THE ROLE OF MACROPHAGES IN PATHOLOGICAL BONE RESORPTION

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Abstract

This thesis examines several aspects of macrophage-osteoclast differentiation in man including, in detail, the manner in which human inflammatory tissue macrophages in periprosthetic tissues surrounding loose prostheses might contribute to osteoclast differentiation and pathological bone resorption associated with aseptic loosening of total joint replacements.

In vitro studies were undertaken to determine whether cells present in periprosthetic tissues surrounding a loose implant could differentiate into osteoclastic bone resorbing cells. The stromal and humoral factors important in this process were studied. These findings were compared to cells isolated from synovial tissue from osteoarthritic (OA) joints. Cultures of periprosthetic macrophages required the presence of osteoblast-like cells and $1,25(OH)_2D_3$ for osteoclast differentiation, but did not require the addition of dexamethasone or exogenous M-CSF. This is in contrast to cultures of OA synovial macrophages where the addition of exogenous M-CSF was found to greatly enhance osteoclast differentiation.

Cell mediators including IL-1β, TNFα, IL-6, M-CSF and PGE_2 are known to stimulate osteoclastic activity *in vivo* and *in vitro*, and are thought to influence the formation of osteoclasts at sites of pathological bone resorption. The levels of these mediators released from periprosthetic cells during coculture with UMR 106 cells was measured using enzyme-linked immunoassays. Substantial levels of M-CSF and IL-6 were found to be released during the first four days of coculture. Small levels of PGE_2 and IL-1β were also released but TNFα release was not detected. Antibodies directed against M-CSF and IL-6 significantly inhibited but did not abolish osteoclast formation and bone resorption. Time course studies showed that these antibodies inhibited the early stages of osteoclast formation from periprosthetic macrophages, most likely osteoclast precursor proliferation.

Serial frozen sections of periprosthetic revision arthroplasty tissue were used to determine the cytokine receptor profile of macrophages and foreign body giant cells (FBGCs) present in these tissues. Using immunohistochemical staining techniques, cells which stained positive for the macrophage-associated antigen CD68 were found to express receptor antigens for IL-1R Type 1, TNFR, IL-6R, M-CSFR and SCFR. Weak staining for GM-CSFR was also detected but IL-3R expression was not found. Imprints of giant cell tumours of bone were also studied to determine the receptor profile of mature osteoclasts. Strong similarities in the cytokine receptor antigen expression on macrophages, FBGCs and osteoclasts were found which possibly reflects the common lineage of these cells.

The direct effect of wear particles on osteoclast formation in an *in vitro* human monocyte-UMR 106 cell coculture system was studied in order to determine whether prosthesis wear particles significantly influence osteoclast differentiation and bone resorption. Phagocytosis of 1.0μm latex beads by human monocytes had no effect on the ability of these cells to undergo osteoclast differentiation and resorb bone. However, metal wear particles of

phagocytosable sizes were found to inhibit osteoclast formation from human monocytes resulting in a decrease in lacunar bone resorption *in vitro*. CoCr and stainless steel particles caused the greatest inhibition. These particles were toxic to the cells. Particles of cpTi and TiAlV also inhibited osteoclast formation and bone resorption although to a lesser degree.

In order to develop an *in vitro* system of human macrophage-osteoclast differentiation which more closely parallels the human situation, a human macrophage-human osteoblastic cell coculture system of osteoclast formation was developed as opposed to the human-rat cell coculture system. Human bone-derived stromal cells were shown to be capable of supporting osteoclast differentiation from periprosthetic cells. The addition of $1,25(OH)_2D_3$ was not found to be essential for osteoclast formation and bone resorption in cocultures of human periprosthetic macrophages and human bone stromal cells. Substantial amounts of PGE_2 were also found to be released early by the human bone-derived stromal cells in coculture. The addition of exogenous PGE_2 was also found to have a strong stimulatory effect on osteoclastic bone resorption in this coculture system.

Finally, a study was undertaken to characterise another joint condition associated with bone destruction, pigmented villonodular synovitis (PVNS). The giant cells in this potentially osteolytic synovial lesion were found to express the phenotypic characteristics of osteoclasts and not macrophage polykaryons.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Susan Dorothy Neale. 8.1.98

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TABLE OF CONTENTS

CHAPTER 1. General Introduction and Review of the Literature

CHAPTER 2. General Methods for *In Vitro* Studies

CHAPTER 3. Characterisation of Osteoclast Differentiation from Periprosthetic Macrophages *In Vitro*

1. General Introduction and Review of the Literature

The literature review first gives an overview of bone cell biology, and what is known of osteoclast origin and function, and control of bone resorption. The role of macrophages in conditions of pathological bone resorption, particularly aseptic loosening of total joint replacements, is then discussed.

1.1. Bone cell biology

There is a continuous process of bone remodelling throughout life in which the balance of resorption and formation of bone is maintained. In the mature skeleton, normal bone undergoes structural remodelling in growth, in response to changing patterns of mechanical stress and under pathological conditions (Frost 1980; Marks and Popoff 1988). The remodelling of bone is largely controlled by the cells that carry out bone formation and resorption, namely osteoblasts and osteoclasts, and the activities of these cells are modulated by systemic hormones and local factors.

1.1.1. Osteoblasts

Osteoblasts are thought to be derived from pluripotent stem cells of the stromal cell system of the bone marrow. These stem cells differentiate into more committed precursors from which different bone stromal cells e.g. osteoblasts, chondrocytes, fibroblasts and adipocytes arise (Freidenstein 1976, Owen 1985). Osteoblasts are the cells responsible for bone formation. They are responsible for the production and subsequent mineralisation of bone matrix and appear in tissue sections as plump cells which contain a single large ovoid nucleus. Their basophilic cytoplasm contains abundant rough endoplasmic reticulum, mitochondria and Golgi apparatus (Holtrop 1975); it is glycogen-rich and contains alkaline phosphatase which is used as a cytochemical marker of osteoblasts (Ham and Cormack 1979; Stein et al 1990). Osteoblasts produce a matrix that is approximately 95% collagen type I as well as several proteins such as osteopontin, osteonectin, osteocalcin, proteoglycans, sialoproteins and growth factors (Franzen and Heinegard 1985; Vaes 1988). Most normal bone surfaces are not undergoing remodelling and are covered by bone lining cells. These are polygonal, flattened

cells which contain little endoplasmic reticulum and are thought to represent quiescent osteoblasts (Miller et al 1989).

Osteoblasts and bone lining cells are thought to mediate the stimulatory effects on osteoclasts of many hormones and cytokines. They are also thought to be responsible for initiating resorption by removing the extracellular barrier, which normally protects bone mineral from resorption, by the secretion of collagenase and tissue plasminogen activator (Delaisse et al 1981; Hamilton et al 1984; Heath et al 1984). Thus, osteoblasts and bone lining cells control several aspects of bone metabolism, permitting close coupling of bone resorption with subsequent bone formation.

1.1.2. Osteocytes

Osteoblasts that become embedded within the bone they have formed are known as osteocytes. They lose many of their protein synthesising organelles, become less active and stop secreting bone matrix. Osteocytes occupy lacunae which are connected with each other and with the surface osteoblasts and bone lining cells by a complex series of canaliculae (Ham and Cormack 1979). The functions of osteocytes are uncertain but it has been proposed that they may form a cellular communication network permeating bone.

1.1.3. Osteoclasts

The osteoclast is the main cell responsible for bone resorption. Osteoclasts appear *in vivo* as large multinucleated cells ranging in size from 20-100μm, with between 2-100 nuclei per cell. They are seen infrequently in normal adult bone but are present at sites of bone resorption often lying close to or within sites of bone excavation, Howship's lacunae. Osteoclasts have a characteristic ruffled border that forms beneath the cell at the site of attachment to bone matrix. This is a specialised area of the plasma membrane which contains many long cytoplasmic processes; this structure is actively involved in the process of bone resorption. Adjacent to the ruffled border, there is an organelle-free area of cytoplasm, the clear zone, which is rich in actin-like filaments and acts to form a seal between the osteoclast and the bone during resorption (Teti et al 1991). Other characteristic ultrastructural features such as large numbers of lysosomes, many

dense granules, prominent vesicles, abundant mitochondria, free ribosomes and extensive Golgi complexes are observed in the osteoclast cytoplasm outside of the clear zone (reviewed in Gothlin and Ericsson 1976; Vaes 1988; Roodman 1991). Once attached to the bone, the ruffled border secretes proteolytic enzymes into an area bounded by the clear zone to form an extracellular vacuole within which an active ion pump produces a low pH that facilitates the action of proteolytic enzymes and dissolution of the bone mineral (Vaes 1988; Blair et al 1993). After a few hours, the osteoclast stops resorption in response to an increase in calcium in the vacuole (Zaidi et al 1991), and moves on to another area of bone, releasing the contents of the vacuole as it moves.

1.1.3.1. Osteoclast formation and life span

Mitotic division has not been observed in osteoclasts, and ^{3}H thymidine incorporation (Gothlin and Ericsson 1976; Feldman et al 1980) and immunostaining by Ki67, a proliferation associated antigen, have not been detected (Apte 1990). It is generally accepted that osteoclasts form by the fusion of their mononuclear precursors. This process occurs rapidly and is subject to systemic and local control.

In the past, osteoclasts were thought to arise from the fusion of osteoblasts (Tonna and Cronkite 1961). A number of studies since have clearly shown that the osteoclast is haematopoietic in origin and not related to bone stromal cells. Parabiosis between irradiated and normal rats showed that the cells that fused to form osteoclasts in a healing fracture in the irradiated rat must have been derived from the non-irradiated rat through the circulation (Gothlin and Ericsson 1973). Experiments with quail-chick chimeric bone grafts made use of the morphological differences between quail and chick nuclei to determine the origin of the cells present in developing bone (Kahn and Simmons 1975; Jotereau and Le Douarin 1978). It was shown that osteoclasts formed by fusion of host mononuclear cells derived from the vasculature of the chorioallantoic membrane, whereas osteoblasts were of local origin. Further evidence came from the mouse model of the inherited bone disease, osteopetrosis (Marks and Walker 1976; Popoff and Marks 1995). Osteopetrosis is characterised by the accumulation of bone throughout the skeleton as a result of deficient osteoclastic bone

resorption. Both parabiosis between osteopetrotic mice (*gl/gl* and *mi/mi* mutants) and normal littermates, and transplantation of haematopoietic stem cell suspensions from normal to affected offspring increased osteoclast numbers and bone resorption. It is now known that multinucleated cells exhibiting all the specific phenotypic characteristics of osteoclasts, including that of lacunar bone resorption, can be formed from isolated cell preparations of the monocyte fraction of peripheral blood and tissue macrophages derived from the synovial membrane (Fujikawa et al 1996a; Fujikawa et al 1996b). Evidence from these studies and all subsequent studies have shown that osteoclasts form by the fusion of circulating cells that are haematopoietic in origin.

Some researchers have proposed a specialised line of osteoclast progenitors derived from bone marrow (Loutit et al 1981; Chambers and Horton 1984a). However, most of the current evidence suggests that the osteoclast forms part of the mononuclear phagocyte system and that osteoclasts, monocytes, and macrophages are derived from the same haematopoietic precursor cell population with differentiation into osteoclasts occurring at a relatively late stage. Consistent with their common haematopoietic origin, osteoclasts share many ultrastructural, cytochemical and morphological characteristics with monocytes and macrophages. Osteoclasts, like monocytes and macrophages, contain many lysosomes and phagosomes and are capable of phagocytosis; they also show trypsin resistant adherence to glass (Chambers 1979). Osteoclasts express CD45 (leukocyte common antigen) and a restricted range of macrophage-associated antigens including CD13, CD15, CD68 and CD54 (Athanasou et al 1987; Athanasou et al 1988; Athanasou and Quinn 1990). However, they do not express other monocyte/macrophage-associated markers such as CD11a, CD11b, CD14, CD18 and HLA-DR (Athanasou et al 1988; Athanasou and Quinn 1990). Unlike osteoclasts, monocytes, macrophages and macrophage polykaryons do not respond to calcitonin or express calcitonin receptors (Nicholson et al 1986; Hattersley and Chambers 1989a). Also they do not express the complex membrane structures of osteoclasts when they are cultured alone on a mineralised substrate (Kahn et al 1978). Monocytes and macrophages are chemotactically attracted to the products of degraded bone matrix (Mundy et al 1978; Mundy and Poser 1983). Mature macrophages are also known to be capable

of degrading both organic and inorganic matrix components of bone particles (Mundy et al 1977; Teitelbaum et al 1979) but unlike osteoclasts are not capable of lacunar bone resorption (Chambers and Horton 1984a).

Osteoclasts appear to have a life span *in vivo* of up to two weeks (Marks and Schneider 1982; Loutit and Townsend 1982) and during this time they can gain and lose nuclei (Marks and Seifert 1985). Recent studies have suggested that osteoclasts undergo apoptosis once the resorbing phase of bone remodelling has finished (Hughes et al 1995; Kameda et al 1995). Apoptosis is the process of programmed cell death which, unlike necrosis, is a genetically controlled response to certain developmental and environmental stimuli. Several factors such as interleukin-1 (IL-1) and macrophage colony stimulating factor (M-CSF) have recently been shown to enhance osteoclast survival *in vitro* whilst others such as oestrogen have been shown to reduce the life span of osteoclasts by promoting apoptosis (Fuller et al 1993; Jimi et al 1995; Kameda et al 1997). Thus, the prevention or stimulation of osteoclast death may be an important mechanism for regulating osteoclastic bone resorption.

1.1.3.2. Cellular factors controlling osteoclastic bone resorption

Osteoclastic bone resorption is thought to be dependent on a number of factors produced in the bone microenvironment by bone stromal/osteoblastic cells. Osteoblasts have been shown to produce factors which are essential for the differentiation of mononuclear precursors to osteoclasts and/or for optimisation of mature osteoclastic activity. Chambers et al (1984b) developed a method of studying bone resorption by isolating osteoclasts and culturing them on thin slices of cortical bone. In the presence or absence of osteoblasts, the extent of lacunar bone resorption was assessed. This model showed that bone resorbing factors, such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and IL-1, require the presence of osteoblasts to induce bone resorption (McSheehy and Chambers 1986; McSheehy and Chambers 1987). The conditioned media of stimulated osteoblasts was also shown to contain factors which induce bone resorption. This led to the view that these hormones and cytokines must first act on osteoblasts which would then be responsible for the activation of pre-existing osteoclasts and the generation of new osteoclasts from

precursors. The fact that osteoclasts do not express receptors for PTH and $1,25(OH)_2D_3$ whereas osteoblasts do express these receptors was further evidence for the role of osteoblasts in osteoclastic bone resorption (Kream et al 1977; Chambers 1980; Rodan and Martin 1981; Rouleau et al 1986; Merke et al 1986). Thus, osteoblasts appear to control the stimulation of osteoclastic activity. However, the process of bone resorption is known to activate and release latent bone-derived factors, such as transforming growth factor beta (TGFβ), and bone metalloproteinases, BMP 1 and 2, which affect the growth, differentiation and activity of osteoblasts (Oursler 1994; Martin 1993). PTH has also been shown to promote the synthesis of insulin growth factor 1 (IGF-1) by osteoblasts which is an important growth factor in bone, stimulating type 1 collagen synthesis and other proteins (Canalis et al 1989; Martin 1993). In this way, osteoclastic resorption releases factors which may act locally to control osteoblast coupling of the processes of resorption and formation.

Tumour cells and inflammatory cells such as macrophages and fibroblasts also release a number of cytokines and prostaglandins which enhance the bone resorptive activity of mature osteoclasts. The effects of these factors are known to be mediated indirectly via osteoblasts. Inflammatory cells may also contribute to osteoclastic bone resorption by the release of proteases (Chambers et al 1984c; Chambers et al 1985a; Vaes 1988), which degrade the organic matrix that covers bone surfaces, and by the release of reactive oxygen metabolites following phagocytosis (Nathan 1987).

1.2. Systemic and local factors influencing osteoclastic bone resorption

1.2.1. Systemic hormones

PTH, $1,25(OH)_2D_3$ and calcitonin are all systemic hormones that play important roles in calcium homeostasis. PTH is a hormone produced by the parathyroid gland largely in response to low blood calcium levels. The presence of PTH in the circulation increases blood calcium levels by acting primarily on bone as well as other parts of the body, such as the intestine and kidney. PTH has potent and rapid effects on bone resorption both *in vivo* and *in vitro* (Holtrop et al 1974; Yates et al 1988). Hyperparathyroidism is characterised by bone loss. PTH acts on

osteoblasts or bone stromal cells, rather than directly on osteoclasts, to stimulate the formation of osteoclasts and mature osteoclastic activity. PTH has been shown to stimulate the recruitment of osteoclast precursors, to induce the differentiation of immature precursors to mature osteoclast precursors, and stimulate their fusion into multinucleated cells (McSheehy and Chambers 1986; Takahashi et al 1988a; Kurihara et al 1991). In the presence of bone stromal cells, PTH has been shown to induce osteoclast formation from haematopoietic precursors via a cyclic AMP and protein kinase A pathway (Greenfield et al 1995; Kim et al 1997). Both IL-6 and IL-11 are thought to be mediators of PTH stimulated bone resorption. PTH stimulates IL-6 production and IL-6 mRNA expression in osteoblasts, and a specific antibody to IL-6 has been shown to inhibit PTH-induced resorption (Greenfield et al 1993; Manolagas 1995). Similarly, IL-11 production from bone marrow stromal cells is stimulated by PTH and a specific antibody to IL-11 blocks PTH-induced osteoclast formation (Girasole et al 1994; Manolagas 1995). PTH may also initiate resorption by stimulating the release of neutral proteases from osteoblasts or by inducing morphological changes in the shape of bone lining cells thereby allowing osteoclasts access to the underlying mineralised matrix to begin resorption (Jones and Boyde 1978).

Similarly, $1,25(OH)_2D_3$ is a steroid hormone which is important for regulating calcium levels. $1,25(OH)_2D_3$ is the active metabolite of vitamin D and is necessary for normal bone mineralisation and skeletal development. Like PTH, $1,25(OH)_2D_3$ is a potent stimulator of osteoclastic resorption and increases osteoclast formation via an osteoblast mediated mechanism (McSheehy and Chambers 1987). Osteoblasts, unlike mature osteoclasts, possess receptors for $1,25(OH)_2D_3$ (Kream et al 1977; Merke et al 1986). Monocytes and macrophages also possess $1,25(OH)_2D_3$ receptors (Gordon 1986). $1,25(OH)_2D_3$ is essential for the *in vitro* differentiation of osteoclast precursors into osteoclastic bone resorbing cells. This effect of $1,25(OH)_2D_3$ may be via the stimulation of cytokines, such as M-CSF, IL-6 and IL-11, from mononuclear phagocytes and bone stromal cells (Kaneki et al 1994; Girasole et al 1994; Romas et al 1996). $1,25(OH)_2D_3$ also acts to enhance monocyte adhesion to substrates, the production of monocyte-specific enzymes and expression of mononuclear phagocyte-associated antigens, as well as their capacity to bind and degrade devitalised bone particles (Bar-Shavit et al

1983). $1,25(OH)_2D_3$ also promotes the fusion of monocytes and macrophages to form macrophage polykaryons (Abe et al 1983).

In contrast to PTH and $1,25(OH)_2D_3$, calcitonin is a peptide hormone which acts to lower blood calcium levels. Calcitonin interacts directly with abundant receptors on mature mammalian osteoclasts to inhibit bone resorption (Chambers and Magnus 1982; Chambers et al 1985c), and has been used therapeutically to treat the growth of giant cell reparative granulomas of the jaw (Harris 1993). Calcitonin causes a rapid disappearance of the osteoclast ruffled border, an abrupt cessation of cell motility, and either physical detachment of the osteoclast from the bone surface or a marked decrease in the contact zone with bone. Changes in carbonic anhydrase location, disruption of actin rings, an increase in cytosolic-free calcium and a reduction in the rate of radiolabelled calcium release from bone have also been reported. When calcitonin is administered to rats, or added to organ cultures, a rapid decrease in the number of osteoclasts in bone is observed (reviewed in Suda et al 1997).

Other systemic hormones which influence bone metabolism include glucocorticoids and sex hormones. Glucocorticoids cause an increase in bone resorption *in vivo* and have been linked to the development of osteoporosis in Cushing's syndrome, as well as therapeutic administration (Reid 1989). *In vitro* dexamethasone has been shown to promote osteogenic differentiation in undifferentiated bone cell populations (Beresford et al 1994; Cheng et al 1994), and is an essential requirement for *in vitro* matrix nodule formation and mineralisation by human marrow-derived stromal cells. Dexamethasone has also been shown to enhance osteoclast formation although its mechanism of action is still unclear. Udagawa et al (1995) has shown that dexamethasone promotes IL-6 induced osteoclast formation by upregulating IL-6 receptor expression in murine osteoblastic cells. Other studies have shown that dexamethasone inhibits the release of certain bone resorbing mediators, including IL-1, TNF, IL-6 and PGE_2 (Russell 1993). Both dexamethasone and $1,25(OH)_2D_3$ have been shown to increase levels of M-CSF released from monocytes and osteoblastic cells (Kaneki et al 1994; Rubin et al 1997).

The importance of sex hormones in regulating bone metabolism is well established based on clinical observations. Oestrogen has an important role in the maintenance of bone mass in women and oestrogen withdrawal is associated with an increase in bone resorption and osteoporosis. There is compelling evidence to suggest that the effects of sex hormones are mediated via IL-6 production by bone stromal cells. Oestrogen receptors have been found on bone stromal and osteoblastic cells, and IL-6 production by these cells is inhibited by oestrogens (reviewed by Manolagas 1995). Oestrogen loss in the gonadectomised mouse model results in an increase in IL-6 production in the marrow and a stimulation of osteoclast progenitor numbers and osteoclast formation (Jilka et al 1992). This is mirrored by an increase in osteoclast numbers in trabecular bone. Further evidence is provided by the finding that an increase in the numbers of osteoclast progenitors or mature osteoclasts was not found in IL-6 deficient mice after ovariectomy. In addition to stimulating osteoclast formation, loss of oestrogen also causes an increase in circulating lymphocytes, neutrophils and monocytes which was mediated by IL-6. An increase in the responsiveness of bone marrow cells to cytokines, such as IL-6 and IL-11, which utilise gp130 for signal transduction was also found (Jilka et al 1995).

1.2.2. Local factors

1.2.2.1. Prostaglandins

Prostaglandins are a group of structurally related molecules derived from arachidonic acid. Many prostaglandins are known to be important local regulators of bone metabolism. PGE_2, in particular, is one of the most potent bone resorbing factors *in vitro* (Klein and Raisz 1970). It is released by a number of cells after activation including monocytes/macrophages, fibroblasts; osteoblasts and bone stromal cells. Production of PGE_2 by osteoblasts is known to be stimulated by mechanical stress implicating it in the remodelling responses to changing patterns of stress (Burger et al 1992). The effects of prostaglandins *in vitro* are dependent on the assay system used. In organ cultures, the addition of PGE_2 stimulates bone resorption at low concentrations (Klein and Raisz 1970). In culture systems of isolated mature osteoclasts, PGE_2 acts directly to inhibit osteoclast spreading and bone resorption (Chambers et al 1985c). However, the

presence of stromal cells in these cultures reverses this effect and stimulates spreading and bone resorption. Thus, PGE_2 can stimulate osteoclastic bone resorption indirectly via an osteoblast-mediated mechanism but can also act transiently on osteoclasts to inhibit directly osteoclast motility and bone resorption in a calcitonin-like manner. A number of bone resorbing factors, such as PTH, IL-1 and TNF, have been shown to stimulate prostaglandin synthesis by osteoblasts and the effects of these factors on bone resorption have been shown to be partly prostaglandin dependent (Sato et al 1986; Akatsu et al 1991; Tashjian et al 1987; Suda et al 1992). Prostaglandins can also affect osteoclastic bone resorption by stimulating the release of collagenase from osteoblasts (Delaisse et al 1981; Heath et al 1984), as well as other bone resorbing factors such as IL-6 and IL-11 (Bertolini et al 1994; Romas et al 1996).

Conflicting results have been reported about the effect of PGE_2 on osteoclast formation. In murine marrow cultures (Collins and Chambers 1991) and mouse spleen-primary osteoblastic cocultures (Akatsu et al 1989), osteoclast formation was stimulated by prostaglandins and also reduced by inhibitors of prostaglandin synthesis indicating that endogenous prostaglandins present may be important. Collins and Chambers (1992) found that prostaglandins could be substituted for $1,25(OH)_2D_3$ as a differentiating factor in murine marrow cultures. Similarly, PGE_2 was found to stimulate osteoclast formation in human marrow cultures (Flanagan et al 1995) but this was in contrast to a study by Chenu et al (1990) reporting that prostaglandins inhibited osteoclast formation. The nature of the osteoclast-like cells formed in this study was queried, however, as their ability to resorb bone was not determined (Flanagan et al 1995). Quinn et al (1997) found that the effect of prostaglandins on osteoclast formation in murine monocyte-stromal cell cocultures was dependent on the nature of the cell line used. Using the cell lines, UMR 106 and ST-2, it was found that prostaglandins both inhibited and stimulated osteoclast formation respectively. This finding confirms the importance of bone stromal cells in prostaglandin-mediated bone resorption.

1.2.2.2. Cytokines

A number of cytokines are known to play an important role in osteoclast formation and activity. Both IL-1β and TNFα are now recognised as potent stimulators of osteoclastic bone resorption (Bertolini et al 1986; Gowen and Mundy 1986; Tashjian et al 1987), and both have been shown to induce osteoclast-like cell formation in human bone marrow cultures (Pfeilschifter et al 1989). These cytokines have been implicated in the osteolysis of several bone diseases including osteoporosis and rheumatoid arthritis (Pacifici et al 1989; Arend and Dayer 1990; Manologas 1995). IL-1β and TNFα are primarily produced by monocytes/macrophages and although receptors for these cytokines have been found on osteoclasts, their stimulatory effects on bone resorption are mediated mainly by osteoblastic or bone stromal cells (Thomson et al 1986; Thomson et al 1987). Both IL-1 and TNF stimulate the release of prostaglandins by osteoblasts and their effects on bone resorption have been shown to be partly prostaglandin dependent (Akatsu et al 1991; Sato et al 1986; Tashjian et al 1987). It is also thought that IL-1 and TNF individually or in combination may enhance osteoclast formation by stimulating the production of IL-6 and IL-11 by osteoblasts (Romas et al 1996; Kim et al 1997).

IL-6 and IL-11 are members of a family of cytokines that use the glycoprotein, gp130, for signal transduction (Taga et al 1989; Kishimoto et al 1992). IL-6 is produced primarily by osteoblastic cells, and factors such as IL-1, TNF and PGE_2 stimulate IL-6 production by these cells (Ishimi et al 1990). Two independent studies have found that IL-6 stimulates *in vitro* bone resorption in fetal mouse metacarpal and calvaria (Lowik et al 1989; Ishimi et al 1990). IL-6 is known to stimulate the development of osteoclast progenitor cells, and in combination with soluble IL-6 receptor (sIL-6R) can increase osteoclast formation and induce osteoclastic bone resorption in cocultures of murine marrow cells and primary osteoblasts (Tamura et al 1993), and human marrow cultures (Kurihara et al 1990). Studies using human IL-6R overexpressing transgenic mice and *in vitro* coculture studies have shown that IL-6 can act directly on osteoblastic cells to induce osteoclast formation (Suda et al 1995). Increased levels of IL-6 and sIL-6R have been detected both systemically and locally in patients with rheumatoid

arthritis, multiple myeloma, Paget's disease, and oestrogen deficiency (Klein et al 1991; Jilka et al 1992; Roodman et al 1992; Kotake et al 1996) .

Recent findings suggest that IL-11, another cytokine produced by bone marrow stromal cells and osteoblastic cells, is important in osteoclast formation *in vitro* (Girasole et al 1994; Romas et al 1996). Both $1,25(OH)_2D_3$ and PTH have been found to stimulate IL-11 production in mouse bone marrow cultures. Addition of a specific antibody directed against IL-11 was found to inhibit osteoclast formation induced by either $1,25(OH)_2D_3$, PTH, IL-1 or TNF. Interestingly, the addition of inhibitors of IL-1 or TNF to the cocultures had no effect on IL-11 stimulated osteoclast formation but the addition of a prostaglandin inhibitor inhibited the effects of IL-11 (Girasole et al 1994; Manolagas 1995). These results suggest that the effects of IL-1 and TNF on osteoclast development are partly mediated by IL-11 produced from bone stromal cells, whereas the effects of IL-11 are independent of IL-1 and TNF but dependent on PGE_2 production. Unlike IL-6, upregulation of IL-11 production has yet to be linked to any pathological disease states. The findings to date suggest that IL-6 appears to be important in osteoclast development in pathological states, whereas IL-11 may be an essential cytokine for osteoclast development in general (Manolagas 1995).

1.2.2.3. Colony stimulating factors

Macrophage colony stimulating factor (M-CSF) was first described as a growth and differentiation factor for cells of the monocyte-macrophage lineage (Stanley and Heard 1977). Studies of the disease osteopetrosis in homozygous *op/op* mice, which is characterised by a severe reduction in osteoclast numbers, have shown that M-CSF is also an essential factor for osteoclast formation. Impaired production of M-CSF by calvarial and other cells and a decrease in the bone marrow macrophage population was found in the *op/op* osteopetrotic mouse but not in the normal littermates (Felix et al 1990; reviewed by Fleisch et al 1993). The cause of this deficiency was found to be a point mutation within the coding region of the M-CSF gene (Yoshida et al 1990). Administration of rhM-CSF to new born *op/op* mice was found to restore *in vivo* bone resorption (Felix et al 1990). The finding that osteoclast-like cells did not form *in vitro* when spleen cells were cocultured with osteoblasts from *op/op* mice unless exogenous M-CSF

was added to the coculture suggested that osteoblastic cells are the main cells responsible for M-CSF production (Kodama et al 1991; Takahashi et al 1991). Both murine osteoblast-like cells and the clonal bone cell line MC3T3-E1 have been shown to release M-CSF *in vitro* (Elford et al 1987) with the release of M-CSF enhanced in the presence of bone resorbing factors such as $1,25(OH)_2D_3$, IL-1, TNF and PTH. Fibroblasts and macrophages may also produce M-CSF (Rambaldi et al 1987; Oster et al 1987; Fibbe et al 1989; Fleisch et al 1993; Kaneki et al 1994).

