immunocytochemical staining using monoclonal antibodies against IL-1β and IL-1β RI and II. The expression of IL-1β was studied in both non-stimulated cells and cells which had been mechanically stimulated at 0.33 Hz, 30 mmHg for 20 minutes. The results demonstrated that IL-1β is present in both non-stimulated and mechanically stimulated cells **(Figure 3.4a)**. The results also suggest that IL-1β expression is higher in non-stimulated cells when compared to mechanically stimulated cells, as the resting cells are more intensely stained when compared to cells stained post mechanical stimulation. It is appreciated however, that immunocytochemistry is, at best only a rough guide, and not a quantitative technique. The expression of IL-1β RI and II was studied only in non-stimulated cells. The results show that HBC in culture express both IL-1β RI and IL-1β RII **(Figure 3.4b)**.

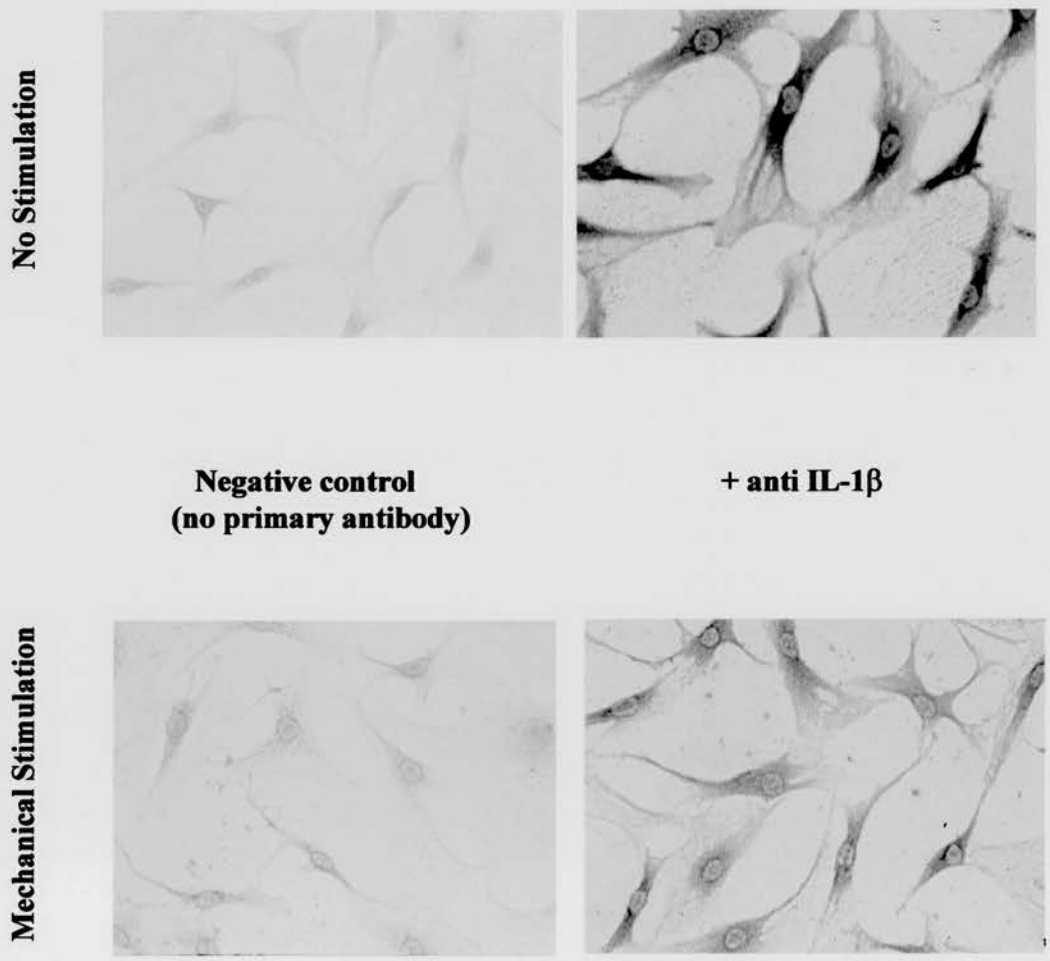
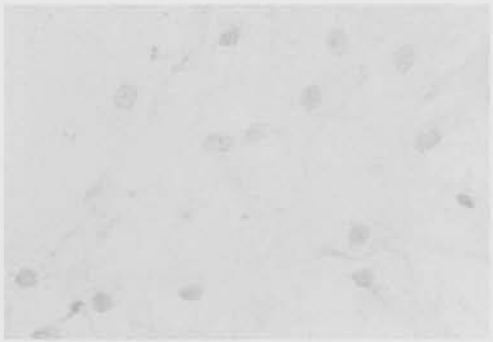
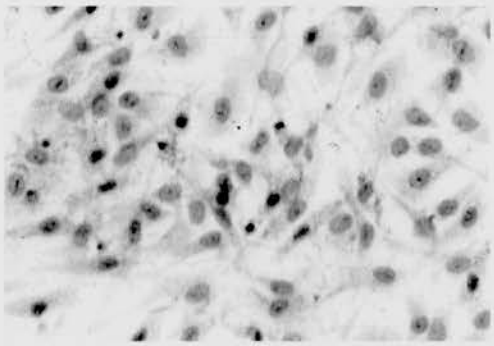


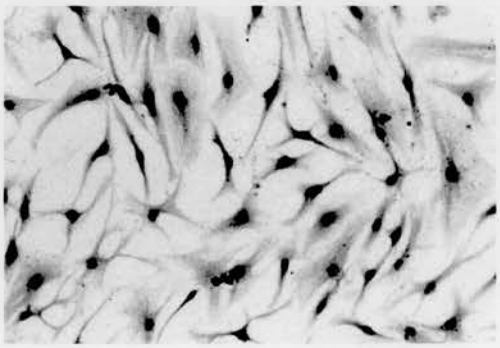
Figure 3.4a – Immunocytochemical staining of cultured HBC: expression of IL-1β in non-stimulated cells and cells stimulated by 0.33 Hz mechanical stimulation (original magnification x 200).



Negative Control
(no Primary Antibody)



Interleukin 1-RI



Interleukin 1-RII

Figure 3.4b – Immunocytochemical staining of cultured HBC: expression of IL-1β RI and II in non-stimulated cells (original magnification x 100).

3.3 Discussion.

3.3.1 HBC Obtained by Outgrowth from Trabecular Bone Fragments Exhibit an Osteoblastic Phenotype.

This study has demonstrated that HBC can be obtained by outgrowth from human trabecular bone fragments, and that these cells exhibit a phenotype which is characteristic of osteoblastic cells. Alkaline phosphatase activity is high in resting osteoblasts and increases following incubation with vitamin D_3. Although the production of alkaline phosphatase is helpful in the identification of osteoblasts, alkaline phosphatase expression is not specific to bone cells and is expressed in a number of different cell types including endothelial cells (Goldring, 1978; Beresford et al, 1984). More important distinguishing features of osteoblastic cells include the production of an extracellular matrix comprising predominantly of type 1 collagen (without type III collagen) and certain bone-specific non-collagenous proteins such as osteocalcin (Auf'mkolk et al, 1985; Robey et al, 1985) and osteopontin. In the present study, RT-PCR has been used to show that the HBC obtained by outgrowth from trabecular bone fragments exhibit a phenotype consistent with that of the osteoblast *in-vivo*. Using RT-PCR, it has been demonstrated that these cells express mRNA for type 1 collagen, osteocalcin, osteopontin and BMP-4.

Every bone explant is extremely heterogeneous, containing a wide range of different cell types. These cell types include osteoblasts, fibroblasts, osteocytes, osteoclasts, marrow stromal cells, endothelial cells and haemopoietic cells. Given this cell type heterogeneity, the method used to extract the bone cells will ultimately determine the characteristics of the resulting culture.

The HBC cultures obtained in these studies are grown directly from trabecular bone fragments which have been washed vigorously in sterile PBS to remove any non-adherent marrow stromal cells, but have not been subjected to any form of enzymatic digestion (Beresford et al, 1984). The resulting culture is heterogeneous containing a mixture of osteoblast-like cells and fibroblasts. Osteocytes are not usually seen as they are buried deep within the trabecular bone, housed within their lacunae. This method of bone cell extraction has several advantages and disadvantages when

compared to other extraction methods. Whereas no *in-vitro* system can truly represent the *in-vivo* environment, it can be argued that this method of bone cell extraction is comparable to the *in-vivo* situation as a mixture of different cell types are present (osteoblast-like cells and fibroblasts) which are very diverse in terms of differentiation state (evidenced by increases in alkaline phosphatase expression following vitamin D_3 incubation). However, depending on the nature of the sample (male versus female, young versus old) there is potential for outgrowth of different populations of cells in varying proportions (Robey et al, 1985), so that cultures from individual donors may be very diverse in terms of the ratios of different cell types present, and therefore difficult to compare. The heterogeneity of the culture also makes it difficult to study the effects of stimuli on osteoblasts specifically, as all the cells in the culture are exposed to the stimuli. This is especially a consideration when performing techniques such as RT-PCR where, following stimulation, all the cells in the culture are lysed and subjected to analysis. Therefore, one cannot be sure which cells in the culture are responding to the stimulation. It is not known if all the cells in the culture respond to the stimulation in a similar or a different manner, or if only a proportion of the cultured cells respond. In the case of electrophysiology, the problem is not quite as prevalent as one can distinguish between osteoblasts and fibroblasts when observing the cells down the microscope, and thus ensure that the desired cell type is impaled. Distinguishing between different cell types becomes more difficult as cell confluency increases, as the close proximity of the cells makes it difficult to distinguish the spindle shaped morphology of the fibroblast from the polygonal morphology of the osteoblast. This is therefore the reason why electrophysiological experiments are performed on sub confluent cultures.

Another method commonly employed in the extraction of bone cells from trabecular bone fragments is the release of cells by sequential digestion with collagenase and trypsin (Luben et al, 1976). Using this technique, osteoblastic cells are typically released at the 1 to 2 hour time points (Wong et al 1975). This technique has the advantage that it can provide cells of a later, more mature osteoblastic phenotype, since less well differentiated outer surface cells have been removed by the enzymatic digestion. In addition, this method minimises contamination by fibroblastic cells,

which reduces the problem of fibroblastic overgrowth in the culture and makes it easier to elucidate osteoblast specific responses. This method has the limitation however that relatively low numbers of osteoblasts are obtained and that in order to achieve sufficient numbers of osteoblasts for experimentation, the cells are subject to lengthy periods of culture and multiple passaging. It has been suggested that older cell cultures may dedifferentiate towards a fibroblastic or preosteoblastic phenotype, as it has been shown that older HBC cultures express increasing levels of collagen III which is known to be expressed in fibroblasts but not in osteoblasts (Scott et al; 1980). A modification of this technique is to eliminate all soft-tissue elements (even though some are of the osteoblast lineage), by pre-treatment of the fragment with collagenase followed by culture of the 'bare bone' explant (Robey et al, 1985; Robey et al 1995). Scanning electron microscopy has documented the emergence of cells from these collagenase treated fragments which have osteoblastic phenotype.

Irrespective of the exact method of bone cell extraction from trabecular bone fragments, it is clear that the cells obtained will be of osteoblastic lineage (Marie, 1989; Gundle, 1995; Voegele, 2000). As such, they serve as useful tools in the determination of bone cell responses to stimuli, the evaluation of intracellular signalling pathways, and can provide a source of protein or mRNA for studies at the protein or genomic level (e.g. Davies et al, 2000).

CHAPTER 4.

THE EFFECT OF CYCLIC MECHANICAL STIMULATION ON HBC IN CULTURE.

4.1 Electrophysiological Response of HBC to Cyclical Mechanical Stimulation.

HBC obtained from adult and juvenile donors were subjected to mechanical stimulation at 0.33 Hz for 20 minutes. Pressure pulses of 0.025 atmospheres above atmospheric pressure (30 mmHg) were used and resulted in ~4000 μstrain on the base of the 58 mm plastic culture dish. The electrophysiological response of both adult and juvenile HBC in culture to this regime of mechanical stimulation was found to be membrane hyperpolarisation. The baseline resting membrane potential and degree of response varied from experiment to experiment when cells from different donors were used. Possible reasons for this are discussed in section 4.4.1.

The data are assumed to be normally distributed, an assumption that seems reasonable given a visual inspection of dotplots compiled from the data **(Figure 4.1),** and to have equality of variances, which also seems reasonable. Therefore, a generalised linear model was fitted to the data collected pre- and post mechanical stimulation from both adult and juvenile HBC **(Appendix 1)**. The model used was;

$$\mu_{ij} = \mathbf{k} + \alpha_i + \beta_j$$

where k, α and β are parameters to be estimated from the data for **i** = c (child) or a (adult) and **j** = b (before MS) or af (after MS). We set $\alpha_c = -\alpha_a$ and $\beta_b = -\beta_{af}$.
This model was used to investigate two hypotheses;

1. Mechanical stimulation has no effect on membrane potential.
2. The membrane potentials of adult and juvenile bone cells are not significantly different.

Initially, a model including an interaction term between donor age category and pre- or post mechanical stimulation was fitted which allowed investigation of the hypothesis that the magnitude of response of adult and juvenile cells is not

significantly different. This interaction term was not found to be significantly different from zero, indicating that both juvenile and adult membrane potentials increase equally following mechanical stimulation. **(illustrated graphically in Figure 4.2)**. Such findings lead to the acceptance of this hypothesis and therefore, as a result, this parameter was excluded from the model. The effects of transforming the data, (i.e. into natural logarithms and square roots) were also investigated. Such transformations did not improve the fit much and so were omitted.

The resulting parameter estimates are given in **Table 4.1**.

Parameter	Estimate	Standard Error	P-Value
k (constant)	40.9194	0.4102	< 0.0001
α_C	-0.9806	0.4102	0.017
α_a	0.9806	0.4102	0.017
β_b	-10.1543	0.4100	< 0.0001
β_{af}	10.1543	0.4100	< 0.0001

Table 4.1 – Parameter estimates for generalised linear model.

The p-values in the above table correspond to the null hypothesis that the parameter is 0. The p-values are all significant, indicating that $\alpha_C \neq \alpha_a$ and $\beta_b \neq \beta_{af}$.
This means that the null hypothesis that the experiment has no effect on membrane potential can be rejected, as μ_{cb} is significantly different from μ_{caf}. Similarily, μ_{ab} is significantly different from μ_{aaf}.
The results also mean that the null hypothesis that the membrane potentials of adult and juvenile bone cells are equal can be rejected, as there is a significant difference between μ_{cb} and μ_{ab} and between μ_{caf} and μ_{aaf}. This difference appears to be small however (estimated at 1.9612). What we **can** say however, is that the effect of MS on membrane potential is the same on both adult and juvenile HBC, due to the rejection of a significant interaction term.

In conclusion:

- **Mechanical stimulation causes significant cell membrane hyperpolarisation in both juvenile and adult cells.**
- **There is a significant difference between the resting membrane potentials levels between adult and juvenile cells. Similarily, there is a significant difference in the membrane potentials observed (Figure 4.2) following mechanical stimulation between adult and juvenile cells.**
- **There is no significant difference in the magnitude of the response to mechanical stimulation when comparing adult and juvenile cells (Figure 4.2).**

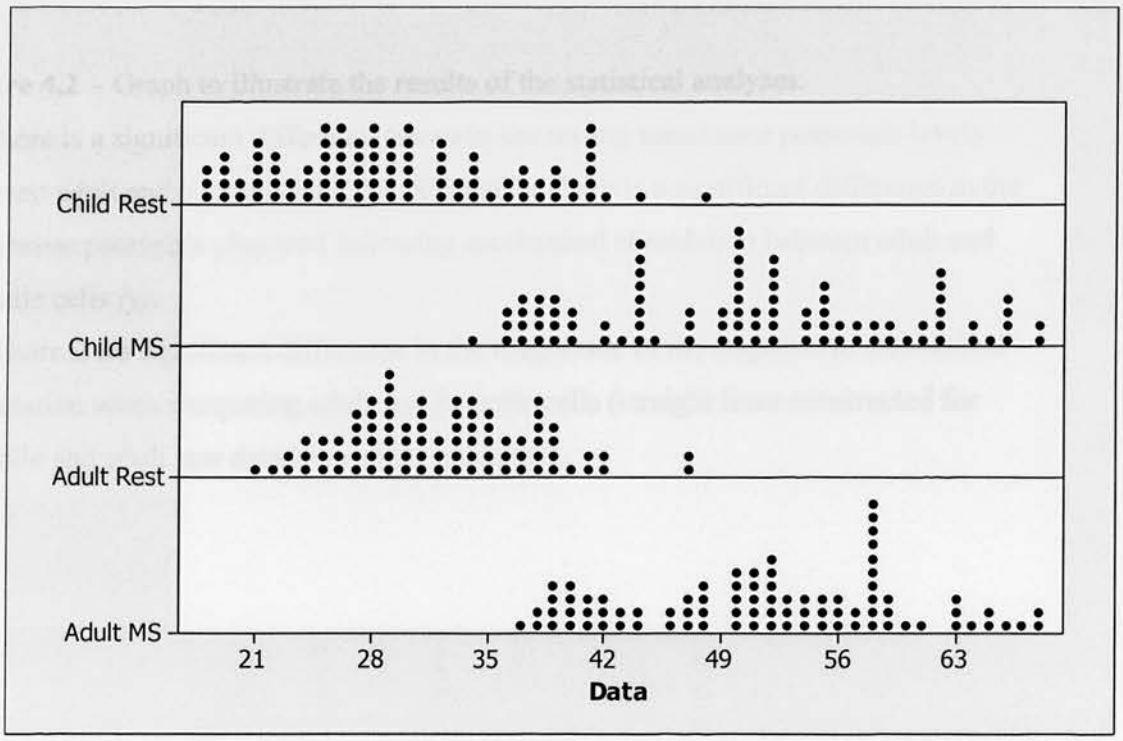


Figure 4.1 – Dotplot to show distribution of data.

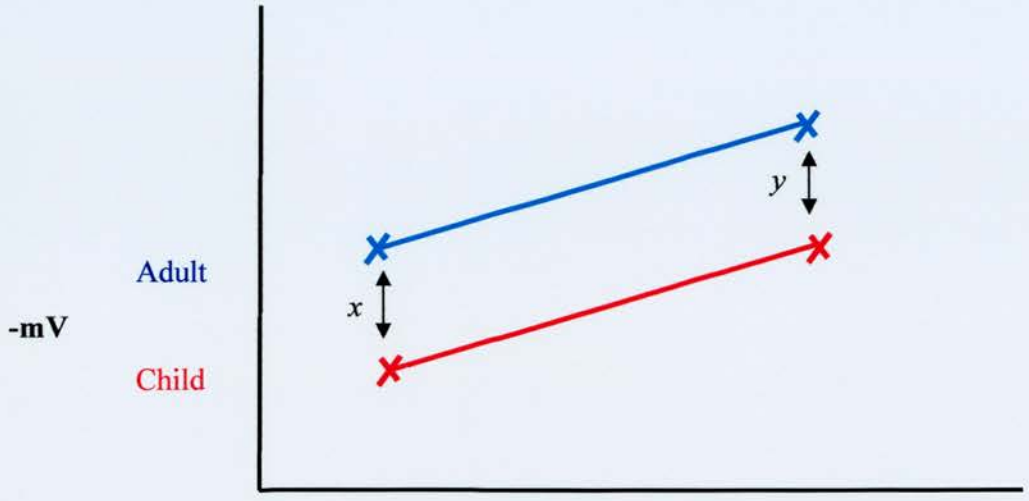


Figure 4.2 - Graph to illustrate the results of the statistical analyses.
(i) There is a significant difference between the resting membrane potentials levels between adult and juvenile cells *(x)*. Similarily, there is a significant difference in the membrane potentials observed following mechanical stimulation between adult and juvenile cells *(y)*.
(ii) There is no significant difference in the magnitude of the response to mechanical stimulation when comparing adult and juvenile cells (straight lines constructed for juvenile and adult raw data have equal gradients).

4.2 Interleukin–1β (IL-1β) is Released From Mechanically Stimulated HBC and Can Induce Membrane Hyperpolarisation.

The full numerical results of individual electrophysiological experiments from which the values given in the Tables in this chapter are derived are given in Appendix I. Results shown in this chapter are from a single representative experiment. Experiments were repeated from HBC derived from at least 3 adult or juvenile donors and were reproducible between donors.

Previous research has identified a transferable factor, shown to be IL-1β, which is released from HBC following mechanical stimulation and which acts in an autocrine / paracrine manner to induce cell membrane hyperpolarisation (Salter et al, 1997; Salter et al, 2000). The stimulation chambers used in the present study are, however, a modification of those used in the prior experiments, and therefore, it was necessary to repeat experiments to ensure that the results obtained using the new stimulation chambers are consistent with those previously obtained.