M-CSF has subsequently been shown to be an essential factor for the proliferation and differentiation of osteoclast precursors (Kodama et al 1991; Takahashi et al 1991; Tanaka et al 1993; Fujikawa et al 1996a; Sarma and Flanagan 1996), and M-CSF can act directly on mature osteoclasts to promote survival (Fuller et al 1993; Sarma et al 1997). Receptors for M-CSF have been found on osteoclast precursors and mature osteoclasts (Kodama et al 1991; Weir et al 1993; Yang et al 1996). The effects of M-CSF on mature osteoclastic bone resorbing activity are unclear. M-CSF was shown to inhibit directly bone resorption by mature rat osteoclasts (Hattersley et al 1988), although a recent study using isolated human osteoclasts found that M-CSF directly stimulated bone resorption by enhancing survival (Sarma et al 1997). Species differences may account for these divergent findings.

The effects of other colony stimulating factors, such as granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 3 (IL-3) and stem cell factor (SCF), on osteoclast formation are still not clear. It has been proposed that these factors may be required in combination with other factors during the very early stages of differentiation of haematopoietic progenitors. IL-3 synergises with IL-6 to stimulate development of colony-forming units for granulocytes/macrophages, from which osteoclast precursors are formed (Kurihara et al 1991). Both GM-CSF and IL-3 have been found to stimulate osteoclast progenitor proliferation although their effects are not as potent as M-CSF (Takahashi et al 1991). Individually, IL-3 and SCF have not been found to have an effect on osteoclast formation or osteoclastic function whilst GM-CSF has been reported to be both a stimulator and inhibitor of osteoclast differentiation (Shinar et al 1990; Takahashi et al 1991; Demulder et al 1992; Suda et al 1995).

1.3. *In vitro* bone resorption assay systems

A number of *in vitro* assay systems have been used to assess bone resorption. The organ culture system has been widely used to examine osteoclastic bone resorption (Gowen and Mundy 1986; Bertolini et al 1986; Tashjian et al 1987). This model involves measuring calcium release from cultures of ^{45}Ca-labelled rat calvaria or long bones and is particularly useful for determining the overall effects of various factors on bone resorption. However, this system is not suitable for determining the mechanism of action of these factors or their individual effects on osteoclast formation because many different cell types are present and interactions between cells and the relative contributions of the various cell types can not be determined.

Culture of isolated disaggregated mature osteoclasts on slices of cortical bone or dentine is a useful model to determine the direct effect of factors on osteoclast function (Chambers et al 1984b). This model has been used to analyse the mechanism of action of systemic hormones such as PTH, $1,25(OH)_2D_3$ and calcitonin on osteoclastic bone resorption. In addition, several local factors such as PGE_2, IL-1 and M-CSF have been shown to have multiple effects on osteoclast activity depending on the cell types present and the culture system used. Examining the effects of these factors on osteoclasts in isolation allowed the direct inhibitory effects of calcitonin and PGE_2, and the effects of M-CSF and IL-1 on osteoclast survival to be demonstrated (Chambers et al 1985c; Fuller et al 1993; Sarma et al 1997). The disadvantage with this model is that it is difficult to obtain resident osteoclasts in sufficient numbers for quantitative studies because of their relative inaccessibility, low numbers, and poor survival following isolation from bone.

The above two models whilst useful do not allow for the study of osteoclast formation as well as mature osteoclast function. Thus, bone marrow culture systems were developed to investigate the origin of osteoclasts and enable osteoclast differentiation to be studied *in vitro*. The mouse marrow culture system is one of the most commonly employed models and involves long term culture of mouse marrow cells on slices of cortical bone devoid of osteoclast

progenitors (Takahashi et al 1988a). Multinucleated cells which form in these cultures have been shown to express all the phenotypic characteristics of osteoclasts, namely TRAP activity, calcitonin receptors and the ability to form resorption pits. This cell culture system has the advantage of being able to generate large numbers of osteoclast-like cells *in vitro*; it also allows osteoclast formation and function and the effects of various bone resorbing factors to be assessed quantitatively.

Subsequently, a cell coculture system was developed to examine the role of the different cell types generated from marrow on osteoclast differentiation. Mouse spleen cells were cocultured with primary osteoblastic cell populations in the presence of $1,25(OH)_2D_3$ on devitalised bone slices (Takahashi et al 1988b). The presence of osteoblastic cells and $1,25(OH)_2D_3$ were found to be essential for osteoclast formation. Later studies showed that stable transformed stromal cell lines, such as MC3T3-G2/PA6, ST2 and UMR 106, could be substituted for primary osteoblastic cells and induce osteoclast differentiation from spleen cells and monocytes (Udagawa et al 1989; Quinn et al 1994). It was also found that osteoclast progenitors were present in the monocyte fraction of mouse peripheral blood, alveolar macrophages and mononuclear cells isolated from the thymus (Udagawa et al 1990; Quinn et al 1994). The fact that not only immature haematopoietic cells but also blood monocytes and some tissue macrophages were capable of differentiating into osteoclasts in the presence of stromal cells supported the earlier studies proposing that osteoclasts form part of the mononuclear phagocyte system.

Using these systems the microenvironment required for osteoclast formation could be studied. It has been shown that osteoclast formation proceeds in two phases, an initial phase of proliferation of osteoclast precursors followed by a second phase in which these cells differentiate into osteoclasts. $1,25(OH)_2D_3$ and M-CSF were both found to be essential for osteoclast proliferation and differentiation (Takahashi et al 1991; Tanaka et al 1993). The importance of the presence of live stromal cells in contact with osteoclast precursors was demonstrated indicating that the stromal cells are responsible for inducing osteoclast formation possibly by the synthesis of a membrane bound factor (Suda

et al 1992). This was challenged by recent studies using enriched haemopoietic progenitor cells which reported that multinucleated osteoclast-like cells can be formed in the absence of osteoblastic stromal cells (Kurihara et al 1991; Matayoshi et al 1996).

Recently, several *in vitro* models of human osteoclast formation have been developed. In comparison to isolated mature osteoclasts, use of these coculture systems enables the generation of substantial numbers of human bone resorbing osteoclasts, providing a means of studying human osteoclast biology *in vitro*. Sarma and Flanagan (1996) developed osteoclasts from human bone marrow. This system involves culturing Ficoll-Paque fractionated marrow mononuclear cells which consisted of a heterogenous population of fibroblasts, adipocytes, macrophages and other haematopoietic cells. In the presence of M-CSF and $1,25(OH)_2D_3$, large numbers of vitronectin receptor positive osteoclast-like cells formed after long term culture and extensive bone resorption was seen. Another human model developed by Fujikawa et al (1996a) found that osteoclast precursors circulate within the monocyte fraction of peripheral blood. This system involves the long term coculture of human monocytes isolated from peripheral blood and rodent osteoblastic cells in the presence of $1,25(OH)_2D_3$ and M-CSF. This model confirmed the importance of contact with osteoblastic cells and the presence of $1,25(OH)_2D_3$ and M-CSF for osteoclast formation. However in a recent study by Matayoshi et al (1996), human osteoclastic bone resorbing cells were formed in the absence of bone stromal cells. Pretreatment of healthy donors with granulocyte colony stimulating factor (G-CSF) resulted in the mobilisation of CD34 positive cells into the circulation. These cells were purified from peripheral blood and cultured in the presence of GM-CSF, IL-1 and IL-3 for up to six weeks. Multinucleated cells with all the phenotypic and functional characteristics of osteoclasts formed in these cultures. Whilst providing valuable information, the lengthy culture time and multiple manipulations involved in this system have not encouraged widespread use. Other researchers have used human cell lines to generate osteoclast-like cells *in vitro*. Yoneda et al (1991) used the human promyelocytic HL-60 cell line, which when cultured in methylcellulose and treated with conditioned media from MH85 tumour cells followed by $1,25(OH)_2D_3$, formed osteoclastic bone resorbing cells. Another

human promyelocytic cell line, FLG 29.1, has been shown to give rise to osteoclast-like cells when treated with phorbol esters (Gattei et al 1992). However, although these multinucleated cells express some distinctive osteoclast phenotypic markers, e.g. response to calcitonin and resorption of bone particles, they have not been shown to be capable of resorption pit formation on cortical bone slices.

1.4. Summary

Normal bone resorption is coupled to bone formation, and numerous systemic and local factors can regulate the recruitment of osteoclast precursors to the bone surface, the formation of new osteoclasts and the activity and survival of resident osteoclasts. The stimulatory effects of these factors on bone resorption and osteoclast formation are effected by osteoblastic cells. It is likely that these factors induce a critical common factor, as yet unknown, to stimulate osteoclast formation and bone resorption. Using *in vitro* and *in vivo* models, the complex cellular and hormonal mechanisms governing the formation and activity of osteoclasts can be determined. This knowledge can in turn be used in understanding the mechanisms related to abnormal bone resorption where an increase in the number or activity of osteoclasts is found.

1.5. Macrophages and pathological bone resorption

Macrophages are commonly observed at sites of increased bone resorption in a number of pathological conditions, including primary or secondary tumours of bone and inflammatory conditions of bone such as osteomyelitis, rheumatoid arthritis and aseptic loosening of total joint replacements. In all these pathological states, the resorption of bone may result from an increase in the number and/or activity of osteoclasts. Inflammatory macrophages release a number of cytokines and prostaglandins, such as IL-1, TNF, IL-6 and PGE_2, which are known to stimulate osteoclastic bone resorption. The effects of these factors are mediated through osteoblasts and stromal cells and may stimulate resorption by resident mature osteoclasts or increase the formation of new osteoclasts from osteoclast precursors. Inflammatory macrophages may also contribute to osteoclastic bone resorption by the release of tissue damaging proteases such as collagenase and plasminogen activator (Vaes 1988; Konttinen et al 1996), which degrade the organic matrix that covers bone surfaces, and by the release of reactive oxygen metabolites following phagocytosis (Nathan 1987; Garrett et al 1990). Factors released by macrophages also promote fibroblast proliferation and activity (Shanbhag et al 1997; Lee et al 1997), and stimulate osteoblasts in turn to release more inflammatory mediators, proteases and chemotactic proteins that promote the recruitment of monocytes into these osteolytic lesions (Malone et al 1982; Zhu et al 1994; Haynes et al 1997).

There is some controversy as to whether macrophages themselves can directly resorb bone. Experimental evidence of bone resorption directly by macrophages has produced contradictory results. Mundy et al (1977) first reported that both monocytes and macrophages induce calcium release from non-viable bone. Although no direct visual evidence of bone resorption was obtained, this increase in calcium release did not appear to be mediated by hormones or cytokines that usually stimulate osteoclastic bone resorption or changes in pH, and the authors concluded that macrophages can directly cause bone resorption. Several other studies supported this finding (Kahn et al 1978; MacArthur et al 1980) until a different criteria for bone resorption activity was used, namely the direct visualisation of resorption pits on mineralised surfaces by scanning electron microscopy (SEM). In several studies, both monocytes and macrophages

failed to produce lacunar resorption pits characteristic of osteoclastic bone resorption (Chambers and Horton 1984a; Pazzaglia and Pringle 1989). A series of experiments with macrophages from a range of pathological tissues described a poorly defined surface roughening as a form of resorption but no well defined pits (Athanasou et al 1992). There is much debate as to whether this form of low grade resorption is clinically significant. Extensive low grade surface resorption by large numbers of mononuclear phagocytes over a long period of time may be clinically significant, and may explain the histological observation of tiny scalloping marks seen at the interface of periprosthetic tissue and bone, and the relative scarcity of osteoclasts in many slowly developing osteolytic lesions.

Tissue macrophages consist of a heterogeneous population of cells with different morphology, function, immunophenotype, and enzyme histochemistry. Numerous *in vitro* studies have shown that there are cells of macrophage phenotype present in this population which are capable of differentiation into functionally mature osteoclasts (Udagawa et al 1990; Quinn et al 1992; Fujikawa et al 1996b). It has been estimated that approximately 5% of isolated monocyte/macrophage populations have the potential for proliferation (van Furth 1989). It may be these more immature cells which represent the fraction that undergoes osteoclast differentiation when recruited to the bone microenvironment. *In vitro*, the presence of stromal cells and the correct hormonal conditions result in the formation of osteoclasts capable of resorbing bone (Takahashi et al 1988b; Quinn et al 1994; Fujikawa et al 1996a). Thus, in many osteolytic lesions containing a heavy infiltrate of inflammatory macrophages, increased release of potent bone resorbing factors, tissue damaging proteases, and the generation of new bone resorbing osteoclasts from within the macrophage infiltrate may all contribute to the abnormal resorption of bone associated with these conditions.

1.6. Aseptic loosening of total joint replacements

As a number of the experiments outlined in this thesis deal with the osteolysis of aseptic loosening, it is appropriate to discuss the pathobiology of aseptic loosening in detail. Joint replacement surgery is now a common surgical treatment for chronic arthritis, and congenital and traumatic joint disorders. The

major cause of late failure of implant components is aseptic loosening. Clinically, aseptic loosening causes pain and disability and the only form of treatment is revision surgery. The mechanism of aseptic loosening is likely to be multifactorial. Mechanical factors such as prosthesis design, implant material, surgical technique, and quality of fixation are known to be important. Late failure of implant components has in many cases been attributed to biological factors amongst which is the tissue response to wear particles arising from the implants (Willert and Semlitsch 1977; Vernon-Roberts and Freeman 1977; Howie 1990; Maloney and Smith 1995).

The wear of implant components, including the articulating surfaces, the surfaces of modular components, and at the prosthesis-bone interface of cemented and cementless components, results in the accumulation of wear particles in the periprosthetic tissues (Willert and Semlitsch 1977; Vernon Roberts and Freeman 1977). Excessive wear due to two or three body wear caused by the presence of particles of the same or other materials between the articulating surfaces may be particularly severe. Particles generated range in size from less than one to over one hundred micrometers. These particles have been found in the lymph nodes draining the hip joint indicating that there is continuous clearance of particles from the tissues around the implant. Continuous production over many years, or excessive production over a shorter period, may result in the accumulation of large numbers of wear particles in the surrounding tissues. For migration of these particles to the bone-implant interface to occur, they must have access. Lack of initial stability and excessive micromotion later in the life of the implant lead to the formation of a fibrous layer of variable thickness between implant and bone. It has been proposed that this fibrous tissue allows movement of fluid and particles to the bone-implant interface (Howie et al 1990; Schmalzreid et al 1992; Aspenberg and Herbertsson 1996). Histological studies of the bone-implant interface at the site of well fixed cemented implants have revealed few wear particles, and no obvious inflammatory response with few macrophages, minimal fibrous tissue and little bone resorption (Charnley 1970). However, longer term review has shown wear particles and bone resorption in the periprosthetic regions of these well fixed prostheses (Malcolm 1988). The bone-implant interface of loose prostheses often has areas of osteolysis associated with

large numbers of wear particles and a prolific macrophage and fibroblast response (Willert and Semlitsch 1977; Vernon-Roberts and Freeman 1977; Howie 1990).

Macrophages are long lived, highly active and adaptive cells that are involved in nearly all aspects of the inflammatory processes of the body. Macrophages utilise their phagocytic capabilities for wound repair and the defence against bacterial infection (Nathan 1987). In the case of total joint replacements, macrophages are recruited from the circulation as a foreign body response to wear particles derived from the artificial implant, and a correlation exists between the number of wear particles present and the degree of macrophage response (Howie 1990). Small particles of metal and polyethylene appear to cause a predominantly macrophage response and larger particles of polyethylene and cement are usually associated with a macrophage and macrophage polykaryon response (Vernon-Roberts and Freeman 1977). The phagocytosis of particles activates the cell leading to the production of enzymes, inflammatory mediators and oxygen metabolites. The indigestible nature of the particles results in a chronic inflammatory state, and the continual release of potent factors leads to the activation of other cells, such as fibroblasts and osteoblasts, and the recruitment of more macrophages into the area. Cell death may also occur if the phagocytosed particles are particularly toxic, and areas of necrosis have been reported in many arthroplasty tissues surrounding cobalt-chrome implants (Howie 1990).

A number of particle-associated factors are believed to determine the intensity of the macrophage response in the arthroplasty tissue and the extent of periprosthetic osteolysis. These include the chemical composition of the implant component, the size, shape and surface area of the wear particles generated as well as the number of particles present (Willert and Semlitsch 1977; Mirra et al 1982; Howie 1990). In a study by Haynes et al (1993), the response of cultured human monocytes to particles of titanium alloy and cobalt-chrome alloy were studied. Clear differences in cell toxicity and mediator release were shown, with cobalt-chrome particles inducing cell toxicity whilst titanium alloy particles induced the release of inflammatory mediators including IL-1, TNF, IL-6 and PGE_2. These particle effects were shown to be dose dependent. Similar *in vitro* studies have shown differences between PMMA, polyethylene and titanium

particles (Glant et al 1993; Horowitz and Gonzales 1997; Shanbhag et al 1995), and even different titanium alloys (Rogers et al 1997). Murray and Rushton (1990) found that phagocytosis of any foreign material that is non-toxic will cause macrophage activation, provided there are enough particles. Particles in the size range of ten microns or less have been shown to be phagocytosable. Although a wide range of particle sizes have been found in arthroplasty tissues adjacent to loose implants, the majority of particles are less than one micron in size. Phagocytosis of these smaller particles have been shown to induce a significantly higher response in macrophages than non-phagocytosed particles (Barth et al 1991; Shanbhag et al 1995). In the case of submicron metallic particles which have a large surface area and are susceptible to intracellular corrosion, a marked stimulation in macrophage activation was found following phagocytosis of these particles (Haynes et al 1996). *In vivo* animal studies, whilst not quantitative, have confirmed differences between particles from different materials and between particles of different sizes (Goodman et al 1988; Gelb et al 1994).

Wear particles may also have a direct effect on other cell types, such as fibroblasts and osteoblasts. Recent studies have shown that titanium particles can induce PGE_2, IL-1 and collagenase release from fibroblasts (Greis et al 1994; Yao et al 1995; Manlapaz et al 1996), and that polyethylene and cobalt chrome particles can inhibit osteoblast function *in vitro* (Allen et al 1997) and *in vivo* (Goodman et al 1995a). The effects of mediators released from wear particle stimulated mononuclear phagocytes may also have a profound effect on these cells. IL-1 and TNF are known to be strong stimulators of fibroblast proliferation. These cytokines also stimulate the release of collagenase from fibroblastic cells, and PGE_2 and IL-6 release from osteoblastic cells (Ohta et al 1994; Horowitz and Purdon 1995; Yao et al 1995; Haynes et al 1997). Studies involving the coculture of wear particle stimulated monocytes and osteoblastic cells have shown that these cells release factors that induce bone resorption (Horowitz and Gonzales 1996). However, the role of mediators such as IL-1, TNF, IL-6 and PGE_2 in these coculture systems is still unclear. Early studies suggested that osteolysis around implants was PGE_2 mediated (Goldring et al 1983). Subsequent studies in which prostaglandins were removed found that bone resorption was not inhibited, and

bone resorption was attributed to cytokines such as IL-1 and TNF (Spector et al 1990; Murray and Rushton 1992; Glant et al 1993; Horowitz et al 1996).

Biochemical and molecular studies of periprosthetic tissue retrieved from around failed implants have identified the presence of mediators that are known to contribute to bone resorption (Goldring et al 1983; Jiranek et al 1993; Chiba et al 1994; Kim et al 1994; Horikoshi et al 1994). Mediator release was compared in revision tissues from patients with and without radiographic evidence of focal osteolysis. The tissues from areas of lysis were found to have more macrophages and particles, and greater IL-1, TNF, IL-6 and PGE_2 activity (Chiba et al 1994; Kim et al 1994; Horikoshi et al 1994). In addition, conditioned media from explant cultures of the tissues was found to induce bone resorption *in vitro* (Ohlin and Lerner 1993). In a study by Jiranek et al (1993), mediator release was associated with macrophages and a general correlation between the amounts released and the amount of debris present was found. Recent studies, however, have shown that the cellular and humoral response to wear debris is not homogeneous throughout the arthroplasty tissue (Goodman et al 1995b). This may explain some of the conflicting findings in the literature regarding levels of mediators present. Multiple tissue sampling may overcome this problem.

In summary, a number of potent bone resorbing mediators have been identified in periprosthetic tissue from around loose prostheses, and these mediators have been shown to be released from wear particle stimulated macrophages both *in vivo* and *in vitro*. Whilst an adverse tissue response to wear particles and the release of inflammatory mediators has been frequently reported, the mechanism whereby wear particles and macrophages provoke bone resorption and implant loosening has remained unclear. Similarly, in other joint conditions associated with bone destruction, for example rheumatoid arthritis and pigmented villonodular synovitis (PVNS), the cellular and humoral mechanisms of bone resorption are poorly understood.

1.7. Hypothesis for osteoclast formation in arthroplasty periprosthetic tissues

Studies to date on the mechanisms of aseptic loosening have shown that macrophages recruited to the bone implant interface phagocytose wear particles and are stimulated to release a number of potent inflammatory factors. These factors act on stromal cells in the bone microenvironment and initiate a complex chain of events ultimately leading to osteolysis and implant loosening. It is known that osteoclasts can form from monocytes/macrophages in other pathological conditions such as rheumatoid arthritis (Fujikawa et al 1996b). Thus, one cellular mechanism whereby bone resorption and implant loosening may occur is by osteoclastic differentiation of precursor cells present in periprosthetic tissue, and increased osteoclastic activity.

The hypothesis for this thesis is that wear particle-induced mononuclear phagocytes present at the bone-implant interface can differentiate into functional osteoclastic cells. When these cells come into contact with bone lined by osteoblasts, and in the microenvironment provided by surrounding stromal cells, they may proliferate and differentiate into osteoclastic cells capable of lacunar bone resorption. Factors which are known to stimulate osteoclastic activity may also contribute to the formation of new osteoclasts at sites of pathological bone resorption.

1.8. Specific aims

I. To investigate whether cells isolated from periprosthetic tissue can differentiate into osteoclastic bone resorbing cells *in vitro*, and the stromal requirements for this to occur.

II. To determine which mediators implicated in osteoclastic bone resorption are released from isolated periprosthetic cells during osteoclast formation and bone resorption *in vitro*.

III. To determine which of these mediators released are important for osteoclast formation from periprosthetic cells, and bone resorption *in vitro*.

IV. To determine whether prosthesis wear particles affect the osteoclast differentiation from human mononuclear phagocytes and whether this is a direct effect or an indirect effect via the release of bone resorbing mediators.

2. General Methods for *In Vitro* Studies

2.1 Criteria for the recognition of osteoclasts

Studies of osteoclast differentiation can only be properly evaluated if the criteria for the recognition of osteoclasts are clearly defined. On histological sections, mature osteoclasts are readily identified as large multinucleated cells that lie in apposition to a bone surface undergoing lacunar resorption. Ultrastructurally, osteoclasts are distinguished by the presence of a ruffled border which is formed when the cell comes in contact with bone. *In vitro*, however, particularly in mixed cell populations, it has proved difficult to distinguish osteoclasts from other cell types, such as macrophages and macrophage polykaryons. It has not been possible to demonstrate ruffled borders on osteoclasts formed in long term cell cultures.

Osteoclasts are rich in certain enzymes, and enzyme histochemistry for the detection of TRAP has been used as a convenient cytochemical marker of osteoclasts (Minkin 1982). However, TRAP has been detected in osteoblasts and activated tissue macrophages, and monocytes and tissue macrophages are known to express TRAP activity after several days in culture (Hattersley and Chambers 1989b; Modderman et al 1991). Therefore, TRAP and other enzyme markers such as type II carbonic anhydrase should be regarded as osteoclast-associated rather than osteoclast-specific markers.

The development of several monoclonal antibodies to macrophage and osteoclast cell surface antigens has aided the identification of osteoclasts and their precursors. Osteoclasts have been shown to highly express the α and β chains of the vitronectin receptor, CD51/61, an antigen involved in both cell-cell and cell-matrix interactions (Horton et al 1985; Horton and Chambers 1986). Like macrophages and macrophage polykaryons, osteoclasts also strongly express CD45, the leukocyte common antigen as well as a number of macrophage-associated antigens, including CD13, CD15, CD68 and CD54 (Athanasou et al 1987; Athanasou et al 1988; Athanasou and Quinn 1990). Osteoclasts do not express some macrophage-associated antigens, such as CD11a, CD11b, CD14, CD18, and HLA-DR, nor receptors for Fc and complement components (Athanasou et al

1988; Athanasou and Quinn 1990). As there is no known cell surface antigen that is specific for osteoclasts, presence or absence of the above antigens are used to distinguish osteoclasts from macrophages and macrophage polykaryons.

There are two specific means of distinguishing mammalian osteoclasts from other cells; the expression of calcitonin receptors and the ability to resorb bone. Osteoclastic activity is directly and specifically inhibited by calcitonin (Chambers and Magnus 1982), and demonstration of calcitonin receptors is considered to be a highly specific marker of mammalian osteoclasts (Hattersley and Chambers 1989a; Nicholson et al 1986). Avian osteoclasts, however, do not express calcitonin receptors (Nicholson et al 1987). By definition an osteoclast is a bone resorbing cell. The unique ability of the osteoclast to form resorption lacunae in bone provides a specific and reliable osteoclast marker. Lacunar resorption pit formation is permanent and unequivocal functional evidence of osteoclast activity. Although other cells, such as tumour cells or inflammatory macrophages, are capable of degrading bone matrix and surface roughening (Teitelbaum et al 1979; Athanasou et al 1992), the osteoclast is the only cell capable of resorbing deep excavations in the bone surface.

For the experiments undertaken in this thesis, identification of osteoclasts was based on morphological, histochemical, immunological and functional evidence. Morphological identification of large multinucleated cells, TRAP positivity, expression of the vitronectin receptor (VNR), and absence of macrophage-associated antigens, CD11b and CD14, which are known not to be present on osteoclasts, were the criteria used, in conjunction with lacunar bone resorption, to identify osteoclasts in culture. Calcitonin receptor expression was not used routinely during the course of the research because visualisation by autoradiography requires unfixed live cells and the expensive radiolabel has a short half-life of approximately one month.

2.2. Preparation of cortical bone slices and glass coverslips.

Blocks of human cortical bone were obtained from the diaphysis of normal tibial and femoral bone derived from amputation specimens. The adherent soft tissues were removed and the blocks cut into pieces and stored at -20°C. Cortical bone slices were prepared by cutting a piece of bone longitudinally into 55µm slices with a low speed bone saw using a diamond wheel (Buehler, Isomet, IL, USA). Each slice was cut further using a scalpel blade into 4mm x 4mm squares. The bone slices were then sonicated in distilled water three times for three minutes each, followed by two rinses in absolute ethanol. The excess ethanol was removed and the bone slices transferred to sterile Petri dishes, allowed to air dry and stored at room temperature indefinitely.

Glass coverslips (Cat. no. M2783-15, Richardsons of Leicester Ltd, UK) were boiled for 5 minutes in 5% Decon. The coverslips were allowed to cool, and then rinsed ten times in distilled water, followed by two rinses in absolute ethanol. The excess ethanol was removed and the coverslips transferred to sterile Petri dishes, allowed to air dry and stored at room temperature indefinitely.

2.3. Histochemical characterisation of cultured cells.

The cultured cells were examined histochemically for the expression of TRAP using a commercially available kit (Cat no. 386, Leukocyte Acid Phosphatase kit, Sigma, UK). Although not a specific marker of osteoclast differentiation, the presence of TRAP activity is a quick and simple method of determining whether potential osteoclast formation had occurred and was used in conjunction with other more specific osteoclast markers. The absence of TRAP is particularly significant and demonstrates that osteoclast differentiation has not occurred.

The principle of the test is based upon the use of naphthol AS-BI phosphate as a substrate. Naphthol AS-BI, released by enzyme hydrolysis, couples immediately with fast garnet GBC salt at acid pH to form an insoluble red deposit at sites of acid phosphatase activity. The presence of tartrate solution during the staining procedure ensures that only cells containing tartrate-resistant acid phosphatase will stain positive. The method used is described below.

1. The glass coverslips with adherent cultured cells were removed from the wells, air dried, and then mounted on glass slides and stained immediately or stored at -20°C.
2. Prior to histochemical staining, the slides were fixed for 30 seconds at room temperature in a dilute citrate/acetone solution (2ml citrate concentrate + 18ml distilled water + 30 ml acetone).
3. The slides were then rinsed in distilled water and allowed to air dry for 15 minutes.
4. An acid phosphatase stain was prepared by adding 2mls of acetate solution, 2mls of naphthol AS-BI phosphoric acid, and 2mls of tartrate solution to 44mls of prewarmed distilled water. The contents of one Fast Garnet GBC salt capsule was added and the solution stirred on a magnetic mixer for 60 seconds.
5. The solution was rapidly filtered into a foil covered Coplin staining jar, placed in a 37°C water bath, and the fixed slides incubated in the solution for 1 hour.
6. The slides were then removed and washed in distilled water for 3 minutes, counterstained in acid haematoxylin solution for 5 minutes, washed for a further 3 minutes in distilled water, air dried and examined by light microscopy.

2.4. Immunohistochemical characterisation of cultured cells.

The antigenic phenotype of the human osteoclast is well characterised. The presence of macrophage associated antigens, CD11b and CD14, which are not detected on osteoclasts, and the presence of the osteoclast associated vitronectin receptor antigen, CD51/61, were determined by immunohistochemical staining with the monoclonal antibodies (McAbs), N306, M132 and 23C6 respectively. The McAbs used were derived from the 4th International Workshop on Human Leukocyte Differentiation Antigens. The McAb, 23C6, was kindly donated by Dr M. Horton, London. An indirect immunoperoxidase method was used for monoclonal antibody staining, as follows (Gatter et al 1984).

1. The glass coverslips with adherent cultured cells were removed from the wells, air dried, fixed in cold (-20°C) acetone for 10 minutes, and then mounted on glass slides and stored at -20°C.
2. Acetone-fixed coverslips stored at -20°C were labelled and allowed to reach room temperature.
3. The coverslips were allowed to rehydrate in PBS for 30 minutes.
4. To block endogenous peroxidase activity, the slides were gently shaken for 30 minutes in an ethanol/0.6% hydrogen peroxide solution.
5. The slides were gently rinsed with PBS from a wash bottle followed by two washes in PBS each for 5 minutes.
6. Mouse anti human McAbs were diluted 1:200 (CD11b, CD14), 1:250 (CD51/61) in PBS containing 2% bovine serum albumin (PBS/BSA), and 30µl added to each appropriate coverslip. Negative controls consisted of PBS/BSA alone with no primary antibody added. The slides were incubated in a moist environment for 30 minutes at room temperature.
7. The slides were removed and rinsed in PBS for a further 10 minutes.
8. The biotinylated secondary antibody (peroxidase conjugated rabbit anti mouse; Cat. no. P0260, Dako Ltd, UK) was diluted 1:50 in PBS/BSA, 30µl applied to each coverslip, and the slides incubated in a moist environment for 30 minutes at room temperature.
9. The slides were removed and rinsed in PBS for 10 minutes.
10. The biotinylated tertiary antibody (peroxidase conjugated swine anti rabbit Ig; Cat. no. P0217, Dako Ltd, UK) was diluted 1:100 in PBS/BSA, 30µl applied to each coverslip, and the slides incubated in a moist environment for 30 minutes at room temperature.
11. The slides were removed and rinsed in PBS for 10 minutes.
12. A solution of 0.5 mg/ml of 3,3 diaminobenzidine (DAB; Cat. no. D-5637, Sigma, UK) containing 0.05% hydrogen peroxide was applied to each coverslip, and the slides incubated for a maximum of 3 minutes.
13. The sections were washed thoroughly in running tap water, counterstained with haematoxylin (Gill's No.3; diluted 1:3 with distilled water), dehydrated through graded alcohols, brought to xylene, and finally mounted with DPX medium and examined by light microscopy.

2.5. Functional evidence of osteoclast differentiation - SEM bone resorption assay.

At the end of each incubation period, the surfaces of the cortical bone slices were examined by SEM for evidence of lacunar bone resorption. To prepare the bone slices for SEM, the slices were rinsed in PBS, trypsinised for 15 minutes to remove the stromal cell layer, washed vigorously in distilled water, and then left overnight in 0.25% ammonium hydroxide to remove the remaining cells. After rinsing in distilled water, the bone slices were dehydrated through graded ethanols, air dried and mounted on SEM stubs (Cat. no. G301, Agar Scientific Ltd, UK) for gold coating prior to examination in a Phillips SEM 505.

The extent of resorption was determined by measuring the number of discrete pits counted on each cortical bone slice. Resorption pits were observed as either individual small pits or large multilocular resorption areas (Figure 1). As such, it was necessary to define a resorption pit as an excavation of the bone surface with a clear rim of unchanged original surface between neighbouring excavations (Figure 2). This method of analysis is limited in that the area of bone resorbed in one small pit and one large convoluted pit is very different. Many studies in this field have quantified bone resorption as the area of bone resorbed by staining the surface of dentine slices with toluidine blue and measuring bone resorption using an image analysis package. However, human cortical bone was used as the substrate for the experiments in this thesis. Human bone was chosen because of its mineral component composition and density, factors which have been shown to affect the depth of pits, and as the studies in this thesis related to diseases occurring in humans, a human resorption assay system was desirable. Although bone has osteocyte lacunae and vascular channels, these could easily be identified and eliminated by a human operator counting pit numbers. Exposed osteocyte lacunae appeared as small holes with irregular well defined margins. Vascular channels were exposed either transversely or longitudinally where they often extended for a considerable distance before disappearing as a tunnel beneath the surface (Figure 3). Both of the above methods involve two dimensional measurements and hence, do not take into account any changes in pit depth and volume changes. There have been reports of a good correlation between area and volume which suggests that volume measurements are not needed, although this can not be assumed for all resorption assays with osteoclasts formed under

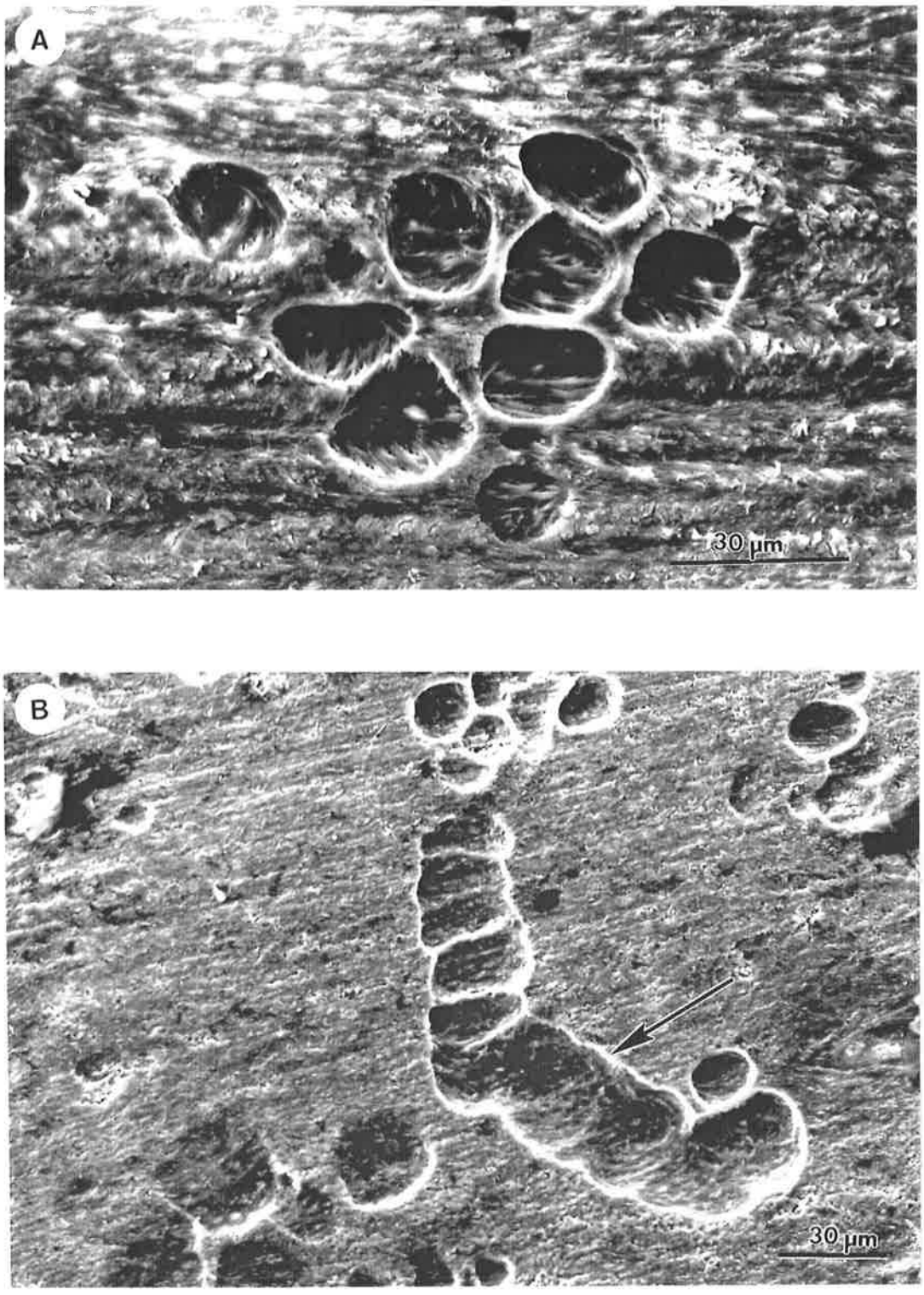

Figure 1. Electron micrographs of human cortical bone slices showing (A) individual small resorption pits and (B) large multilocular resorption areas (arrowed).

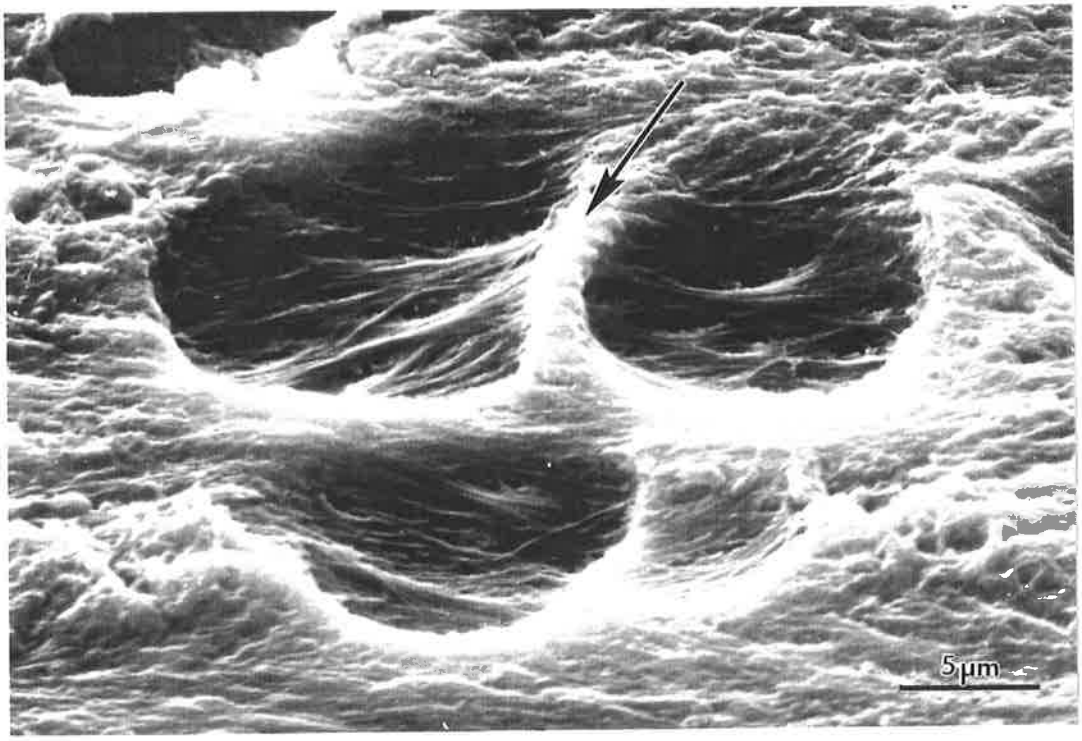

Figure 2. Higher magnification of human cortical bone slices showing resorption pits with exposed collagen fibrils. Note the clear rim of unchanged original surface between neighbouring excavations (arrowed).

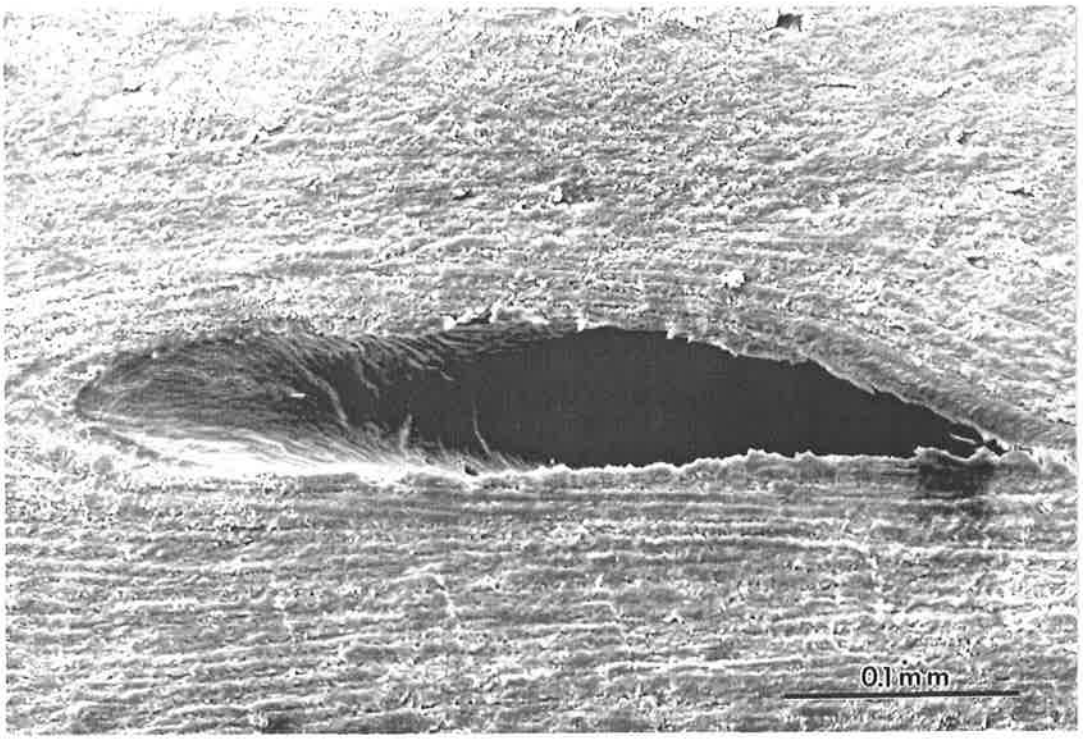

Figure 3. Electron micrograph of a longitudinally exposed vascular channel. These extend for a variable but usually considerable distance along the bone surfaces before disappearing as a tunnel beneath the surface. Also note the otherwise smooth surface of the bone slice showing parallel saw markings.

different conditions. For this thesis, the results are expressed as the number of resorption pits per bone slice. For single pits, a good correlation has been shown with the total surface area of bone resorbed as the volume and area of individual resorption pits is known to fall within a defined range (Chambers et al 1984b). The limitations of this method of assaying resorption pit formation are understood, and the conclusions based on the results generated reflect this.

3. Characterisation of Osteoclast Differentiation from Periprosthetic Macrophages *In Vitro*

3.1. Introduction

A predominant histological feature of periprosthetic tissue surrounding aseptically loose implant components is the presence of a prominent foreign body macrophage response to prosthesis-derived wear particles (Willert and Semlitsch 1977; Vernon-Roberts and Freeman 1977; Howie 1990). A number of potent bone resorbing mediators have been identified in periprosthetic tissue from around loose prostheses (Goldring et al 1983; Kim et al 1994; Chiba et al 1994; Horikoshi et al 1994), and these mediators have been shown to be released from wear particle stimulated macrophages both *in vivo* and *in vitro* (Jiranek et al 1993; Haynes et al 1993; Glant et al 1993). However, the cellular mechanisms underlying aseptic loosening of implant components and the manner in which this foreign body macrophage infiltrate in periprosthetic tissues contributes to osteolysis remain uncertain.

A recent study has shown that murine monocytes and inflammatory macrophages responding to prosthesis wear particles are capable of differentiating into osteoclastic bone resorbing cells (Quinn et al 1992). Thus, one cellular mechanism whereby bone resorption and implant loosening may occur is by osteoclastic differentiation of precursor cells present in periprosthetic tissue. Osteoclasts are known to form part of the mononuclear phagocyte system and it has recently been shown that mononuclear phagocyte osteoclast precursors are present in the monocyte fraction of human peripheral blood (Fujikawa et al 1996a). It is also known that human osteoclasts can form from monocytes/macrophages in other pathological conditions such as rheumatoid arthritis (Fujikawa et al 1996b). These studies investigating osteoclast differentiation from cultures of human monocytes and RA synovial macrophages have found that the presence of bone stromal cells, $1,25(OH)_2D_3$ and the addition of exogenous M-CSF are all essential factors for osteoclast formation and bone resorption.

The aim of this study was to determine whether human wear particle associated macrophages present in periprosthetic tissues around loose implants are capable of differentiating into osteoclastic bone resorbing cells. A time course study was undertaken to determine the optimal incubation periods for the formation of osteoclastic bone resorbing cells from periprosthetic macrophages *in vitro*. The cellular and humoral requirements for periprosthetic macrophage-osteoclast differentiation were also determined. As a control, the requirements for osteoclast formation from periprosthetic macrophages were compared with the requirements for osteoclast formation from cells isolated from osteoarthritis (OA) synovium.

3.2. Methods for the characterisation of osteoclast differentiation from periprosthetic and OA macrophages

3.2.1. Histology of tissue specimens

Specimens of acetabular and femoral periprosthetic tissue were obtained from patients undergoing revision surgery for aseptic loosening. Frozen sections of all the aseptic arthroplasty tissue specimens obtained were examined histologically prior to digestion to determine whether the tissue was suitable for use. Tissue showing evidence of infection by the presence of polymorphonuclear cells were rare and were excluded. Tissue specimens which contained an obvious macrophage and macrophage polykaryon response to prosthetic wear debris were used, although it was noted that the extent of cellular response did vary between patients. Attempts to correlate the cellular response seen histologically with the amount of bone resorption seen *in vitro* were unsuccessful. In some cases, the specimens were also found to contain tiny fragments of bone.

As it was not possible to obtain specimens of normal synovium from human subjects, specimens of OA synovium were used as control joint tissue. These were obtained from patients undergoing primary total hip arthroplasty surgery. Histologically, the OA synovial membrane shows patchy intimal thickening and subintimal fibrosis and oedema. This tissue does not contain a heavy macrophage infiltrate but may contain a variable light or moderate macrophage infiltrate as well as in some cases small bone fragments derived from the eroded articular surface.

The clinical details of all of these patients are shown in Appendix 1.

3.2.2. Isolation of cells from human tissue specimens

The tissue specimens obtained were washed thoroughly with sterile phosphate buffered saline (PBS) before being cut into small pieces and digested in Minimal Essential Media Eagle (αMEM; Cat no. M-2279, Sigma, UK) containing 1mg/ml collagenase type 1 (Cat no. C-0130, Sigma, UK) for 30 minutes at 37°C, and 5 mls of trypsin (Cat no. T-4424, Sigma, UK) for 1 hour. The digested tissue was then filtered with a 70μm cell strainer, and the filtrate centrifuged at 1500rpm for 5 minutes. After two washes in αMEM only, the pellet was resuspended in αMEM supplemented with 10% foetal calf serum (FCS; Cat no. 10106-169, Gibco, UK), 100U/ml Penicillin and 100μg/ml Streptomycin sulphate (Cat no. 15140-114, Gibco, UK) and 2mM L-glutamine (Cat no. 25030-024, Gibco, UK) (αMEM+10%FCS media). The cell suspension was finally counted in a haemocytometer after lysis of the red blood cells using a 5% (v/v) acetic acid solution.

3.2.3. Preparation of macrophage cocultures on coverslips and bone slices

A concentration of the macrophage cell suspensions ($1x10^5$ cells/100μl) was added to 7mm wells of a 96 well tissue culture plate. These wells contained either glass coverslips or prewetted cortical bone slices. Half the coverslips and bone slices were seeded 24 hours earlier with the stromal cells, rat osteoblast-like UMR-106 cell line (donated by Professor T.J. Martin, Melbourne, Australia) at a concentration of $2x10^4$ cells/100μl. After 1-2 hours incubation at 37°C in 5% CO_2, the bone slices and coverslips were removed from the wells, washed vigorously in αMEM to remove the non-adherent cells, and placed in larger 16mm wells containing 1ml of αMEM+10% FCS media. These cultures were incubated for up to 14 days in the presence of 10^{-7}M $1,25(OH)_2D_3$ (Calcitriol; Solvay Duphar, Netherlands) and, in some experiments, 10^{-8}M dexamethasone (Cat. no. D-4902, Sigma, UK) and 25ng/ml M-CSF (Cat. no. 216-MC-010, R&D Systems, UK). The culture medium was changed every 3-4 days and fresh factors added.

3.3. Time course study

To determine the optimal incubation periods for the formation of osteoclastic bone resorbing cells *in vitro*, adherent cells were isolated from tissue specimens obtained from three patients undergoing revision arthroplasty surgery (ADM1-3; Appendix 1), as described earlier. The isolated cells were cultured in the presence and absence of UMR 106 cells, with $1,25(OH)_2D_3$ and dexamethasone added to all the cultures. The coverslips and bone slices were removed from the cultures after 1, 4, 7, 10 or 14 days incubation. The coverslips were characterised histochemically for the expression of TRAP, and immunohistochemically for VNR expression and expression of the macrophage associated antigens, CD11b and CD14. Bone resorption was measured as the number of resorption pits per bone slice. Each time period was studied in triplicate for each tissue specimen.

3.3.1. Histochemical and immunohistochemical characterisation of the cultured cells

Numerous adherent cells isolated from the arthroplasty tissue were found to express strongly the macrophage cell surface antigens, CD11b and CD14, after 24 hours incubation. These 24 hour cultures were largely negative for TRAP and VNR multinucleated cells although a few TRAP and VNR positive mononuclear cells were present. In some specimens, occasional TRAP and VNR positive multinucleated cells were present. After four days incubation, cocultures of periprosthetic macrophages and UMR 106 cells, incubated in the presence of $1,25(OH)_2D_3$ and dexamethasone, contained TRAP and VNR positive mononuclear and occasional multinucleated cells. After seven days incubation, there were substantially greater numbers of TRAP and VNR positive multinucleated cells present in the cocultures. TRAP positive cells were particularly numerous and included not only scattered mononuclear and multinucleated cells but also small and large clusters of TRAP positive cells. VNR positive cells were mainly seen as large isolated cells. The formation of osteoclastic cells over this time period is shown in Figure 4. The numbers of TRAP and VNR positive multinucleated cells did not appear to increase after ten days incubation. After 14 days incubation when the covering confluent layer of UMR 106 cells began to detach from the surface of the coverslip, large

multinucleated cells were more readily identifiable. Numerous CD11b and CD14 positive mononuclear cells were seen in the cocultures throughout the incubation period. In the absence of UMR 106 cells, scattered TRAP and VNR positive mononuclear cells and occasional multinucleated cells were seen in the cultures from day one to day 14 (Figure 4).

3.3.2. Formation of lacunar resorption pits on cortical bone slices

Lacunar bone resorption was not seen on 16 of the 18 bone slices in the 24 hour cultures of periprosthetic macrophages alone or in the presence of UMR 106 cells. In the two bone slices on which bone resorption was noted, there were three small resorption pits. However, after four days incubation, lacunar bone resorption was evident on eight of the nine bone slices with up to 17 pits per bone slice counted when periprosthetic macrophages were cocultured with UMR 106 cells, in the presence of $1,25(OH)_2D_3$ and dexamethasone. The number of resorption pits per bone slice increased with time (Figure 5), and after seven days coculture of periprosthetic macrophages and UMR 106 cells, in the presence of $1,25(OH)_2D_3$ and dexamethasone, extensive lacunar bone resorption was seen on all nine cortical bone slices. By day 14, single pits and large convoluted areas of lacunar excavation consisting of multiple pits were seen (Figure 6). Lacunar bone resorption was not seen on seven of the nine bone slices when periprosthetic macrophages were cultured in the absence of UMR 106 cells. The remaining two bone slices on which bone resorption was noted had three and seven small resorption pits formed after 14 days culture in the absence of UMR 106 cells.

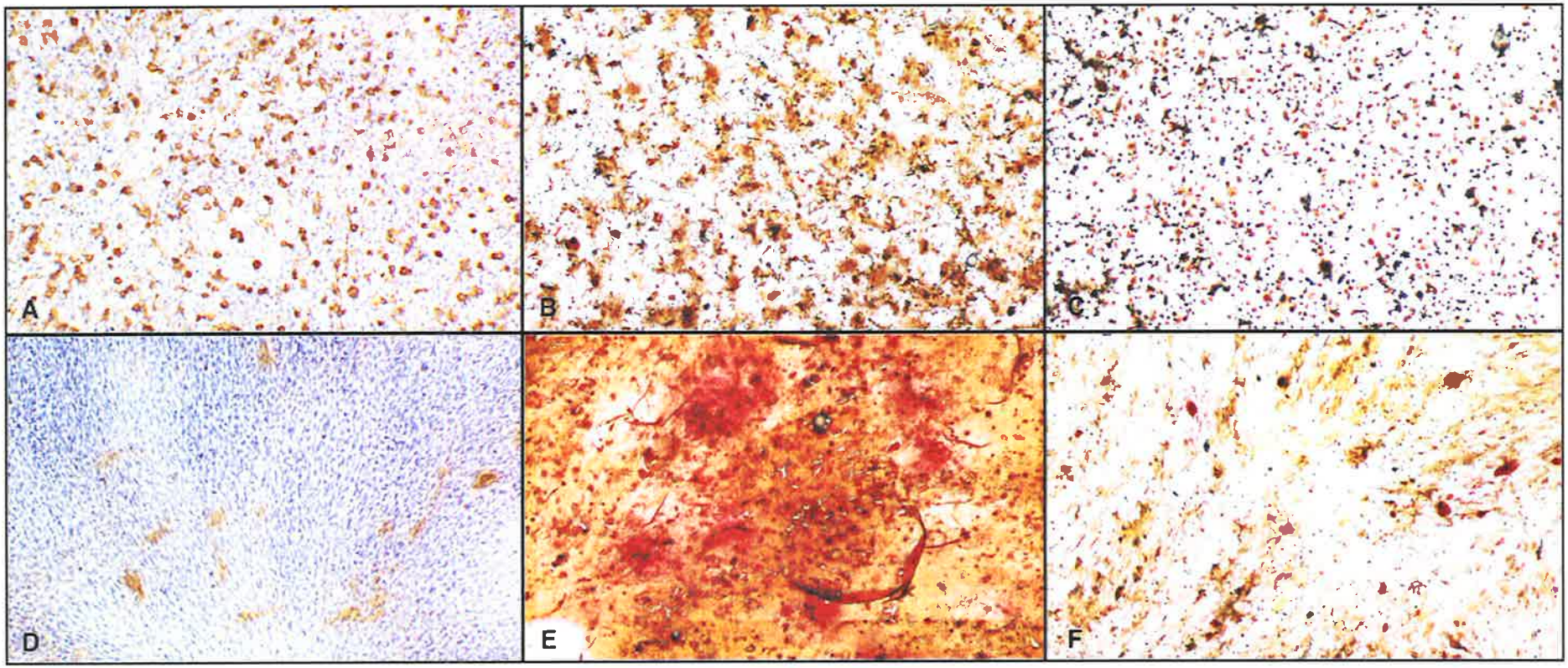

Figure 4. 24 hour and seven day cocultures of arthroplasty derived macrophages and UMR 106 cells incubated in the presence of $1,25(OH)_2D_3$ and dexamethasone showing (A) numerous CD14 positive cells after 24 hours coculture in the presence of UMR 106 cells (x100); (B) scattered mononuclear TRAP positive cells after 24 hours coculture in the presence of UMR 106 cells (x40); (C) scattered mononuclear cells after 24 hours culture in the absence of UMR 106 cells (x40); (D) large VNR positive cells after seven days coculture in the presence of UMR 106 cells (x40); (E) clusters of large TRAP positive cells after seven days coculture in the presence of UMR 106 cells (x40); and (F) scattered mononuclear cells after seven days culture in the absence of UMR 106 cells (x40). Counterstained with haematoxylin.

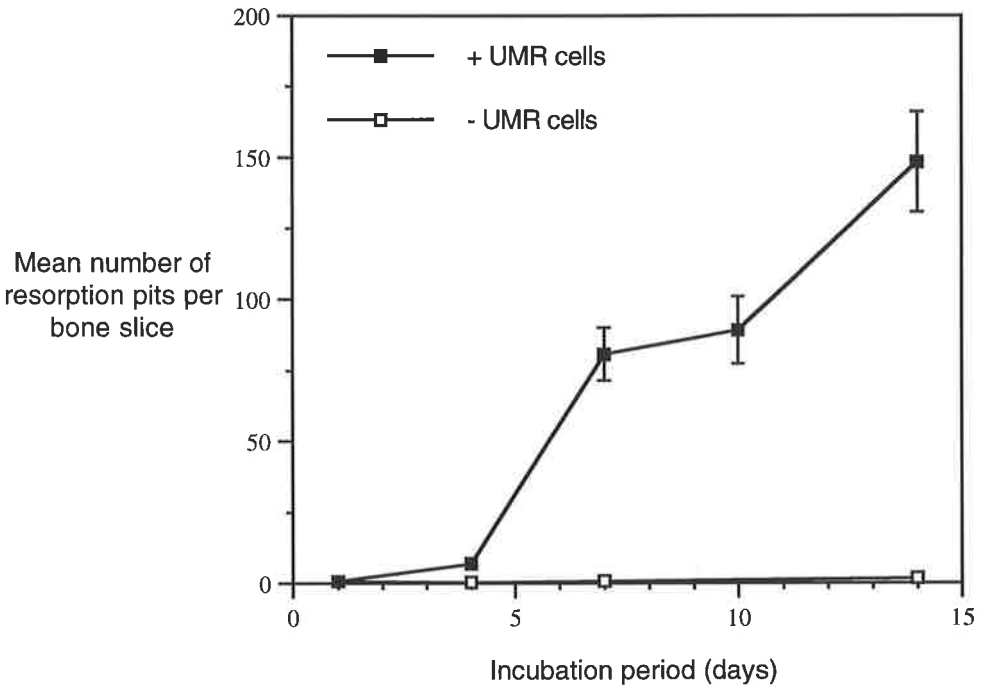

Figure 5. The formation of lacunar resorption pits in arthroplasty derived macrophage-UMR 106 cell cocultures over an incubation period of 14 days.