Conditioned Medium (CM) experiments were performed to confirm that, following mechanical stimulation, a transferable factor (IL-1β) is released by the cells which can cause cell membrane hyperpolarisation when transferred to resting cells. For these experiments, a dish of HBC was stimulated by 0.33 Hz cyclic mechanical stimulation and the medium (CM) immediately transferred to a dish of unstimulated HBC, membrane potential being assessed 20 minutes after transfer of media. In all studies, at least three experiments were performed on different days under each condition.

The results show that CM from cells stimulated by 0.33Hz mechanical stimulation, when added to unstimulated bone cells for 20 minutes, resulted in cell membrane hyperpolarisation of these cells **(Table 4.2)**. These observations confirm the presence of a soluble transferable factor secreted by 0.33 Hz mechanically stimulated HBC. Recombinant IL-1β (10 pg/ml) when added to resting HBC for 10 minutes,

induced a membrane hyperpolarisation response that was similar in extent to that seen following 0.33 Hz mechanical stimulation or CM transfer **(Table 4.3)**. Previous experiments, utilising neutralizing anticytokine antibodies against IL-1β, have confirmed that IL-1β is necessary for the membrane hyperpolarisation response to mechanical stimulation (Salter et al 2000).

Table 4.2 – Effect of CM from HBC mechanically stimulated for 20 minutes, on the membrane potential of previously unstimulated HBC.

		Membrane potential (-mV) (mean ± 1 SEM)			
Stimulus	*n*	*Resting*	*Post MS (% change)*	*Resting*	*With-CM (% change)*
0.33 Hz MS	5	27.6 ± 1.65	43.0 ± 3.26 (+56%)*	24.8 ± 1.03	37.8 ± 1.07 (+52%)*

Results shown are from a single experiment and are consistent between experiments and between cells from 4 different donors (Appendix I).

* $p < 0.05$. MS = mechanical stimulation.

Table 4.3 – Effect of recombinant IL-1β on HBC membrane potential.

		Membrane Potential (-mV) (mean ± 1 SEM)		
Reagent	*n*	*Resting*	*With Reagent*	*% change*
IL-1β	5	29.4 ± 1.33	51.4 ± 2.42	+74.8*

Results shown are from a single experiment and are consistent between experiments and between cells from 3 different donors (Appendix I).

* $p < 0.001$.

4.3 The Effect of Mechanical Stimulation of HBC on Relative Levels of Collagen type 1 and Bone Morphogenetic Protein - 4 (BMP-4) mRNA Expression.

Semi-quantitative RT-PCR was performed to investigate if mechanical stimulation has any effect on the relative levels of type 1 collagen (coll 1) and bone morphogenetic protein-4 (BMP-4) mRNA expression in HBC. Throughout the semi-quantitative RT-PCR experiments, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. GAPDH is a suitable control for these experiments, as GAPDH transcription levels do not alter following mechanical or cytokine stuimulation. Therefore, the use of GAPDH primers serve as an internal reference, allowing the experimenter to determine whether the transcription levels of the mRNA of interest have altered in response to the experimental / test conditions.

Before commencing the semi-quantitative RT-PCR experiments, it was necessary to determine the logarithmic phase of amplification for each primer pair used. This was necessary to ensure that subsequent semi-quantitative RT-PCR experiments are carried out within the exponential range for both the GAPDH primer pair and the primer pair of interest.

4.3.1 Determination of the Logarithmic Phase of Amplification for PCR Primer Pairs.

Prior to performing semi-quantitative RT-PCR, the exponential range of amplification was determined for the GAPDH, collagen type 1 and BMP-4 primer pairs. In the cases of GAPDH and collagen type 1, PCR products were removed upon completion of every second cycle between 18 and 34. In the case of BMP-4, PCR products were removed upon completion of every cycle between 22 and 36. PCR products were separated upon a 1% agarose gel and quantified using the EASY imaging software.

For GAPDH, cycle numbers that lie between 22 and 30 fall in the exponential range **(Figure 4.3)**.

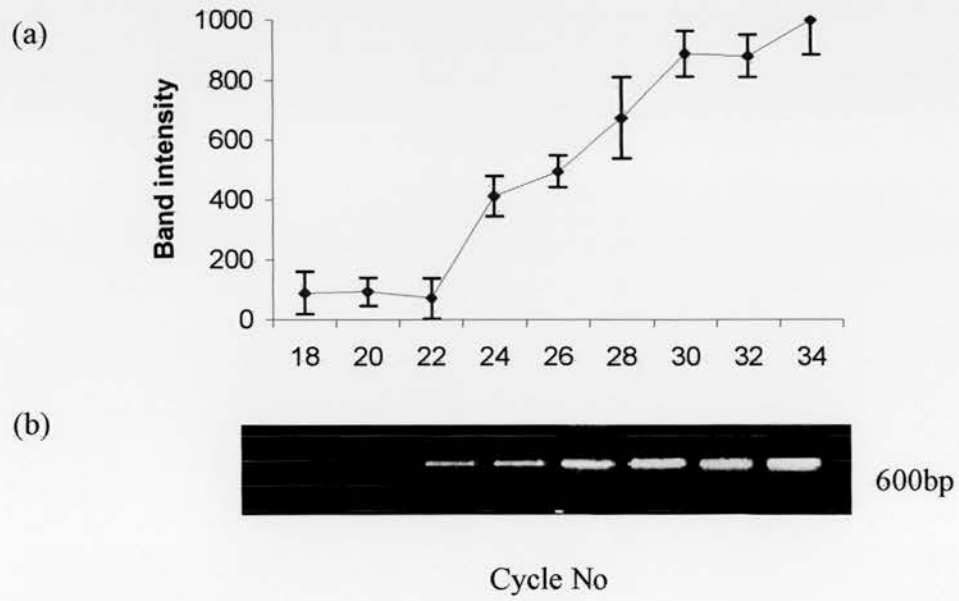


Figure 4.3 – PCR titration to determine the exponential range of amplification for GAPDH primers. (a) Pooled data for 3 donors performed in duplicate (n=6). (b) Representative scanned agarose gel.

For collagen type 1, cycle numbers that lie between 22 and 30 fall in the exponential range **(Figure 4.4)**.

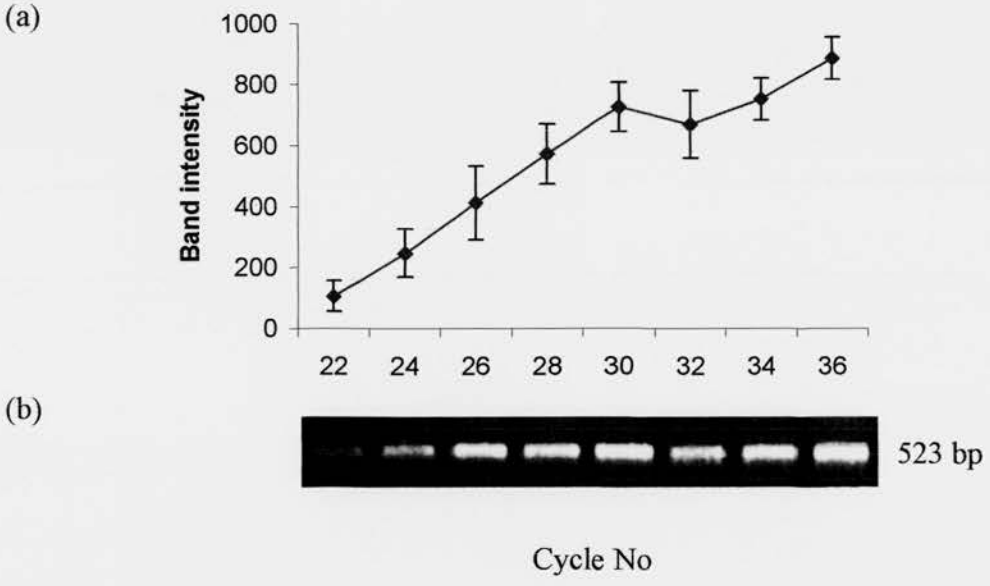


Figure 4.4 – PCR titration to determine the exponential range of amplification for collagen type 1 primers. (a) Pooled data for 3 donors performed in duplicate (n=6). (b) Representative scanned agarose gel.

For BMP-4, cycle numbers that lie between 26 and 34 fall in the exponential range. **(Figure 4.5)**.

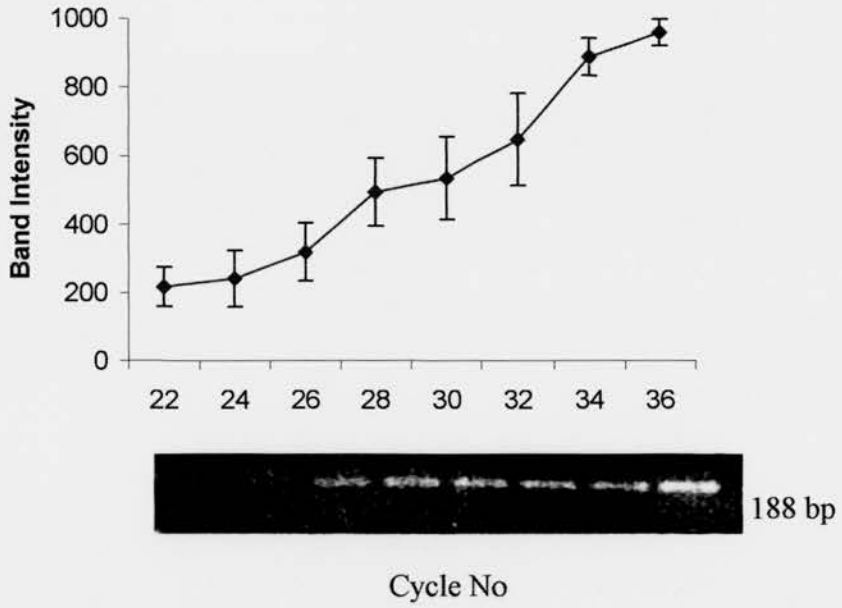


Figure 4.5 – PCR titration to determine the exponential range of amplification for BMP-4 primers. (a) Pooled data for 3 donors performed in duplicate (n=6).

(b) Representative scanned agarose gel.

4.3.2 Collagen type 1 mRNA Expression is Upregulated Following Cyclical Mechanical Stimulation.

HBC, cultured in 58 mm culture dishes, were mechanically stimulated at 0.33 Hz, 30 mmHg for 20 minutes. Following stimulation, HBC were incubated at 37°C, 95% CO_2, 5% O_2 for various lengths of time (0, 1,3, 6 or 24 hours). Following this incubation period, RNA was extracted and quantified. RNA was reverse transcribed to obtain cDNA. Semi-quantitative PCR was than performed to investigate the effect of mechanical stimulation on collagen 1 mRNA levels in HBC.

The results are shown in **Figure 4.6**. On the basis of previous experiments to obtain the logarithmic phase of amplification for the GAPDH and collagen type 1 primers, PCR samples were removed following either 26 or 30 cycles of amplification, separated on a 1% agarose gel and quantified using the EASY imaging software. **Figure 4.6** demonstrates that collagen type 1 mRNA expression is increased in response to 20 minutes 0.33 Hz mechanical stimulation after 1, 3, 6 and 24 hours incubation compared to the non-stimulated (NS) control, although these increases do not reach statistical significance (Mann-Whitney U-test). The increase in collagen type 1 mRNA levels is gradual, reaching a peak 6 hours after stimulation. Collagen type 1 mRNA levels are reduced at 24 hours post stimulation (compared to 6 hours) although this decrease does not reach statistical significance (Mann-Whitney U-test).

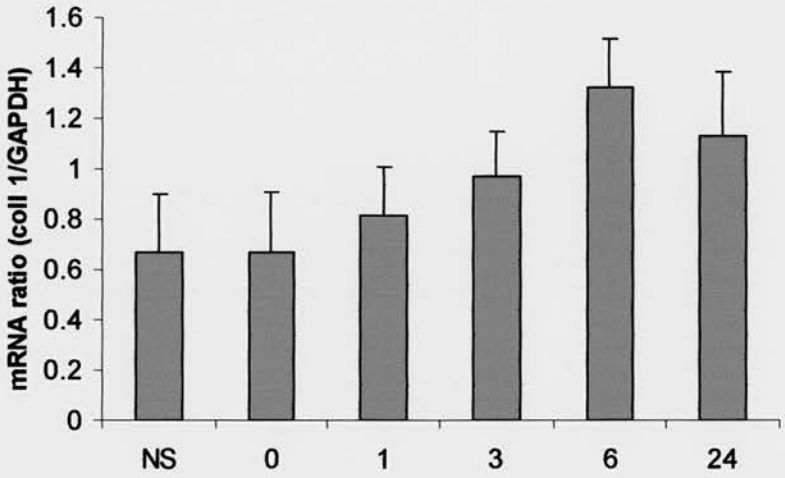


Figure 4.6 – The effect of mechanical stimulation on collagen type 1 mRNA expression in HBC. Increased collagen type 1 mRNA expression following mechanical stimulation. Pooled data for 3 donors performed in duplicate (n=6). Error bars are +1 SEM. NS: non-stimulated; 0, 1, 3, 6 and 24: time in hours after 0.33 Hz mechanical stimulation.

4.3.3 BMP-4 mRNA Expression is Decreased Following Mechanical Stimulation.

HBC, cultured in 58 mm culture dishes, were mechanically stimulated at 0.33 Hz, 30 mmHg for 20 minutes. Following stimulation, HBC were incubated at 37°C, 95% CO_2, 5% O_2 for various lengths of time (0, 1, 3, 6 or 24 hours). Following this incubation period, RNA was extracted and quantified. RNA was reverse transcribed to obtain cDNA. Semi-quantitative PCR was than performed to investigate the effect of mechanical stimulation on BMP-4 mRNA levels in HBC.

The results are shown in **Figure 4.7**. On the basis of previous experiments to obtain the logarithmic phase of amplification for the GAPDH and BMP-4 primers, PCR samples were removed following either 26 or 30 cycles of amplification, separated on a 1% agarose gel and quantified using the EASY imaging software. **Figure 4.7** demonstrates that BMP-4 mRNA expression is significantly decreased ($p < 0.01$) in response to 20 minutes 0.33 Hz mechanical stimulation after 3, 6 and 24 hours incubation compared to the non-stimulated (NS) control (Mann-Whitney U-test). There is also a decrease in BMP-4 mRNA expression 1 hour after mechanical stimulation but this decrease does not reach statistical significance.

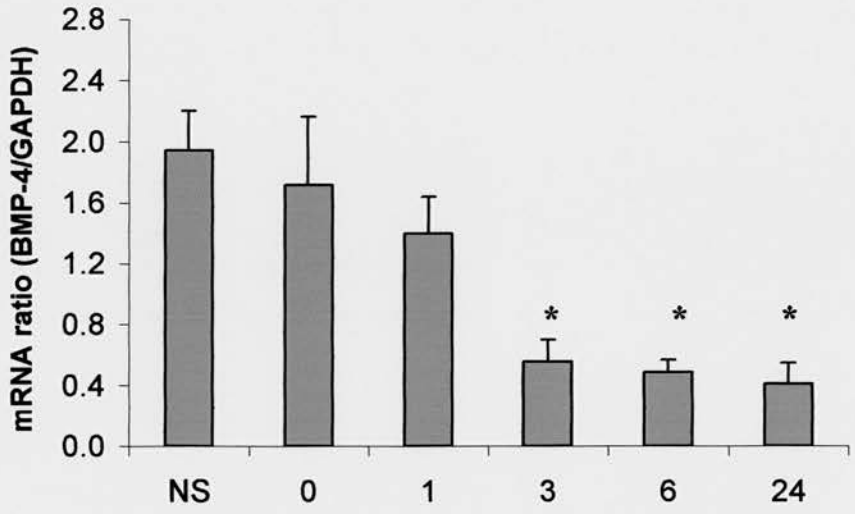


Figure 4.7 – The effect of mechanical stimulation on BMP-4 mRNA expression in HBC. Decreased BMP-4 mRNA expression following mechanical stimulation ($p < 0.01$). Pooled data for 3 donors performed in duplicate (n=6). Error bars are +1 SEM. NS: non-stimulated; 0,1,3,6 and 24: time in hours after 0.33 Hz mechanical stimulation.

4.4 Discussion.

4.4.1 Mechanical Stimulation Activates an Integrin Dependant Signalling Pathway in HBC.

It has previously been demonstrated that mechanical stimulation at 0.33 Hz results in membrane hyperpolarisation of HBC (Salter et al; 1997). The mechanotransduction pathway involved has been shown to include α_5 β_1 integrin, stretch activated ion channels, the actin cytoskeleton and tyrosine kinase activity. It has also been shown that, following 0.33 Hz mechanical stimulation, IL-1β is released which acts in an autocrine / paracrine way to initiate a signalling cascade which involves phospholipase C, diacylglycerol (DAG) and protein kinase C (PKC), leading to the activation of small conductance Ca^+ activated K^+ (SK) channels and resultant membrane hyperpolarisation (Salter et al; 2000). Experiments utilising neutralising antibodies have demonstrated that antibodies to IL-1β but not to other cytokines studied, inhibited the hyperpolarisation response to mechanical stimulation (Salter et al; 2000). It has also been shown that specific blockade of the $\alpha_5\beta_1$ heterodimer with inhibitory antibodies, incubation with Arg-Gly-Asp (RGD) containing peptides, or disruption of the actin cytoskeleton with cytochalasin D inhibits mechanically induced membrane hyperpolarisation but has no effect on the hyperpolarisation produced by exogenously added IL-1β (Salter et al, 2000). These results suggest that the integrin antibodies and RGD peptides have a role upstream of IL-1β release in the mechanotransduction pathway, and also that $\alpha_5\beta_1$ integrin acts as an HBC mechanoreceptor, causing the activation of a distinct signalling pathway which leads to the release of IL-1β.

The mechanical stimulation system used in the present study is a modification of that described previously (Wright et al, 1992; Wright et al, 1996; Salter et al 1997, Wright et al, 1997; Salter et al, 2000; Lee et al, 2000), and therefore a range of experiments were repeated to confirm that the results obtained were consistent with those previously reported. The new mechanical stimulation system differs from the old system in several ways.

In the new system a 58 mm culture dish of HBC is supported on a rubber 'O' ring in the stimulation chamber, creating a chamber above and below the culture dish. The lid is screwed down and holds the dish in position. The lid and the 'O'-ring ensure an airtight seal between the chambers below and above the dish. When pressure is applied to the system, the gas enters the chamber below the culture dish via an inlet valve. This causes an increase in pressure in the chamber below the culture dish, which causes the base of the culture dish to curve upwards. As a consequence the cells are subjected to strain (measured in μstrain).

In the old system, the culture dish was supported on a small cylinder, which was attached to the base of the stimulation chamber. A tight seal was present between the top of the cylinder and the culture dish. A space of volume 8 ml was present between the base of the culture dish and the base of the chamber and the gas entered this space via 18 holes, each 2.2 mm in diameter in the small cylinder. Cyclical pressurisation of this system induced deformation and strain of the base of the culture dish and its adherent cells due to a differential rate of rise of pressure above and below the tissue culture dish.

The new system has several advantages when compared to the old system. The culture dish is deformed as a result of increased gas pressure on the underside of the dish while the pressure of the upper side remains unchanged. Therefore, the cells are subjected to stretch without being subjected to an increase in pressure or exposure to nitrogen gas. Both systems have the disadvantage, however, that they do not have the 95% O_2, 5% CO_2 humidified atmosphere of the tissue culture incubator, and therefore cells being exposed to mechanical stimulation are deprived of both O_2 and CO_2 throughout the course of the stimulation. It could therefore be argued that CO_2 and O_2 starvation (and not the cyclical mechanical stimulation) may account for the change in membrane potential observed following 20 minutes stimulation. This problem is overcome by placing the non-stimulated control plates in the stimulation incubator alongside dishes being stimulated, which exposes them to the same O_2 and CO_2 conditions as the stimulated dishes. As there is not a significant change to the membrane potential of the control dishes following exposure to these conditions, it

can be concluded that changes in the membrane potential of test plates must be as a direct result of the cyclical mechanical stimulation. Both stimulation systems also have the added complication that fluid shear may contribute to the strain sensed by the cells, and thus to the response initiated by the cells. During the cyclical mechanical stimulation, the strain on the base of the culture dish is 4000 μstrain, which is equivalent to 0.40% deformation. Movement of fluid is slight but noticeable to the naked eye, but it is not known how much this fluid shear contributes to the mechanical stimulation sensed by the cells.

The present studies have demonstrated that HBC derived from trabecular bone show a consistent and reproducible membrane hyperpolarisation response to 0.33 Hz when mechanically stimulated using the modified system. CM experiments also suggest that following mechanical stimulation, IL-1β is released and acts as a transferable factor. These results are confirmed by experiments which utilise recombinant IL-1β in place of mechanical stimulation. Attempts to quantify IL-1β concentrations using commercially available ELISA kits have proved unsuccessful. Possible reasons for this include (a) that the concentration of IL-1β is below the limit of detection for the assay and (b) that IL-1β release is a tightly regulated process and that, upon its release, IL-1β is immediately taken up by other HBC. In the present studies, immunocytochemistry has been used to demonstrate the presence of IL-1β in both unstimulated and mechanically stimulated HBC. The presence of IL-1 Receptors I and II have also been demonstrated in HBC. Whilst these observations are useful, a quantitative assay would have provided more information.