The results are expressed as the mean number of resorption pits per bone slice ± SEM (n=9). All the cultures were incubated in the presence of 10^{-7}M $1,25(OH)_2D_3$ and 10^{-8}M dexamethasone.

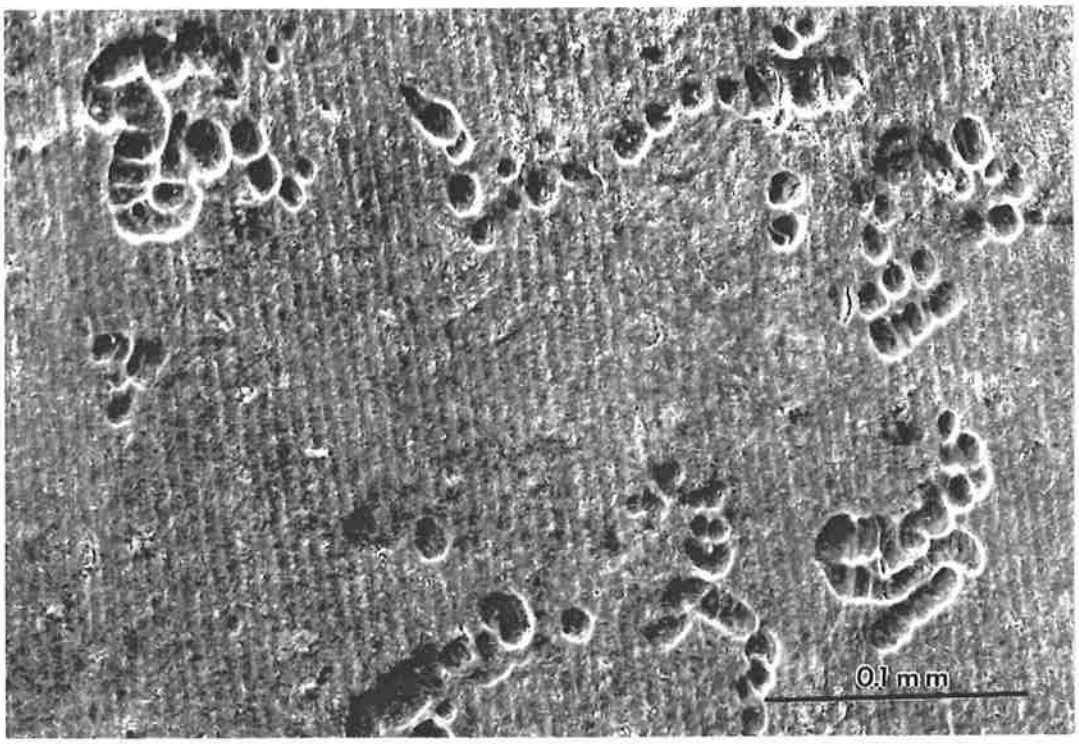

Figure 6. Fourteen day coculture of arthroplasty derived macrophages and UMR 106 cells incubated in the presence of 10^{-7}M $1,25(OH)_2D_3$ and 10^{-8}M dexamethasone on human bone slices. The cells have been removed to reveal evidence of lacunar bone resorption.

3.4. Cellular and humoral requirements for osteoclast differentiation from periprosthetic macrophages

For this series of experiments, periprosthetic macrophages were isolated (as described above in section 3.2.) from tissue specimens obtained from three patients undergoing revision arthroplasty surgery (ADM1, ADM2 and ADM4; Appendix 1). OA synovial macrophages were similarly isolated from the synovium of two patients (OA1 and OA2; Appendix 1) undergoing primary total hip arthroplasties.

To determine the cellular and humoral requirements for osteoclast formation from these two different cell populations, the isolated adherent cells were cultured in (a) the absence of UMR 106 cells; (b) the absence of $1,25(OH)_2D_3$; (c) the absence of dexamethasone; and (d) the absence of M-CSF, as shown in Tables 1 and 2. The coverslips were removed after 24 hours and seven days incubation and characterised histochemically for the expression of TRAP. The cortical bone slices were removed after 24 hours and 14 days incubation and the number of resorption pits per bone slice was counted.

3.4.1. Characterisation of 24 hour cocultures

In the 24 hour cocultures of periprosthetic macrophages and UMR 106 cells, scattered TRAP positive mononuclear and multinucleated cells were present although either only a few (less than six pits) or no lacunar resorption pits were seen (mean of 1.2 pits per bone slice). Similarly, in the 24 hour cocultures of OA synovial macrophages and UMR 106 cells, occasional TRAP positive mononuclear and multinucleated cells were present. Bone resorption was not seen on four of the six bone slices, and the two bone slices on which resorption pits were noted both had three small pits.

3.4.2. Periprosthetic macrophage-UMR 106 cocultures

In the seven and 14 day cocultures of periprosthetic macrophages and UMR 106 cells, in the presence of $1,25(OH)_2D_3$ and dexamethasone, large TRAP positive cells formed and extensive lacunar bone resorption was evident on all the bone slices in all three specimens studied (Table 1). Lacunar bone resorption was not seen when periprosthetic macrophages were cultured in the absence of UMR 106

cells (i.e. only $1,25(OH)_2D_3$ and dexamethasone added) or in the absence of $1,25(OH)_2D_3$ (i.e. only UMR 106 cells and dexamethasone added). Only occasional TRAP positive multinucleated cells were evident in these cocultures. Omitting dexamethasone from the cocultures (i.e. periprosthetic macrophages cocultured with UMR 106 cells and $1,25(OH)_2D_3$) had no effect on the numbers of resorption pits compared with cocultures that had dexamethasone present. Although there was no effect on bone resorption, an increase in the numbers of large TRAP positive cells was noted when dexamethasone was not added to the cocultures compared with cocultures that had dexamethasone added (Figure 7).

Table 1. Requirements for osteoclastic differentiation from periprosthetic macrophages.

Treatments				Results	
UMR cells	$1,25(OH)_2D_3$	Dex	M-CSF	Bone resorption	TRAP activity
+	+	+	-	145.5 ± 17.5	++
-	+	+	-	3.4 ± 1.7	-/+
+	-	+	-	0.8 ± 0.6	-/+
+	+	-	-	139.7 ± 12.7	+++

Bone resorption results are expressed as the mean number of resorption pits per bone slice ± SEM (n=9). Each treatment was studied in triplicate for each specimen.

-/+ = none or few TRAP positive cells present

++ = >20 large individual and clusters of small TRAP positive cells per coverslip

+++ = >30 large individual and clusters of small TRAP positive cells per coverslip

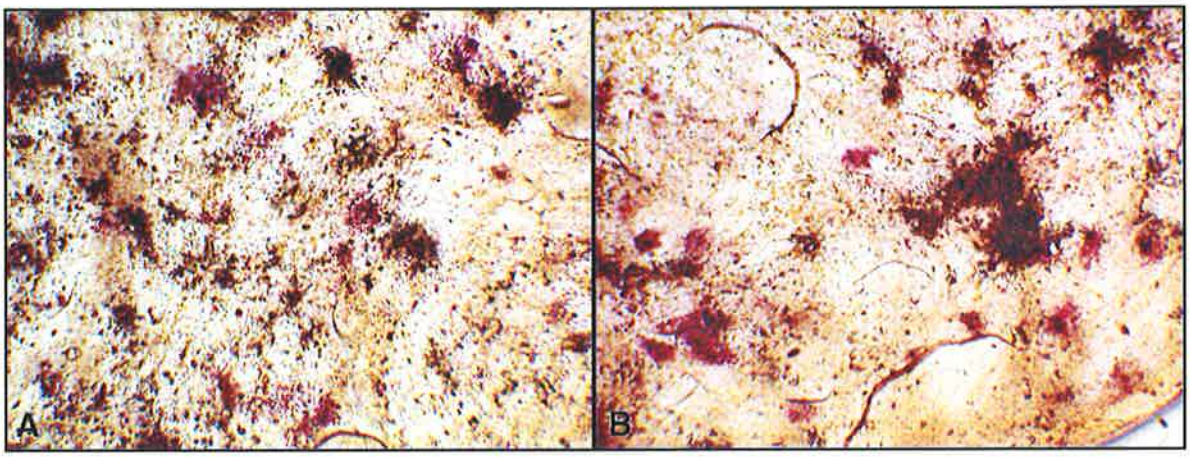

Figure 7. Seven day cocultures of arthroplasty derived macrophages and UMR 106 cells incubated in the presence of $1,25(OH)_2D_3$ showing clusters of large TRAP positive cells in the presence (A) and absence (B) of dexamaethasone (x40).

Table 2. Requirements for osteoclastic differentiation from OA synovial macrophages.

Treatments				Results	
UMR cells	$1,25(OH)_2D_3$	Dex	M-CSF	Bone resorption	TRAP activity
+	+	+	-	26.7 ± 5.8	+
+	+	+	+	130.7 ± 24.1	++
-	+	+	+	3.3 ± 3.3	-/+
+	-	+	+	ND	ND
+	+	-	+	38.3 ± 4.5	+

Bone resorption results are expressed as the mean number of resorption pits per bone slice ± SEM (n=6). Each treatment was studied in triplicate for each specimen.

ND = not determined

-/+ = none or few TRAP positive cells present

+ = approx. 5 large individual and clusters of small TRAP positive cells per coverslip

++ = >20 large individual and clusters of small TRAP positive cells per coverslip

3.4.3. OA synovial macrophage-UMR 106 cocultures

In the seven day cocultures of OA synovial macrophages and UMR 106 cells, in the presence of $1,25(OH)_2D_3$ and dexamethasone, that is the absence of M-CSF, occasional TRAP positive mononuclear and multinucleated cells were seen. In the 14 day cocultures, resorption pits were seen on all of the bone slices (range of 12 to 42 pits per slice). However, in the presence of 25 ng/ml M-CSF, the numbers of TRAP positive cells in the seven day cocultures were substantially increased and extensive lacunar bone resorption was seen after 14 days coculture (Table 2). In the absence of UMR 106 cells, occasional TRAP positive mononuclear and multinucleated cells were seen in the seven day cultures and a small number of

resorption pits were seen on the bone slices after 14 days culture (less than 10 pits per slice). The absence of dexamethasone in the cocultures (i.e. OA synovial macrophages cocultured with UMR 106 cells, $1,25(OH)_2D_3$ and M-CSF) substantially reduced but did not abolish osteoclast formation and bone resorption compared with cocultures that had dexamethasone present.

The few small isolated resorption pits that formed in the absence of UMR 106 cells in the 24 hour and 14 day cultures were most probably produced by osteoclasts associated with bone particles embedded in the pseudomembrane. This is supported by the finding of scattered TRAP positive multinucleated cells in the 24 hour and 14 day cultures. A small amount of lacunar bone resorption by osteoclasts isolated from periprosthetic tissues has previously been reported (Athanasou et al 1992). The OA synovium also typically contains fragments of degenerative cartilage and bone from the eroded articular surface. In the cultures of OA synovial cells, occasional TRAP positive multinucleated cells were seen and a small number of resorption pits formed in both the 24 hour and 14 day cultures.

3.5. Discussion

The results of these studies suggest that there are cells present in the macrophage-rich periprosthetic tissues surrounding a loose implant which are capable of differentiating into osteoclastic bone resorbing cells. Large TRAP and VNR positive cells appeared in cocultures of periprosthetic macrophages and UMR 106 cells after seven days, and extensive lacunar bone resorption was seen after seven days but was greatest in the 14 day cocultures.

Cultures of periprosthetic macrophages required the presence of osteoblastic cells and $1,25(OH)_2D_3$ for osteoclast differentiation, but did not require the addition of exogenous M-CSF. This is in contrast to cultures of OA synovial macrophages where the addition of exogenous M-CSF was found to greatly enhance osteoclast differentiation. Recent similar studies investigating osteoclast differentiation by cultures of human monocytes and RA synovial macrophages have also found

that M-CSF is an essential factor for osteoclast formation and bone resorption (Fujikawa et al 1996a; Fujikawa et al 1996b). One possible explanation for this is that any M-CSF required for periprosthetic macrophage-osteoclast differentiation is produced endogenously by cells present in the periprosthetic tissues. M-CSF is produced by a large number of cell types including fibroblasts, bone marrow stromal cells, osteoblasts, and activated monocytes/macrophages (Rambaldi et al 1987; Oster et al 1987; Fibbe et al 1988; Takahashi et al 1991; Roth and Stanley 1992; Kaneki et al 1994). Prosthesis wear particles present in arthroplasty tissue are known to stimulate macrophages and fibroblasts to release a number of potent bone resorbing mediators, however, the levels of M-CSF produced by these cells after exposure to wear particles have not been determined.

In addition to the presence of bone stromal cells, $1,25(OH)_2D_3$ was the only other factor required for osteoclast formation from periprosthetic macrophages. $1,25(OH)_2D_3$ is likely to be present in increased amounts at the bone-implant interface. Activated inflammatory foreign body macrophages are known to convert the comparatively inactive form of vitamin D_3, 25 hydroxyvitamin D_3, to its most biologically active form, $1,25(OH)_2D_3$ (Rigby 1988). As well as enhancing monocyte proliferation, which has been attributed to stimulated M-CSF release, $1,25(OH)_2D_3$ has also been found to enhance monocyte/macrophage maturation and fusion (Abe et al 1983).

Dexamethasone was not found to be required for osteoclast differentiation from periprosthetic macrophages. Dexamethasone enhanced osteoclast differentiation in the OA synovial macrophage cocultures and has been shown to enhance osteoclast differentiation from human monocytes *in vitro* (Fujikawa et al 1996a), although its mechanism of action is still unclear. Udagawa et al (1995) have shown that dexamethasone promotes IL-6 induced osteoclastogenesis by upregulating IL-6 receptor expression in murine stromal cells. Other studies have shown that dexamethasone inhibits the release of certain bone resorbing mediators, including IL-6, TNF and PGE_2 (reviewed in Russell 1993). Both dexamethasone and $1,25(OH)_2D_3$ have been shown to increase levels of M-CSF released from monocytes and osteoblastic cells (Kaneki et al 1994; Rubin et al 1997). Whilst dexamethasone enhanced osteoclast formation and bone resorption

from OA synovial macrophages, the presence of dexamethasone was not found to have an inhibitory or stimulatory effect on osteoclast formation and bone resorption from periprosthetic macrophages. Thus, all the future experiments using periprosthetic macrophage-UMR 106 cell cocultures were carried out in the absence of dexamethasone.

In summary, these findings indicate that one cellular mechanism whereby bone resorption and implant loosening may occur is by osteoclastic differentiation of mononuclear phagocyte osteoclast precursor cells present in periprosthetic tissue surrounding loose implant components. The requirements for this to occur were similar but not the same as those required for osteoclast differentiation from human monocytes (Fujikawa et al 1996a) and OA and RA synovial macrophages (Fujikawa et al 1996b). The addition of exogenous M-CSF was not required for periprosthetic macrophage-osteoclast differentiation. These findings have important implications for periprosthetic bone resorption and aseptic loosening. In the context of the prolific foreign body macrophage response to wear particles at the bone-implant interface, macrophage-osteoclast differentiation may represent an important cellular mechanism whereby bone resorption leads to prosthesis loosening.

3.6. The role of cell mediators in periprosthetic macrophage-osteoclast differentiation and bone resorption

3.6.1. Introduction

Several mediators implicated in bone resorption, such as IL-1β, TNFα, IL-6 and PGE_2, have been identified in periprosthetic tissue (Goldring et al 1983; Kim et al 1994; Chiba et al 1994; Horikoshi et al 1994). These mediators have been implicated in the inflammatory response to wear particles and the osteolysis of aseptic loosening. *In vivo* and *in vitro* studies have shown that these mediators are released from wear particle stimulated macrophages (Jiranek et al 1993; Haynes et al 1993; Glant et al 1993), and it is thought that they induce osteoclastic bone resorption through their effects on osteoblasts (Thomson et al 1986; Thomson et al 1987; Horowitz et al 1994).

Recent *in vitro* studies have also shown that these mediators promote osteoclast formation from marrow precursors. Both IL-1 and TNF have been shown to induce osteoclast-like cell formation in human bone marrow cultures (Pfeilschifter et al 1989). IL-1 and TNF stimulate the release of prostaglandins and IL-6 by osteoblasts, and it is thought these cytokines may induce osteoclast formation by stimulating the release of IL-6 and another gp130 mediated cytokine, IL-11, from osteoblasts (Romas et al 1996; Kim et al 1997). The importance of M-CSF in osteoclast formation has been demonstrated *in vitro* using both human and murine models (Tanaka et al 1993; Fujikawa et al 1996a; Sarma and Flanagan 1996) and *in vivo* using the op/op mouse (Yoshida et al 1990; Felix et al 1990). M-CSF was found to be essential for osteoclast precursor proliferation and differentiation, and the finding in the study above that M-CSF is important for osteoclast formation from OA synovial macrophages supports this. However, the humoral factors required for human osteoclast differentiation by periprosthetic macrophages have not been characterised.

The hypothesis for this study was that cell mediators implicated in periprosthetic bone resorption promote the differentiation of periprosthetic macrophages into osteoclastic cells capable of lacunar bone resorption. Thus, the aims of this study were firstly to determine whether mediators implicated in periprosthetic bone resorption are released by activated macrophages and fibroblasts isolated from

arthroplasty tissue during osteoclast formation and bone resorption, and secondly to determine the effect of antibodies directed against these mediators on periprosthetic macrophage-osteoclast differentiation and bone resorption.

3.6.2. Mediator release during osteoclast differentiation of periprosthetic macrophages

The aim of this study was to determine whether cell mediators implicated in periprosthetic bone resorption are released by cells isolated from arthroplasty tissue during osteoclast formation and bone resorption.

3.6.2.1. Methods

To measure the levels of mediators released, supernatants were collected during media changes from five periprosthetic macrophage-UMR 106 cell cocultures incubated in the presence of $1,25(OH)_2D_3$. Supernatants were collected from days 1, 4, 7 and 10. At days 1, 4 and 7, supernatants were collected from wells containing bone slices and coverslips. At day 10, only supernatants from wells containing bone slices were available. The supernatants were centrifuged to remove wear particles and cell debris, and stored at -20°C until ready for testing. Levels of human IL-1β, TNFα, M-CSF and IL-6 were measured by enzyme linked immunoassays (ELISAs) purchased from R&D Systems, UK. Human and rat PGE_2 were measured using an ELISA purchased from Amersham International, UK.

3.6.2.2. Results

Extensive lacunar bone resorption was seen on the bone slices cocultured with UMR 106 cells in the presence of $1,25(OH)_2D_3$ for 14 days. The mean number of resorption pits ranged from 39.3 to 243.3 pits per bone slice.

Table 3. Release of mediators from cells isolated from periprosthetic tissue during osteoclast formation and bone resorption.

Incubation period (days)	IL-1b (pg/ml)	TNFa (pg/ml)	M-CSF (pg/ml)	IL-6 (pg/ml)	PGE_2 (pg/ml)
Day 1#	3.9 (range 0-8.0)	-	32.0 (range 0-76)	647.5 (range 550-900)	24.0 (range 3.6-64)
Day 4#	12.6 (range 3.0-63)	-	151.4 (range 60-400)	3954.4 (range 580-8000)	169.5 (range 20-640)
Day 7#	1.8 (range 0-4.2)	-	216.6 (range 98-510)	616.7 (range 76-1600)	9.8 (range 0-25)
Day 10##	1.2 (range 0-5.0)	-	129.1 (range 115-155)	411.8 (range 92-1000)	9.5 (range 0-21)

Periprosthetic macrophages were isolated from tissue specimens from patients ADM2, ADM5-ADM8 (Appendix 1). Results are expressed as the mean level of mediator release.

corresponds to n=10, ## corresponds to n=5

- = not detectable

Increased levels of IL-1β were released by isolated arthroplasty cells after four days coculture although these levels were small. No detectable levels of TNFα were measured in any of the supernatants. TNFα is an early phase cytokine with maximal release after four hours stimulation. It is possible that increased levels of TNFα were present in the supernatants but that these levels were not detected after 24 hours or longer incubations.

Levels of M-CSF in the supernatants were substantially increased after four and seven days coculture (Table 3). In addition to activated macrophages and fibroblasts, it is possible that osteoclasts formed in the cocultures may be contributing to the levels of soluble M-CSF present at the later time point as these cells have been shown to release M-CSF *in vitro* and *in vivo*. Substantial levels of IL-6 were also detected in the supernatants after four days coculture. Although still present, levels of IL-6 were reduced in the supernatants after this time point. Similarly, increased levels of PGE_2 were detected in the supernatants after four days coculture. These levels decreased to very small amounts in the supernatants from the seven and ten day cocultures.

3.6.2.3. Discussion

The results of this study have shown that mediators which have been implicated in human and murine osteoclast formation and bone resorption, namely M-CSF, IL-6 and PGE_2, are released by adherent cells isolated from periprosthetic tissue surrounding loose implant components. Detectable levels of IL-1β were also released by isolated arthroplasty cells after four days coculture although these levels were small.

It has been shown recently that the addition of exogenous M-CSF is an essential requirement for osteoclast differentiation from human monocyte-UMR 106 cocultures (Fujikawa et al 1996a). The results of the above study show that M-CSF required for osteoclast differentiation from human periprosthetic macrophage-UMR 106 cocultures is released endogenously by cells isolated from arthroplasty tissue. Other mediators such as IL-6 and PGE_2 were also released during the early stages of osteoclast differentiation. IL-6 is known to stimulate the development of

osteoclast progenitor cells, and in combination with soluble IL-6R can increase osteoclast formation and induce osteoclastic bone resorption in cocultures of murine marrow cells and primary osteoblasts (Tamura et al 1993) and human bone marrow cultures (Kurihara et al 1990). Conflicting results have been reported about the effect of PGE_2 on osteoclast formation. In murine marrow cultures (Collins and Chambers 1991) and mouse spleen-primary osteoblastic cocultures (Akatsu et al 1989), osteoclast formation was stimulated by prostaglandins and also reduced by inhibitors of prostaglandin synthesis indicating that endogenous prostaglandins present may be important. Similarly, PGE_2 was found to stimulate osteoclast formation in human marrow cultures (Flanagan et al 1995). Recently, it has been shown that the effect of prostaglandins on osteoclast formation in murine monocyte-stromal cell cocultures was dependent on the nature of the stromal cell used (Quinn et al 1997). Using cell lines rather than primary osteoblastic cells, it was found that prostaglandins both inhibited and stimulated osteoclast formation. This finding confirms the importance of bone stromal cells in prostaglandin mediated bone resorption.

A large variation in the levels of mediator release was seen between different tissue specimens. Some of this variation could be attributed to errors in measurement techniques, and differences in surface area between bone slices and coverslips. Most of this variation, however, was attributed to differences in the cellular inflammatory response evident between tissue specimens. IL-6 release, in particular, was consistently higher in cocultures in which a large number of pits formed after 14 days incubation. The pattern of mediator release during the incubation period, however, was similar for all five tissue specimens studied.

There are several limitations to this study. Apart from the PGE_2 immunoassay which detects PGE_2 from both human and rodent cells, the immunoassays for measurement of the other factors are specific for human cytokine detection, and will not measure rodent cytokines released by the UMR 106 cells. This can be an advantage, however, as the aim of this study was to determine which mediators implicated in osteoclast formation and bone resorption are released from cells isolated from arthroplasty tissue. Thus, the increased levels of M-CSF and IL-6 detected in this study were human in origin and therefore derived from cell

types present in the periprosthetic tissue, most likely macrophages and stromal cells. The release of M-CSF and PGE_2 from human osteoblastic cells during periprosthetic macrophage-osteoclast differentiation was determined in a later study described in Chapter 6 of this thesis. ELISAs will also only detect the release of soluble cytokines. Recent studies have shown that it is the membrane-bound form of M-CSF which may be important for osteoclast formation, despite the fact that this form of the cytokine only represents a small proportion of the total amount present (Sun et al 1997). It was not possible to measure membrane-bound M-CSF during the course of this study.

3.6.3. The effect of cell mediators on human periprosthetic macrophage-osteoclast differentiation and bone resorption

3.6.3.1. Introduction

It was shown in the previous study that bone resorbing mediators are released by activated macrophages and fibroblasts isolated from periprosthetic arthroplasty tissue from around loosened implants. The second part of this study was to determine which of these mediators released by activated cells in the periprosthetic tissues affect osteoclast formation and bone resorption in this coculture system. Specific antibodies to human IL-1ß, TNFα, M-CSF and IL-6 were used to determine the effect of these cytokines on the number of resorption pits formed. Many of these cytokines are known to stimulate bone resorption through a mechanism involving PGE_2 production. The role of PGE_2 was examined by the addition of indomethacin, a prostaglandin inhibitor. The addition of either cytokine antibody or inhibitor will block both soluble and membrane-bound mediators present.

In the final part of this study, the effects of these mediators on osteoclast precursor proliferation and maturation, differentiation, and mature osteoclast function were determined by adding the cytokine antibodies at specific time points during the coculture period.

3.6.3.2. Methods

Adherent cells were isolated from tissue specimens obtained from patients undergoing revision arthroplasty surgery. Positive and negative controls consisted of periprosthetic macrophages cultured in the presence and absence of UMR 106 cells respectively. All the cultures had $1,25(OH)_2D_3$ added throughout the incubation period. Specific polyclonal antibodies to IL-1, TNF, M-CSF and IL-6 were purchased from R&D Systems, Abingdon, UK. Indomethacin was purchased from Sigma, UK (Cat. no. 17378). Either cytokine antibody or indomethacin was added to the cocultures at the beginning of each experiment and every media change. The coverslips and bone slices were removed from the cultures after seven days and 14 days incubation respectively. The coverslips were

characterised separately for the expression of TRAP and VNR. The bone slices were assessed quantitatively for resorption pit formation.

3.6.3.3. Preliminary experiments

Preliminary experiments were undertaken examining a range of antibody and inhibitor concentrations. The concentration which neutralised levels of mediators absolutely and which reduced bone resorption most effectively was determined. The preliminary experiments were carried out on human bone slices only and bone resorption was the only marker of osteoclast formation assessed.

Adherent cells were isolated from three patients (ADM9 acetab., ADM9 fem., ADM10, ADM11; Appendix 1) undergoing revision arthroplasty surgery for Experiments 1-4 respectively. To determine the effects of IL-1β, TNFα, M-CSF and IL-6, concentrations of antibody directed against either human IL-1β (anti IL-1β), human TNFα (anti TNFα), human M-CSF (anti M-CSF), or human IL-6 (anti IL-6) ranging from one to 20ug/ml were added at the beginning of each experiment and every media change. To determine the effect of PGE_2, concentrations of indomethacin (Indo) ranging from 10^{-5}M to 10^{-8}M were added.

Supernatants were collected at the first media change (Day 4), centrifuged to remove cell debris, and stored at -20°C until ready for mediator level testing. Levels of mediators were measured using commercially available ELISA kits (Amersham International, UK, and R&D Systems, UK).

The results of the preliminary experiments are shown in Figure 9. Concentrations of 5ug/ml or greater of anti IL-1β neutralised the presence of human IL-1β to undetectable levels compared with a mean of 5.65pg/ml IL-1β in the untreated control. No detectable levels of TNFα were measured in either the four day untreated controls or wells which had anti TNFα antibody added. The addition of either anti IL-1β or anti TNFα appeared to have no effect on

osteoclastic bone resorption, although the addition of 5μg/ml anti IL-1β resulted in a partial reduction in the number of resorption pits formed.

A concentration of 10ug/ml or greater of anti M-CSF neutralised the presence of human M-CSF to undetectable levels. After the addition of 5ug/ml of anti M-CSF, the mean level of M-CSF in the four day cocultures was 20pg/ml compared with a mean of 166.3pg/ml for the untreated control. The addition of anti M-CSF caused a dose dependent reduction in osteoclastic bone resorption (Figure 9). A concentration of 10ug/ml of antibody resulted in the greatest reduction in resorption pit formation.

A concentration of 10ug/ml or greater of anti IL-6 substantially reduced the presence of human IL-6 in the four day cocultures. After the addition of 10ug/ml of anti IL-6, the mean level of IL-6 in the four day cocultures was 3.2pg/ml compared with a mean of 13.6pg/ml after the addition of 5ug/ml of anti IL-6, and 6825pg/ml for the untreated control. The addition of anti IL-6 substantially reduced osteoclastic bone resorption in a dose dependent manner (Figure 9). A concentration of 10ug/ml of antibody resulted in the greatest reduction in resorption pit formation.

A concentration of 10^{-6}M or greater of indomethacin substantially reduced the presence of PGE_2 in the four day cocultures. After the addition of 10^{-6}M Indo, the mean level of PGE_2 in the four day cocultures was 33pg/ml compared with a mean of 350pg/ml for the untreated control. The addition of indomethacin appeared to induce a slight increase in osteoclastic bone resorption in a dose dependent manner (Figure 9).