It was noted whilst performing electrophysiological experiments that there was a degree of variation between the baseline resting membrane potential when comparing HBC obtained from different donors, although the degree of hyperpolarisation observed following cyclical mechanical stimulation did not differ significantly between donors. Cell confluency is thought to contribute to this, and although cells were plated at the same density (5 x10^4 cells/ml) for every electrophysiological experiment performed, after the 48-hour incubation period there were noticeable variations in cell confluency between donors. Biological variance

(i.e. age and sex of donor) is the most likely explanation for these differences. It has been previously reported that resting membrane potential increases as the density of the culture increases (Bard et al, 1974) **(Figure 4.8)**, and it has been suggested that, as cell confluency increases, there may be alterations in the permeability of the cell membrane to sodium, chloride or potassium, the three ions principally responsible for maintaining and altering membrane potential levels. It has also been shown that the substrate on which cells are cultured can have effects on resting membrane potential level (Bard et al, 1974), with fibroblastic cells cultured on collagen having higher resting membrane potentials than the same cells cultured on plastic. Thus, confluent cells may have higher resting membrane potentials, as they are likely to have produced a considerable amount of extracellular matrix which they will have forged strong attachments to.

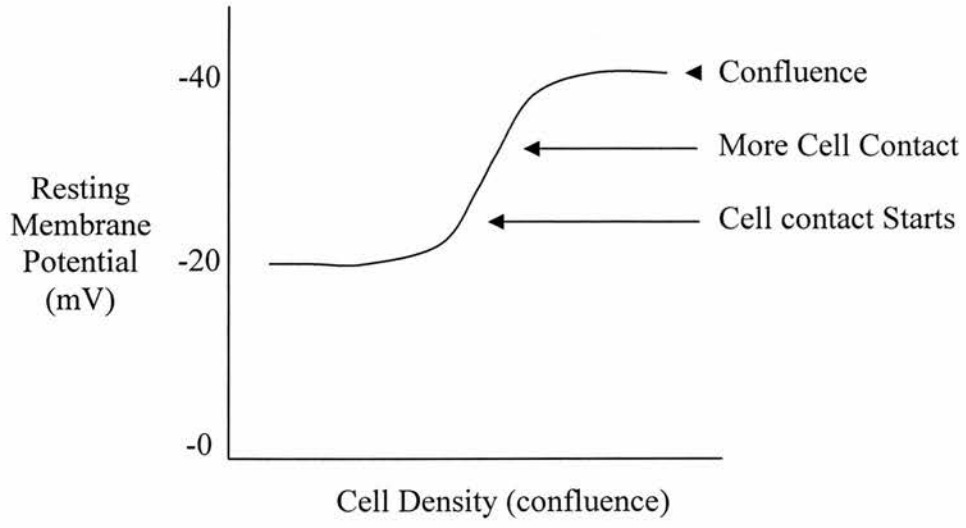


Figure 4.8 – Effect of cell confluency on resting membrane potential.

4.4.2 Collagen type 1 mRNA Expression is Upregulated Following Cyclical Mechanical Stimulation.

This study has demonstrated that, following a single 20 minute period of 0.33 Hz mechanical stimulation, collagen type 1 mRNA levels increase and remain elevated for up to 24 hours post stimulation.

Several studies, utilising several different osteoblast-like cells and different *in-vitro* mechanical stimulation techniques, have provided somewhat variable evidence regarding the effect of mechanical stimulation on collagen type 1 synthesis and secretion. Some studies have reported increases in collagen type 1 synthesis following mechanical stimulation whereas others have observed a decrease in collagen type 1 synthesis following mechanical stimulation.

One study (Harter et al, 1995) reported that, following 48 hours mechanical stimulation at a frequency of three cycles per minute, there is a 3.4 fold increase in collagen type 1 mRNA expression compared to non-stimulated controls. This study also utilised immunofluorescence to demonstrate that the increase in collagen type 1 mRNA can be attributed to an increase in the production of procollagen. Another study (Buckley et al, 1990) has measured the synthesis and secretion of collagenous and non-collagenous proteins following 24 - 72 hours of mechanical stimulation at a frequency of 3 cycles per minute. They reported that, following 24 hours of mechanical stimulation, there is a small but not-significant increase in cellular collagen type 1 synthesis. Following 72 hours of mechanical stimulation there was a significant increase in collagen type 1 synthesis, although there did not appear to be any significant changes noted in collagen secretion.

In contrast to the above results and those in the present study, several studies have presented evidence that collagen type 1 synthesis is decreased following mechanical stimulation (Ozawa et al, 1990; Brighton et al, 1991). One study (Brighton et al; 1991) reported that calvarial bone cells isolated from rats responded to mechanical stimulation with increased proliferation but decreased production of collagen type 1

and alkaline phosphatase. They also noted that these effects could be elicited by strain durations of as little as 15 minutes per day.

These conflicting observations of levels of collagen type 1 synthesis are not unique. It would appear that the response of the osteoblast to mechanical strain is variable on a number of levels. Several studies have reported increases in cell proliferation (Buckley et al, 1988; Hasagawa et al, 1985) and total protein production (Hasagawa et al, 1985), whereas others have reported a decrease in these responses following mechanical stimulation (Burger et al, 1992; Veldhuijzen et al, 1979). The lack of a consistent response may be attributed to several factors, which vary considerably between these investigations.

1. The source of the osteoblast like cells are likely to be an important factor, as is whether the cultures were derived from primary cells or a transformed cell line.
2. The method of cell extraction utilised and the purity of the resulting cultures are extremely important to the characteristics of the culture and are likely to affect the results obtained.
3. Stimulation frequency has been shown to be extremely important in the responses of cells to mechanical stimuli, and it is known that different intracellular signalling pathways are activated by different stimulation frequencies. It has previously been shown that HBC mechanically stimulated at 0.33 Hz for 20 minutes undergo membrane hyperpolarisation via a pathway involving $\alpha_5\beta_1$ integrin and Ca^{++} activated K^+ channels, but that the same cells, stimulated for 20 minutes at 0.104 Hz at the same pressure undergo membrane depolarisation via a separate pathway involving $\alpha_V\beta_5$ integrin and tetrodotoxin-sensitive Na^{++} channels (Salter et al, 1997).
4. The system used to stimulate the cells is of importance when considering their response. Many systems have been developed to allow mechanical stimulation of cells *in-vitro*, while attempting to recreate the *in-vivo* environment (Brown, 2000). Such systems include compressive loading systems, which allow direct pressurisation of cells in a contained vessel (Bourret et al, 1976; Lippiello et al, 1985; Klein-Nulend et al, 1986; Burger et al, 1992; Brighton et al 1996), substrate stretch systems in which cells are grown on a deformable substrate (Ives et al, 1986;

Bottlang et al, 1997), out-of-place circular substrate distension systems (as used in the present study) and fluid shear systems (Hermann et al, 1997; Chun et al, 1997).

5. It has been proposed that the magnitude of mechanical stimulation is of great importance to the cellular responses (Burger and Veldhuijzen, 1993). At high levels of strain, it is believed that the osteoblast responds with proliferative activity and a decrease in the production of osteoblast phenotyopic markers such as alkaline phosphatase and bone matrix proteins, but that at lower levels of strain the response of the osteoblast indicates a more differentiated state, with an increase in alkaline phosphatase activity and matrix protein production, and a decrease in proliferation. The cells in the present study are subjected to approximately 4000 μstrain, which results in 0.4% deformation on the base of the tissue culture plate. This strain magnitude is extremely small when compared to previous studies. Harter et al (1995), applied deformations of 12% to the base of the culture dish, which was equivalent to 120,000 μstrain, whereas Buckley et al (1990), applied deformations of 24% to the base of the tissue culture dish, which is equivalent to 240,000 μstrain. Both of these studies reported an increase in collagen type 1 synthesis and secretion, which may appear to contradict the hypothesis of Burger and Veldhuijzen. Both studies have used the Flexercell ® system however, which applies a heterogeneous magnitude of strain across the base of the tissue culture plate which falls to 0 μstrain at the centre of the dish (Gilbert et al, 1994). Therefore, in actuality, the strain delivered to the osteoblasts is significantly lower than the maximum strain across the majority of the tissue culture plate. It has previously been demonstrated that the heterogeneity of strain across the base of the culture dish affects collagen type 1 secretion (Harter et al, 1995). Collagen type 1 synthesis appeared to be much higher in areas of the tissue culture plate that experienced moderate levels of strain. In addition, at the edge of the plate, where osteoblasts experienced the greatest strain, there was little or no secretion of collagen type 1. In the present study, which utilises strain levels much lower than those previously reported, most of the cells will be exposed to moderate or low strain magnitudes. Therefore, this is a possible explanation as to why collagen type 1 levels increase following 20 minutes 0.33 Hz, 30 mmHg cyclical mechanical stimulation.

4.4.3 Bone Morphogenetic Protein – 4 (BMP- 4) mRNA Expression is Decreased Following Cyclical Mechanical Stimulation.

The present study has demonstrated that following a single 20 minute period of 0.33 Hz mechanical stimulation, BMP-4 mRNA levels are decreased and remain depressed for up to 24 hours post stimulation.

BMP's are a family of multifunctional proteins, which were originally identified as substances that initiate bone and cartilage formation (Reddi, 1992; Reddi, 1994). Several proteins belong to the BMP family, including BMP-2-6 (Wozney et al, 1988; Celeste et al, 1990) and osteogenic proteins (OP) 1 and 2 (Özkaynak et al, 1990; Özkaynak et al, 1992). BMP's have various biological effects on different cell types. They promote differentiation and maturation of preosteoblastic cells and up-regulate features of the mature osteoblast, including alkaline phosphatase activity, collagen synthesis, and osteocalcin expression, whereas in chondroblasts, they stimulate proteoglycan synthesis (Vukicevic et al, 1989).

Although it is well established that BMP family members play important roles in the patterning and development of skeletal tissues during embryogenesis, many studies have failed to show any BMP-4 expression in adult bone tissue. One exception, demonstrated the presence of the BMP-4 gene in normal bone tissue from neonatal animals using RT-PCR (Ikegame et al; 2001). This study also demonstrated that mechanical stimulation induces osteoblast differentiation in mouse calvarial sutures in culture, which was accompanied by an increase in BMP-4 gene expression as evaluated by RT-PCR. These results prompted the investigation of the effect of cyclic mechanical stimulation on BMP-4 mRNA levels in HBC in the present study.

The results obtained contradict those published previously (Ikegame et al, 2001). It was found that following 20 minutes cyclic mechanical stimulation at 0.33 Hz there was a significant decrease in BMP-4 mRNA levels when compared to the non-stimulated controls. Although the reasons why these results are different from those obtained by Ikegame et al, (2001), are not known for certain, there are several major differences between the two studies.

The method of mechanical stimulation is different when comparing the two studies. In the present study, cells were subjected to cyclic mechanical stimulation for 20 minutes at a frequency of 0.33 Hz and RNA extracted 0h, 1h, 3h, 6h or 24h after stimulation, whereas in the study of Ikegame et al, (2001), cells were subjected to continuous tensile stress for 1h, 6h, 24h or 48h before RNA extraction. This difference in stimulation regimes is likely to be significant as degree and duration of stress may be important to the activation of pathways responsible for BMP-4 mRNA expression.

The cells used in the present study are derived from human long bones, whereas the cells used by Ikegame et al, (2001) were obtained from mouse calvarial sutures. This is likely to be significant not only due to the species difference but also as long bones and calvarial sutures are formed by different forms of ossification (endochondral versus intramembranous), which may give rise to differing signalling pathways. Also, the cells derived from the calvarial sutures are likely to be of a very immature osteoblast phenotype and high in BMP-4 levels whereas those used in the present study are more mature and are likely to contain lower BMP-4 levels. The fact that mechanical stimulation causes a decrease in relative BMP-4 mRNA levels which is accompanied by an increase in relative type 1 collagen levels may indicate that the mechanical stimulus that is being provided in the current model is inducing a differentiation / anabolic response, driving the cells towards a more mature active bone cell phenotype.

CHAPTER 5.

THE EFFECT OF MTX ON NORMAL HBC RESPONSES TO CYCLICAL MECHANICAL STIMULATION.

5.1 Methotrexate (MTX) Dose Dependently Inhibits the Electrophysiological Response of HBC to Mechanical Stimulation.

There is a great deal of evidence to suggest that chemotherapy has effects on normal bone growth and mass *in-vivo* (Halton, 1996; Arikoski et al, 1999; Kaste, 1999). Several clinical studies have demonstrated that patients who chemotherapeutic regimes incorporating MTX often experience fractures and pain throughout the course of the treatment, which is commonly accompanied by a decrease in bone mass (O'Regan et al 1973; Preston et al, 1993; Eckland, 1997; Warner, 1999; Stevens et al, 2001; Wijnands et al, 2001). A seperate study (May et al; 1994) demonstrated that low-dose MTX, when administered to rats, caused significant osteopenia. The results also demonstrated that this occurred as a result of suppression of osteoblast activity and stimulation of osteoclast recruitment, resulting in increased bone resorption. *In-vitro* studies have also provided evidence that MTX has inhibitory effects on osteoblast function. It has been shown that MTX diminishes osteoblastic cell function by significantly decreasing osteocalcin levels, matrix calcification and cell adhesion to the culture substrate (May et al, 1996). Similarily, it has also been reported that MTX suppresses bone formation in-vitro in human and mouse cell lines by inhibiting the differentiation of osteoblastic cells (Uehara et al, 2001).

Bone responds to mechanical stimulation with increases in bone mass and osteoblast function (Rubin et al, 1985). Therefore, the possibility exists that MTX may interfere with normal bone cell responses to mechanical stimuli.

The full numerical results of individual experiments from which the values given in the Tables in this chapter are derived are given in Appendix I. Results shown in this chapter are from a single representative experiment. Experiments were repeated from HBC derived from at least 3 adult or juvenile donors and were reproducible between donors.

Experiments were performed to investigate the effects of three concentrations of MTX (1 μM, 50 nM and 1 nM) on the responses of HBC in culture to 0.33Hz mechanical stimulation. These concentrations were chosen, based on the studies of May et al (1996), in which a similar concentration range of MTX had significant effects on mouse osteoblastic cell function *in-vitro*.

The resting membrane potential of 5 cells was assessed prior to the addition of 1 μM, 50 nM or 1 nM MTX. HBC were incubated with MTX for 30 minutes at 37°C following which time, the membrane potentials of another 5 cells were measured. The cells were then subjected to cyclical mechanical stimulation for 20 minutes at 0.33 Hz, 30 mmHg. Following the mechanical stimulation, the membrane potential of a further 5 cells was assessed. MTX itself was found to have no effect on the membrane potential of resting cells, but it dose dependently inhibited the hyperpolarisation response which is normally induced by cyclical mechanical stimulation. Both 1 μM and 50 nM MTX completely inhibited the hyperpolarisation response to cyclical mechanical stimulation and although a degree of hyperpolarisation was observed in mechanically stimulated HBC which had been incubated with 1 nM MTX this hyperpolarisation did not consistently reach control levels **(Table 5.1)**.

Table 5.1 – Effect of MTX on the electrophysiological response of HBC to cyclical mechanical stimulation.

Reagent	*n*	*Membrane Potential (-mV) (mean ± SEM)*			
		Resting Cells	*+ MTX*	*Post 0.33Hz MS*	*% Change (Resting – Post MS)*
Nil	5	32.6 ± 1.29	n/a	54.4 ± 2.87	+ 67% *
MTX;					
1 μM	5	46.2 ± 1.96	45.0 ± 3.99	42.0 ± 3.89	-10% ns
50 nM	5	41.4 ± 2.48	43.2 ± 5.26	39.6 ± 2.69	- 4% ns
1 nM	5	38.0 ± 3.17	37.0 ± 2.30	50.4 ± 4.94	+ 33% *

Results shown are from a single experiment and are consistent between experiments and between cells from 3 different donors (Appendix I).

*$p < 0.05$; ns = not significant. MS = mechanical stimulation.

5.2 The Inhibitory Effects of MTX on the Electrophysiological Response of HBC to Mechanical Stimulation is not a Result of MTX Cytotoxicity.

The results presented above suggest that MTX dose dependently inhibits the HBC electrophysiological response to mechanical stimulation. Three different types of cell toxicity assay were performed to investigate whether this observation was a result of a cytotoxic effect of MTX.

5.2.1 Lactate Dehydrogenase Assays.

Lactate dehydrogenase assays were performed to assess levels of cell necrosis following exposure to differing concentrations of MTX. Lactate dehydrogenase is a stable cytosolic enzyme, which is released from a cell upon its death. Therefore, the concentration of lactate dehydrogenase present in cell culture supernatants can be used to determine the viability of the culture. Lactate dehydrogenase levels were assessed in supernatants removed from control (no MTX) cells, and cells that had been incubated with 1 μM, 50 nM or 1 nM MTX for 90 minutes at 37°C **(Figure 5.1)**. The results show that MTX, at the concentrations studied, and over a 90-minute incubation period does not cause cell necrosis in HBC. Results are expressed as a percentage of the negative control (no MTX). Triton-X 100 (0.2%) was included as a positive control. Other concentrations of MTX and other incubation periods were not investigated.

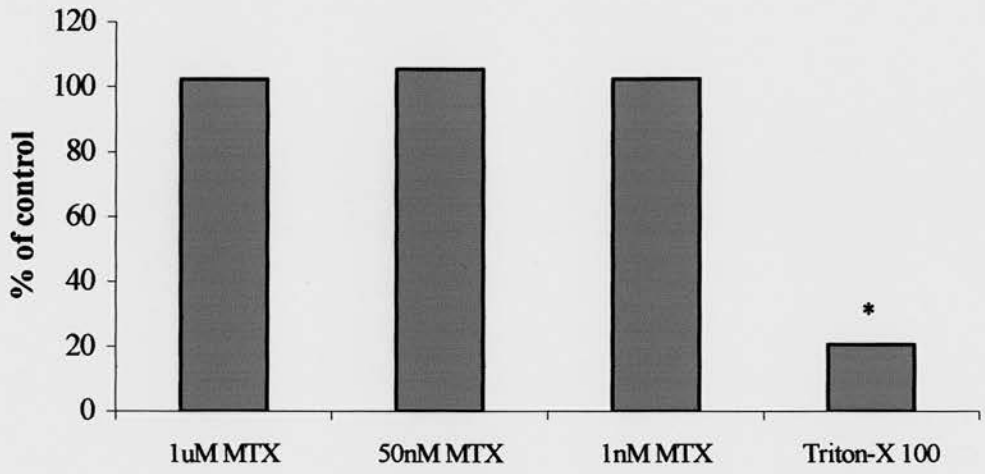
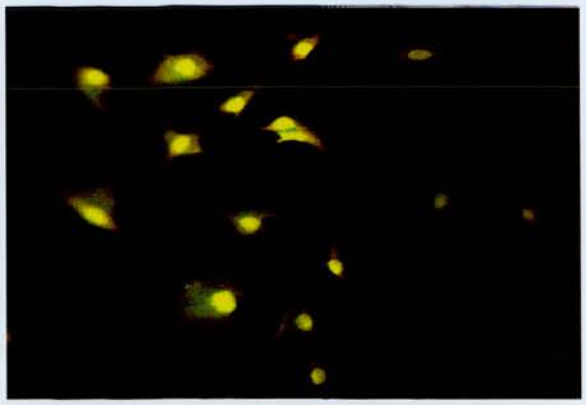


Figure 5.1 – Lactate dehydrogenase assays to investigate any cytotoxic effect of 1 μM, 50 nM or 1nM MTX on HBC over a 90 minute period. Results are expressed as a percentage of the negative control (no MTX). Triton-X 100 is included as a positive control. $n = 4$. $p < 0.01$.

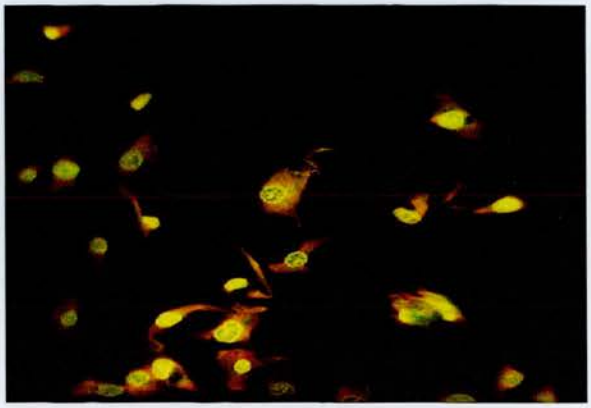
5.2.2 Acridine Orange Staining.