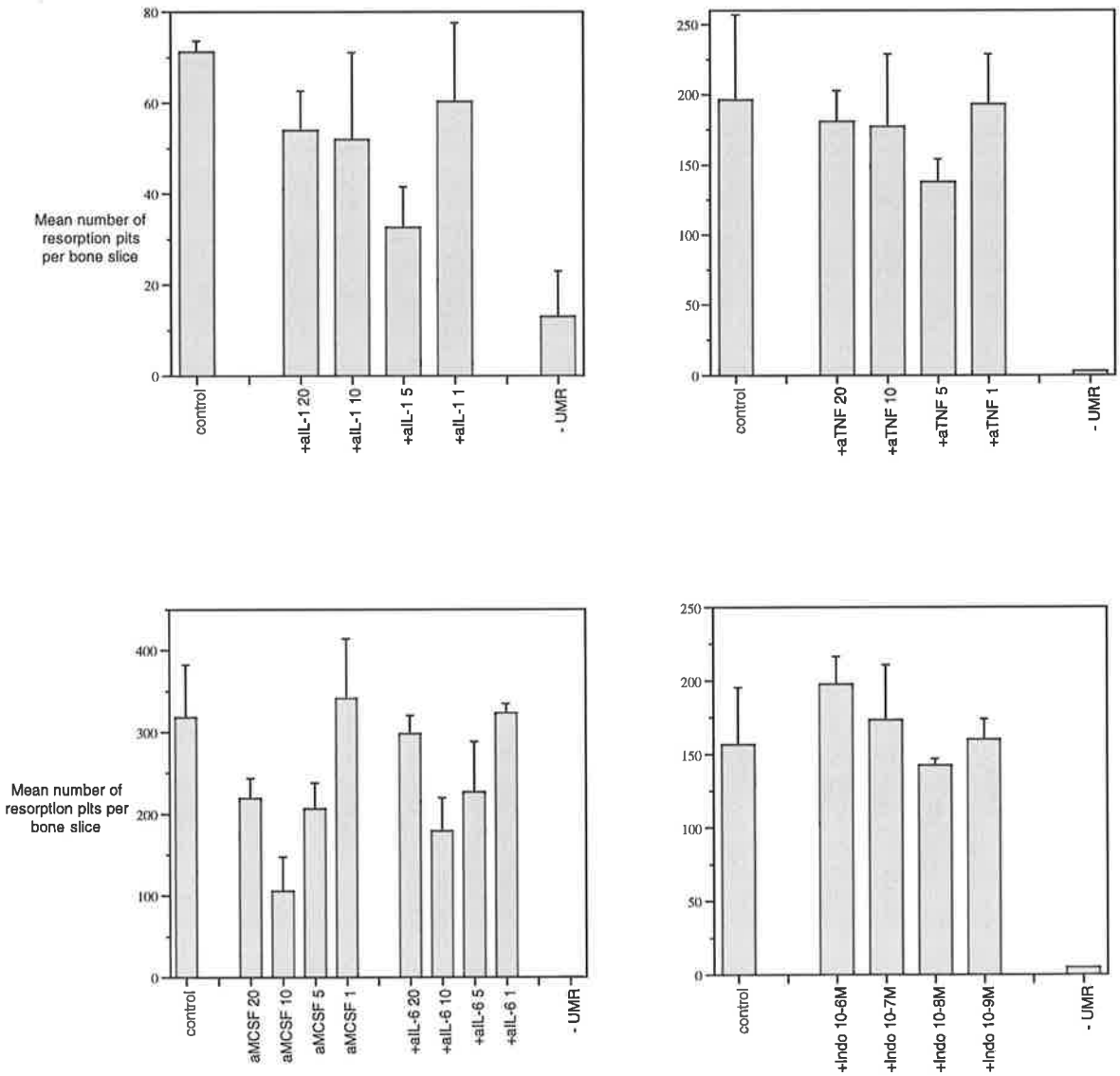

Figure 9. Dose response curves for the effects of anti IL-1, anti TNF, anti M-CSF, anti IL-6 and indomethacin on lacunar bone resorption in arthroplasty derived macrophage-UMR 106 cell cocultures after 14 days incubation.

The results are expressed as the mean number of resorption pits per bone slice ± SD (n=3).

3.6.3.4. Effects of cell mediators on periprosthetic macrophage-osteoclast differentiation and bone resorption

3.6.3.4.1. Introduction

Using the results from these preliminary experiments, the role of each mediator in periprosthetic macrophage-osteoclast differentiation and bone resorption was confirmed in a further five patients. The concentrations of antibodies and inhibitor chosen were based on the above experiments which determined the amount of antibody or inhibitor necessary to neutralise endogenous mediator present and the concentration which reduced bone resorption most effectively.

3.6.3.4.2. Methods

Adherent cells were isolated from tissue obtained from five patients (ADM12, ADM13, ADM10, ADM14, ADM6; Appendix 1) undergoing revision arthroplasty surgery. The following concentrations of antibodies were added at the beginning of each experiment and every media change; 5µg/ml anti IL-1b, 5µg/ml anti TNFa, 10 and 5µg/ml anti M-CSF, and 10µg/ml anti IL-6. Indomethacin at a concentration of 10^{-6}M was added to determine the importance of PGE_2.

3.6.3.4.3. Results

After seven days coculture of periprosthetic macrophages and UMR 106 cells, in the presence of $1,25(OH)_2D_3$, numerous large TRAP and VNR positive cells were seen on the control coverslips. Extensive lacunar bone resorption was seen on the control bone slices after 14 days coculture. The mean number of resorption pits ranged from 63.7 to 318.3 pits per bone slice. In the absence of UMR 106 cells, only occasional TRAP and VNR positive cells were seen on the coverslips after seven days incubation, and few if any resorption pits formed on the bone slices after 14 days incubation. The mean number of resorption pits ranged from 0.3 to 3 pits per bone slice.

The presence of antibodies to either IL-1β or TNFα did not appear to inhibit osteoclast formation, as determined by TRAP and VNR expression, or significantly reduce lacunar bone resorption in this *in vitro* coculture system ($p=0.756$ and $p=0.708$ respectively). In contrast, the addition of anti M-CSF

antibody caused a dose dependent inhibition of osteoclast formation and lacunar bone resorption. At a concentration of 10 µg/ml, anti M-CSF substantially reduced the numbers of TRAP and VNR positive cells present in the seven day cocultures (Figure 10) and significantly reduced ($p=0.0007$) but did not abolish resorption pit formation. Anti IL-6 was less effective at reducing osteoclast formation and bone resorption compared with the addition of anti M-CSF but at a concentration of 10µg/ml, anti IL-6 substantially reduced the numbers of TRAP and VNR positive cells present in the seven day cocultures and significantly reduced ($p=0.0013$) but did not abolish resorption pit formation. The addition of 10^{-6}M indomethacin to the coculture system did not appear to affect TRAP and VNR expression, and caused a slight but not significant increase in lacunar bone resorption ($p=0.419$). Effects on lacunar bone resorption by the addition of antibody or inhibitor for each individual experiment are shown in Table 4. The results were finally combined and expressed as the mean number of resorption pits per bone slice for all five experiments ± SEM (Figure 11).

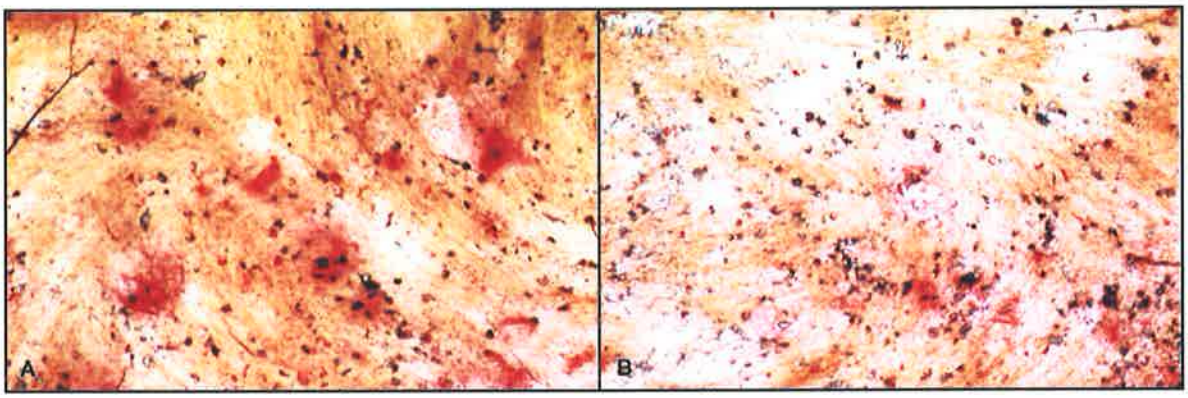

Figure 10. Seven day cocultures of arthroplasty derived macrophages and UMR 106 cells incubated in the presence of $1,25(OH)_2D_3$ showing (A) TRAP positive cells in control cocultures (x40); and (B) TRAP positive cells in cocultures incubated in the presence of 10μg/ml anti M-CSF (x40).

Table 4. Effects of cell mediators on periprosthetic macrophage-osteoclast differentiation and bone resorption

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
No treatment (control)	100% (mean=214.6)	100% (mean=142.0)	100% (mean=318.3)	100% (mean=63.7)	100% (mean=196.3)
+anti IL-1β 5μg/ml	95.3	69.7	121.4	85.9	94.2
+anti TNFα 5μg/ml	78.4	78.5	118.9	73.3	110.5
+anti MCSF 10μg/ml	ND	ND	33.3	38.1	44.6
+anti MCSF 5μg/ml	85.6	45.0	64.9	72.7	66.0
+anti IL-6 10μg/ml	54.3	85.0	56.3	37.7	45.6
+Indo 10^{-6}M	ND	122.0	132.9	106.8	101.8

Results are expressed as a percentage of the control. Mean value of the control for each experiment is given in brackets. Each experiment was done in triplicate.

ND = not determined

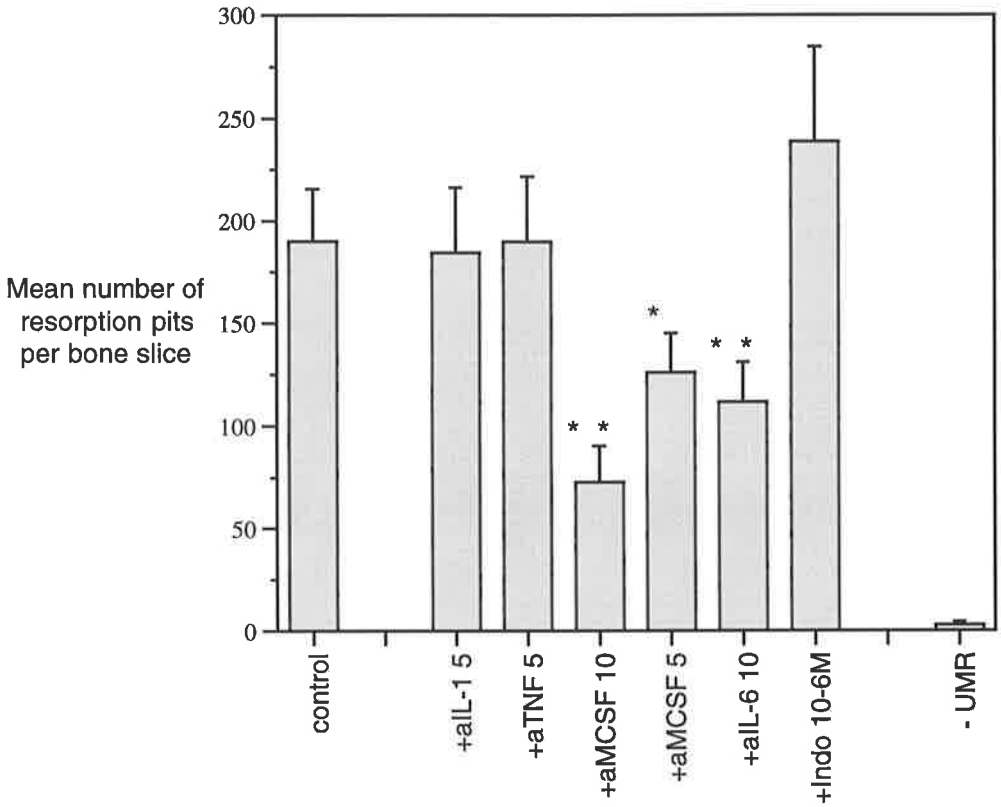

Figure 11. Effects of cytokine antibodies (5µg/ml anti IL-1, 5µg/ml anti TNF, 10 and 5µg/ml anti M-CSF, 10µg/ml IL-6) and the prostaglandin inhibitor, 10^{-6}M indomethacin, on lacunar bone resorption in arthroplasty derived macrophage-UMR 106 cell cocultures after 14 days incubation.

The results are expressed as the mean number of resorption pits per bone slice for all five experiments ± SEM. Statistical analyses were performed using a two factor with replication ANOVA test.
**$p<0.005$, *$p<0.05$ compared to control

3.6.3.5. The effects of M-CSF and IL-6 on proliferation, differentiation and mature osteoclast function

The earlier time course experiments examining periprosthetic macrophage-osteoclast differentiation (Chapter 3.3.) showed that proliferation and maturation of osteoclast precursors occurred mainly in the first four days of the coculture period. After four days incubation, osteoclast-like cells were seen in the periprosthetic macrophage-UMR 106 cocultures and a small number of resorption pits had formed. This result correlates well with previous studies using murine marrow cells and murine monocytes which have shown that osteoclast formation proceeds in two phases, an initial phase of proliferation of osteoclast precursors, and a second phase in which these cells differentiate into osteoclasts. In the murine system, osteoclast proliferation occurs during the first four days of incubation (Tanaka et al 1993; Quinn et al 1994).

By adding antibodies to M-CSF and IL-6 at specific time points during the coculture period it was possible to identify whether these factors were predominantly influencing osteoclast precursor proliferation and maturation, differentiation and/or mature osteoclast activity. Additional wells were set up in Experiments 3 and 5 whereby anti M-CSF (10μg/ml) or anti IL-6 (10μg/ml) were added to the cocultures for the first time at day four of the incubation period.

Numbers of TRAP positive cells and the extent of resorption pit formation were compared firstly to cocultures which had no antibody treatment (positive control), and secondly to cocultures which had anti M-CSF or anti IL-6 added at the beginning of the experiment (Day 0) and every media change.

The effects of antibodies to M-CSF and IL-6 on TRAP activity and lacunar bone resorption are shown in Figures 12 and 13. No effect on the numbers of TRAP positive cells and no significant reduction in lacunar bone resorption was seen when the cytokine antibodies were added for the first time on Day 4 of the incubation period compared with the untreated positive control ($p=0.254$ and $p=0.548$ respectively). This was in contrast to a substantial reduction in the numbers of TRAP positive cells and a significant reduction in lacunar bone resorption when either anti M-CSF or anti IL-6 were present from the beginning

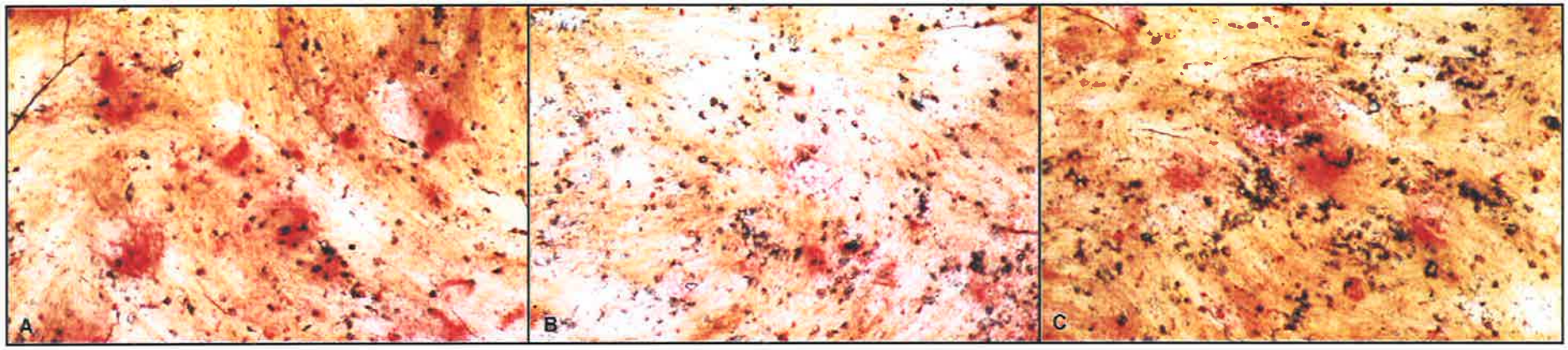

Figure 12. Seven day cocultures of arthroplasty derived macrophages and UMR 106 cells incubated in the presence of $1,25(OH)_2D_3$ showing (A) TRAP positive cells in control cocultures (x40); (B) TRAP positive cells in cocultures incubated in the presence of 10µg/ml anti M-CSF from the beginning of the experiment (x40); and (C) TRAP positive cells in cocultures incubated in the presence of 10µg/ml anti M-CSF from Day 4 of the incubation period (x40).

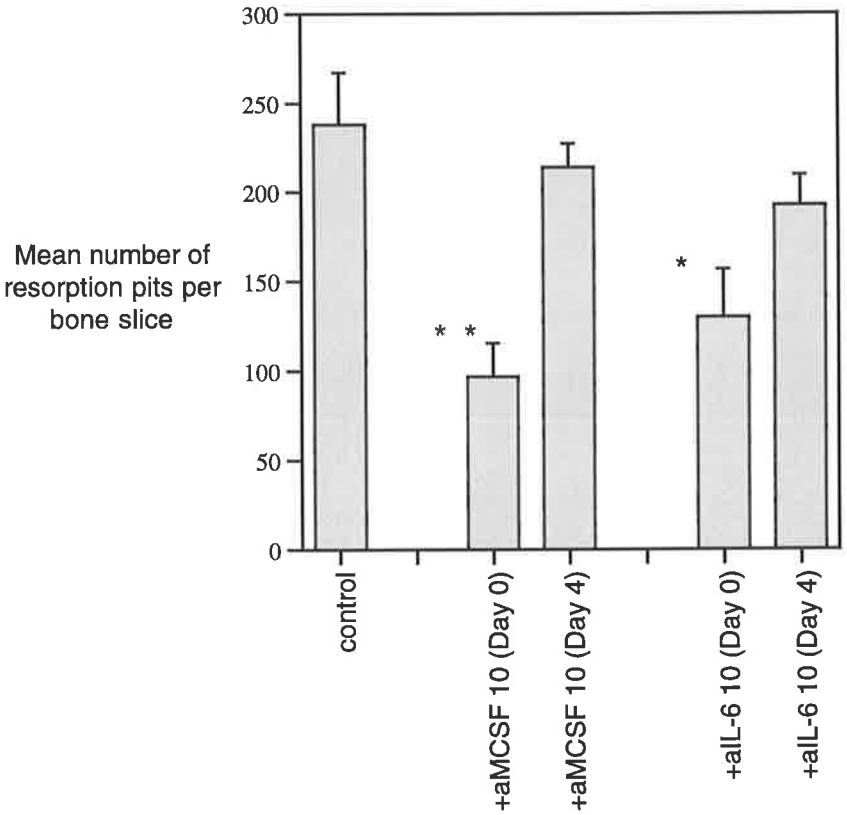

Figure 13. The effects of cytokine antibodies (10µg/ml anti MCSF and 10µg/ml anti IL-6) added at different time points on lacunar bone resorption in arthroplasty derived macrophage-UMR 106 cell cocultures after 14 days incubation.

The results are expressed as the mean number of resorption pits ± SEM (n=6). Statistical analyses were performed using a two factor with replication ANOVA test.
** $p<0.005$, * $p<0.05$ compared to control

of the incubation period compared with the untreated positive control (p=0.003 and p=0.013 respectively). This result suggests that both M-CSF and IL-6 act early in osteoclast development.

3.6.3.6. Effects of antibodies to M-CSF and IL-6 in combination

The effects of anti M-CSF and anti IL-6 on lacunar bone resorption when added in combination were also determined in Experiment 5. Additional wells were set up whereby both anti M-CSF (10µg/ml) and anti IL-6 (10µg/ml) were added to the cocultures throughout the incubation period. No difference in the inhibition of bone resorption was found when antibodies to M-CSF and IL-6 were added alone (mean inhibition of 44.6% and 45.6% respectively) or in combination (mean inhibition of 43.3%). This result suggests that there are other factors present which are important for osteoclast formation. Factors such as IL-3 and IL-11 were not examined in this study.

3.6.3.7. Discussion

Both IL-1β and TNFα are known to be potent bone resorbing agents *in vivo* and *in vitro*, and have been shown in previous studies to promote osteoclast formation from human precursors (Gowen and Mundy 1986; Bertolini et al 1986; Tashjian et al 1987; Pfeilschifter et al 1989). In this current study, detectable levels of IL-1β were released by isolated arthroplasty cells after four days incubation *in vitro*. These levels were small, however, and the addition of a specific antibody to IL-1β had no effect on osteoclast formation or bone resorption. Detectable levels of TNFα were not measured in the supernatants, and the addition of a specific antibody to TNFα had no effect on osteoclast formation or bone resorption. It must be remembered that the cytokine antibodies used in this study will only block human cytokines present endogenously. However, it is unlikely that the rat UMR 106 cells will release substantial amounts of IL-1β and TNFα as these cytokines are predominantly released by monocytes/macrophages. Although IL-1β and TNFα may promote osteoclast formation in combination with mediators such as M-CSF, the results of this current study suggest that these

cytokines are not essential for periprosthetic macrophage-osteoclast differentiation and bone resorption.

Increased levels of M-CSF, IL-6 and PGE_2 were found to be released by adherent cells isolated from periprosthetic tissue from around loose implants. Of these mediators released, M-CSF and IL-6 were shown to play important roles in the early stages of osteoclast formation from periprosthetic macrophages. Both M-CSF and IL-6 have been shown in previous studies to promote osteoclast formation from human and murine precursors. However, the mechanisms of action of these cytokines are still unclear. M-CSF has been shown to be an essential factor for the proliferation and differentiation of osteoclast precursors (Kodama et al 1991; Takahashi et al 1991; Tanaka et al 1993; Fujikawa et al 1996a; Sarma and Flanagan 1996), and also enhances survival of mature human osteoclasts thereby increasing bone resorption (Sarma et al 1997). IL-6 has been shown to stimulate the development of osteoclast progenitor cells, increase recruitment of osteoclast precursors and promote murine osteoclast differentiation *in vitro* (Kurihara et al 1990; Tamura et al 1993). *In vivo*, IL-6 has been linked to a number of bone diseases including rheumatoid arthritis, Paget's disease, oestrogen deficiency and multiple myeloma (Klein et al 1991; Jilka et al 1992; Roodman et al 1992; Kotake et al 1996). Previous studies have shown that increased levels of IL-6 are released by wear particle stimulated monocytes/macrophages, and osteoblastic cells indirectly stimulated by wear particle conditioned media (Haynes et al 1993; Haynes et al 1997). Whilst the importance of M-CSF in osteoclast formation is now widely recognised, the results of this study suggest that substantial amounts of IL-6 are released from cells present in arthroplasty tissue and that this cytokine may also play a major role in osteoclast formation and bone resorption at the bone-implant interface. M-CSF and IL-6 were found to act at the same stages of osteoclast formation from mononuclear precursors present in arthroplasty periprosthetic tissues, and were not found to act synergistically. Although the antibodies used in this study were specific for human cytokines only, rodent M-CSF produced by the UMR 106 cells is known not to interact with the human M-CSF receptor (Roussel et al 1988). Thus, any M-CSF produced by the UMR 106 cells will not affect osteoclast formation in this *in vitro* coculture system. Rodent IL-6, however, is capable of

stimulating human cells, and osteoblastic cells have been shown to release large amounts of IL-6 after stimulation (Ohta et al 1994). The levels of IL-6 produced by the rodent UMR 106 cells in this system were not determined. Thus, any effect by IL-6 released from the stromal cells was not taken into account, and the role of IL-6 in this coculture system may have been underestimated.

The addition of indomethacin was used to determine the effects of endogenous prostaglandins on periprosthetic macrophage-osteoclast differentiation. Elevated levels of PGE_2 were released by isolated arthroplasty cells after four days coculture. Inhibition of both human and rat PGE_2 levels by Indomethacin resulted in a slight increase in bone resorption suggesting that endogenous PGE_2, as well as other prostaglandins present, may act to inhibit bone resorption in this coculture system. This result was not unexpected as previous studies have shown that PGE_2 inhibits osteoclast formation in murine monocyte-UMR 106 cell cocultures (Quinn et al 1997). Quinn et al (1997) found that this inhibitory effect was dependent on the nature of the stromal cell supporting osteoclast differentiation as PGE_2 was shown to stimulate osteoclast formation in murine monocyte-ST2 cell cocultures. This work strongly suggests that PGE_2 mediates its effects on osteoclast formation through the stromal cell component of these cultures despite the fact that PGE_2 is known to inhibit directly, and transiently, mature osteoclast activity. The results of this current study support the previous findings with UMR 106 cell cocultures that small levels of PGE_2 present endogenously inhibit osteoclast formation. However, the contrary findings reported depending on the stromal cell line used make it difficult to relate these *in vitro* findings to the *in vivo* situation. These effects of PGE_2 are further examined in Chapter 6 of this thesis when human bone stromal cells derived from trabecular bone explants are used to support periprosthetic macrophage-osteoclast differentiation.

It was not surprising that anti M-CSF inhibited bone resorption given the previous results for OA synovial macrophage-osteoclast differentiation (Chapter 3.4), and earlier work which demonstrated the importance of M-CSF in human osteoclast differentiation (Fujikawa et al 1996a; Sarma and Flanagan 1996). However, the addition of anti M-CSF did not totally abolish lacunar bone

resorption. Although, it is known that rodent M-CSF does not interact with the human M-CSF receptor, UMR 106 cells are still an essential requirement for human macrophage-osteoclast differentiation. This is confirmed by other studies which have found that M-CSF stimulates osteoclast formation only in the presence of osteoblasts (Fujikawa et al 1996a) and that *in vivo* administration of rhM-CSF to op/op mice only partially restores the animal's ability to form osteoclasts (Felix et al 1990). These findings suggest that there is another factor(s) produced by the bone stromal cells which is essential for osteoclast formation. Recent work by others have shown that cell-cell contact is required between the stromal cells and osteoclast precursors indicating that it is a membrane-bound factor which is important (Jimi et al 1996). The aim of this study was not to determine the identity of this factor. Rather the aim of this study was to determine which mediators released by activated cells in the periprosthetic tissues affect osteoclast formation and bone resorption in this coculture system. It was found that mediators implicated in osteoclast formation and osteoclastic bone resorption, namely M-CSF, IL-6 and PGE_2, are released from cells isolated from arthroplasty periprosthetic tissues from around loosened implants. Of these mediators present, M-CSF and IL-6 were found to be important *in vitro* for the proliferation and differentiation of precursor cells into osteoclastic bone resorbing cells. These results suggest that these mediators may be important for the formation of new osteoclasts at sites of pathological bone resorption.

4. Cytokine Receptor Profile of Macrophages and Foreign Body Giant Cells in Periprosthetic Revision Arthroplasty Tissue, and Mature Osteoclasts

4.1 Introduction

Numerous cytokines including IL-1β, TNFα, M-CSF and IL-6 are known to influence the bone resorbing activity of osteoclasts and their formation from haematopoietic and mononuclear phagocyte precursors. There is evidence that several of these cytokines may play a role in development of osteolysis associated with aseptic loosening of total joint arthroplasties. The effects of cytokines on cells are mediated by specific cell surface receptors. The distribution of these receptors and changes in their number and activity provide an important mechanism for regulating responses to cytokines.

The aim of this study was to determine the cytokine receptor profile of macrophages and foreign body giant cells (FBGCs) in periprosthetic revision arthroplasty tissue, and mature osteoclasts derived from giant cell tumours of bone.

4.2. Methods

4.2.1. Clinical details

Human periprosthetic arthroplasty tissue was obtained from two patients undergoing revision total joint arthroplasty surgery. Histologically, both specimens contained biomaterial wear debris, and large numbers of macrophages and FBGCs were present.

Tissue specimens of giant cell tumours of bone were obtained from two patients. The multinucleated giant cells present in these tissues have all the characteristics of osteoclasts and are commonly used in studies of osteoclasts *in vitro*. They possess abundant calcitonin receptors, respond to calcitonin with a rise in cyclic adenosine monophosphate, they are positive for tartrate-resistant acid phosphatase, have an antigenic phenotype identical to that of osteoclasts, and are capable of lacunar bone resorption (Horton et al 1985; Chambers et al 1985b; Goldring et al 1986; Athanasou et al 1988; Grano et al 1994; Athanasou 1996).

4.2.2. Monoclonal antibodies

The monoclonal antibodies (McAbs) used in this study are listed in Appendix 2, and were derived from the Cytokine Panel of McAbs analysed in the 6th International Workshop on Human Leukocyte Differentiation Antigens. The identity of tissue macrophages, FBGCs and osteoclasts was established using the anti-CD68 antibody, EBM/11, which is present on all three cell types, and a monoclonal antibody to the vitronectin receptor, 23C6, an osteoclast-associated marker.

4.2.3. Immunohistochemical staining method

The specimens were obtained fresh and snap-frozen in liquid nitrogen. With the use of a cryostat, serial frozen 6μm sections were collected onto multiwell glass slides (Cat. no. PH019, Hendley, Essex, UK), fixed in cold (-20°C) acetone for 20 minutes and air dried at room temperature. Imprints of osteoclasts were prepared by pressing a freshly cut piece of tissue from a giant cell tumour of bone onto multiwell glass slides. The imprints were air dried then fixed for 20 minutes in cold acetone. An indirect immunoperoxidase method was used for monoclonal antibody staining (Gatter et al 1984) as follows:

1. Acetone-fixed frozen sections and imprints stored at -20°C were labelled and allowed to reach room temperature.
2. Mouse anti human McAbs were diluted 1:10 in PBS containing 2% bovine serum albumin (PBS+2% BSA), and 90μl added to each appropriate section or imprint. Negative controls consisted of no primary antibody (PBS+2% BSA alone). The slides were incubated in a moist environment overnight at 4°C. The slides were gently rinsed with PBS from a wash bottle followed by two washes in PBS each for 5 minutes.
3. To block endogenous peroxidase activity, the slides were gently shaken for 30 minutes in an ethanol/0.6% hydrogen peroxide solution.
4. The slides were removed and rinsed in PBS for a further 10 minutes.
5. The biotinylated secondary antibody (peroxidase conjugated rabbit anti mouse; Cat. no. P0260, Dako Ltd, UK) was diluted 1:50 in PBS+2% BSA, 90μl applied to

each section and imprint, and the slides incubated in a moist environment for 30 minutes at room temperature.