The lactate dehydrogenase assays suggest that over the concentration range / incubation period studied, MTX does not induce cell necrosis. These experiments are not sufficient to investigate whether MTX has apoptotic effects on HBC. To investigate this, acridine orange staining was performed. Acridine orange is a non-specific nucleic acid stain, which can be used to demonstrate the presence of apoptotic nuclei (Carmichael et al, 1980). Apoptotic cells have small, condensed nuclei, which are dense and often fragmented. Acridine orange staining was performed in control cells (no MTX) and also in cells which had been incubated with 50 nM MTX for 90 minutes at 37°C.

The results demonstrate that at this concentration and over a 90 minute time period, MTX does not induce apoptosis in HBC, as nuclear size and density do not vary significantly from control cells **(Figure 5.2)**.



(a) Control
(no MTX)



(b) 50 nM
MTX

Figure 5.2 – Acridine orange staining of HBC to investigate whether 50 nM MTX causes apoptosis. (a) Control cells – (no MTX) (b) 50 nM MTX. Original magnification x 100.

5.2.3 MTT Assays

MTT assays were performed to investigate the effect of 50 nM MTX over a 24-hour period in unstimulated HBC. The MTT assay is a colometric assay, which allows the assessment of viable cells in drug toxicology studies. The assay is based on the ability of mitochondrial succinate dehydrogenase to catalyse conversion of MTT to a blue formazan product.

Cells were incubated with MTX for 1 hour, 3 hours, 6 hours or 24 hours prior to performing the assay. Sterile PBS and 0.2 % Triton-X 100 were used as negative and positive controls respectively.

The results show that, over a 24-hour incubation period, MTX does not have cytotoxic effect on HBC in culture (Mann-Whitney U-test) **(Figure 5.3)**.

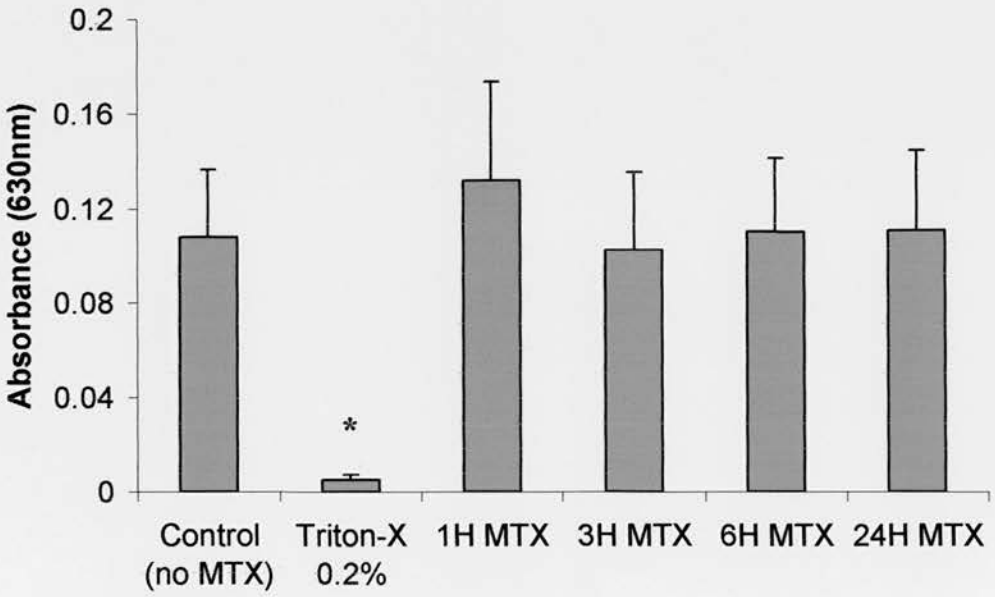


Figure 5.3 – The effect of MTX on HBC. MTX was added to cells for the appropriate times and all time points were assayed after 24 hours. Triton-X 100 was added to HBC for 1 hour and is included as a positive control.
Error bars are +1 SEM, n = 3, * $p < 0.05$ (Mann-Whitney U test).

5.3 MTX inhibits IL-1β release but has no effect on IL-1β signalling.

Electrophysiological experiments have shown that MTX dose dependently inhibits hyperpolarisation responses occurring in HBC following 0.33 Hz mechanical stimulation. Toxicity assays have confirmed that the inhibition of cell signalling by MTX is not a result of cell toxicity.

At this stage, it is not known at which point or by what mechanism MTX exerts its inhibitory effects on the cell-signalling pathway following stimulation. MTX may affect any stage of the signalling pathway - it may inhibit integrin function, focal adhesion formation, actin cytoskeleton organisation, tyrosine phosphorylation, the release of IL-1β, the binding of IL-1β to the IL-1 receptor, the action of PLC, DAG or PKC or the activation of the small conductance Ca^{2+} activated K^+ (SK) channels.

To elucidate whether MTX acts upstream or downstream of IL-1β, a series of CM experiments involving MTX were performed as follows.

(a) CM was transferred from mechanically stimulated cells, which had been incubated with 50 nM MTX for 30 minutes at 37 °C prior to stimulation to unstimulated cells.

(b) CM was transferred from mechanically stimulated cells to unstimulated cells, which had been incubated with 50 nM MTX for 30 minutes at 37°C.

When CM is transferred from mechanically stimulated cells, which have been incubated with MTX prior to stimulation, no hyperpolarisation is observed in the unstimulated cells following the media transfer **(Table 5.2a)**. This suggests either that IL-1β is not present in the transferred media or that the presence of MTX in the media inhibits IL-1β signalling in the unstimulated cells.

When CM is transferred from mechanically stimulated cells to cells, which have been incubated with MTX for 30 minutes at 37°C, hyperpolarisation is observed in the unstimulated cells **(Table 5.2b)**. These results suggest that MTX does not inhibit

IL-1β signalling in unstimulated cells. Therefore, these results, in conjunction with those presented in **Table 5.2a** provide evidence that MTX acts in the upstream part of the pathway and that IL-1β release is inhibited.

Table 5.2(a) – CM from HBC mechanically stimulated for 20 minutes induces membrane hyperpolarisation when transferred to previously unstimulated HBC. If HBC are incubated with 50 nM MTX prior to mechanical stimulation, no transfer of hyperpolarising activity is observed when CM is transferred to unstimulated cells.

		Membrane potential (-mV) (Mean ± SEM)						
Reagent	*n*	*Resting*	*+ MTX*	*Post MS*	*% Change (Rest - Post MS)*	*Resting*	*With CM*	*% Change (Rest – Post MS)*
Nil (control)	5	27.6 ± 1.7	n/a	43.0 ± 3.3	+56%*	24.8 ± 1.0	37.8 ± 1.1	+52%**
MTX (50 nM)	5	27.6 ± 1.8	29.4 ± 1.6	26.2 ± 2.2	- 5%ns	26.8 ± 2.0	31.8 ± 1.6	+19%ns

Results shown are from a single experiment and are consistent between experiments and between cells from 3 different donors.

* $p < 0.005$; $p < 0.0005$; ns = not significant.

Table 5.2 (b) - CM from HBC's mechanically stimulated for 20 minutes induces membrane hyperpolarisation when transferred to previously unstimulated HBC's. Incubation of unstimulated (CM) cells with MTX has no effect of the ability of these cells to be activated following transfer of media from mechanically stimulated cells.

		Membrane potential (-mV) (Mean ± SEM)						
Reagent	*n*	*Resting*	*Post MS*	*% Change (Rest - Post MS)*	*Resting*	*+ MTX*	*With CM*	*% Change (Rest – Post MS)*
Nil	5	33.2 ± 2.2	55.4 ± 3.5	+67 %*	29.6 ± 2.6	n/a	62.6 ± 2.6	+111%**
50 nM MTX	5	39.2 ± 2.2	58.4 ± 2.1	+49 %*	35.0 ± 1.8	38.0 ± 2.7	56.6 ± 2.8	+49%***

Results shown are from a single experiment and are consistent between experiments and between cells from 3 different donors.

* $p < 0.001$; **$p < 0.0005$; ***$p<0.005$.

The effect of 50 nM MTX on recombinant IL1-β induced membrane hyperpolarisation of HBC was then examined. Resting membrane potentials were obtained following which, the cells were incubated with 50 nM MTX for 30 minutes at 37°C. Consistent with previous results, MTX has no effect on resting membrane potential. Recombinant IL1-β (10 ng/ml) was then added to the cells and they were incubated for a further 10 minutes at 37°C.

The results show that in the presence of 50 nM MTX, HBC show a membrane hyperpolarisation response to IL1-β, which is similar in magnitude to control (no MTX) cells **(Table 5.3)**. These results are consistent with those presented in **Table 5.2** and provide further evidence that the action of MTX lies upstream of IL-β and thus in some way is inhibiting the release IL1-β.

Table 5.3 – Effect of MTX on the membrane hyperpolarisation response of HBC to IL-1β.

			Membrane Potential (-mV) (mean ± SEM)			
Stimulus	*Reagent*	*n*	*Resting*	*+ MTX*	*IL1-β*	*% Change (Resting–Post-IL1-β)*
IL-1β	Nil	5	29.4 ± 1.3	n/a	51.4 ± 2.4	+74.8*
IL-1β	50 nM MTX	5	31.2 ± 2.0	29.4 ± 2.8	51.4 ± 2.8	+74.8**

Results shown are from a single experiment and are consistent between experiments and between cells from 3 different donors.

* $p < 0.0005$; **$p < 0.005$.

The results presented to this point indicate that MTX prevents the release of IL-1β by inhibiting component(s) of the signalling pathway, which lie upstream of its release,

thus disrupting normal signal transduction. To gain further clarification of where in the signal transduction pathway MTX acts, experiments were performed which allowed study of effects of MTX on specific components of the integrin-associated signalling pathway known to lie upstream of IL-1β.

5.4 MTX Decreases HBC Adhesion to Fibronectin and Type 1 Collagen Coated Surfaces.

To investigate the effect, if any, of MTX on $\alpha_5\beta_1$ integrin function, methylene blue cell adhesion assays were performed. The adhesion of HBC to the ECM proteins fibronectin and type 1 collagen was studied following the addition of MTX. It is known that the $\alpha_5\beta_1$ integrin binds fibronectin and that type 1 collagen is bound by integrin heterodimers which contain the β_1 subunit.

HBC were incubated with 1 μM, 50 nM or 1 nM MTX for 90 minutes before being allowed to adhere to either fibronectin or type 1 collagen coated surfaces. After 3 hours, non-adherent cells were removed by pouring off the supernatant and gently washing the adherent cells with PBS. HBC were stained using methylene blue, a non-specific DNA stain. The amount of staining is proportional to the number of bound cells **(Figure 2.11)**.

The results show that HBC adhesion to both fibronectin **(Figure 5.4)** and type 1 collagen **(Figure 5.5)** coated surfaces is significantly decreased to a similar magnitude by all concentrations of MTX studied. There is not any significant difference in the magnitude of decreased cell adhesion when comparing different concentrations of MTX. Experiments were performed in triplicate, using cells from three different donors (Appendix I).

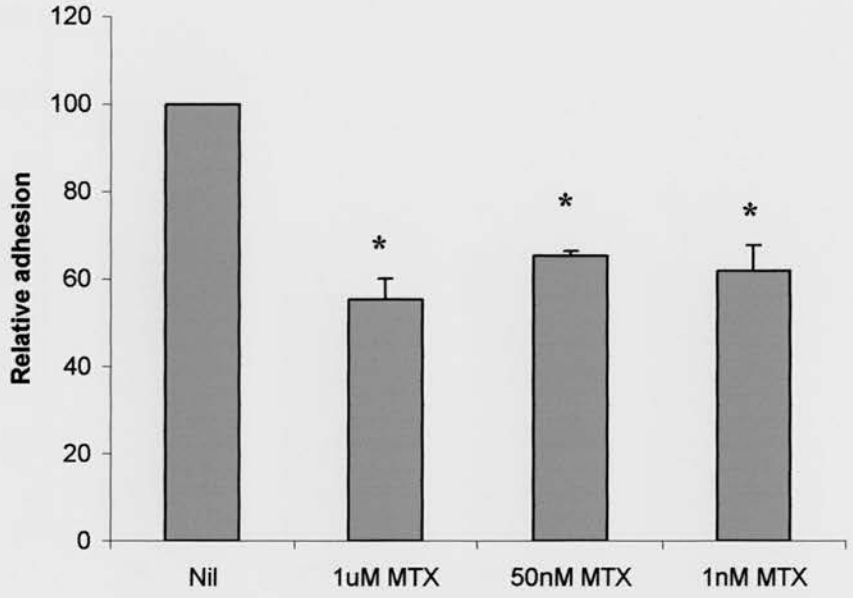


Figure 5.4 – The effect of MTX on the adhesion of HBC to fibronectin (FN).
The adhesion of HBC to FN was compared to the adhesion of cells treated with 1 μM, 50 nM or 1 nM MTX for 90 minutes at 37°C. The values shown represent the mean of 3 experiments carried out in triplicate +1 SEM. All concentrations of MTX tested significantly decrease the adhesion of HBC to FN when compared to adhesion in the absence of MTX. Raw data can be found in Appendix I.
* $p < 0.01$ (Mann Whitney U test).

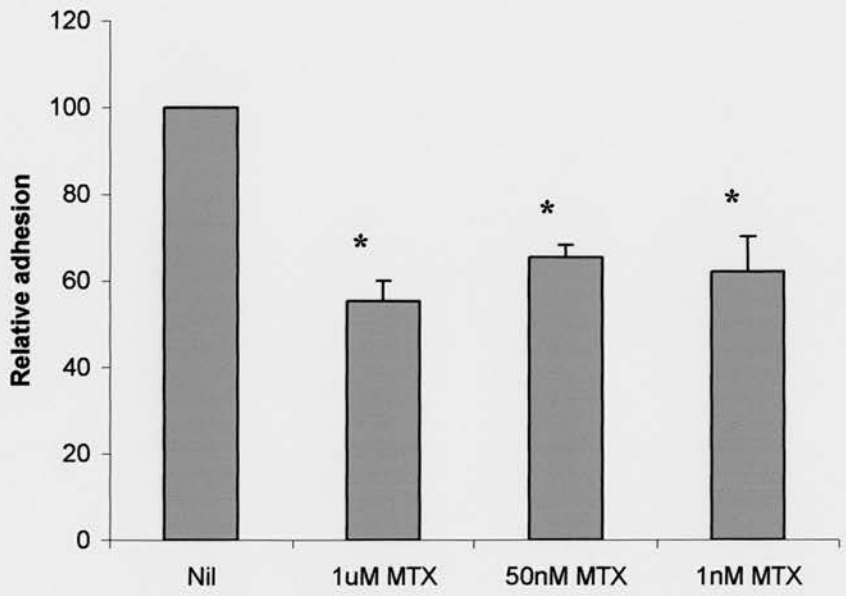


Figure 5.5 - The effect of MTX on the adhesion of HBC to type 1 collagen (coll 1). The adhesion of HBC to coll 1 was compared to the adhesion of cells treated with 1 μM, 50 nM or 1 nM MTX for 90 minutes at 37°C. The values shown represent the mean of 3 experiments +1 SEM. All concentrations of MTX tested significantly decrease the adhesion of HBC to type 1 collagen when compared to adhesion in the absence of MTX. Raw data can be found in Appendix I.

* $p < 0.01$ (Mann Whitney U test).

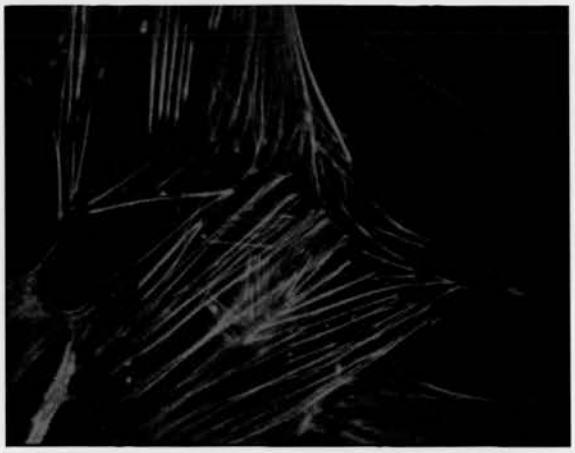
5.5 MTX Does Not Disrupt the Organisation of the Intracellular Actin Cytoskeleton.

Integrin signalling is known to involve the actin cytoskeleton (Clarke et al, 1995), and previous work has shown that the disruption of the actin cytoskeleton with cytochalasin D inhibits cell membrane hyperpolarisation following mechanical stimulation (Wright et al, 1997). Experiments were therefore undertaken to investigate if MTX has any effect on the organisation of the actin cytoskeleton.

The actin cytoskeleton was visualised using rhodamine (TRITC) labelled phalloidin in unstimulated HBC which had been incubated with 50 nM MTX for 90 minutes at 37°C, 95% O_2, 5% CO_2. HBC, which had not been incubated with MTX, were used as controls. No differences were observed when comparing the conformation of the actin cytoskeleton in control and MTX treated cells. The actin stress fibres appear well organised and are tethered to sites of focal adhesions at the plasma membrane of the cells **(Figure 5.6)**.

The conformation of the actin cytoskeleton was then visualised in HBC which had been mechanically stimulated for 20 minutes at 0.33 Hz in the presence / absence of 50 nM MTX **(Figure 5.7)**. As in **Figure 5.6**, there are no significant differences observed when comparing the conformation of the actin cytoskeleton in control and MTX treated cells.

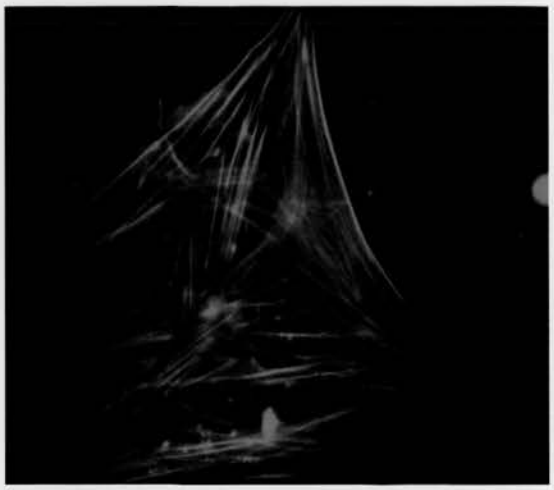
Therefore, these results suggest that 50 nM MTX has no effect on the conformation of the actin cytoskeleton in resting or mechanically stimulated HBC.



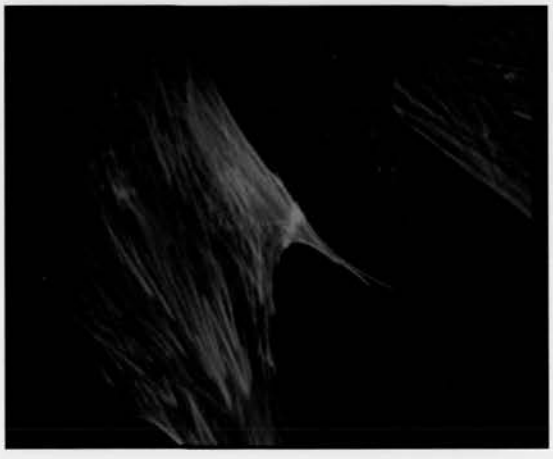
(a) No Stimulation
(no MTX).

(b) No Stimulation
+ 50 nM MTX.

Figure 5.6 – Visualisation of the actin cytoskeleton using rhodamine (TRITC) labelled phalloidin in unstimulated cells in the absence or presence of 50 nM MTX (original magnification x 630).



(a) Stimulation
(no MTX).



(b) Stimulation
+ 50 nM MTX.

Figure 5.7 – Actin cytoskeleton visualisation. Rhodamine (TRITC) labelled phalloidin was used to visualise the organisation of the intracellular actin cytoskeleton in mechanically stimulated cells ± 50 nM MTX. The results show that MTX has no effect on actin cytoskeleton conformation in mechanically stimulated HBC's (original magnification x 630).

5.6 Effects of MTX on Type 1 Collagen mRNA Expression Following Mechanical Stimulation

This study has shown that type 1 collagen mRNA expression is upregulated following mechanical stimulation and remains elevated for up to 24 hours post stimulation **(Figure 4.4)**. Although the increase in type 1 collagen mRNA expression is statistically non-significant, a clear trend can be observed. The effect of 50 nM MTX on type 1 collagen mRNA expression following mechanical stimulation has been examined. MTX was added to HBC for 30 minutes prior to the induction of mechanical stimulation at 0.33 Hz for 20 minutes. Following stimulation, HBC were incubated at 37°C for 0, 1, 3, 6 or 24 hours. Following this incubation period, RNA was extracted and quantified. RNA was reverse transcribed to obtain cDNA. Semi-quantitative PCR was than performed to investigate whether 50 nM MTX had an effect on type 1 collagen mRNA expression in HBC following mechanical stimulation.

The results are shown in **Figure 5.8**. PCR samples were removed following either 26 or 30 cycles of amplification. The results demonstrate that in the presence of 50 nM MTX, type 1 collagen mRNA expression is increased in RNA extracted from HBC 0, 1, 3, 6 and 24 hours after a single 20 minutes period of 0.33 Hz mechanical stimulation, when compared to the non-stimulated (NS) control. These results only reach statistical significance at the 6-hour and 24 hour time points ($p < 0.05$; Mann-Whitney U-test). The increase in type 1 collagen mRNA levels with time is gradual, reaching a peak 6 hours after stimulation. If the results from **Figure 5.8** are presented together with those in **Figure 4.4 (Figure 5.9)** it is clear that although MTX does not inhibit the increases in type 1 collagen mRNA expression observed following mechanical stimulation the relative levels of type 1 collagen mRNA are lower following incubation with MTX, although the decrease in type 1 collagen levels observed in the presence of MTX is not statistically significant.

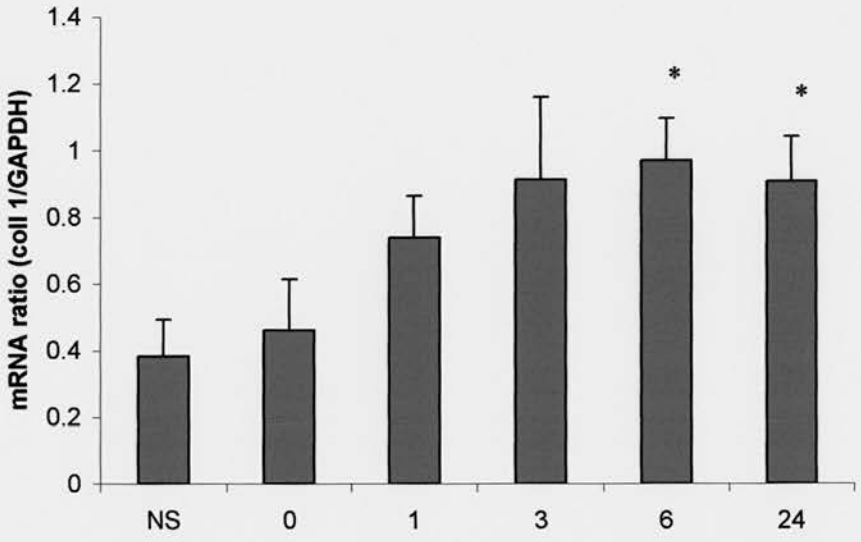


Figure 5.8 – The effect of mechanical stimulation on type 1 collagen mRNA expression in the presence of 50 nM MTX. Type 1 collagen mRNA expression increases with respect to time following 20 minutes 0.33 Hz mechanical stimulation. These increases only reach statistical significance at the 6 and 24 hour time points (p Mann-Whitney U-test). Pooled data for 3 donors performed in duplicate (n=6). Error bars are +1 SEM. * = $p < 0.05$.

NS: non-stimulated; 0,1,3,6 and 24: time in hours after 0.33 Hz mechanical stimulation.

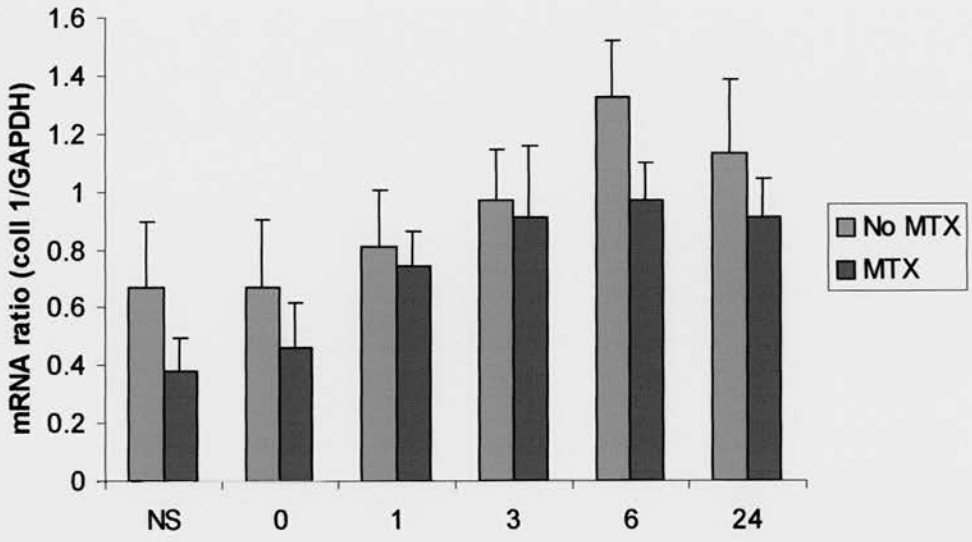


Figure 5.9 – The effect of 20 minutes, 0.33 Hz mechanical stimulation on HBC type 1 collagen mRNA expression in the absence and presence of 50 nM MTX. Error bars are +1 SEM.

NS: non-stimulated; 0,1,3,6 and 24: time in hours after 0.33 Hz mechanical stimulation.

5.7 Effects of MTX on BMP-4 mRNA Expression Following Mechanical Stimulation.

This study has shown that levels of BMP-4 mRNA expression in HBC, are significantly decreased following 20 minutes cyclic mechanical stimulation at the 3, 6 and 24 hour time points **(Figure 4.5)**. The effect of 50 nM MTX on levels of BMP-4 mRNA expression following mechanical stimulation has also been examined. As before, the HBC were incubated with 50 nM MTX for 30 minutes at 37°C prior to the induction of 20 minutes, 0.33 Hz mechanical stimulation. Following stimulation, HBC were incubated at 37°C for 0, 1, 3, 6 or 24 hours. Following this incubation period, RNA was extracted and quantified. RNA was reverse transcribed to obtain cDNA. Semi-quantitative PCR was than performed to investigate whether 50 nM MTX had an effect on BMP-4 mRNA expression in HBC following mechanical stimulation.

The results are shown in **Figure 5.10**. PCR samples were removed following either 26 or 30 cycles of amplification. The results show, that in the presence of 50 nM MTX, BMP-4 mRNA expression is significantly decreased in response to 20 minutes 0.33 Hz mechanical stimulation after 3, 6 and 24 hours incubation compared to the non-stimulated (NS) control ($p < 0.05$; Mann-Whitney U-test). If the results from **Figure 5.10** are presented together with those in **Figure 4.5 (Figure 5.11)**, it is clear that MTX does not alter the decreases in BMP-4 mRNA expression observed following mechanical stimulation.

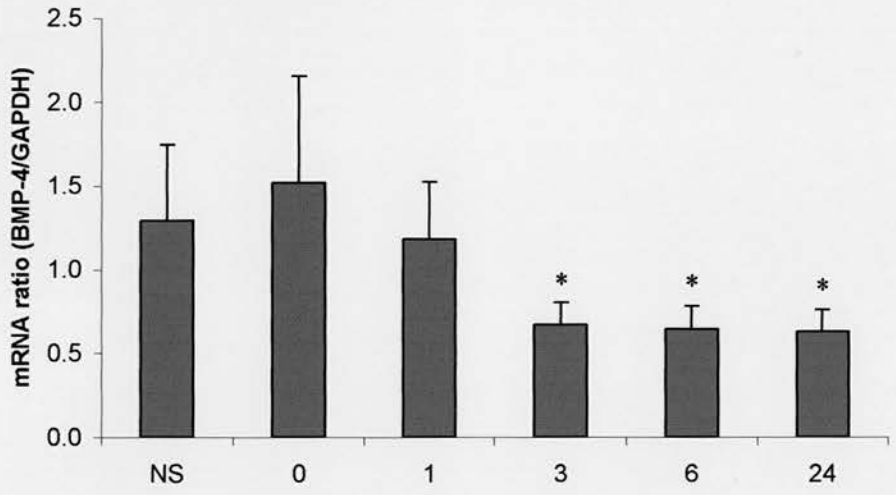


Figure 5.10 – The effect of mechanical stimulation on BMP-4 mRNA expression in the presence of 50 nM MTX. BMP-4 mRNA expression decreases with respect to time following 20 minutes 0.33 Hz mechanical stimulation. These increases reach statistical significance at the 3, 6 and 24 hour time points (p Mann-Whitney U-test). Pooled data for 3 donors performed in duplicate (n=6). Error bars are +1 SEM. * = $p < 0.05$.
NS: non-stimulated; 0,1,3,6 and 24: time in hours after 0.33 Hz mechanical stimulation

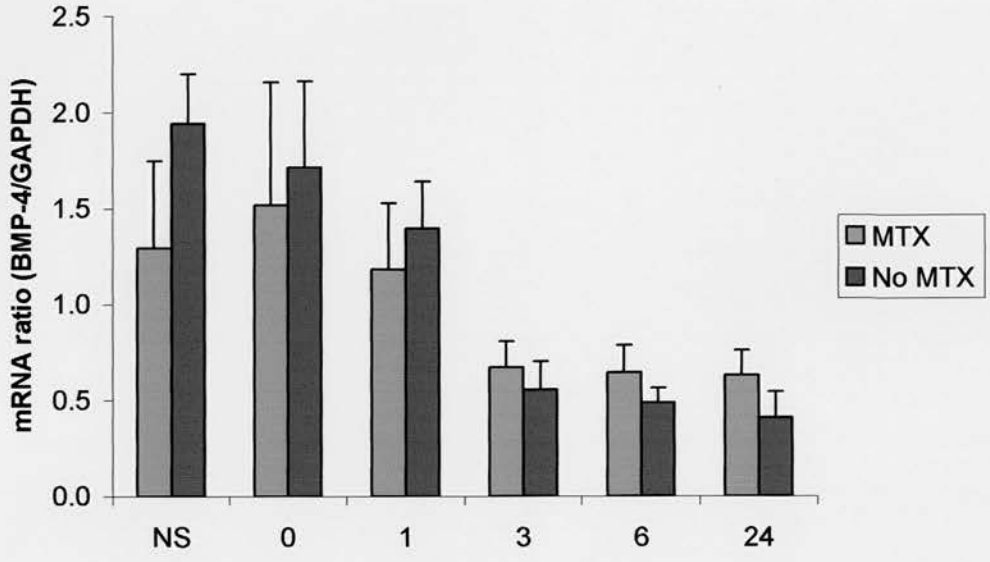


Figure 5.11 – The effect of 20 minutes, 0.33 Hz mechanical stimulation on HBC BMP-4 mRNA expression in the absence and presence of 50 nM MTX. Error bars are +1 SEM. NS: non-stimulated; 0, 1, 3, 6 and 24: time in hours after 0.33 Hz mechanical stimulation.

5.8 Discussion

5.8.1 The Effect of MTX on IL-1β Production / Release from Mechanically Stimulated HBC.

The aims of the present study were to determine if MTX has any effects on the responses of HBC in culture to 0.33 Hz cyclical mechanical stimulation.
The results show that MTX blocks the hyperpolarisation response of HBC to 0.33 Hz mechanical stimulation in a dose dependant manner but that it has no effect on membrane hyperpolarisation induced by a transferable factor, IL-1β (medium transfer) or by the addition of recombinant IL-1β. These results suggest that the mechanisms responsible for MTX mediated inhibition of hyperpolarisation are involved early in the mechanotransduction pathway; upstream of IL-β, or that the secretion of IL-1β from the cell is affected.

Whilst it is generally accepted that MTX has effects on IL-1β / IL-1β signalling (Segal et al, 1989, Novaes et al, 1996, Bendele et al, 1999, Williams et al, 1999) opinion in the scientific literature is divided upon whether MTX affects the production and subsequent secretion of IL-1β or whether it affects the ability of IL-1β to bind to its receptor thus inhibiting IL-1β signalling without altering its production or secretion. The present study appears to be the first time that the effects of MTX on bone cell mechanotransduction have been studied. Results on the effects of MTX on IL-1β secretion and signalling are largely confined to *in-vivo* and *in-vitro* models of RA and inflammation (Novaes et al, 1996, Bendele et al, 1999). In agreement with the results presented in this study, Novaes et al, (1996), have demonstrated that MTX reduces IL-1β production in the early phase of antigen induced arthritis in rabbits. Similarly, Williams et al, (1999), have reported that several liposomally conjugated MTX formulations are potent inhibitors of lipopolysaccharide induced IL-1β release. In contrast, Segal et al, (1989), have demonstrated that MTX has no significant effect on IL-1β synthesis *in-vitro* or in RA patients or mice treated with MTX, but concluded that the activity of IL-1β was affected. This work did not address whether the effect of MTX on IL-1β activity was direct or indirect although suggestions have been made that there may be a

physical or chemical interaction between MTX and IL-1β, that MTX may down regulate IL-1 receptors or that there may be a post receptor inhibition of intracellular mechanisms requiring IL-1 (Brody et al, 1993). There is also some evidence that MTX causes an increase in adenosine production (Cronstein et al, 1992; Cronstein et al, 1993; Cronstein et al, 1997) and subsequent adenosine signalling via adenosine receptors may promote the IL-1 receptor antagonist (IL-1Ra) transcription and presumably its production (Seitz et al, 1995). Recent studies seem to confirm this possibility because it has been shown that MTX treatment generates a less inflammatory type of circulating monocyte in RA patient by inhibiting IL-1 and IL-8.

5.8.2 The Effects of MTX on Cell Adhesion to Fibronectin and Type 1 Collagen.

It was noted in the present study that, whilst performing electrophysiological experiments involving MTX, that following mechanical stimulation some cells appeared loosely adherent, rounded and often detached upon impalement. Such observations were not noted in the control (no MTX) dish. Loss of cell adhesion in mouse osteoblastic cells following MTX administration has been previously observed (May et al, 1996). This group reported that following exposure to various concentrations of MTX (0.6 μM – 0.5 nM) for 7 days, caused mouse osteoblastic cells to lose adhesion and become rounded, with total cell detachment by day 14. The degree of cell detachment varied dose dependently with the concentration of MTX added.

In the present study methylene blue cell adhesion assays were performed to assess effects of MTX on HBC adhesion to the ECM proteins fibronectin (FN) and Type 1 collagen (coll 1). These assays have confirmed that, following exposure to MTX (1 μM, 50 nM or 1 nM) there is a significant decrease in cell adhesion to fibronectin and collagen 1 coated surfaces when compared to control (no MTX) HBC. The degree of the decrease in cell adhesion did not vary with MTX concentration. These results raise the possibility that integrin function (in particular the β_1 integrin subunit) may be affected by MTX as it is known that the $\alpha_5\beta_1$ integrin heterodimer

binds to fibronectin and that type 1 collagen is bound by integrin heterodimers which contain the β_1 subunit.

There is limited evidence regarding the effects of MTX on integrin function. Although the mechanism of MTX action in RA is at the present time unclear (Cutolo et al, 2001), there is some evidence to suggest that MTX may decrease neutrophil chemotaxis and adhesion at sites of inflammation by inhibiting conformational changes in the β_2 integrin CD11b/CD18, which are necessary for adhesion (Cronstein et al, 1992). The authors speculate that MTX induced increases in adenosine concentrations are responsible for this, although no explanation is offered regarding the link between adenosine signalling and integrin function. There does not appear to be any published data regarding the effects of MTX on $\alpha_5\beta_1$ integrin function.

Studies have also been undertaken to investigate MTX effects on cell adhesion molecules. There have been reports that MTX decreases the expression of the adhesion molecules E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in patients with RA (Dolhain et al, 1998), and as a result there is a decrease in the influx of various leucocytes to the site of inflammation. It is also known that the expression of adhesion molecules on vascular endothelium can be upregulated by cytokines such as TNF-α and IL-1β (Klein et al, 1994). Therefore, the changes in adhesion molecule expression in synovial tissue during therapy with MTX might be explained by a reduction in cytokine production, which would be consistent with the inhibition of IL-1β release observed in this study.

5.8.3 MTX Does Not Disrupt the Conformation of the Actin Cytoskeleton.

The conformation of the actin cytoskeleton is extremely important to integrin mediated signalling (Wang et al, 1993, Miyamoto et al, 1995; Yamada, 1995). Integrins act as a transmembrane link between the ECM, cytoskeletal proteins and actin filaments. Binding of integrins to the ECM causes the formation of complex protein structures called focal adhesions, which tether actin stress fibres to the

cytoplasmic face of the plasma membrane. At these sites the cytoplasmic domains of the integrin subunits associate with the cytoskeletal proteins talin and α-actinin, serving to link the ECM to the actin cytoskeleton (Burridge et al, 1988; Jockusch et al, 1995). Focal adhesions however, play more than a structural role in anchoring the cell to the ECM, as ligand binding to the integrins leads to activation of a range of biochemical signalling events / pathways (Longhurst et al, 1998), including that activated by 0.33 Hz cyclic mechanical stimulation in HBC (Salter et al, 1997, Salter et al, 2000). Previous work by our group has shown that disruption of the actin cytoskeleton by cytochalasin D inhibits membrane hyperpolarisation in response to 0.33 Hz mechanical stimulation (Wright et al, 1997). Therefore it is possible that MTX mediated inhibition of mechanically induced signalling in HBC may be due to disruption of the intracellular actin cytoskeleton. In the present study the organization of the actin cytoskeleton was studied in unstimulated and mechanically stimulated HBC in the presence and absence of 50 nM MTX. No effect of MTX on actin cytoskeleton organisation was observed in either unstimulated or mechanically stimulated cells. The stress fibres are visible and remain well organised, tethered to focal adhesions. Therefore, it is unlikely that the inhibitory effects of MTX arise as a result of disruption of the actin cytoskeleton.

5.8.4 MTX Effects on Type 1 Collagen 1 mRNA Levels Following Mechanical Stimulation.

Type 1 collagen mRNA levels are increased in HBC following 20 minutes 0.33 Hz mechanical stimulation, increasing gradually post stimulation and reaching a maximum 6 hours after stimulation. In the presence of 50 nM MTX type 1 collagen mRNA expression increases following mechanical stimulation and the trend observed is almost identical to that seen in the absence of MTX. Although MTX had no effect on relative levels of type 1 collagen following mechanical stimulation, levels were consistently decreased in the presence of MTX raising the possibility of effects on gene expression through different routes. These results also indicate that the mechanotransduction pathway by which type 1 collagen gene expression is regulated is not reliant on IL1-β function. In the present study however it is not possible to ascertain whether these responses are mediated via $\alpha_5\beta_1$ or dependent

upon other mechanically responsive membrane components such as stretch activated ion channels.

It is known that *in*-vivo MTX has considerable effects on type 1 collagen levels (Crofton et al, 1998; Crofton et al, 2000). Much of the published work investigating effects of MTX on the levels of type 1 collagen has been performed at the protein rather than the molecular level. Several *in-vivo* studies investigating effects of chemotherapy on bone turnover and growth in children with acute lymphoblastic leukaemia have reported effects on type 1 collagen synthesis. One study reported that during high dose MTX treatment, children had reduced C-terminal propeptide of type 1 collagen (PICP) levels and alkaline phosphatase levels and increased C-terminal telopeptide of type 1 collagen (ICTP), compared to those not so treated (Crofton et al, 1998). PICP quantitatively reflects type 1 collagen synthesis and is a marker of the osteoblast in its early proliferative and undifferentiated state. Alkaline phosphatase (AP) is a marker of the differentiated osteoblast whereas ICTP reflects the breakdown of type 1 collagen largely, although not exclusively from bone. These results suggest that during high dose MTX therapy there is reduced bone formation and increased bone resorption.

A subsequent study carried out in a later non-intensive period of chemotherapy reported that AP levels remained depressed throughout this period but that linear growth and PICP levels had returned to normal (Crofton et al, 2000). The authors therefore conclude that chemotherapy has an adverse effect of osteoblast maturation and speculate that the component of chemotherapy most likely to be responsible for this is weekly oral MTX administration. It has been shown in many animal models and also in many *in-vitro* studies that MTX treatment suppresses osteoblast activity and bone formation (May et al, 1996; Uehara et al, 2000; Davies et al; 2002), whilst enhancing osteoclast activity and bone resorption, resulting in reduced bone mass and trabecular bone volume (May et al, 1994; Moëll et al, 1995; Wheeler et al, 1995). Therefore it would appear that the smaller oral doses administered during continuing, less intensive chemotherapy may be sufficient to affect osteoblast differentiation (AP) but not it ability to synthesise collagen (PICP) nor the activity

of osteoclasts in degrading collagen (ICTP). The authors speculate that the consequence of this is likely to be either ineffective collagen synthesis, in which newly synthesised collagen is degraded without being incorporated into the bone matrix or that normal amounts of collagen are laid down in growing bone followed by suboptimal mineralisation. Either scenario may account for the fractures and pain or the low BMD observed throughout chemotherapy.