6. The slides were rinsed in PBS for 10 minutes.
7. The biotinylated tertiary antibody (peroxidase conjugated swine anti rabbit Ig; Cat. no. P0217, Dako Ltd, UK) was diluted 1:100 in PBS+2% BSA, 90µl applied to each section and imprint, and the slides incubated in a moist environment for 30 minutes at room temperature.
8. The slides were rinsed in PBS for 10 minutes.
9. A solution of 0.5 mg/ml of DAB (Cat. no. D-5637, Sigma, UK) containing 0.05% hydrogen peroxide was applied to each section and imprint, and the slides incubated for 3 minutes.
10. The sections were washed thoroughly in running tap water, counterstained with haematoxylin (Gill's No.3; diluted 1 in 3 with distilled water), dehydrated through graded alcohols, brought to xylene, and finally mounted with DPX medium and examined by light microscopy.

4.3. Results

The revision arthroplasty specimens contained abundant polyethylene wear debris in association with tissue macrophages and foreign body giant cells. These cells stained strongly with the anti-macrophage CD68 antibody (Figure 14). The GCT of bone imprints contained large numbers of osteoclasts identifiable by their size and multinuclearity. Small mononuclear cells were also present. Both the osteoclasts and mononuclear cells in the imprints showed strong cytoplasmic staining and membrane staining to CD68 and the vitronectin receptor antibody (Figure 14).

The pattern of staining by the cytokine receptor McAbs on both inflammatory macrophages and FBGCs was almost identical to that of osteoclasts. A summary of the staining pattern is shown in Table 5. Macrophages, FBGCs and osteoclasts reacted with antibodies directed against IL-1R type 1, IL-2Rα and γ, IL-4R, IL-6R, gp130, TNFR, GM-CSF and SCFR. All of these antibodies reacted with the macrophage, FBGC and osteoclast cell membrane but the more strongly staining antibodies (anti IL-1R type 1, anti IL-2R, anti IL-4 R, anti IL-6R and TNFR) also

showed some cytoplasmic staining (Figure 15). Blood vessels in the revision arthroplasty tissues showed strong staining for antibodies directed against SCFR and IL-7R (Figure 15). Macrophages, FBGCs and osteoclasts did not react with antibodies directed against IL-3Rα and IL-8R.

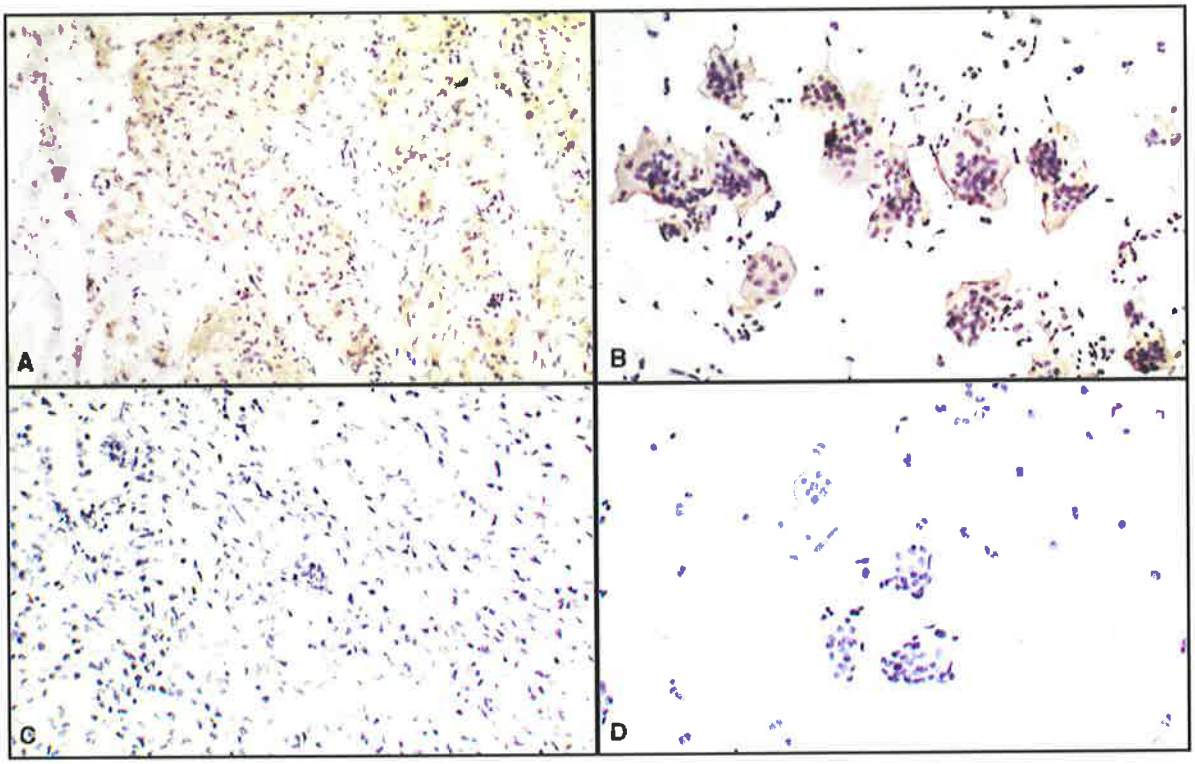

Figure 14. Indirect immunoperoxidase staining of (A) macrophages and FBGCs in revision arthroplasty tissue for CD68 expression (x100); and (B) osteoclasts from GCT imprints for VNR expression (x100). (C) Arthroplasty tissue negative control (no antibody) (x100) and (D) GCT imprint negative control (x100). Counterstained with haematoxylin.

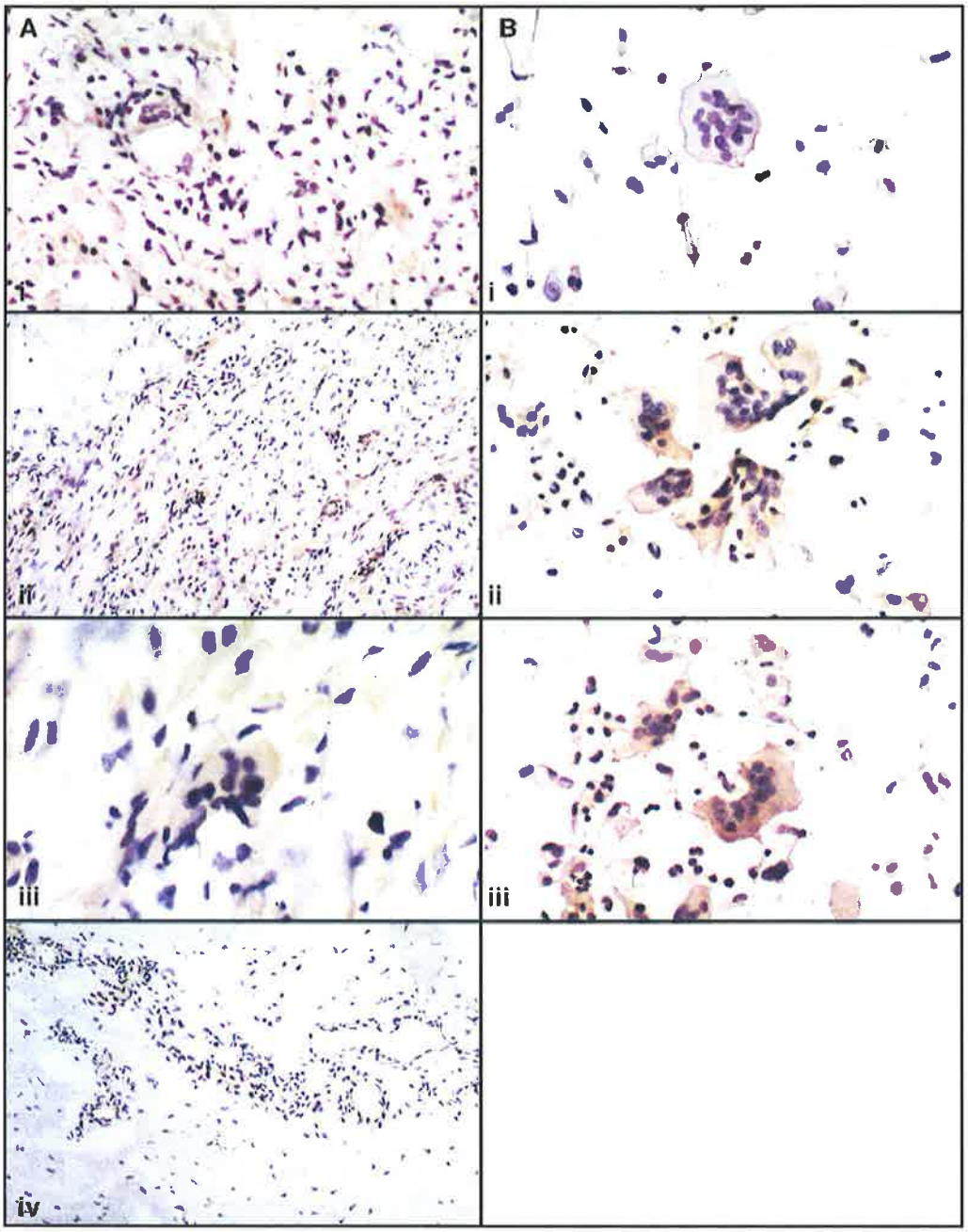

Figure 15. Cytokine receptor expression on (A) macrophages and FBGCs in revision arthroplasty tissue and (B) osteoclasts from GCT imprints. Counterstained with haematoxylin.

(i) IL-1 Type 1 receptor expression (x200 and x200 respectively)
(ii) IL-6 receptor expression (x100 and x200 respectively)
(iii) M-CSF receptor expression (x400 and x200 respectively)
(iv) SCF receptor expression (x100). Note blood vessels stained strongly.

Table 5. Cytokine receptor profile of macrophages and FBGCs in periprosthetic revision arthroplasty tissue, and mature osteoclasts.

mAb code	Reactivity	Isotype	Revision A	Revision B	GCT C	GCT D
C-79	IL-1R Type1	G1	+	+	+	+
C-25	IL-2Rα	G2a	w	-w	+	+
C-53	IL-2Rα	M	+	+	+	+
C-54	IL-2Rα	M	+	+	+	+
C-90	IL-2Rα	G1	+	w	+	+
C-89	IL-2Rγ	G2b	+	w	+	+
C-67	IL-3Rα	G1	-	-	-	-
C-4	IL-4R	G1	+	+	w	-w
C-81	IL-4R	G1	+	+	+	+
C-74	IL-6R	G1	+	+	-w	+
C-75	IL-6R	G1	+	+	-w	w
C-63	IL-6R		+	w	+	w
C-88	IL-6R	G2a	+	w	+	+
C-5	IL-7R	G	- (bv+)	- (bv+)	+	+
C-77	IL-8R	M	-	-	-	-
C-94	TNFR/55kD	G1	+	+	+	+
C-95	TNFR/75kD	G1	+	+	+	+
C-13	gp130	G1	+	+	+	+
C-16	gp130	G1	w	+	w	w
C-18	gp130	G1	w	-	+	+
C-24	gp130	G2a	w	-w	+	+
C-34	gp130	G1	w	-w	+	w
	M-CSFR		+	+	+	+
C-48	SCFR	G1	+	bv+	w	+
C-29	SCFR	G1	+	-w	w	w
C-68	SCFR	G1	w	w	+	+
C-69	SCFR	G1	w	w	+	+
C-72	SCFR	M	+ (bv+)	+	+	+
C-45	SCFR	G1	w (bv+)	- (bv+)	-	w
C-46	SCFR	G1	w (bv+)	-w (bv+)	-	-
C-3	GM-CSF-R	G1	-w	-	-w	-
C-57	GM-CSF-R	G1	+	w	-	-w

+ = strong positive staining

w = weak staining

w- = very weak to almost no staining

- = no staining

(bv+) = cells lining blood vessels stained positive

4.4. Discussion

The findings in this study show that a number of cytokine receptors are present on macrophages, FBGCs in revision tissue, and osteoclasts with a strong correlation between cytokine receptor antigen expression on macrophages, FBGCs and osteoclasts. It is widely accepted that FBGCs are formed by the fusion of mature macrophages. FBGCs are considered to be distinct from osteoclasts despite a number of common features, including their probable origin from bone marrow precursors. The strong correlation between cytokine receptor antigen expression on macrophages, FBGCs and osteoclasts possibly reflects the common lineage of these cells.

The presence of IL-1 and TNF receptors on macrophages in revision tissue and osteoclasts is consistent with the hypothesis that these cytokines are important in periprosthetic bone resorption and implant loosening. Both IL-1 and TNF are potent inducers of bone resorption in calvarial organ cultures (Bertolini et al 1986; Gowen and Mundy 1986; Tashjian et al 1987). In addition, IL-1 and TNF also stimulate osteoclast formation in long-term human marrow cultures (Pfeilshifter et al 1989). Thus, IL-1 and TNF appear to stimulate osteoclastic activity and osteoclast formation. The mechanism by which these cytokines act is complex, however, as these and other factors have multiple and overlapping effects. Both IL-1 and TNF act in an autocrine manner on macrophages to stimulate their own production, as well as the synthesis and secretion of other cell mediators such as IL-6 and PGE_2. Although osteoclasts were shown to possess receptors for IL-1 and TNF, there is strong evidence that these cytokines act through osteoblasts or bone marrow-derived stromal cells rather than on osteoclasts directly (Thomson et al 1986; Thomson et al 1987). However, IL-1 has been shown to be important for osteoclast survival (Jimi et al 1995).

Strong expression of IL-6 receptors on macrophages, FBGCs in revision tissue and osteoclasts was shown in this study. Previous studies have shown IL-6 receptors on monocytes/macrophages (Munck-Petersen et al 1990), and giant cells isolated from osteoclastomas (Ohsaki et al 1992). IL-6 has been shown to have a direct effect on osteoclasts *in vitro* (Ohsaki et al 1992), and IL-6 also acts to increase the

recruitment, proliferation and differentiation of osteoclast progenitors from haematopoeitic precursors (Kurihara et al 1990; Roodman et al 1992; Tamura et al 1993). Recent studies have shown that the effect of IL-6 on osteoclast formation appears to be mediated via osteoblasts or bone marrow stromal cells (Udagawa et al 1995; Tamura et al 1993), and expression of IL-6 receptors on osteoblasts has been demonstrated. In contrast to IL-1 and TNF, which are produced primarily by monocytes/macrophages, IL-6 is produced primarily by osteoblasts and bone marrow stromal cells (Ishimi et al 1990). IL-6, IL-1 and TNF have all been implicated in the osteolysis of several bone diseases including Paget's disease, osteoporosis, multiple myeloma and rheumatoid arthritis (Pacifici et al 1989; Arend and Dayer 1990; Jilka et al 1992; Manolagas 1995; Roodman et al 1992). IL-1 and TNF are known to stimulate the synthesis and secretion of IL-6 and this mechanism of action has been proposed for the osteolysis associated with these diseases. IL-6 is known to exert its activity through a cell surface receptor which consists of two components, a membrane-bound IL-6 receptor and gp130. When the IL-6 receptor is occupied, the ligand receptor complex binds to gp130 then transduces the IL-6 signals (Taga et al 1989). Strong expression of gp130 was shown on macrophages and FBGCs in the revision arthroplasty tissues, and on osteoclasts in this study. In addition to IL-6, other cytokines such as IL-11, leukaemia inhibitory factor and oncostatin M utilise gp130 as a signal transducer (Kishimoto et al 1992). IL-11, in particular, has recently been shown to be important factor in osteoclast formation although receptor expression of this cytokine was not determined in this study (Girasole et al 1994).

Other receptors for cytokines which influence osteoclast formation from mononuclear osteoclast precursors such as M-CSF and SCF were also present on macrophages and FBGCs in revision tissue, and osteoclasts in this study. Of these, M-CSF has been shown to be an essential cofactor for the proliferation of osteoclast progenitors and differentiation into mature osteoclasts from mononuclear precursors. Studies of the genetically defective osteopetrotic mouse (*op/op*) have shown that its defective osteoclastic bone resorption was due to a mutation in the M-CSF gene and an inability to produce functional M-CSF (Yoshida et al 1990; Felix et al 1990). *In vitro* studies using long-term mouse marrow culture systems (Kodama et al 1991; Tanaka et al 1993) and more recently

using human culture systems (Fujikawa et al 1996a; Sarma et al 1996) have confirmed the importance of M-CSF in osteoclast formation. M-CSF has also been shown to enhance osteoclast survival (Fuller et al 1993; Jimi et al 1995), and receptors for M-CSF have recently been shown on osteoclasts (Kodama et al 1991; Weir et al 1993; Yang et al 1996). The expression of M-CSF receptors on macrophages in revision tissue and osteoclasts in our study confirms that these cells are potential targets of M-CSF. SCF in combination with other haematopoietic growth factors also probably plays a role in the regulation of osteoclast progenitor formation, although unlike M-CSF, SCF does not affect osteoclast formation individually (Demulder et al 1992).

Weak staining for GM-CSF receptors on macrophages, FBGCs in revision tissue and osteoclasts was shown in this study. The role of GM-CSF in osteoclast formation is unclear. GM-CSF has been reported to be both a stimulator and inhibitor of osteoclast differentiation (Takahashi et al 1991; Shinar et al 1990; Suda et al 1995). GM-CSF has been shown to stimulate IL-1 production and induce HLA-DR expression on monocytes (Xu et al 1989) and has recently been shown to be released from monocytes after wear particle stimulation (Horowitz and Purdon 1995). This latter response may be due to increased levels of IL-1, IL-6 and TNF as these cytokines are known to stimulate GM-CSF production. GM-CSF acts by binding to the GM-CSF receptor and then forms a complex with a distinct molecule, KH97, for signal transduction. Other cytokines including IL-3 and IL-5 also utilise KH97 as a signal transducer. The absence of IL-3 receptor expression in this study may reflect the fact that IL-3 acts primarily on early progenitor cells.

Strong expression of the α and γ components of the IL-2 receptor on macrophages, FBGCs and osteoclasts was also shown in this study. IL-2 primarily causes the expansion and proliferation of the lymphocyte population but also acts on macrophages to enhance their biological activities. The IL-2 receptor consists of an $\alpha\beta\gamma$ complex and the IL-2 receptor γ chain is also used by the cytokines IL-4, IL-7 and IL-9 as part of their respective receptor complexes. The expression of IL-4 receptors on macrophages in revision tissue and osteoclasts is of interest as IL-4

has been shown to inhibit osteoclast formation from both marrow and mononuclear phagocyte precursors (Shioi et al 1991; Lacey et al 1995). This inhibitory effect may be directed against the differentiation of mononuclear precursors to mature osteoclasts or inhibition of proliferation of osteoclast precursors (Jansen et al 1990). Strong expression of these receptors on blood vessels in revision arthroplasty tissue was found in this study. No expression of IL-7 receptors on macrophages and FBGCs in revision arthroplasty tissue was found, although osteoclasts were found to express IL-7 receptors.

In summary, macrophages and FBGCs in periprosthetic revision arthroplasty tissue were found to express receptor antigens for cytokines implicated in osteoclastic bone resorption including IL-1, TNF and IL-6, and cytokines known to be involved in osteoclast recruitment, proliferation and differentiation from osteoclast precursors including IL-1, TNF, IL-6, M-CSF and SCF. These receptor antigens were also found to be present on osteoclasts. These findings suggest that these cells may be targets for cytokines implicated in osteoclast formation and osteoclastic bone resorption associated with aseptic loosening. The widespread distribution of these receptors may account for the diversity of the biological activities of these cytokines, although many of the cytokines exert their effects through common signal molecules such as gp130, the IL-2 receptor γ chain and KH97.

5. The Effect of Prosthesis Wear Particles on Osteoclast Differentiation and Bone Resorption *In Vitro*

5.1. Introduction

In the previous experiments, a large variation in the mean number of resorption pits counted per bone slice was seen between different tissue specimens despite the fact that the concentration of cells added remained the same throughout the experiments. It is known that the intensity of the macrophage response to particulate wear debris and the extent of periprosthetic osteolysis varies greatly between patients. Arthroplasty periprosthetic tissue contains a mixture of cell types, including macrophages and stromal cells, and a number of wear particles of varying sizes generated from different materials. Thus, although wear particles are clearly important in the recruitment of foreign body macrophages to the site of inflammation, it is difficult to determine from the previous experiments (Chapter 3) whether prosthesis wear particles significantly influence osteoclast differentiation and bone resorption.

To determine the effects of prosthesis wear particles on macrophage-osteoclast differentiation and bone resorption, a human monocyte-osteoblast-like cell coculture system was used. Adherent mononuclear phagocytes (monocytes) isolated from peripheral blood have been used to study cellular responses to wear particles *in vitro* (Bennett et al 1991; Shanbhag et al 1995; Blaine et al 1996; Rogers et al 1997), and the indirect effects of these particles through wear particle conditioned media on other cell types, such as osteoblastic and fibroblastic cells (Ohta et al 1994; Horowitz and Purdon 1995; Yao et al 1995; Haynes et al 1997). One main advantage is that the effects of individual wear particles of a controlled size and number can be determined using monocytes from normal healthy donors without the conflicting problems of pre-exposure to wear particles and/or various drug treatments that are associated with human arthroplasty tissues. Human monocytes can be isolated in large numbers from peripheral blood with greater than 95% purity (Rogers et al 1997). Although minor differences between monocytes and tissue macrophages in their phagocytic capability and their ability to release the mediators, IL-1 and PGE_2, in relation to wear particles (Glant and Jacobs 1994; Howie et al 1996), and the levels of lysosomal enzyme released (Cline

1970) have been reported, these cells like macrophages avidly phagocytose wear particles.

Recently, it has been shown that a sub-population of cells isolated from human peripheral blood are capable of differentiating into large multinucleated cells exhibiting all the specific phenotypic characteristics of osteoclasts, including that of lacunar bone resorption (Fujikawa et al 1996a). Contact with bone stromal cells, and the presence of $1,25(OH)_2D_3$ and exogenous M-CSF were all found to be essential for human monocyte-osteoclast differentiation. Using this *in vitro* coculture system, the effect on macrophage-osteoclast differentiation and bone resorption of particle phagocytosis and wear particles similar to those found at the bone-implant interface was determined.

As mediator release has been shown to be stimulated by wear particles phagocytosis, the hypothesis for this study was that prosthesis wear particles would similarly stimulate the differentiation of human peripheral blood monocytes into osteoclastic cells, and increase resorption of cortical bone *in vitro*. Thus, the aims were firstly to set-up and characterise the cellular and humoral requirements for osteoclast formation in this *in vitro* coculture system; secondly to determine whether these cells could phagocytose particles, a distinct macrophage function, and still undergo osteoclast differentiation and retain the ability to resorb bone; and finally to determine the effects of different prosthesis wear particles on osteoclast differentiation and bone resorption. Direct effects of prosthesis wear particles on human monocyte-osteoclast differentiation and bone resorption, and indirect effects of wear particle-stimulated human monocyte conditioned media on osteoclast differentiation and bone resorption were determined.

5.2. General methods for the isolation of human monocytes and *in vitro* coculture

5.2.1. Isolation of cells from human peripheral blood

Human mononuclear cells were isolated from the peripheral blood of healthy volunteers using Ficoll-Paque sedimentation. Each 10 mls of blood collected was diluted 1:1 with αMEM only, layered over 8 mls of Ficoll-Paque (Cat. no. 17-0840-02, Pharmacia Biotech, UK), and centrifuged at 630g for 15 minutes. The white layer of cells at the interface was removed, washed two times in αMEM only, and finally resuspended in 2 mls αMEM+FCS media. The concentration of cells was determined by counting the number of cells in a haemocytometer after lysis of red blood cells using a 5% (v/v) acetic acid solution.

5.2.2. Preparation of human monocyte-UMR 106 cell cocultures on coverslips and human bone slices

A concentration of $5x10^5$ cells/100µl of the cell suspension was added to 7mm wells of a 96 well tissue culture plate. These wells contained either glass coverslips or prewetted cortical bone slices. Half the coverslips and bone slices were seeded 24 hours earlier with the stromal cells, rat osteoblast-like UMR-106 cell line, at a concentration of $2x10^4$ cells/ml. After 1-2 hours incubation at 37°C in 5% CO_2, the bone slices and coverslips were removed from the wells, washed vigorously in αMEM to remove the non-adherent cells, and placed in larger 16mm wells containing 1ml of αMEM+10%FCS media. These cultures were incubated for up to 21 days in the presence of 10^{-7}M $1,25(OH)_2D_3$, 10^{-8}M dexamethasone and 25 ng/ml M-CSF, with the culture medium changed and factors added every 3-4 days.

The incubation periods required for human monocyte-osteoclast differentiation were considerably longer than those required for periprosthetic macrophage-osteoclast differentiation. Preliminary experiments found that only a mean of 18.7 resorption pits per bone slice (n=3) was seen on cortical bone slices after 14 days coculture under appropriate conditions for osteoclast formation. The number of resorption pits had greatly increased, however, in the 21 day cocultures (mean of 179.7 pits per bone slice; n=3). The reason for this is

unknown but may reflect the fact that periprosthetic cells may be further down the differentiation pathway than human monocytes.

5.3. Cellular and humoral requirements for osteoclast differentiation from human monocytes

To determine the cellular and humoral requirements for osteoclast formation, human monocytes were isolated from the peripheral blood of three healthy volunteers, as described above. The isolated adherent cells were cultured on coverslips and human bone slices in (a) the absence of UMR 106 cells; (b) the absence of $1,25(OH)_2D_3$; (c) the absence of dexamethasone; and (d) the absence of M-CSF, as shown in Table 6. The coverslips were removed from the cultures after 24 hours and 14 days incubation and characterised histochemically for the expression of TRAP, and immunohistochemically for VNR expression and expression of the macrophage associated antigens, CD11b and CD14. The cortical bone slices were removed from the cultures after 24 hours and 21 days incubation and the number of resorption pits per bone slice was counted. Each treatment was studied in triplicate for each blood donor.

5.3.1. Results

In the 24 hour cocultures on glass coverslips, the isolated cells were positive for the macrophage markers, CD11b and CD14, and entirely negative for the osteoclast markers, TRAP and VNR (Figure 16). No lacunar resorption pits were seen after the cells were cocultured on bone slices. However, in the 14 day and 21 day cocultures of human monocytes and UMR 106 cells, in the presence of $1,25(OH)_2D_3$, dexamethasone, and exogenous M-CSF, numerous large TRAP and VNR positive cells formed on coverslips (Figure 17) and extensive lacunar bone resorption was evident on all the nine bone slices studied (Figure 18).

When dexamethasone was omitted from the cultures, it was noted that numerous cells, both mononuclear and multinucleated, with abundant foamy cytoplasm were visible through gaps in the covering UMR 106 cell layer. These cells only faintly expressed TRAP and VNR. However, it was noted that a few multinucleated cells (approx. 10 cells per coverslip) in the 14 day cocultures

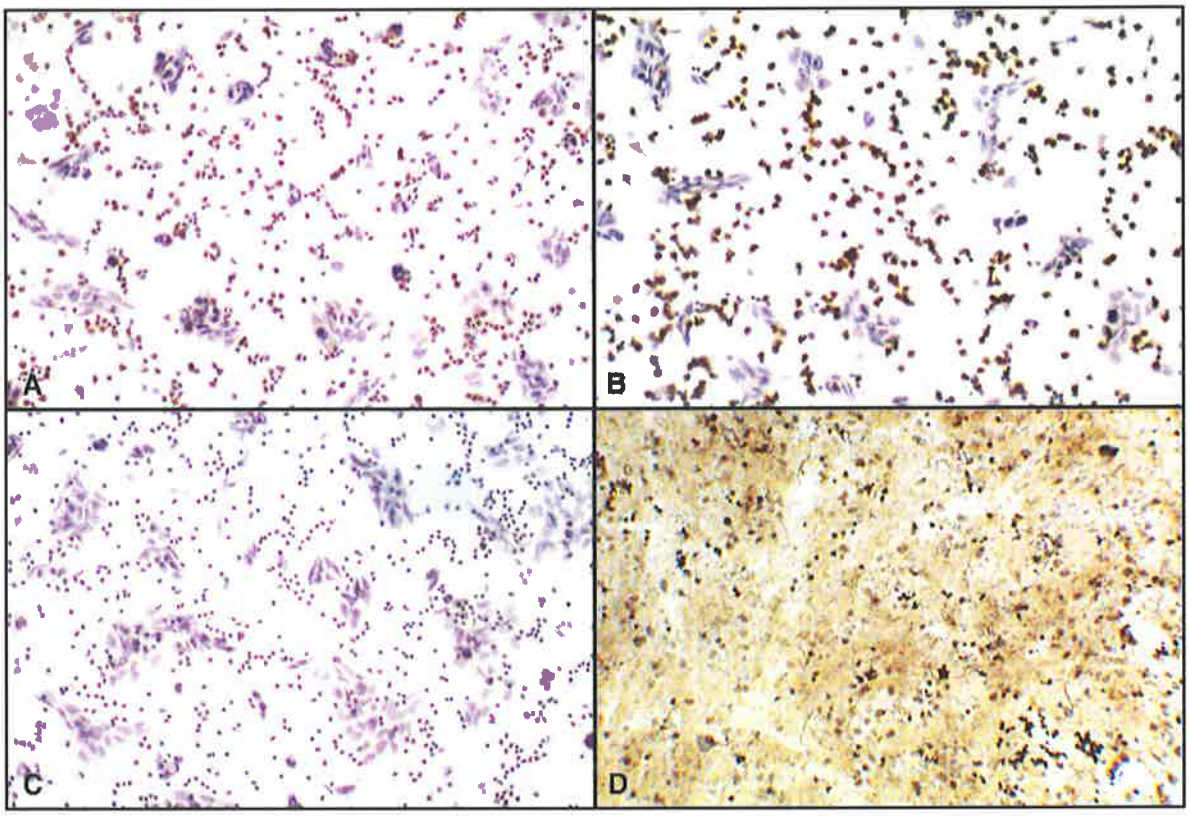

Figure 16. Human monocyte-UMR 106 cell cocultures after 24 hours incubation showing (A) numerous CD11b positive mononuclear cells (x100); and (B) numerous CD14 positive mononuclear cells (x100). These cells were found to be entirely negative for the osteoclast markers, VNR expression (C) (x100) and TRAP activity (D) (x40). Counterstained with haematoxylin.