5.8.5 MTX Effects on Relative Bone Morphogenetic Protein–4 (BMP-4) mRNA Levels Following Mechanical Stimulation

BMP-4 mRNA levels decrease in HBC following 20 minutes 0.33 Hz mechanical stimulation. In the presence of 50 nM MTX BMP-4, mRNA expression decreases following mechanical stimulation and there is no significant difference in relative levels of BMP-4 when comparing the control (no MTX) and MTX treated cells. These results suggest that BMP-4 gene expression is regulated by a mechanotransduction pathway that is not reliant on IL-1β and is unaffected by MTX.

CHAPTER 6.

ADENOSINE AFFECTS NORMAL HBC RESPONSES TO CYCLICAL MECHANICAL STIMULATION.

It is widely acknowledged that adenosine production is increased in response to MTX treatment and that increases in adenosine may account, at least in part, for the anti-inflammatory effects of MTX in rheumatoid arthritis although the exact mechanisms behind this are unclear (Cronstein et al, 1992; Cronstein et al, 1993; Montesinos et al, 2000; Cronstein et al, 2001). The present study has shown that MTX inhibits the response of HBC in culture to 20 minutes of 0.33 Hz mechanical stimulation and that MTX mediates this effect by inhibiting a component of the mechanical stimulation pathway, which lies upstream of the release of the transferable factor IL-1β. Although the exact mechanisms involved in MTX mediated inhibition of HBC responses to mechanical stimulation are unclear, results of methylene blue cell adhesion assays indicate that integrin function may be affected by MTX. There is also some evidence to suggest that the anti-inflammatory effects of MTX in rheumatoid arthritis may be due to decreased neutrophil adhesion at sites of inflammation, and that this decreased neutrophil adhesion arises as a consequence of inhibitory effects of adenosine on integrin conformation (Cronstein et al, 1992). Therefore it is possible that adenosine signalling may play a role in MTX inhibition of HBC responses to cyclical mechanical stimulation, and experiments have been performed to investigate this.

6.1 Adenosine inhibits HBC Electrophysiological Responses to Mechanical Stimulation

The full numerical results of individual experiments from which the values given in the Tables in this chapter are derived are given in Appendix I. Results shown in this chapter are from a single representative experiment. Experiments were repeated from HBC derived from at least 3 adult or juvenile donors and were reproducible between donors.

Experiments were performed to investigate the effects of adenosine (100 μM) on HBC responses to 20 minutes of 0.33 Hz cyclical mechanical stimulation (Shimegi et al, 1998).

The resting membrane potential of 5 HBC was assessed prior to the addition of 100 μM adenosine. HBC were incubated with adenosine for 20 minutes at 37°C, following which time the membrane potential of a further 5 HBC was assessed. The HBC were then mechanically stimulated for 20 minutes at 0.33 Hz. Following stimulation, the membrane potentials of another 5 HBC were measured.

Adenosine itself was found to have no effect on the membrane potential of resting cells, but it inhibited cell membrane hyperpolarisation following 20 minutes of 0.33 Hz cyclical mechanical stimulation **(Table 6.1)**.

Table 6.1 – Adenosine inhibits HBC responses to cyclic mechanical stimulation.

		Membrane Potential (-mV) *(mean ± SEM)*			
Reagent	*n*	*Resting*	*+ Aden*	*Post MS*	*% Change (Resting – Post MS)*
Nil	5	26.8 ± 1.2	n/a	39.4 ± 0.8	+47%*
Aden (100 μM)	5	27.2 ± 1.6	33.0 ± 2.4	28.2 ± 1.46	+3.6% ns

Results shown are from a single experiment and are consistent between experiments and between cells from 3 different donors (Appendix I).

* $p < 0.001$; ns = non-significant.

6.2 Adenosine Deaminase (ADA) Restores Normal HBC Responses to MTX Treated Cells.

The results in **Table 6.1** show that 100 μM adenosine inhibits HBC responses to mechanical stimulation. To test the hypothesis that adenosine signalling is involved in MTX mediated inhibition of HBC mechanotransduction, electrophysiological experiments were performed which incorporated both MTX and adenosine deaminase (ADA), an enzyme responsible for the breakdown of adenosine to inosine.

Initially, the effect of ADA on HBC was examined in resting HBC and in HBC subjected to 20 minutes of 0.33 Hz mechanical stimulation. The resting membrane potential of 5 HBC was assessed prior to incubation with ADA (0.125 U/ml) for 10 minutes at 37°C. Following the incubation period, the membrane potential of a further 5 HBC were assessed and the cells were then mechanically stimulated at 0.33 Hz for 20 minutes. Following stimulation, the membrane potentials of another 5 HBC were assessed. ADA itself was found to have no effect on HBC resting membrane potential and HBC mechanically stimulated in the presence of ADA hyperpolarised as normal **(Table 6.2)**.

The effect of ADA on MTX mediated inhibition of HBC responses to mechanical stimulation was then assessed. The resting membrane potential of 5 HBC was assessed prior to the addition of ADA. Following the 10 minute incubation period, the membrane potential of a further 5 HBC was assessed. The cells were then incubated with 50 nM MTX for 30 minutes at 37°C. Following this incubation period, the membrane potentials of another 5 HBC were assessed and the cells were then subjected to 20 minutes of 0.33 Hz mechanical stimulation. Following this time, post stimulation membrane potentials of another 5 HBC were recorded

HBC hyperpolarised following mechanical stimulation in the presence of ADA and MTX **(Table 6.2)**.

Table 6.2 - Adenosine deaminase restores normal HBC responses to mechanical stimulation to MTX treated cells.

		Membrane Potential (-mV) (mean ± SEM)				
Reagent	*n*	*Resting*	*+ ADA*	*+ MTX*	*Post MS*	*% Change (Resting – Post MS)*
Nil	5	36.6 ± 1.6	n/a	n/a	51.4 ± 3.7	+ 40%*
ADA	5	32.8 ± 2.0	30.0 ± 3.1	n/a	46.2 ± 0.7	+ 40%*
MTX	5	32.2 ± 1.5	n/a	34.0 ± 1.2	28.8 ± 2.4	- 10%ns
ADA + MTX	5	29.2 ± 2.6	32.4 ± 2.7	25.8 ± 1.1	53.4 ± 5.0	+ 83%*

Results shown are from a single experiment and are consistent between experiments and from cells from 2 different donors.

* $p < 0.01$, ns = non-significant.

6.3 Adenosine Decreases HBC Adhesion to Fibronectin and Type 1 Collagen Coated Surfaces.

The present study has previously been shown that 1 μM, 50 nM and 1 nM MTX decrease HBC adhesion to FN and type 1 collagen surfaces, and it has been proposed that integrin function (in particular the β_1 integrin subunit) may be affected by MTX.

To investigate whether the decrease in HBC adhesion to FN and type 1 collagen observed following MTX addition is mediated via mechanisms involving adenosine, methylene blue cell adhesion assays were performed to investigate the effect of 100 μM adenosine on HBC adhesion to FN and type 1 collagen.

HBC were incubated with 100 μM adenosine for 90 minutes before being allowed to adhere to either fibronectin or type 1 collagen coated surfaces. After 3 hours, non-adherent cells were removed by pouring off the supernatant and gently washing the adherent cells with PBS. HBC were stained using methylene blue. The amount of staining is proportional to the number of bound cells **(see Figure 2.11)**.

The results show that HBC adhesion to both fibronectin **(Figure 6.1)** and type 1 collagen **(Figure 6.2)** coated surfaces is significantly decreased by incubation with 100 μM adenosine. The decrease in cell adhesion observed is of similar magnitude to that seen following incubation with 50 nM MTX. Experiments were performed in triplicate, using cells from three different donors (Appendix I).

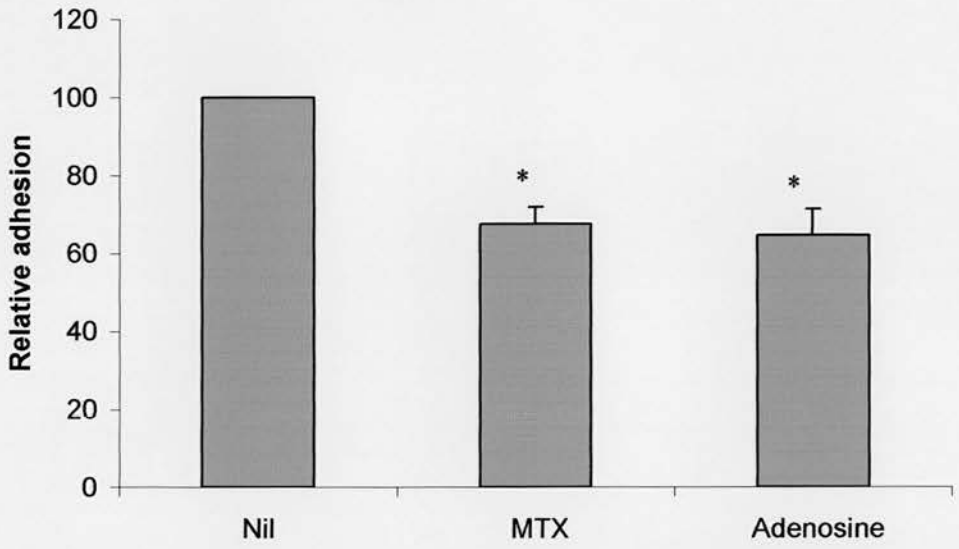


Figure 6.1 – The effect of adenosine on the adhesion of HBC to fibronectin (FN). The adhesion of HBC to FN was compared to the adhesion of cells treated with 50 nM MTX or 100 μM adenosine for 90 minutes at 37°C. The values shown represent the mean of 3 experiments carried out in triplicate +1 SEM. Both MTX and adenosine decrease the adhesion of HBC to FN when compared to adhesion in the absence of either adenosine or MTX.

* $p < 0.05$ (Mann Whitney U test). Raw data can be found in Appendix I.

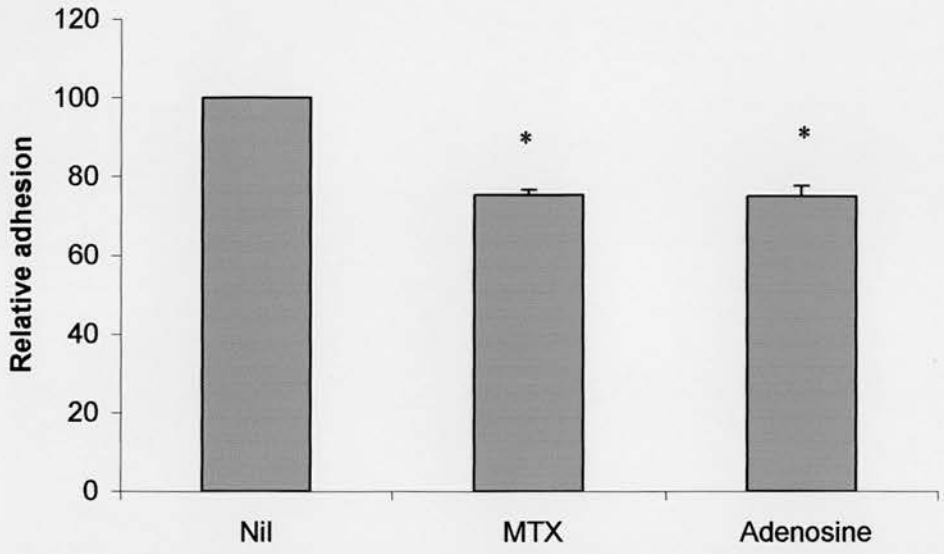


Figure 6.2 – The effect of adenosine on the adhesion of HBC to type 1 collagen (coll 1). The adhesion of HBC to Type 1 collagen was compared to the adhesion of cells treated with 50 nM MTX or 100 μM adenosine for 90 minutes at 37°C. The values shown represent the mean of 3 experiments carried out in triplicate +1 SEM. Both MTX and adenosine decrease the adhesion of HBC to coll 1 when compared to adhesion in the absence of either adenosine or MTX. Raw data can be found in Appendix I.
* $p < 0.05$ (Mann Whitney U test).

6.4 A_1, A_{2A} and A_3 but not A_{2B} Receptor Antagonists Reverse the Inhibitory Effects of MTX on HBC Responses to Mechanical Stimulation.

To further test the hypothesis that adenosine signalling is involved in MTX mediated inhibition of HBC mechanotransduction, electropysiological experiments were performed which incorporated both MTX and an adenosine receptor antagonist, specific to one of the four adenosine receptors (A_1, A_{2A}, A_{2B} or A_3).

The resting membrane potential of 5 HBC was assessed prior to incubation with each of the specific adenosine receptor antagonists at the appropriate concentration for 10 minutes at 37°C. Following the incubation period, the membrane potential of a further 5 HBC were assessed, and the cells were then mechanically stimulated at 0.33 Hz for 20 minutes. Following stimulation, the membrane potentials of another 5 HBC were assessed. The antagonists themselves were found to have no effect on the resting membrane potential, and HBC mechanically stimulated in the presence of the adenosine receptor antagonists hyperpolarised as normal **(Table 6.3)**.

The effect of each of the adenosine receptor antagonists on MTX mediated inhibition of HBC responses to mechanical stimulation was then assessed. The resting membrane potential of 5 HBC was assessed prior to the addition of antagonists. Following the 10 minute incubation period, the membrane potential of a further 5 HBC was assessed. The cells were then incubated with 50 nM MTX for 30 minutes at 37°C. Following this incubation period, the membrane potentials of another 5 HBC were assessed and the cells were then subjected to 20 minutes of 0.33 Hz mechanical stimulation. Following this time, post stimulation membrane potentials of another 5 HBC were recorded

In the presence of the A_1 receptor antagonist, 8-Cyclopentyl –1, 3-dipropylxanthine (DPCPX), the A_{2A} receptor antagonist 8-(3-chlorostyryl)-caffeine (CSC), and the A_3 receptor antagonist MRS-1191, MTX treated cells hyperpolarised significantly following mechanical stimulation, suggesting that adenosine signalling via these

receptors is involved in MTX inhibition of HBC responses to cyclical mechanical stimulation. These results are shown in **Table 6.3, Table 6.4 and Table 6.6**.

In the presence of the A_{2B} receptor antagonist, alloxazine, MTX treated cells do not hyperpolarise following cyclical mechanical stimulation. These results would suggest that MTX treatment does not induce signalling via A_{2B} adenosine receptors, or that signalling via A_{2B} adenosine receptors is not a component of MTX inhibition of HBC responses to cyclical mechanical stimulation **(Table 6.5).**

In summary, these results suggest that the inhibitory G_i-protein linked A_1 and A_3 receptors and also the stimulatory G_s-protein linked A_{2A}, are involved in MTX inhibition of HBC signalling. The results suggest that A_{2B} receptors do not play a role in this response.

Table 6.3 - The A_1 receptor, 8-Cyclopentyl –1, 3-dipropylxanthine (DPCPX), restores normal HBC responses to mechanical stimulation to MTX treated cells.

		Membrane Potential (-mV) (mean ± SEM)				
Reagent	*n*	*Resting*	*+ DPCPX*	*+MTX*	*Post MS*	*% Change (Rest – Post MS)*
Nil	5	25.6 ± 1.43	n/a	n/a	40.6 ± 1.63	+ 59%*
DPCPX (1 μM)	5	29.4 ± 3.05	30.0 ± 2.99	n/a	52.8 ± 2.63	+ 79%*
MTX (50 nM)	5	34.6 ± 2.37	n/a	27.6 ± 1.72	28.8 ± 2.41	- 17%ns
DPCPX (1 μM) + MTX (50 nM)	5	28.4 ± 3.52	26.4 ± 2.29	25.6 ± 2.25	49.6 ± 3.13	+ 75%*

Results shown are from a single experiment and are consistent between experiments and from cells from 3 different donors (Appendix I).

* $p < 0.0005$, ns = non-significant.

Table 6.4 - The A_{2A} receptor, 8-(3-chlorostyryl) caffeine (CSC), restores normal HBC responses to MTX treated cells.

Reagent	*n*	*Membrane Potential (-mV) (mean ± SEM)*				
		Resting	*+ CSC*	*+MTX*	*Post MS*	*% Change (Rest – Post MS)*
Nil	5	29.4 ± 2.01	n/a	n/a	50.0 ± 3.64	+ 70%*
CSC (1 μM)	5	31.8 ± 1.62	34.6 ± 2.22	n/a	50.6 ± 4.44	+ 60%*
MTX (50 nM)	5	35.8 ± 2.26	n/a	35.4 ± 2.13	37.6 ± 1.69	+ 5%ns
CSC(1 μM) + MTX (50 nM)	5	28.4 ± 3.32	27.0 ± 1.22	36.6 ± 2.26	46.6 ± 2.94	+ 64%*

Results shown are from a single experiment and are consistent between experiments and from cells from 2 different donors (Appendix I).

* $p < 0.0005$, ns = non-significant.

Table 6.5 - The A_{2B} receptor, Alloxazine does not restore normal HBC responses to MTX treated cells.

		Membrane Potential (-mV) *(mean ± SEM)*				
Reagent	*n*	*Resting*	*+ Alloxazine*	*+MTX*	*Post MS*	*% Change*
Nil	5	34.8 ± 1.85	n/a	n/a	48.2 ± 3.33	+ 39%*
Allozazine (1 μM)	5	32.8 ± 1.93	33.6 ± 0.81	n/a	45.6 ± 2.18	+ 39%*
MTX (50 nM)	5	38.0 ± 1.34	n/a	37.6 ± 1.69	34.2 ± 2.20	- 10%ns
Alloxazine (1 μM) + MTX (50 nM)	5	39.4 ± 1.77	37.6 ± 2.04	34.8 ± 0.91	34.0 ± 2.48	- 14%ns

Results shown are from a single experiment and are consistent between experiments and from cells from 2 different donors (Appendix I).

* $p < 0.05$, ns = non-significant.

Table 6.6 - The A_3 receptor, MRS-1191 restores normal HBC responses to MTX treated cells.

		Membrane Potential (-mV) (mean ± SEM)				
Reagent	*n*	*Resting*	*+ MRS-1191*	*+MTX*	*Post MS*	*% Change*
Nil	5	30.0 ± 1.92	n/a	n/a	49.4 ± 3.88	+ 65%*
MRS-1191 (100 nM)	5	31.6 ± 2.29	33.0 ± 2.07	n/a	45.2 ± 4.23	+ 43%**
MTX (50 nM)	5	35.2 ± 1.98	n/a	33.6 ± 1.69	31.8 ± 1.82	- 9%ns
MRS-1191 (100 nM) + MTX (50 nM).	5	35.8 ± 1.85	24.4 ± 1.21	36.0 ± 1.92	51.4 ± 1.99	+ 44%*

Results shown are from a single experiment and are consistent between experiments and from cells from 2 different donors (Appendix I).

* $p < 0.005$, **$p < 0.05$, ns = non-significant.

6.5 Discussion.

6.5.1 Adenosine inhibits HBC Electrophysiological Responses to Mechanical Stimulation.

Adenosine is found in all living cells and its concentration is regulated by a variety of pathophysiological conditions. Under appropriate conditions adenosine is released from the cell where it can interact with specific cell surface adenosine receptors to modulate cell function in an autocrine or paracrine manner. These receptors were originally classified as P_1 or P_2 purinergic receptors, based upon their preference for adenosine or adenine nucleotides respectively (Burnstock, 1978). The relative potencies for P_1 sites are, adenosine $\geq$ AMP $\geq$ ADP $\geq$ ATP, whereas the potencies for the P_2 sites are, ATP $\geq$ ADP $\geq$ AMP $\geq$ adenosine (Olah et al, 1992). Activation of P_1 receptors by adenosine has been known to regulate a diverse set of physiological functions including cardiac rate and contractility, smooth muscle tone, sedation, neural activity, platelet function, lipolysis and renal function (Collis et al, 1993).

Although much evidence shows the ubiquity of P_1 (adenosine) receptors, there is limited data available regarding their expression in osteoblasts. Lerner et al, (1987) reported that A_2 like receptors are linked to cAMP stimulation in rat calvarial bone and in isolated osteoblastic cells from the calvaria, but with no real relation to cell function. Similarly Shimegi et al (1998), showed that A_1 receptor stimulation by adenosine induces proliferation of MC3T3-E1 osteoblastic cells, but the signalling pathways involved could not be determined.

Despite the widespread use of MTX in the treatment of RA, its action in the treatment of inflammation is still unclear. A number of clinical observations suggest that MTX does not suppress inflammation in rheumatoid arthritis by inhibition of DHFR alone. Several clinical trials have demonstrated that neither folate supplementation nor administration of reduced folate (folinic acid) reversed the therapeutic effects of MTX in the therapy of RA, indirect evidence against inhibition of DHFR (Hanrahan et al, 1988, Morgan et al, 1990). Also, several side effects of MTX commonly associated with DHFR inhibition are not observed following low

dose MTX administration. Indeed, bone marrow toxicity and leukopenia, common due to inhibition of DNA synthesis, do not occur, a finding which is consistent with the hypothesis that the anti-inflammatory effects of MTX are not primarily due to DHFR inhibition.