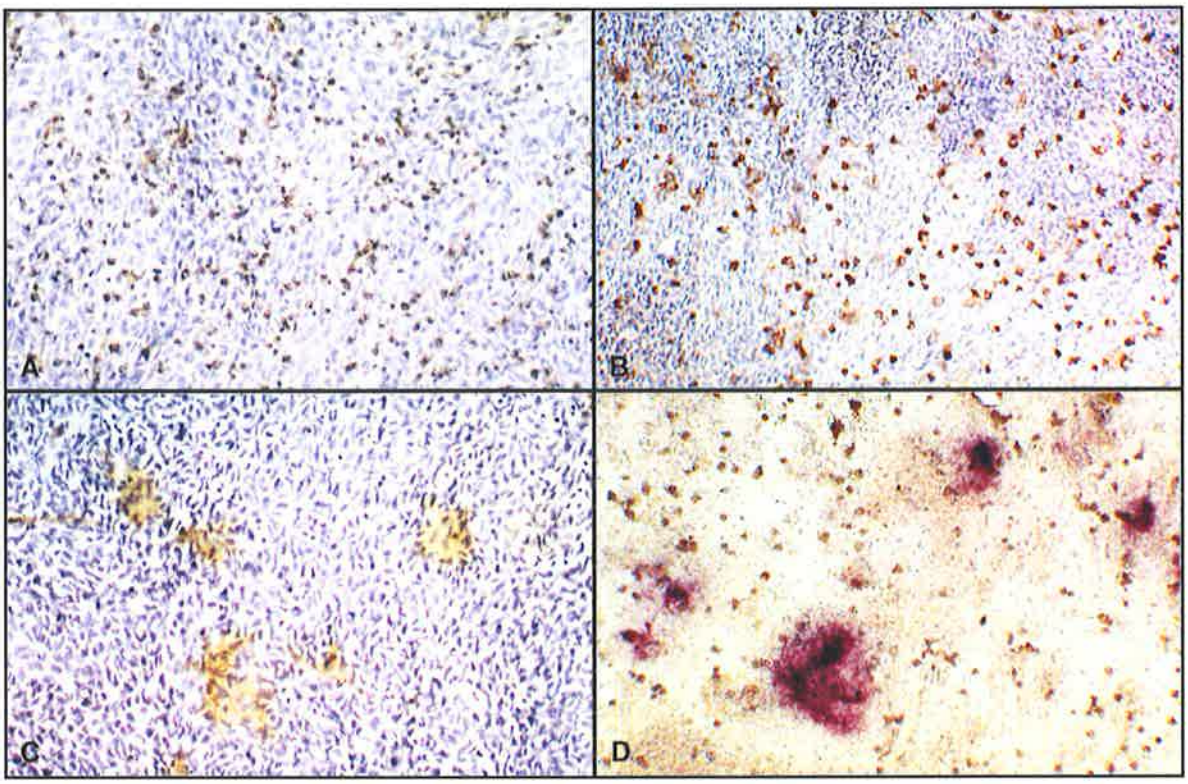

Figure 17. Human monocyte-UMR 106 cell cocultures after 14 days incubation in the presence of $1,25(OH)_2D_3$, dexamethasone and M-CSF showing (A) numerous CD11b positive mononuclear cells (x100); and (B) numerous CD14 positive mononuclear cells (x100). Large multinucleated cells positive for VNR expression (C) (x100) and TRAP activity (D) (x40) were also present in the cocultures. Counterstained with haematoxylin.

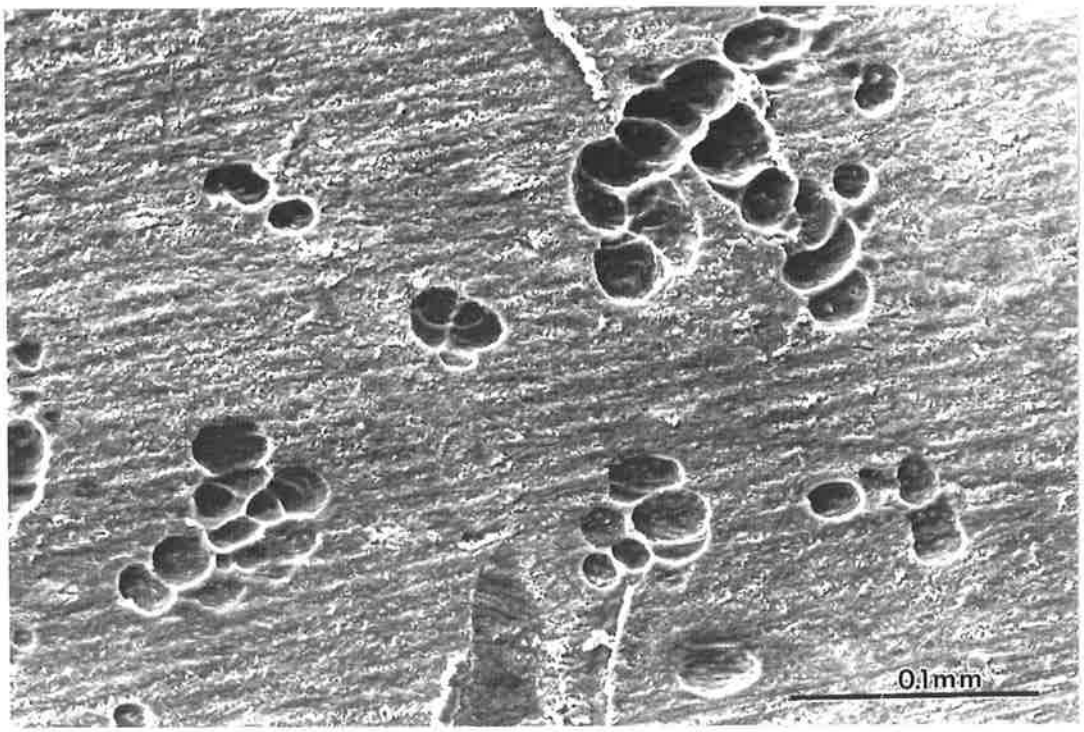

Figure 18. Human monocytes and UMR 106 cells cocultured for 21 days in the presence of 10^{-7}M $1,25(OH)_2D_3$, 10^{-8}M dexamethasone and 25ng/ml M-CSF on human bone slices. The cells have been removed to reveal evidence of lacunar bone resorption.

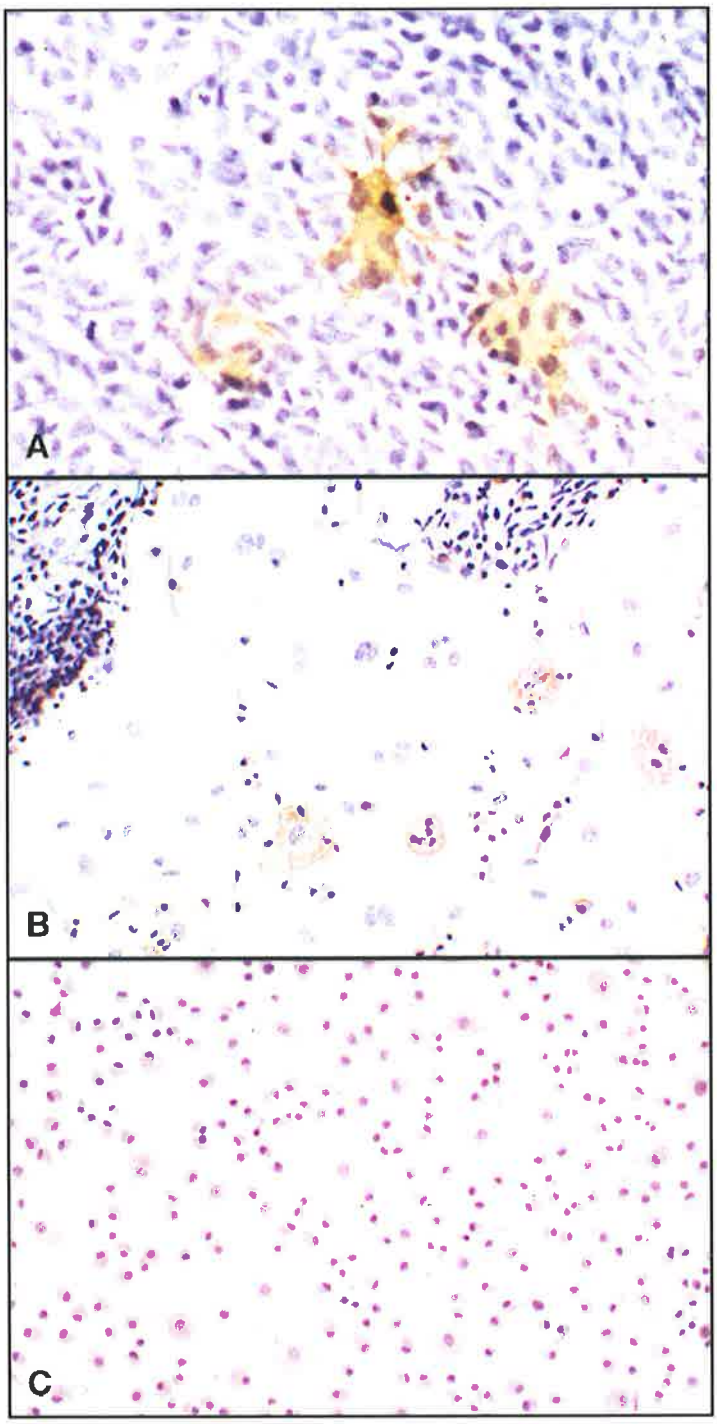

Figure 19. Human monocyte-UMR 106 cell cocultures after 14 days incubation. (A) Cocultures incubated in the presence of $1,25(OH)_2D_3$, dexamethasone and M-CSF showing large VNR positive cells (x200). (B) Numerous VNR negative multinucleated cells were visible through gaps in the covering UMR 106 layer when dexamethasone was omitted from the cocultures. A few strongly VNR positive cells were also present (x200). (C) Cultures incubated in the absence of UMR 106 cells showing small and large VNR negative mononuclear cells (x200). Counterstained with haematoxylin.

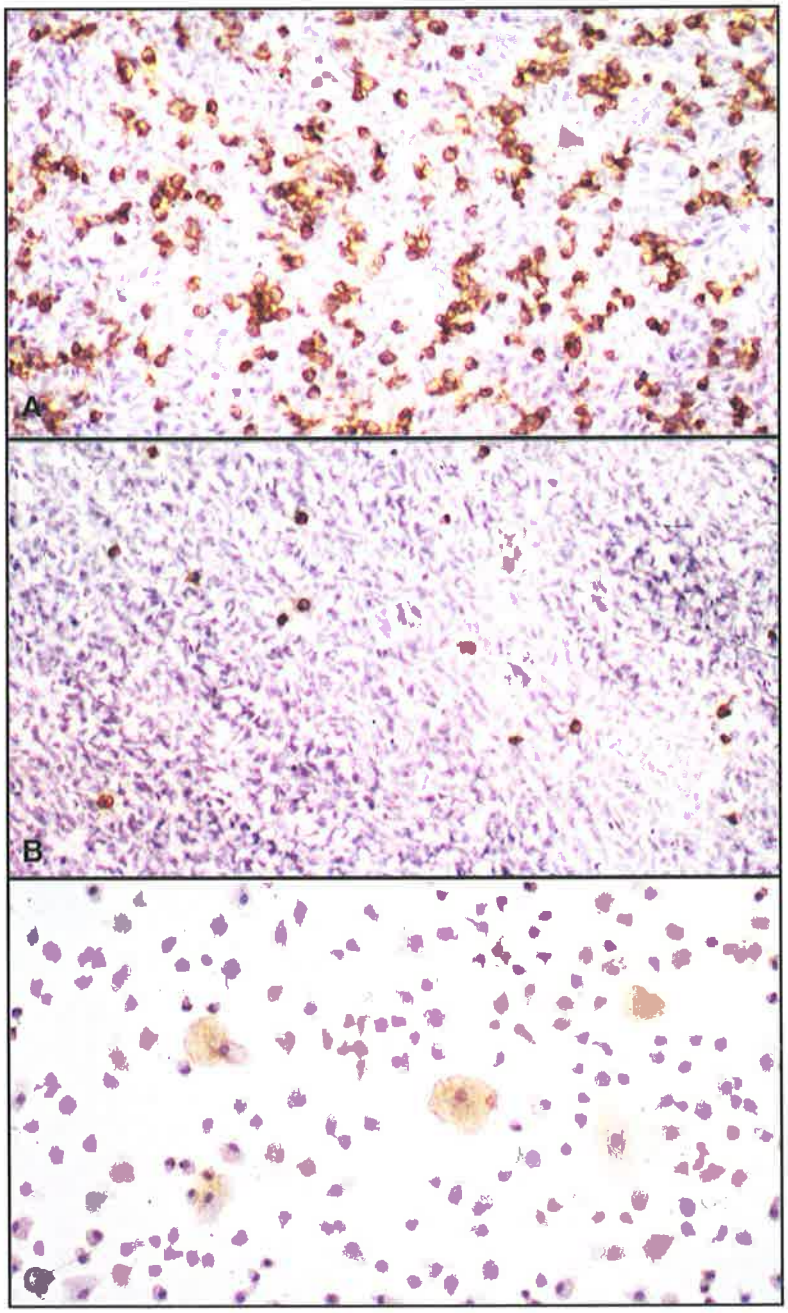

Figure 19a. Human monocyte-UMR 106 cell cocultures after 14 days incubation. (A) Cocultures incubated in the presence of $1,25(OH)_2D_3$, dexamethasone and M-CSF showing numerous CD14 positive mononuclear cells (x200). (B) Few CD14 positive cells were present when M-CSF was omitted from the cocultures (x200). (C) Cultures incubated in the absence of UMR 106 cells showing small and large CD14 positive mononuclear cells (x200). Counterstained with haematoxylin.

showed strong staining for the osteoclast markers, TRAP activity and VNR expression (Figure 19). In the 21 day cocultures, the omission of dexamethasone greatly reduced but did not completely abolish the formation of resorption pits (Table 6).

Table 6. Cellular and humoral requirements for osteoclast differentiation from human monocytes.

Treatments				Results	
UMR cells	$1,25(OH)_2D_3$	Dex	M-CSF	Bone resorption	VNR activity
+	+	+	+	246.8 ± 44.3	++
+	+	-	+	25.9 ± 6.8	+
+	-	+	+	0 ± 0	0
+	+	+	-	0 ± 0	0
-	+	+	+	0 ± 0	0

Bone resorption results are expressed as the mean number of resorption pits per bone slice ± SEM (n=9). Each treatment was studied in triplicate for each donor.

0 = no VNR positive cells present

+ = approx. 10 large VNR positive cells per coverslip

++ = >50 large VNR positive cells per coverslip

TRAP and VNR positive cells and lacunar bone resorption were not seen when human monocytes were cultured in the absence of $1,25(OH)_2D_3$ (only UMR 106 cells, dexamethasone and M-CSF added) or in the absence of M-CSF (only UMR 106 cells, $1,25(OH)_2D_3$ and dexamethasone added). When UMR 106 cells were omitted from the cultures, large mononuclear and multinucleated cells which were faintly TRAP and VNR positive were seen after 14 days culture although no resorption pits formed on any of the bone slices. These cells were morphologically similar to those formed in cocultures when dexamethasone was omitted with abundant foamy cytoplasm (Figure 19).

Numerous CD11b and CD14 positive mononuclear cells were seen in all the 14 day cultures which had exogenous M-CSF added with the numbers of positively stained cells increased compared to the 24 hour cultures. The cells appeared to have acquired a macrophage-like morphology with plump cytoplasm. They also showed more membrane than cytoplasmic staining. In the 14 day cultures, the numbers of CD11b and CD14 positive cells were greater in the cocultures with UMR 106 cells present than in cultures of human monocytes alone with the exception of those cocultures which did not have M-CSF added (Figure 19a). In the absence of M-CSF (only UMR 106 cells, $1,25(OH)_2D_3$ and dexamethasone added), very few CD11b and CD14 positive mononuclear cells were seen in the 14 day cocultures.

5.3.2. Discussion

These results confirm the work of Fujikawa et al (1996a) and show that osteoclast precursors are present in the monocyte fraction of human peripheral blood and that these precursors express a monocyte/macrophage phenotype. These precursors were found to be entirely negative for the osteoclast associated markers, TRAP and VNR expression and lacunar bone resorption. However, long term *in vitro* culture involving contact with UMR 106 cells, and the presence of $1,25(OH)_2D_3$ and exogenous M-CSF resulted in osteoclast formation and extensive lacunar bone resorption.

The addition of dexamethasone was not found to be an essential requirement for osteoclast differentiation, although the presence of dexamethasone substantially enhanced the number of resorption pits formed. This increase in bone resorption correlated well with the numbers of strongly staining TRAP and VNR positive osteoclast-like cells which formed. In the absence of dexamethasone, only a few osteoclast-like cells strongly positive for TRAP and VNR expression formed after long term culture reflecting the reduction in bone resorption seen. The formation of large numbers of macrophage and macrophage polykaryons in the cultures when dexamethasone was omitted suggests that dexamethasone may play a role in determining the differentiation pathway taken. Macrophages, macrophage polykaryons and osteoclasts all form from a common precursor although the hormonal influences which determine the pathway chosen are as yet unknown. Dexamethasone has been shown to promote osteogenic differentiation in undifferentiated bone cell populations, and is an essential requirement for *in vitro* matrix nodule formation and mineralisation by human marrow-derived stromal cells (Beresford et al 1994; Cheng et al 1994). The results of this study suggest that dexamethasone may also promote osteoclast differentiation. This finding may have important implications for the pathophysiology of osteoporosis. The therapeutic use of glucocorticoids followed by the development of osteoporosis, and an association between glucocorticoid excess and osteoporosis in Cushing's syndrome have been well documented (Reid 1989). Although it has been postulated that the anti-anabolic actions of glucocorticoids contribute to osteoporosis, the effects of dexamethasone shown in this study suggest that it may also selectively stimulate osteoclast differentiation from a relatively undifferentiated mononuclear phagocyte cell population. This may also explain the lack of effect of dexamethasone in the cocultures of periprosthetic macrophages and UMR 106 cells. Cells isolated from arthroplasty periprosthetic tissue with a macrophage morphology were found to be weakly positive for TRAP and VNR expression which suggests that these cells may be already be taking the osteoclastic differentiation pathway.

It is not surprising that faint TRAP and VNR staining was found on the macrophage and macrophage polykaryon-like cells after long term culture. Both monocytes and tissue macrophages are known to become weakly positive for

TRAP after several days in culture (Hattersley and Chambers 1989b; Modderman et al 1991), and expression of receptors for vitronectin is likely to be necessary for cellular adhesion, particularly cell-glass interactions. The faint expression of these markers could easily be distinguished from the strong membrane and cytoplasmic staining of osteoclast-like cells, however.

Both $1,25(OH)_2D_3$ and exogenous M-CSF were found to be essential requirements for *in vitro* human monocyte-osteoclast differentiation and bone resorption. $1,25(OH)_2D_3$ is known to promote monocyte-macrophage proliferation, maturation and also polykaryon formation (Abe et al 1983), hence the presence of $1,25(OH)_2D_3$ may explain why macrophage polykaryons formed after long term culture in the absence of UMR 106 cells. The effect of M-CSF was also significant. Substantially fewer CD11b and CD14 cells were present in the 14 day cocultures when M-CSF was omitted. Whilst M-CSF has been shown to be essential for osteoclast formation, its mechanism of action is still unclear. M-CSF is a potent stimulator of monocyte/macrophage proliferation and differentiation, and promotes osteoclast survival (Kodama et al 1991; Takahashi et al 1991; Tanaka et al 1993; Fuller et al 1993; Jimi et al 1995). Receptors for M-CSF have been found on both mature osteoclasts and osteoclast precursors (Kodama et al 1991; Weir et al 1993; Yang et al 1996). It is known that approximately 5% of monocytes/macrophages are capable of proliferation (van Furth 1989). These cells are morphologically indistinguishable from mature monocytes and macrophages and it has been proposed that it is this fraction of relatively immature monocytes which may be capable of undergoing osteoclast differentiation (Athanasou 1996). The findings from this current study suggest that M-CSF is acting directly on these mononuclear osteoclast precursors in the cocultures to stimulate proliferation and/or promote the survival of these cells during long term culture.

In summary, the results of this study have shown that a sub-population of circulating human peripheral blood monocytes exhibiting the phenotypic features of mononuclear phagocytes is capable of differentiating into osteoclastic bone resorbing cells. Contact with bone stromal cells, in this case UMR 106 cells, and the presence of $1,25(OH)_2D_3$ and exogenous M-CSF were all essential

requirements for osteoclast differentiation and bone resorption. The presence of dexamethasone, whilst not essential, greatly enhanced the amount of lacunar bone resorption seen.

5.4. The effect of particle phagocytosis on osteoclast differentiation and bone resorption

5.4.1. Introduction

The second aim of this study was to determine whether particle phagocytosis affects osteoclast differentiation and bone resorption. To determine the effect of particle phagocytosis on osteoclast formation and bone resorption, latex beads, 1μm in diameter, were added to human monocytes and the cells were cultured under conditions which result in the formation of osteoclasts and bone resorption. Latex particles consist of spherical polystyrene beads which are considered to be inert, non-toxic and non-inflammatory to macrophages unless used in very high concentrations (Murray and Rushton 1990). The beads are readily phagocytosed and can easily be visualised within the cells after Giemsa staining using light microscopy.

5.4.2. Methods

Human monocytes were isolated from the peripheral blood of two healthy donors, as described above. The isolated adherent cells were cocultured with UMR 106 cells on coverslips and human bone slices in the presence of 10^{-7}M1,25(OH)$_2$D$_3$, 10^{-8}M dexamethasone and 25ng/ml M-CSF. A single dose of 1.0μm latex beads (Cat. no. LB-11, Sigma, UK) at a concentration of $1x10^7$ particles per ml was added to the cocultures at the beginning of the incubation period. Cocultures with no latex particles added were included as positive controls. The coverslips were removed after 24 hours and 14 days incubation and characterised histochemically for the expression of TRAP, and immunohistochemically for VNR expression and expression of the macrophage-associated antigens, CD11b and CD14. The cortical bone slices were removed after 24 hours and 21 days incubation and the number of resorption pits per bone slice was counted. Each treatment was studied in triplicate for each blood donor.

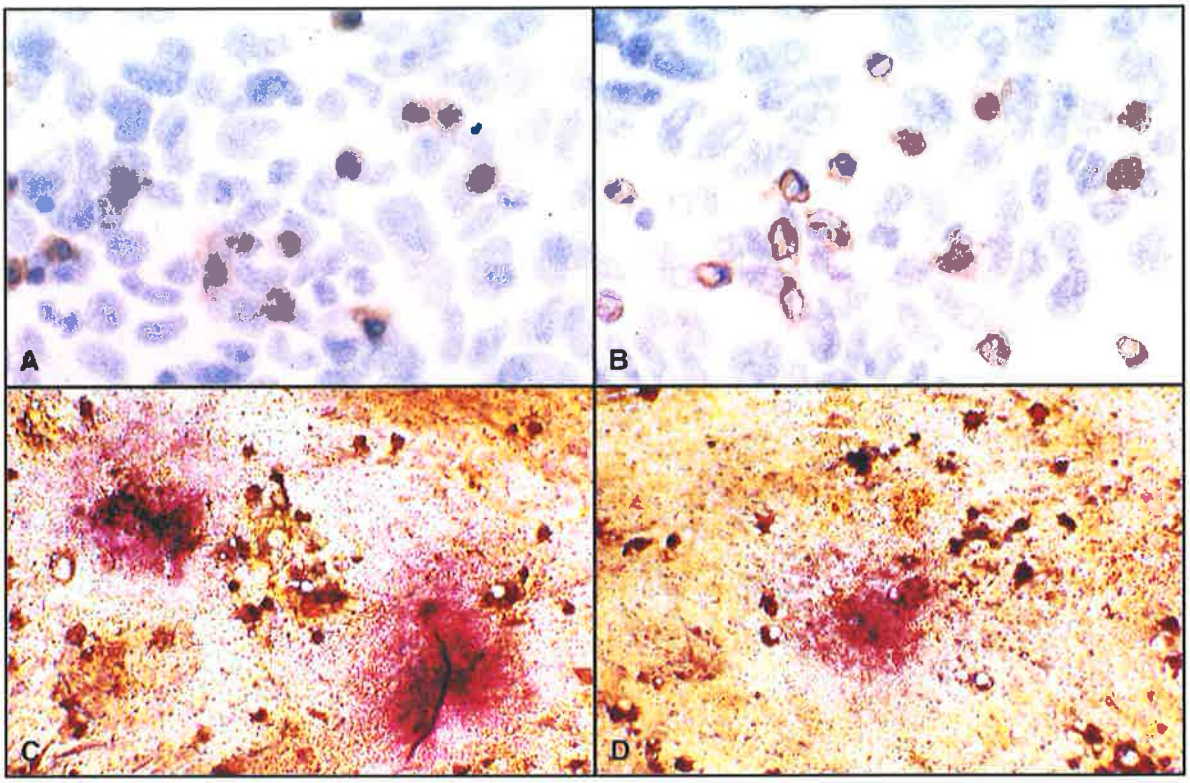

Figure 20. 24 hour and 14 day cocultures of human monocyte and UMR 106 cell incubated in the presence of $1,25(OH)_2D_3$, dexamethasone and M-CSF showing (A) CD14 positive mononuclear cells in control 24 hour cocultures (x400); (B) CD14 positive mononuclear cells containing latex particles after 24 hours coculture (x400); (C) large TRAP positive cells in control 14 day cocultures (x100); and (D) a large TRAP positive cell in 14 day cocultures after exposure to latex beads (x100).

The 24 hour cocultures were examined closely under light microscopy to determine whether all the cells stained positively for the macrophage-associated antigens, CD11b and CD14, had phagocytosed latex particles or whether there was a sub-population which had not. Similarly, the 14 day cocultures were examined to determine whether the large VNR and TRAP positive cells present contained visible latex particles.

5.4.3. Results

Giemsa staining of the 24 hour cocultures showed that the latex beads were readily phagocytosed and that all the cells appeared full with an excess of particles present. The latex particle-containing cells were shown to express the macrophage markers, CD11b and CD14, and were TRAP and VNR negative (Figure 20). No evidence of lacunar bone resorption was seen after 24 hours coculture.

After 14 days incubation, numerous large TRAP and VNR positive cells formed in the presence of UMR 106 cells, $1,25(OH)_2D_3$, dexamethasone and M-CSF. After 21 days incubation, lacunar bone resorption was seen on all the cortical bone slices. The addition of 1.0μm latex beads did not significantly inhibit osteoclast formation as determined by TRAP and VNR expression (Figure 20), or the number of resorption pits formed. The mean number of resorption pits per bone slice was 195.7 ± 26.1 for the untreated positive control compared with 205.6 ± 24.7 for the cocultures which had latex beads added (mean±SEM; n=6).

5.4.4. Discussion

The results of this study show that particle phagocytosis by human monocytes does not abrogate the ability of these cells to undergo osteoclast differentiation. It has been shown previously that latex particle phagocytosis by macrophages stimulates PGE_2 release and bone resorption (Murray and Rushton 1990). However, prosthesis wear particles, including PMMA and polyethylene particles, were shown to stimulate a significantly greater increase in PGE_2 release and bone resorption than latex particles. Although phagocytosis of latex beads did not increase human monocyte-osteoclast differentiation in this current study,

phagocytosis of prosthesis wear particles which are known to be more stimulatory to macrophages, may increase osteoclast formation.

5.5. The effect of prosthesis wear particles on human monocyte-osteoclast differentiation and bone resorption

5.5.1. Introduction

The hypothesis for this study was that prosthesis wear particles stimulate the differentiation of human peripheral blood monocytes into osteoclastic cells, and increase resorption of cortical bone *in vitro*. Direct effects of prosthesis wear particles on osteoclast formation and resultant bone resorption, and indirect effects of wear particle-stimulated human monocyte conditioned media on osteoclast formation and bone resorption were determined. This effect should be distinguished from that of the release of factors which stimulate mature osteoclastic activity by macrophages which have phagocytosed wear particles.

A number of particle-associated factors are believed to determine the intensity of the macrophage response in the arthroplasty pseudomembrane and the extent of periprosthetic osteolysis (Willert and Semlitsch 1977; Mirra et al 1982; Howie 1990). These include the chemical composition of the implant component, the size, shape and surface area of the wear particles as well as the number of particles present. The implant materials most commonly used in hip and knee replacements are metals such as titanium alloy (TiAlV), commercially pure titanium (cpTi), cobalt-chrome alloy (CoCr), and stainless steel (316L SS), or ceramics either articulating against themselves or a polymer, ultra high molecular weight polyethylene (UHMWPE). Although a wide range of particle sizes have been found in tissues adjacent to loose implants, the majority of particles are less than one micron in size (Howie 1990; Kossovsky et al 1992; Margevicius et al 1994; Shanbhag et al 1994). Phagocytosis of these smaller particles which have a large surface area and are susceptible to intracellular corrosion has been associated with macrophage activation and the release of factors that promote inflammation and bone resorption (Haynes et al 1996).