Several studies have provided evidence to suggest that the therapeutic effects of MTX may be linked to the capacity of MTX to increase adenosine concentrations (Baggott et al, 1986; Cronstein et al, 1991; Cronstein et al, 1993; Montesinos et al, 2000). Studies have shown that MTX accumulates within cells and inhibits the enzyme AICAR transformylase, resulting in increased AICAR concentrations. Increased AICAR concentrations promote, by a complex mechanism, the increased release of adenosine **(Figure 1.9)** (Gruber et al 1989, Barankiewicz et al, 1990), which is believed to act in an anti-inflammatory manner.

The experiments in the current study, were performed to investigate if adenosine signalling is involved in the inhibitory effects of MTX on HBC responses to cyclical mechanical stimulation.

The results presented in **Table 6.1** demonstrate that adenosine, inhibits HBC membrane hyperpolarisation in response to 0.33 Hz cyclic mechanical stimulation. These results are similar to those seen following the same regime of cyclical mechanical stimulation in the presence of MTX, which may indicate that adenosine is involved in the inhibitory effects of MTX on HBC. Further evidence is provided by the observation that normal hyperpolarising activity can be restored to MTX treated cells by the addition of ADA, an enzyme involved in the breakdown of adenosine to inosine.

6.5.2 A_1, A_{2A} and A_3 but not A_{2B} Receptor Antagonists Reverse the Inhibitory Effects of MTX on HBC Responses to Mechanical Stimulation.

Limited evidence exists regarding the expression of adenosine receptors in bone cells. It is known that A_1 and A_2 receptors are espressed in osteoblasts but not in

osteoclasts Experiments utilising adenosine receptor antagonists were undertaken to investigate the specific adenosine receptor subtypes involved in MTX mediated inhibition of HBC responses to mechanical stimulation.

The results of the specific anagonist experiments performed in this study have demonstrated that the inhibitory effects of MTX can be reversed by an A_1, A_{2A} or A_3 receptor antagonist but <u>not</u> by an A_{2B} receptor antagonist. These results are somewhat unexpected as A_1 and A_3 receptors are negatively linked to adenylate cyclase via inhibitory G_i proteins while the A_{2A} receptor is positively linked to adenylate cyclase via a stimulatory G_s protein. Therefore, one would expect that differing signalling cascades are activated following receptor binding.
There are several possible explanations for the results obtained. It is possible that the signalling pathways activated are distinct from the classical positive / negative coupling to adenylate cyclase. For example, there is much evidence to indicate that the participation of actvation of adenosine receptors may promote the activation of the mitogen-activated-protein kinases (MAPK), which are known to play are essential role in processes such as cell differentiation, survival, proliferation (van Corven et al, 1993; Shimegi et al, 1998) and death. MAPK are typically activated via receptor tyrosine kinases, but can also be activated by G protein coupled receptors including adenosine recptors.

A lack of antagonist specificity could also account for the unexpected results obtained in this study. For example DPCPX, the 'specific' A_1 recptor antagonist may have actions on more than one type of adeosine receptor. This A_1 antagonist is extremely selective for the rat A_1 adenosine receptor versus the rat A_2 receptor (1000 fold), but its potency is decreased at the human A_1 recptor versus the human A_{2A} receptor (30 fold) (Klotz et al, 1998). Moreover, it has also been demonstrated that this antagonist has some affinity for the A_{2B} receptor (Linden et al, 1999). Similarily, CSC is a functionally specific antagonist at A_{2A} receptors versus A_1 receptors (500 fold), although the selectivity of CSC for A_{2A} receptors via A_{2B} receptors is yet to be established.

6.5.3 Adenosine Decreases HBC Cell Adhesion to Type 1 Collagen and FN – Possible Effects on Integrin receptors?

Gruber et al, (1989) have demonstrated that administration of acadesine, the non-phosphorylated precursor of AICAR, prevents accumulation of neutrophils in ischemic myocardial tissue in a canine reperfusion injury model. Parallel with the diminished neutrophil accumulation was the observation of diminished infarct size and increased adenosine release from the ischemic myocardium. Similarly, Cronstein et al, have demonstrated that, at pharmacologically relevant concentrations, MTX, like acadesine, promotes adenosine release from cultured fibroblasts and endothelial cells (Cronstein et al, 1991). This study also demonstrated that the adenosine released from MTX treated cells, in turn, inhibits neutrophil adherence to connective tissue cells, a critical step for infiltration or injury by neutrophils. Neutrophils are known to express both A_1 and A_2 adenosine receptors and using specific A_1 and A_2 receptor agonists, Cronstein et al, (1992), have provided evidence to suggest that adenosine, binding to the A_2 receptor inhibits adherence of stimulated neutrophils to the endothelium. Neutrophils adhere to the endothelium via a variety of adhesion molecules and receptors and can alter their adhesiveness in response to agents such as IL-1 (Harlan et al, 1985). Intact CD11b/CD18 (β_2-integrin) molecules on the surface of the neutrophils are known to be critical for the adherence of FMLP-stimulated neutrophils to endothelial cells, gelatin and fibrinogen because an antibody directed against the β_2-integrin inhibited neutrophil adhesion to all of these surfaces (Cronstein et al, 1992).

Although it is unlikely that β_2-integrin expression is decreased by adenosine, there is evidence to suggest that adenosine may inhibit upregulation of β_2-integrin expression on stimulated neutrophils (Wollner et al, 1993), or that occupancy of A_2 receptors inhibits conformational changes in the β_2-integrin subunit, which are necessary for neutrophil adhesion to endothelial cells. At present, there is no data describing similar effects of adenosine on other integrin receptor subunits including the β_1 integrin, although it is possible that adenosine may affect other integrin receptors by a similar mechanism, and this may explain the decreases in HBC adhesion to

fibronectin and type 1 collagen observed following adenosine (and MTX) treatment. Signalling pathways involved remain to be elucidated.

Cell surface expression of an integrin alone is not sufficient to guarantee adhesion. It is now known, for many cell types, that integrin mediated cell binding to extracellular ligands can be enhanced dramatically by certain stimuli, which activate intracellular signal transduction cascades. Activation of these signalling transduction cascades can 'activate' integrins from the cytoplasmic face of the membrane in order to bind to their ligands, a phenomenon known as 'inside-out integrin signalling'. For example, for β_2 integrins to mediate cell-cell interactions (Dustin et al, 1989), and for lymphocyte β_1 integrins to mediate adhesion efficiently, leukocytes must be activated. Soluble agonists such as thrombin or adenine nucleotides activate platelet and leukocyte integrins. These agonists probably do not act directly on the integrins, but bind to cellular receptors leading to engagement of 'classical' signal transduction systems. Cellular receptors that couple to non-receptor tyrosine kinases are known to activate integrin dependant adhesion pathways in leukocytes whereas seven transmembrane proteins such as thrombin (or adenosine) receptors, couple to downstream elements through the activation of heterotrimeric G-proteins. Both types of receptors stimulate pathways which involve the activation of phospholipases, phosphatidylinositol lipid breakdown and activation of cellular protein kinases (Hynes et al, 1992, Lub et al; 1995). It is still unclear exactly how intracellular signal transduction events alter the affinity of ligand binding to integrins, although cytoskeletal interactions are believed to be important (Otey et al, 1990; Lewis et al, 1995; Dedhar et al, 1996). The cytoplasmic portions of both the α-integrin (Kassner et al, 1993; Kassner et al, 1994) and β-integrin chains (Chen et al, 1992, Pasqualini et al, 1994) are believed to participate in the inside-out integrin signalling process. Certain motifs present on the cytoplasmic tails of both α and β subunits are believed to be targets for inside-out signalling and are thought to be responsible for regulating integrin affinity states. One of these motifs is the highly conserved NPxY (Asn-Pro-x-Tyr; where x represents any amino acid) (O'Toole et al, 1995) found in the cytoplasmic domain of the integrins β_1 - β_7, with the exception of β_4. Point mutations in this motif can abolish integrin activation (O'Toole et al,

1995). Another motif is the conserved membrane-proximal sequence KLLxxxD (Lys-Leu-Leu-xxx-Asp) of the β cytoplasmic domains, which is also involved in regulation of integrin affinity state as deletion of part of this sequence results in a constitutively active integrin (Tozer et al, 1996).

As HBC adhesion is altered following MTX treatment and the results obtained are consistent with a role for adenosine in this response, it may be possible that the $\alpha_5\beta_1$ integrin is modulated by inside-out signalling mechanisms which are activated as a result of adenosine binding to G-protein coupled adenosine receptors. The proposed mechanisms are summarised in **Figure 6.3**.

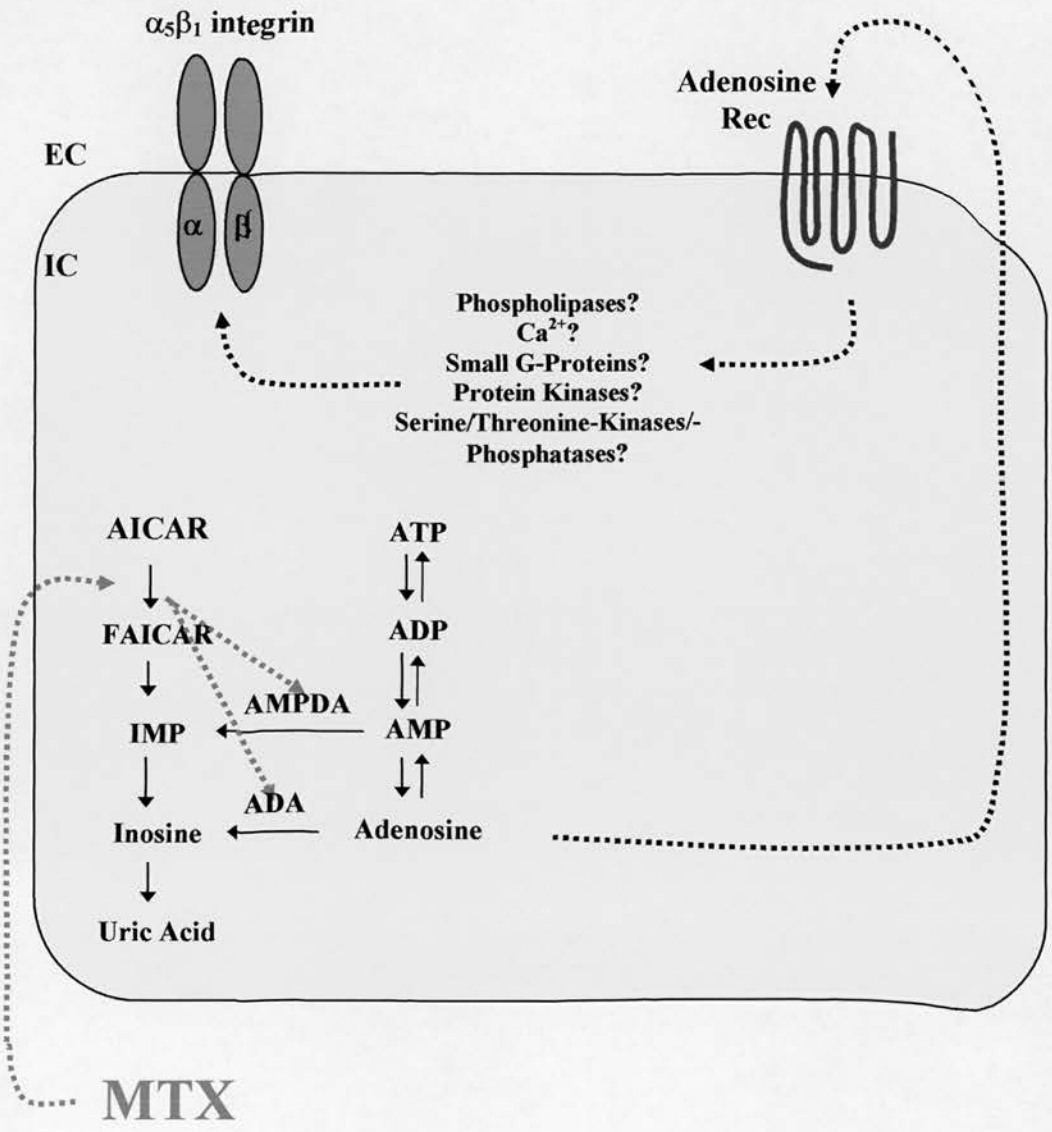


Figure 6.3 – Proposed pathway by which the adhesiveness of the β_1 integrin is moduated by MTX. AICAR = 5-aminoimidazole-4-carboxamide ribonucleotide, FAICAR = formyl-AICAR, ATP = adenosine trisphosphate, ADP = adenosine diphosphate, AMP = adenosine monophosphate, AMPDA = adenosine monophosphate deaminase, ADA = adenosine deaminase.

CHAPTER 7.

SUMMARY AND GENERAL DISCUSSION.

- Cyclical mechanical stimulation at a frequency of 0.33 Hz results in a hyperpolarisation of HBC, via activation of small conductance Ca^{++} activated K^+ (SK) channels. The mechanotransduction pathway involves the $\alpha_5\beta_1$ integrin receptor, stretch-activated ion channels, the actin cytoskeleton and tyrosine kinases with subsequent release of IL-1β. PLC, DAG, IP_3 and PKC are known to be involved in the downstream signalling events initiated by IL-1β.

- This study has demonstrated that, following a single 20 minute period of 0.33 Hz mechanical stimulation, collagen type 1 mRNA levels increase and remain elevated for up to 24 hours post stimulation. It is known that bone mass increases in response to mechanical stimulation (Rubin et al, 1984, Frost, 1987, Sessions 1989), and therefore it is expected that type 1 collagen mRNA levels would increase in response to mechanical stimulation as type 1 collagen comprises 95% of the organic part of bone. BMP-4 mRNA levels are decreased following a single 20 minute period of 0.33 Hz mechanical stimulation and remain depressed for up to 24 hours post stimulation. The fact that mechanical stimulation causes a decrease in relative BMP-4 mRNA levels which is accompanied by an increase in relative type 1 collagen levels may indicate that the mechanical stimulus that is being provided in the current model is inducing a differentiation / anabolic response, driving the cells towards a more mature active bone cell phenotype.

- MTX dose dependently inhibits HBC responses to 0.33 Hz cyclical mechanical stimulation. The results obtained suggest that the mechanisms responsible for MTX mediated inhibition of hyperpolarisation are involved early in the mechanotransduction pathway; upstream of IL-β, or that the secretion of IL-1β from the cell is affected. Investigation of components of

the signalling pathway which lie upstream of IL-β secretion, indicate that MTX does not affect the conformation of the actin cytoskeleton. There is a significant decrease in HBC adhesion to the ECM proteins fibronectin and type 1 collagen following MTX treatment, which may indicate that β_1 integrin function is affected by MTX.

- The responses of type 1 collagen and BMP-4 to 0.33 Hz cyclical mechanical stimulation are unaffected by MTX, suggesting that the mechanotransduction pathways controlling the expression of type 1 collagen and BMP-4 are distinct from the $\alpha_5\beta_1$ integrin mediated pathway and are not reliant on IL-1β.

- It has been demonstrated that adenosine inhibits HBC hyperpolarisation following 0.33 Hz cyclical mechanical stimulation, and that the inhibitory effects of MTX on HBC responses to mechanical stimulation can be prevented by ADA. Cell adhesion to fibronectin and type 1 collagen is decreased in the presence of adenosine, suggesting that β_1 integrin function is affected by adenosine. Although these results suggest that adenosine signalling may be involved in MTX mediated inhibition of HBC responses to cyclical mechanical stimulation, further work is required to determine the exact mechanisms involved.

This study has allowed the investigation of MTX on the responses of a heterogeneous culture of HBC to mechanical stimulation. The results presented in Chapter 3, clearly show that the cultures obtained consist of cells which posses an 'osteoblast-like phenotype' although the heterogeneity of the culture means that care must be taken when interpreting the results as one cannot be certain which cell types in the culture are responsible for the responses observed. While other extraction methods, such as enzymatic digestion, may yield a purer population of 'osteoblastic' cells, such protocols also have limitations. For example, enzymatic digestion typically produces few cells, which may result in lengthy periods in culture and increased possibility of cell dedifferentiation.

The results of this study have clearly shown that primary HBC from both juvenile and adult donors respond to 0.33 Hz cyclical mechanical stimulation with cell membrane hyperpolarisation. Statistical analyses have shown that the hyperpolarisation observed is significant but that there is no significant difference in magnitude of hyperpolarisation when comparing HBC from juvenile and adult donors. Although membrane hyperpolarisation is used throughout these studies as an indicator of mechanically induced cell signalling, the actual significance of the membrane hyperpolarisation response is still unclear, nor is it known if membrane hyperpolarisation is the 'end-point' of this mechanotransduction pathway or simply an intermediate step leading to further signalling pathways which are yet to be determined. At the present time, is also unknown if mechanically induced membrane hyperpolarisation has any functional role in the cell, or if the cell derives any benefit from potassium efflux and a more negatively charged environment. As the membrane hyperpolarisation response following stimulation is transient (only lasting 10 -15 minutes), it is also unclear which mechanisms are involved in restoring the membrane potential to its 'normal' resting state. Although these studies concentrate on the study and manipulation of the $\alpha_5\beta_1$ mediated pathway; it is not assumed that this is the only pathway which is activated by 0.33 Hz mechanical stimulation. Indeed, these studies have shown that both collagen 1 and BMP-4 gene expression can be mechanically regulated by 0.33 Hz mechanical stimulation but that the signalling pathways involved are distinct from the $\alpha_5\beta_1$ mediated pathway. At

present the mechanotransduction pathways responsible for alterations in type 1 collagen and BMP-4 gene expression following 0.33 Hz are unknown and would benefit from further investigation.

These studies have shown that MTX dose dependently inhibits the hyperpolarisation response to mechanical stimulation. It was extremely important to perform several toxicity assays on the MTX treated cells as it was necessary to ensure that inhibition of stimulation induced hyperpolarisation is not a result of cell toxicity. This is extremely important, as the therapeutic benefits of MTX in malignancy rely on the ability of this drug to inhibit DNA replication and kill cells. Three kinds of toxicity assay which investigate both necrosis and apoptosis were performed which provided conclusive evidence that, at the concentrations utilised, MTX did not have a toxic effect on HBC.

This study has also shown that the inhibitory effects of MTX lie in the upstream part of the mechanotransduction pathway prior to IL-1β release. Experiments have shown that the conformation of the actin cytoskeleton is not affected by MTX, but that cell adhesion is decreased following MTX administration, and that this is likely a result of MTX effects on the β_1-integrin subunit. It is not known whether decreased cell adhesion to the substrate results in inadequate propagation of the signal form the substrate to the integrin receptor or whether effects on the β_1-integrin subunit may affect integrin clustering, focal adhesion formation, recruitment of proteins and / or tyrosine kinase mediated events. It would have been useful to investigate focal adhesion formation using immunofluorescence and to investigate focal adhesion proteins such as FAK, which are known to be tyrosine phosphorylated following mechanical stimulation.

It is interesting that MTX affected levels of collagen 1 following mechanical stimulation (although not significantly). Although it is widely recognised that MTX has effects on type 1 collagen levels, these effects are normally only seen in patients subjected to long term high dose MTX therapy. Therefore, it is of interest that MTX causes a decrease in type 1 collagen mRNA levels after an incubation period of only

30 minutes. From these results, it is not possible to ascertain what effect (if any) MTX has on type 1 collagen protein levels in HBC and therefore it may have been interesting to investigate type 1 collagen protein levels following mechanical stimulation. Caution must also be taken when comparing these results to those obtained from *in-vivo* studies. Although studies estimate that following intramuscular MTX administration the concentrations observed in serum, synovium and bone are 0.6 μM, 0.28 nM and 0.29 nM respectively (Kremer et al, 1986; Preston et al, 1993), it is extremely difficult to accurately assess what concentration of MTX, individual bone cells are exposed to. It is also likely that HBC in monolayer may be more susceptible to both strain and MTX that osteoblasts *in-vivo*, owing to the fact that they are 'less protected' than osteoblasts *in-vivo* which are surrounded by a rich ECM and strong framework and which have contact with other bone cell types such as osteocytes.

Although MTX has been used for many decades in the treatment of rheumatoid arthritis, the exact mechanism of action of MTX remains unclear. A great deal of evidence however, points toward a mechanism of action which is distinct from DHFR inhibition, as the concentrations required to attain clinical efficacy are much lower than those used to treat malignancy and cell toxicity is not commonly observed. Many studies have proposed a role for adenosine as an anti-inflammatory mediator, whose concentration is increased by MTX administration. Although it is known that adenosine (and adenosine receptors) are found in all living cells, there is limited data about adenosine receptor expression in osteoblasts. The present studies have indicated that adenosine may have a role in MTX inhibition of stimulation induced hyperpolarisation in HBC. Previous studies have suggested an interaction between MTX and adenosine signalling in inflammatory cells, but this study is the first time that MTX and adenosine signalling has been studied in bone cells. The studies have shown that adenosine inhibits stimulation induced hyperpolarisation to a similar magnitude to MTX and that the effects of MTX are reversed by adenosine deaminase, suggesting that the effects of MTX on HBC may be mediated via adenosine. In addition, adenosine decreases HBC adhesion to both fibronectin and collagen type 1 suggesting that adenosine may also have inhibitory effects on the β_1-

integrin subunit. It is not known whether MTX and adenosine exclusively affect the β_1-integrin subunit, or whether several integrin subunits may be affected. The present studies failed to determine which adenosine subunits are involved in MTX induced adenosine signalling. As mentioned previously, this may be due to a lack of specificity of the adenosine receptor antagonists used or it may be that the signalling pathways activated are distinct from the common positive / negative coupling to adenylate cyclase. Experiments which measure levels of cAMP before and after mechanical stimulation would perhaps provide information as to which adenosine receptors are involved. Similarly, inhibition of adenylate cyclase with a selective inhibitor would determine if adenylate cycalse has a role in this pathway or whether an atypical or novel adenosine signalling pathway is responsible. Once this has been established experiments can be performed to elucidate the exact signalling mechanisms involved. At the present time, it is unclear how such signalling pathways might lead to alterations in integrin conformation and / or expression. There is limited evidence to suggest that adenosine can reduce the adhesion of stimulated neutrophils to endothelial cells by inhibiting upregulation of β_2-integrin subunits or by preventing conformational changes which are necessary for adhesion. It is most likely that adenosine exerts its effects on the integrin subunits via an 'inside-out' signalling mechanism. It is thought that 'inside-out' integrin signalling is as crucial as 'outside-in' integrin signalling, and that 'inside-out' signalling is responsible for ensuring that the integrin subunits are in an 'appropriate' conformation to be activated by 'outside-in' signalling. At present, the exact mechanisms involved in 'inside-out' signalling are poorly defined but it is believed that various intracellular signalling pathways including phosphatidylinositol lipid breakdown, protein kinase C and small G-proteins may be involved in activation (or repression) of integrins via certain highly conserved motifs which are located on the cytoplasmic domains of the integrin subunits. Further investigation of these signalling pathways may involve transfection of bone cells with integrin subunits with point mutations or deletions of the cytosolic consensus sequences. The usefulness of performing such experiments on transfected human bone cell lines are limited by the fact that human bone cell lines examined in the preliminary stages of

this study did **not** hyperpolarise following mechanical stimulation (results not shown).

APPENDIX I

Original data from which the results given in the Tables of Chapters 4, 5 and 6 are calculated. The Table number corresponds to that in the Results chapters.

A = HBC grown from trabecular bone fragment obtained from adult donors.

C = HBC grown from trabecular bone fragment obtained from juvenile donors.

Table 4.1 – Raw data from which parameters in generalised linear model were estimated.

(a) Raw data collected from juvenile HBC.

Resting Cells (-mV)	Following MS (-mV)
34	62
32	44
25	50
27	52
29	49
30	66
26	55
21	58
21	57
30	52
34	62
32	44
25	50
27	52
29	49
30	66
26	55
21	56
21	57
30	52
34	62
32	44
25	50
27	52
29	49
37	64
39	54
44	44
42	59
36	55

19	68
19	52
22	44
26	37
18	62
19	68
19	52
22	44
26	37
18	62
40	66
38	50
41	61
39	50
41	51
28	39
24	34
23	37
28	43
18	39
48	66
38	50
41	61
39	50
41	51
28	38
27	40
37	45
26	44
37	47
20	65
24	42
23	50
20	38
25	51
22	38
30	36
25	36
22	37
28	36
40	62
41	54
35	44
33	55
34	42
21	38
29	47
26	40
24	39
28	39

27	50
25	54
30	56
32	40
36	47
29	60
32	59
28	55
41	64
33	58

n = 90.

(b) Raw data collected from adult HBC.

Resting Cells (-mV)	Following MS (-mV)
31	58
29	46
35	52
36	63
32	53
39	58
31	52
29	48
38	51
29	68
31	58
29	46
35	52
36	63
32	53
33	58
37	51
38	55
34	54
33	50
39	58
31	52
29	48
28	51
29	68
33	58
37	51
38	55
34	54
33	50

34	40
27	41
27	40
25	56
25	38
23	43
34	60
26	56
28	58
24	42
28	40
30	42
26	39
27	39
23	37
25	57
22	52
28	63
30	66
39	50
29	58
27	43
38	44
34	50
35	54
21	38
29	47
26	40
24	39
28	39
40	65
34	48
42	57
36	53
31	67
28	44
33	59
35	41
24	58
27	48
33	59
41	52
31	41
32	42
37	47
42	65
30	50
30	55
35	59
38	61

41	56
38	58
47	51
39	64
47	47

n = 85.

Table 4.2 – Effect of CM from HBC mechanically stimulated for 20 minutes, on the membrane potential of previously unstimulated HBC.

		Membrane potential (-mV)			
Donor	n	Resting	Post MS	Resting	With-CM
12542 A	5	34, 27, 27, 25, 25	40, 41, 40, 56, 38	23, 27, 25, 22, 27	38, 36, 39, 41, 35
12485 C	5	30, 26, 21, 21, 30	66, 55, 58, 57, 52	22, 33, 30, 34, 33	55, 67, 65, 50, 57
12507 A	5	39, 31, 29, 38, 29	58, 52, 48, 51, 68	24, 39, 29, 26, 30	60, 59, 64, 58, 72
12779 C	5	19, 19,22, 26, 18	68, 52, 44, 37, 62	32, 29, 28, 31, 33	51, 48, 38, 42, 46

Table 4.3 – Effect of recombinant IL-1β on HBC membrane potential.

		Membrane Potential (-mV)	
Donor	n	Resting	+ IL-1β
12485 C	5	34, 32, 25, 27, 29	62, 44, 50, 52, 49
12551 C	5	40, 38, 41, 39, 41	66, 50, 61, 50, 51
12542 A	5	23, 34, 26, 28, 24	43, 60, 56, 58, 42

Table 5.1 – Effect of MTX on the electrophysiological response of HBC to cyclical mechanical stimulation.

Donor	Reagent	n	Membrane Potential (-mV)		
			Resting Cells	+ MTX	Post 0.33Hz MS
12507 A	Nil	5	31, 29, 35, 36, 32	n/a	58, 46, 52, 63, 53
	1 μM MTX	5	46, 42, 52, 49, 42	55, 36, 42, 38, 54	52, 50, 40, 32, 36
	50 nM MTX	5	38, 45, 42, 48, 34	63, 34, 35, 40, 44	33, 40, 46, 34, 45
	1 nM MTX	5	37, 31, 48, 32, 42	35, 45, 32, 39, 34	57, 38, 45, 46, 66
12443 C	Nil	5	28, 27, 37, 26, 37	n/a	38, 40, 45, 44, 47
	1 μM MTX	5	40, 41, 33, 34, 31	38, 38, 36, 40, 30	38, 40, 42, 40, 42
	50 nM MTX	5	34, 33, 36, 32, 33	35, 32, 43, 35, 49	42, 36, 39, 37, 50
	1 nM MTX	5	34, 44, 38, 35, 36	39, 34, 38, 45, 43	50, 56, 46, 53, 48

Table 5.1 (continued) – Effect of MTX on the electrophysiological response of HBC to cyclical mechanical stimulation.

			Membrane Potential (-mV)		
Donor	Reagent	n	Resting Cells	+ MTX	Post 0.33Hz MS
12551 C	Nil	5	28, 24, 23, 28, 18	n/a	39, 34, 37, 43, 39
	1 μM MTX	5	35, 37, 42, 32, 31	39, 36, 40, 31, 30	40, 40, 38, 40, 46
	50 nM MTX	5	35, 36, 36, 33, 38	33, 50, 36, 46, 44	38, 36, 36, 34, 35
	1 nM MTX	5	39, 32, 34, 30, 28	31, 46, 38, 41, 32	40, 26, 39, 26, 27

Table 5.2 (a) – CM from HBC mechanically stimulated for 20 minutes induces membrane hyperpolarisation when transferred to previously unstimulated HBC. If HBC are incubated with 50 nM MTX prior to mechanical stimulation, no transfer of hyperpolarising activity is observed when CM is transferred to unstimulated cells.

			Membrane Potential (-mV)				
Donor	Reagent	n	Resting Cells	+ MTX	Post 0.33Hz MS	Resting	With CM
12542 A	Nil	5	34, 27, 27, 25, 25	n/a	40, 41, 40, 56, 38	23, 27, 25, 22, 27	38, 36, 39, 41, 35
	50 nM MTX	5	31, 28, 23, 32, 24	25, 35, 28, 30, 29	21, 24, 24, 28, 34	24, 28, 21, 28, 33	27, 34, 34, 35, 29
12507 A	Nil	5	33, 37, 38, 34, 33	n/a	58, 51, 55, 54, 50	42, 44, 39, 40, 33	56, 54, 59, 60, 54
	50 nM MTX	5	43, 35, 44, 45, 35	34, 33, 35, 32, 43	30, 36, 38, 33, 32	30, 45, 34, 37, 33	33, 48, 38, 35, 32
12648 C	Nil	5	20, 24, 23, 20, 25	n/a	65, 42, 50, 38, 51	23, 27, 23, 31, 19	41, 55, 54, 54, 57
	50 nM MTX	5	31, 40, 32, 36, 33	38, 19, 27, 26, 23	43, 23, 26, 38,32	33, 36, 29, 38, 21	42, 28, 28, 24, 38

Table 5.2 (b) - CM from HBC's mechanically stimulated for 20 minutes induces membrane hyperpolarisation when transferred to previously unstimulated HBC's. Incubation of unstimulated (CM) cells with MTX has no effect of the ability of these cells to be activated following transfer of media from mechanically stimulated cells.

			Membrane Potential (-mV)				
Donor	Reagent	n	Resting Cells	Post 0.33Hz MS	Resting	+ MTX	With CM
12507 A	Nil	5	39, 31, 29, 38, 29	58, 52, 48, 51, 68	24, 39, 29, 26, 30	n/a	60, 59, 64, 58, 72
	50 nM MTX	5	42, 43, 33, 43, 35	54, 56, 56, 60, 66	30, 32, 40, 38, 35	35, 33, 46, 43, 33	48, 52, 62, 61, 60
12485 C	Nil	5	30, 26, 21, 21, 30	66, 55, 56, 57, 52	22, 33, 30, 34, 33	n/a	55, 67, 65, 50, 57
	50 nM MTX	5	32, 26, 23, 25, 24	56, 58, 62, 51, 71	35, 32, 26, 26, 36	36, 29, 26, 29, 34	59, 47, 67, 50, 51
12779 C	Nil	5	19, 19, 22, 26, 18	68, 52, 44, 37, 62	32, 29, 28, 31, 23	n/a	51, 48, 38, 42, 46
	50 nM MTX	5	41, 20, 28, 23, 40	53, 70, 60, 42, 51	29, 20, 22, 29, 26	23, 26, 27, 27, 19	54, 39, 37, 66, 46

Table 5.3 – Effect of MTX on the membrane hyperpolarisation response of HBC to IL-1β.

			Membrane Potential (-mV)		
Donor	Reagent	n	Resting Cells	+ MTX	IL1-β
12485 C	Nil	5	34, 32, 25, 27, 29	n/a	62, 44, 50, 52, 49
	50 nM MTX	5	35, 24, 29, 30, 38	25, 42, 25, 24, 31	46, 52, 61, 56, 42
12485 C	Nil	5	37, 39, 44, 42, 36	n/a	64, 54, 44, 59, 55
	50 nM MTX	5	37, 37, 48, 41, 38	45, 40, 38, 40, 49	50, 43, 54, 51, 63
12251 C	Nil	5	40, 38, 41, 39, 41	n/a	66, 50, 61, 50, 51
	50 nM MTX	5	33, 33, 37, 39, 30	38, 34, 47, 36, 33	52, 61, 53, 53, 46

Figure 5.4 – The effect of MTX on the adhesion of HBC to fibronectin (FN). Results are expressed as % of control (No MTX)

	12443 C	12592 C	13929 A	Mean
No MTX	100.0	100.0	100.0	100.0
1 μM MTX	53.0	72.0	68.0	64.3
50 nM MTX	63.0	65.0	68.0	65.3
1 nM MTX	52.0	68.0	76.0	65.3

Figure 5.5 - The effect of MTX on the adhesion of HBC to type 1 collagen (coll 1). Results are expressed as % of control (no MTX).

	12648 C	12779 C	13929 A	Mean
No MTX	100.0	100.0	100.0	100.0
1 μM MTX	54.0	48.0	64.0	55.3
50 nM MTX	60.0	70.0	66.0	65.3
1 nM MTX	64.0	47.0	75.0	62.0

Table 6.1 –Adenosine inhibits HBC responses to cyclic mechanical stimulation.

			Membrane Potential (-mV)		
Donor	**Reagent**	**n**	**Resting**	**+ Aden**	**Post MS**
13862 A	Nil	5	28, 30, 26, 27, 23	n/a	40, 42, 39, 39, 37
	Aden (100 μM)	5	26, 24, 31, 31, 24	29, 39, 36, 35, 26	28, 29, 33, 24, 27
13841 A	Nil	5	25, 22, 28, 30, 39	n/a	57, 52, 63, 66, 50
	Aden (100 μM)	5	23, 27, 24, 26, 23	33, 25, 26, 23, 24	30, 31, 35, 32, 24
13897 C	Nil	5	22, 30, 25, 22, 28	n/a	38, 36, 36, 37, 36
	Aden (100 μM)	5	26, 25, 20, 28, 28	29, 25, 21, 21, 24	20, 19, 17, 22, 20

Table 6.2 - Adenosine deaminase restores normal HBC responses to mechanical stimulation to MTX treated cells.

			Membrane Potential (-mV)			
Donor	Reagent	n	Resting	+ ADA	+ MTX	Post MS
13897 C	Nil	5	40, 41, 35, 33, 34	n/a	n/a	62, 54, 44, 55, 42
	ADA	5	40, 37, 30, 30, 37	24, 24, 40, 25, 30	n/a	44, 48, 46, 46, 47
	MTX	5	36, 30, 32, 35, 28	n/a	30, 35, 37, 33, 35	23, 26, 37, 31, 27
	ADA + MTX	5	22, 27, 27, 36, 34	26, 31, 42, 29, 34	28, 26, 22, 25, 28	52, 40, 46, 62, 67
13880 A	Nil	5	29, 27, 38, 34, 35	n/a	n/a	58, 43, 44, 50, 54
	ADA	5	30, 30, 24, 36, 36	35, 27, 30, 30, 24	n/a	52, 50, 44, 39, 45
	MTX	5	26, 24, 36, 36, 24	n/a	34, 41, 33, 40, 46	43, 33, 29, 38, 44
	ADA + MTX	5	32, 40, 27, 32, 28	36, 42, 29, 29, 29	29, 29, 28, 29, 36	40, 54, 54, 57, 64

Figure 6.1 – The effect of adenosine on the adhesion of HBC to fibronectin (FN). Results are expressed as % of control (no reagent).

	12648 C	13800 A	13920 A	Mean
No Reagent	100.0	100.0	100.0	100.0
100 μM Adenosine	58.0	78.0	58.0	64.7
50 nM MTX	62.0	76.0	65.0	67.7

Figure 6.2 – The effect of adenosine on the adhesion of HBC to type 1 collagen (coll 1). Results are expressed as % of control (no reagent).

	12648 C	13821 A	13920 A	Mean
No Reagent	100.0	100.0	100.0	100.0
100 μM Adenosine	68.0	79.0	78.0	75.0
50 nM MTX	72.0	76.0	78.0	75.3

Table 6.3 - The A_1 receptor, 8-Cyclopentyl –1, 3-dipropylxanthine (DPCPX), restores normal HBC responses to mechanical stimulation to MTX treated cells.

			Membrane Potential (-mV)			
Donor	Reagent	n	Resting	+ DPCPX	+MTX	Post MS
13891 C	Nil	5	21, 29, 26, 24, 28	n/a	n/a	38, 47, 40, 39, 39
	DPCPX	5	41, 30, 26, 24, 26	27, 41, 23, 29, 30	n/a	50, 62, 55, 47, 50
	MTX	5	28, 35, 39, 39, 34	n/a	31, 32, 23, 27, 25	35, 23, 25, 34, 27
	DPCPX + MTX	5	24, 41, 28, 29, 20	29, 20, 29, 22, 32	27, 20, 21, 28, 32	42, 57, 43, 50, 56
13900 A	Nil	5	33, 30, 39, 29, 38	n/a	n/a	50, 64, 53, 70, 63
	DPCPX	5	40, 45, 33, 44, 46	44, 31, 36, 35, 31	n/a	53, 51, 49, 59, 49
	MTX	5	32, 36, 38, 38, 29	n/a	30, 38, 38, 39, 34	38, 42, 45, 34, 40
	DPCPX + MTX	5	25, 31, 29, 28, 40	29, 39, 40, 36, 38	33, 27, 24, 34, 33	65, 39, 68, 47, 58

Table 6.3 (continued) - The A_1 receptor, 8-Cyclopentyl –1, 3-dipropylxanthine (DPCPX), restores normal HBC responses to mechanical stimulation to MTX treated cells.

			Membrane Potential (-mV)			
Donor	Reagent	n	Resting	+ DPCPX	+MTX	Post MS
13883 A	Nil	5	40, 34, 42, 36, 31	n/a	n/a	65, 48, 57, 53, 67
	DPCPX	5	34, 45, 31, 30, 33	43, 38, 33, 33, 35	n/a	66, 53, 61, 59, 52
	MTX	5	30, 38, 41, 39, 42	n/a	32, 48, 45, 37, 32	34, 36, 26, 29, 28
	DPCPX + MTX	5	29, 26, 28, 24, 34	36, 31, 36, 32, 42	29, 35, 35, 48, 38	47, 51, 47, 46, 56

Table 6.4- The A_{2A} receptor, 8-(3-chlorostyryl) caffeine (CSC), restores normal HBC responses to MTX treated cells.

			Membrane Potential (-mV)			
Donor	Reagent	n	Resting	+ CSC	+MTX	Post MS
13883 A	Nil	5	28, 33, 35, 24, 27	n/a	n/a	44, 59, 41, 58, 48
	CSC	5	30, 32, 30, 29, 38	40, 31, 40, 30, 32	n/a	66, 52, 46, 50, 39
	MTX	5	30, 40, 41, 31, 37	n/a	36, 28, 39, 40, 34	38, 36, 28, 37, 38
	CSC + MTX	5	22, 30, 32, 29, 30	26, 27, 30, 29, 23	32, 41, 42, 37, 31	53, 39, 51, 40, 50
13956 C	Nil	5	29, 32, 28, 41, 33	n/a	n/a	60, 59, 55, 64, 58
	CSC + MTX	5	31, 37, 34, 42, 34	38, 30, 40, 39, 30	29, 35, 39, 40, 30	60, 62, 51, 40, 46

Table 6.5 - The A_{2B} receptor, Alloxazine, does not restore normal HBC responses to MTX treated cells.

			Membrane Potential (-mV)			
Donor	Reagent	n	Resting	+ Alloxazine	+MTX	Post MS
13921 A	Nil	5	33, 41, 31, 32, 37	n/a	n/a	59, 52, 41, 42, 47
	Alloxazine	5	34, 27, 31, 34, 38	32, 33, 35, 36, 32	n/a	43, 47, 45, 40, 53
	MTX	5	38, 37, 35, 37, 43	n/a	33, 41, 40, 40, 34	30, 30, 39, 32, 40
	Alloxazine + MTX	5	40, 42, 44, 37, 34	44, 32, 36, 40, 36	34, 37, 37, 33, 33	40, 28, 30, 42, 30
13909 A	Nil	5	42, 30, 30, 35, 38	n/a	n/a	65, 50, 55, 59, 61
	Allozazine + MTX	5	32, 37, 38, 36, 37	43, 37, 38, 45, 35	43, 40, 42, 41, 46	37, 38, 45, 48, 39

Table 6.6 - The A_3 receptor, MRS-1191 restores normal HBC responses to MTX treated cells.

			Membrane Potential (-mV)			
Donor	Reagent	n	Resting	+ MRS-1191	+MTX	Post MS
13891 C	Nil	5	27, 25, 30, 32, 36	n/a	n/a	50, 54, 56, 40, 47
	MRS-1191	5	29, 40, 28, 28, 33	36, 33, 39, 29, 28	n/a	38, 50, 48, 51, 40
	MTX	5	32, 38, 41, 30, 35	n/a	30, 34, 35, 30, 39	30, 31, 30, 29, 39
	MRS-1191 + MTX	5	36, 30, 35, 34, 40	21, 26, 27, 26, 22	34, 42, 32, 33, 39	48, 54, 49, 58, 48
13909 A	Nil	5	41, 38, 47, 39, 47	n/a	n/a	56, 58, 51, 64, 47
	MRS-1191 + MTX	5	37, 43, 46, 37, 38	38, 40, 35, 45, 44	38, 40, 44, 44, 43	57, 66, 56, 47, 48

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