There are problems in interpreting the results of different studies because of differences in materials and methods of preparation of the materials used for testing. Earlier work in this laboratory led to the development of a reproducible method for the production and characterisation of metal particles of known size, shape and concentration (Rogers et al 1993). Particular attention was given to producing metal particles that closely resembled particles found around human prostheses. Particles that were 0.5-3.0μm in diameter were isolated by differential sedimentation, and the distribution of particle sizes determined with the use of a Coulter Multisizer. Chemical composition was assessed by atomic absorption spectrophotometry, and electron microscopy was used to characterise particle shape. The particles generated were found to be irregular in shape, and in the size range of 0.5-3.0μm diameter. TiAlV, cpTi, CoCr and 316L SS particles were produced and all were found to have the same chemical composition as their respective original alloy. These particles were used in the following experiments at clinically relevant concentrations. Metal levels in tissue retrieved during revision surgery for aseptic loosening have been measured with an average of 818 μg per gram of tissue (Agins et al 1988; Lombardi et al 1989; Huo et al 1992). Assuming a particle size of one micron, this corresponds to an average $4x10^8$ particles per gram of tissue of TiAlV.

5.5.2. Methods

Human peripheral blood monocytes were isolated from healthy volunteer donors by Ficoll-Paque sedimentation and adherence, as described previously. The isolated adherent cells were cocultured with UMR 106 cells on coverslips and human bone slices in the presence of 10^{-7}M $1,25(OH)_2D_3$, 10^{-8}M dexamethasone and 25ng/ml M-CSF. The effects of the following prosthesis wear particles, CoCr, 316L SS, TiAlV and cpTi particles were determined. Four concentrations of the particles, $4x10^7$, $1x10^7$, $2.5x10^6$ and $6.25x10^5$ particles per ml, were added to the cocultures as a single dose at the beginning of the incubation period. Cocultures with no wear particles added were included as positive controls.

The coverslips were removed after 14 days incubation and characterised immunohistochemically for VNR and either CD11b or CD14 expression. For comparison of cell numbers, two additional coverslips were included consisting

of cocultures without wear particles added. These were removed after 24 hours incubation. These coverslips were assessed for the numbers of CD11b and CD14 positive monocytes present at the beginning of the incubation period. The cortical bone slices were removed after 21 days incubation and the number of resorption pits per bone slice was counted. Each treatment was studied in triplicate for each blood donor.

To measure cytotoxicity, supernatants were collected during the first media change, centrifuged to remove wear particles and cell debris, and stored at -20°C until ready for testing. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH), a marker of cell damage, was measured using a commercially available Cytotox 96 kit (Cat no. G1780, Promega, Madison, WI). Each supernatant was tested in duplicate.

5.5.3. Results

All four types of wear particles were readily phagocytosed by the monocytes (Figure 21). After four days coculture, cell toxicity after exposure to the different particle preparations was found. Both CoCr and 316L SS particles at concentrations of $4x10^7$ and $1x10^7$ particles per ml caused an increase in LDH release compared with the unexposed control (Figure 22). This result was not unexpected as the toxic effects of CoCr and 316L SS particles of a phagocytosable size both *in vivo* and *in vitro* are well known (Haynes et al 1993; Howie and Vernon-Roberts 1988; Rae 1981). However, TiAlV and cpTi were also shown to cause cell toxicity after four days coculture although to a lesser degree and only at the highest concentration tested ($4x10^7$ particles per ml).

A reduction in the numbers of CD11b or CD14 positive cells present in the 14 day cocultures after exposure to prosthesis wear particles was also observed (Figure 23). This reduction in macrophage cell numbers was dose dependent. No positively staining cells were seen after exposure to $4x10^7$ particles per ml of CoCr and 316L SS particles. Very few CD11b or CD14 positive cells were seen after exposure to $1x10^7$ particles per ml of CoCr and 316L SS particles. Larger numbers of positive cells were present after exposure to $2.5x10^6$ particles per ml of CoCr and 316L SS particles and although the numbers of positive cells were substantially

greater after exposure to $6.25x10^5$ particles per ml of CoCr and 316L SS particles, the macrophage cell numbers were not as great as the control cocultures. Numbers of CD11b or CD14 positive cells, whilst present, were also reduced after exposure to concentrations of $4x10^7$ particles per ml of TiAlV and cpTi particles. Substantially greater numbers of positive cells were seen after exposure to $1x10^7$ and $2.5x10^6$ particles per ml. Concentrations of $6.25x10^5$ particles per ml of TiAlV and cpTi particles had no obvious effect on the numbers of positively stained cells present compared to the control cocultures.

Exposure to all four wear particle preparations caused a dose dependent reduction in the numbers of VNR positive cells present in the 14 day cocultures (Figure 24), and a dose dependent reduction in the bone resorption produced by these cells (Figure 25). At a concentration of $4x10^7$ particles per ml, both CoCr and 316L SS particles totally abolished osteoclast formation and resultant resorption pit formation. Very few VNR positive cells were seen in the 14 day cocultures after exposure to $1x10^7$ particles per ml of CoCr particles compared to the control cocultures. The number of resorption pits formed was significantly reduced after exposure to $1x10^7$ particles per ml of CoCr particles ($p=0.007$). Similarly, very few VNR positive cells were seen on the 14 day coverslips after exposure to the same concentration of 316L SS particles, and lacunar bone resorption was significantly reduced ($p=0.0001$). The number of VNR positive cells present and the number of resorption pits formed after exposure to the lower concentrations of CoCr and 316L SS particles (i.e. $2.5x10^6$ and $6.25x10^5$ particles per ml) were increased compared to the higher concentrations. The numbers of VNR positive cells and resultant bone resorption, however, was not as great as the control cocultures. At a concentration of $4x10^7$ particles per ml, TiAlV particles substantially reduced but did not abolish osteoclast formation, and significantly reduced resorption pit formation ($p=0.0001$). Exposure to $4x10^7$ particles per ml of cpTi particles also reduced the numbers of VNR positive cells present and significantly reduced the number of resorption pits formed ($p=0.008$) but this reduction was not as great as that caused by CoCr, 316L SS and TiAlV particles. Exposure to $1x10^7$ particles per ml of TiAlV and cpTi particles resulted in approximately half the number of VNR positive cells compared to the unexposed controls. Resorption pit formation was also reduced. Substantially greater numbers of VNR positive cells

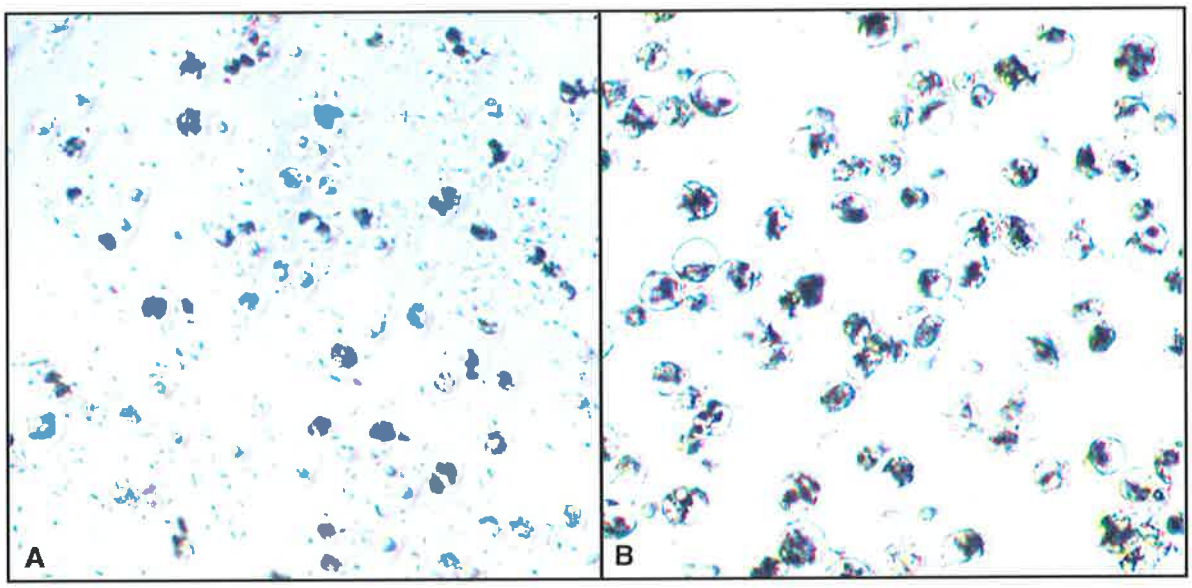

Figure 21. Photomicrographs of human monocytes after 3 days culture in the presence of $1,25(OH)_2D_3$, dexamethasone and M-CSF. (A) Human monocytes cultured with $1x10^7$ particles/ml of 316L SS particles (x200); (B) Human monocytes cultured with $1x10^7$ particles/ml of cpTi particles (x200). Unstained.

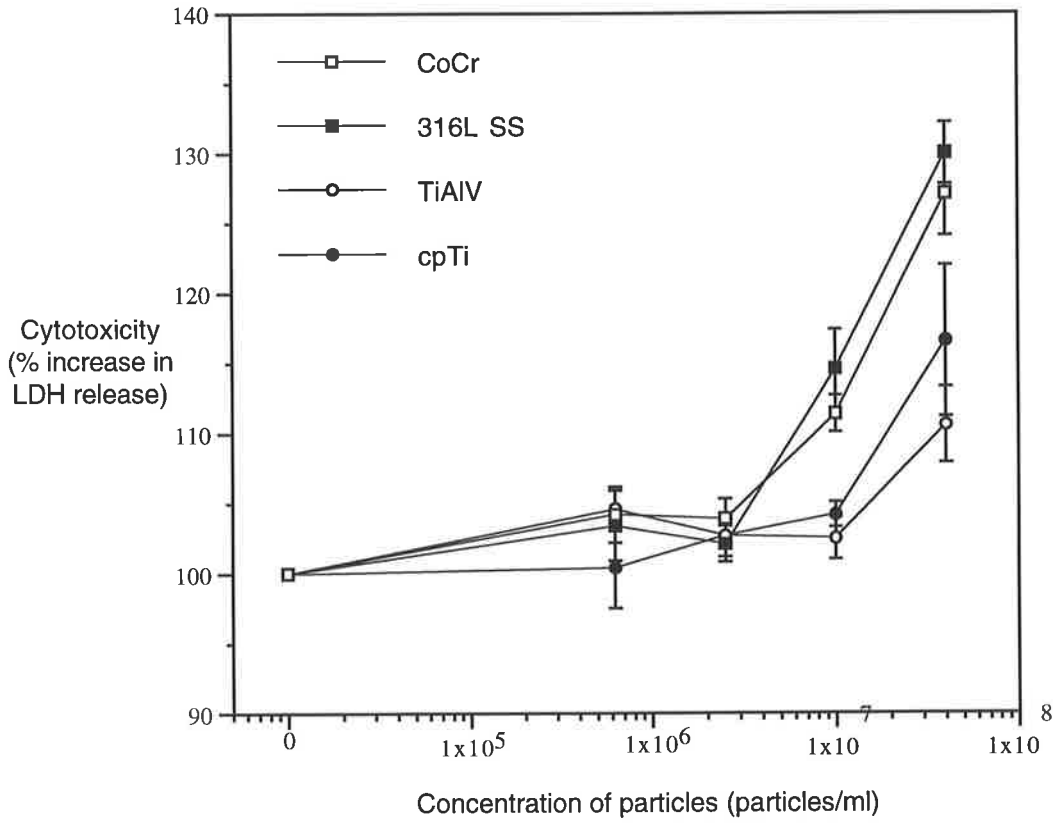

Figure 22. The effect of metal prosthesis wear particles on cell toxicity in human monocyte-UMR 106 cell cocultures after four days incubation.

The results are expressed as the mean percentage increase in LDH release compared to the control (no particles) ± SEM (n=6). Each supernatant was tested in duplicate.

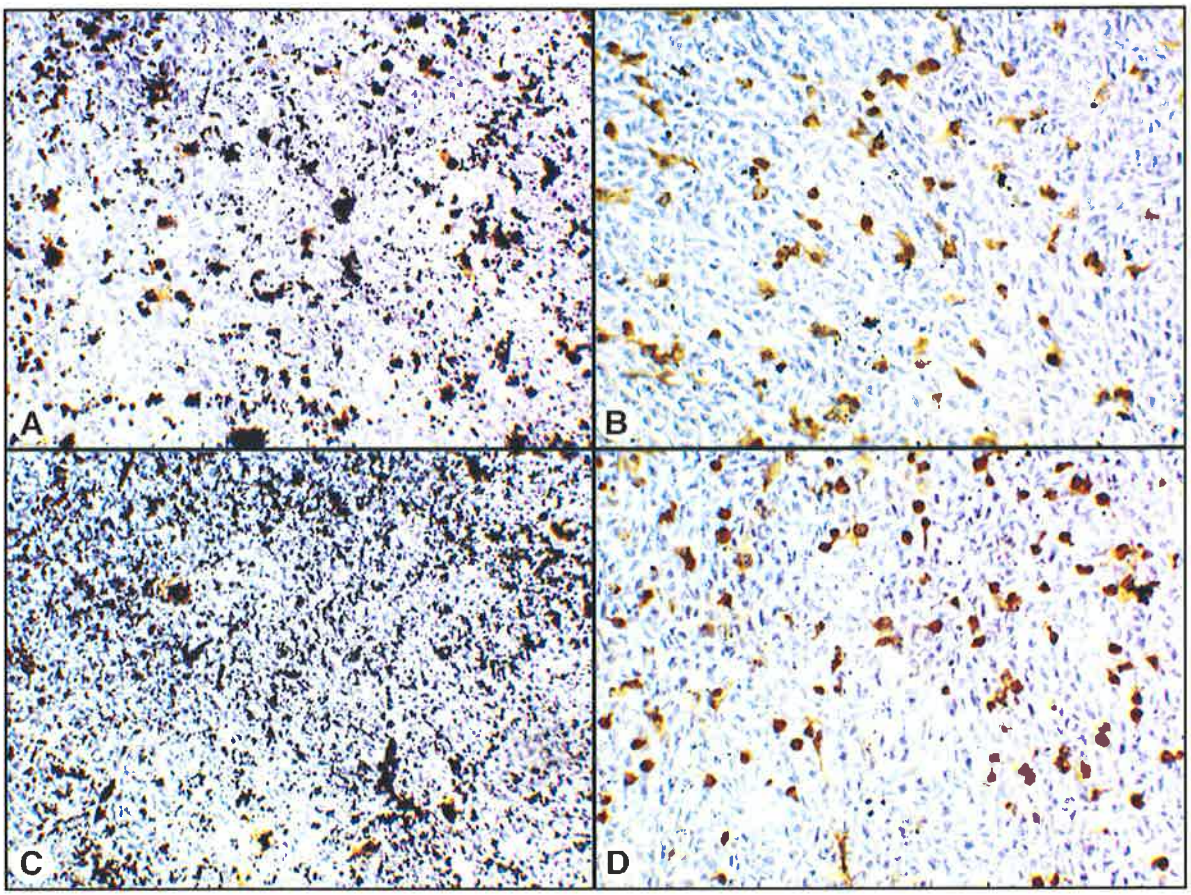

Figure 23. 14 day cocultures of human monocytes and UMR 106 cells incubated in the presence of 1,25$(OH)_2D_3$, dexamethasone and M-CSF showing (A) CD14 expression in cocultures exposed to $4x10^7$ particles/ml of TiAlV particles (x200); (B) CD14 expression in cocultures exposed to $2.5x10^6$ particles/ml of TiAlV particles (x200); (C) CD14 expression in cocultures exposed to $4x10^7$ particles/ml of CoCr particles (x200); (B) CD14 expression in cocultures exposed to $2.5x10^6$ particles/ml of CoCr particles (x200). Counterstained with haematoxylin.

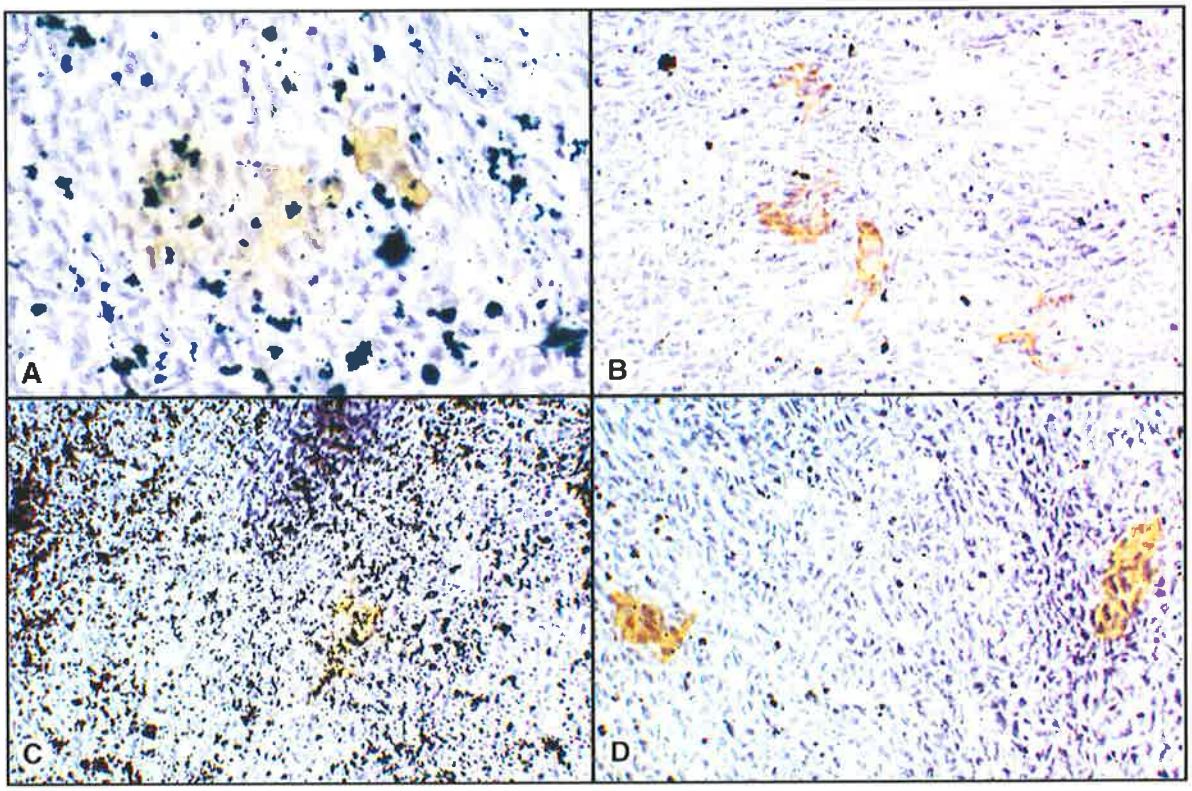

Figure 24. 14 day cocultures of human monocytes and UMR 106 cells incubated in the presence of $1,25(OH)_2D_3$, dexamethasone and M-CSF showing (A) VNR expression in cocultures exposed to $4x10^7$ particles/ml of TiAlV particles (x200); (B) VNR expression in cocultures exposed to $2.5x10^6$ particles/ml of TiAlV particles (x100); (C) VNR expression in cocultures exposed to $4x10^7$ particles/ml of CoCr particles (x100); (B) VNR expression in cocultures exposed to $2.5x10^6$ particles/ml of CoCr particles (x100). Counterstained with haematoxylin.

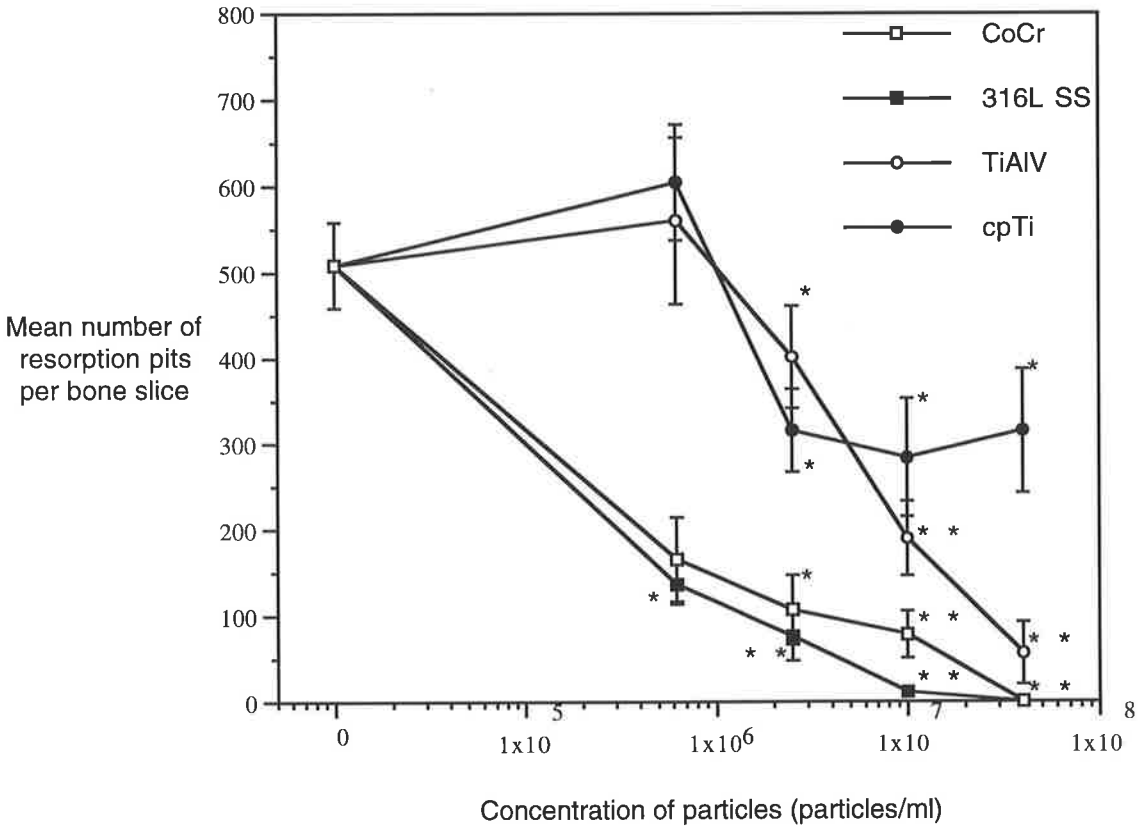

Figure 25. The effect of metal prosthesis wear particles on lacunar bone resorption in human monocyte-UMR 106 cell cocultures after 21 days incubation.

The results are expressed as the mean number of resorption pits per bone slice ± SEM (n=9). Statistical analyses were performed using a two factor with replication ANOVA test.
**p<0.005, *p<0.05 compared to control (no particles)

were seen after exposure to $2.5x10^6$ particles per ml. Exposure to concentrations of $6.25x10^5$ particles per ml of TiAlV and cpTi particles did not have an obvious effect on the numbers of VNR positively stained cells present compared to the control cocultures. Extensive resorption pit formation was seen after exposure to the lower concentrations of TiAlV and cpTi particles and no significant differences between these cocultures and the unexposed controls were found ($p=0.292$ and $p=0.151$ respectively).

5.5.4. Discussion

The results of this study have not supported the primary hypothesis that prosthesis wear particles stimulate the differentiation of human peripheral blood monocytes into osteoclastic cells, and increase resorption of cortical bone *in vitro*. Rather prosthesis wear particles were shown to directly inhibit osteoclast formation resulting in a decrease in lacunar bone resorption.

The fact that no VNR positive osteoclastic cells formed in culture after exposure to high concentrations of CoCr and 316L SS particles is most likely due to the toxic nature of these particles. Cell toxicity after exposure to CoCr and 316L SS particles of a phagocytosable size has previously been shown both *in vitro* and *in vivo* (Haynes et al 1993; Howie and Vernon-Roberts 1988; Rae 1981). In the current study, high concentrations of these particles were shown to be toxic to the cells after four days coculture, and very few macrophages were present after 14 days coculture.

Previous studies have shown that TiAlV and cpTi particles are relatively well tolerated by monocytes/macrophages after short term culture *in vitro* (Haynes et al 1993). However, longer culture periods of four days in this study showed that exposure to $4x10^7$ particles per ml of TiAlV and cpTi particles caused cell toxicity although the levels of LDH release were not as great as those measured after exposure to CoCr and 316L SS particles. Lower concentrations of TiAlV and cpTi particles were not toxic to the cells. A reduction in the numbers of macrophages present and the number of VNR positive cells formed in the 14 day cocultures after exposure to high concentrations of TiAlV and cpTi particles was also seen. After exposure to lower non-toxic concentrations, however, osteoclast formation

and bone resorption was reduced despite the presence of large numbers of CD11b or CD14 positive cells in the 14 day cocultures. As well as long term cell damage, reduced adhesion, impaired motility or an inability of the monocytes to proliferate after exposure to these wear particles may result in the reduction in osteoclast formation seen.

Previous studies examining the effect of wear particles on mouse monocyte-osteoclast differentiation have shown an increase in osteoclast formation and bone resorption after exposure to polymethylmethacrylate (PMMA) wear particles (Sabokbar et al 1997). Differences in the dose, size and type of particles used may account for the different results. The results of this current study have shown that the chemical composition of the particles is particularly important. Latex beads had no effect on osteoclast formation and bone resorption whereas metallic wear particles of a similar concentration and size caused a reduction in osteoclast formation and bone resorption. Differences were seen between the different metal particles which was partly dependent on the toxicity of each particle type. Larger non-phagocytosable particles which are not as susceptible to corrosion, particularly intracellular corrosion (Haynes et al 1996), may induce a different cellular response. In addition, polyethylene particles which are relatively non-toxic but highly inflammatory *in vivo* may stimulate an increase in osteoclast formation and bone resorption *in vitro*.

5.6. Indirect effects of prosthesis wear particles on human monocyte-osteoclast differentiation and bone resorption

5.6.1. Introduction

The results of the previous study have shown that metal prosthesis wear particles can directly inhibit osteoclast formation and thereby lacunar bone resorption. In the final part of this study, the effect of wear particle-stimulated human monocyte conditioned media on osteoclast formation was determined. Wear particles have been shown to induce mediator release from monocytes/macrophages both *in vitro* and *in vivo*. M-CSF has been shown to be released by activated macrophages (Rambaldi et al 1987; Oster et al 1987; Kaneki et al 1994). Therefore, it was hypothesised that prosthesis wear particles may

increase osteoclast formation and bone resorption indirectly via the release of mediators that promote monocyte-osteoclast differentiation.

TiAlV at a concentration of $4x10^7$ particles per ml was chosen as the wear particle and optimal concentration to determine any indirect particle effects. Previous *in vitro* studies have shown a large increase in bone resorbing mediator release by human monocytes and rodent macrophages after exposure to TiAlV particles at this concentration (Haynes et al 1993; Rogers et al 1997). The levels of IL-1, TNF, IL-6 and PGE_2 released from human monocytes after exposure to TiAlV particles were shown to be significantly greater than the levels released from human monocytes after exposure to the same concentration of cpTi particles (Rogers et al 1997). The ability of TiAlV particle-stimulated monocytes to release M-CSF and thereby stimulate osteoclast formation was determined by incubating the cocultures in the absence of M-CSF.

5.6.2. Methods

Wear particle-stimulated human monocyte conditioned media was prepared 48 hours prior to the start of the experiment as follows. Human peripheral blood monocytes were isolated from a healthy volunteer donor by Ficoll-Paque sedimentation. A suspension of $4x10^6$ cells per ml was pipetted into wells of a 24 well plate and the monocytes allowed to adhere at 37°C in an humidified atmosphere. After one hour, the non-adherent cells were removed by gentle washing and a concentration of $4x10^7$ particles per ml of TiAlV particles in αMEM+10%FCS was added to half of the wells. The remaining wells had no particles added. The cells were cultured for 48 hours at 37°C in 5% CO_2. After 48 hours, the supernatants were removed, centrifuged, and the supernatants filtered through 0.22μm filters (Cat. no. SLGP R25 LS, Millipore UK Ltd, UK) to remove cell debris and particles. The resultant supernatants were aliquoted and stored at -20°C until use.

To determine the effects of the conditioned media on osteoclast formation, human peripheral blood monocytes were isolated from a healthy volunteer donor by Ficoll-Paque sedimentation and adherence, as described previously. The isolated adherent cells were cocultured with UMR 106 cells on coverslips and

human bone slices in the presence of 10^{-7}M1,25$(OH)_2D_3$, and 10^{-8}M dexamethasone. Half of the wells had 25ng/ml M-CSF added. Conditioned media from TiAlV-stimulated monocytes (CM TiAlV) and conditioned media from non-stimulated cells (CM 0) were added to the cocultures, in the presence and absence of M-CSF, at a 1 in 5 dilution from the beginning of the incubation period and every subsequent media change until Day 14. Cocultures, in the presence and absence of M-CSF, with no conditioned media added were included as positive and negative controls respectively.

The coverslips were removed after 14 days incubation and characterised immunohistochemically for VNR and CD14 expression. For comparison of cell numbers, two additional coverslips were included consisting of cocultures without conditioned media added. These were removed after 24 hours incubation. These coverslips were assessed for the numbers of CD14 positive monocytes present at the beginning of the incubation period. The cortical bone slices were removed after 21 days incubation and the number of resorption pits per bone slice was counted. Each treatment was studied in triplicate.

To determine if particle-stimulated conditioned media stimulates M-CSF, supernatants from cocultures incubated in the absence of M-CSF were collected at the first media change (Day 4), centrifuged to remove cell debris, and stored at -20°C. Levels of M-CSF were measured using a commercially available ELISA kit (R&D Systems, Abingdon, UK).

5.6.3. Results

Numerous CD14 positive mononuclear cells were seen in all the 14 day cultures that had exogenous M-CSF added with the numbers of cells present substantially increased compared to the 24 hour cultures. No difference in the number of CD14 positive cells present in the 14 day cocultures was found between cocultures that had M-CSF added and cocultures that had M-CSF and conditioned media added, either CM TiAlV or CM 0. Numerous VNR positive cells were also present in all the 14 day cocultures that had exogenous M-CSF added. The addition of conditioned media, either CM TiAlV or CM 0, did not appear to have an effect on the numbers of VNR cells present. However, the addition of TiAlV particle-

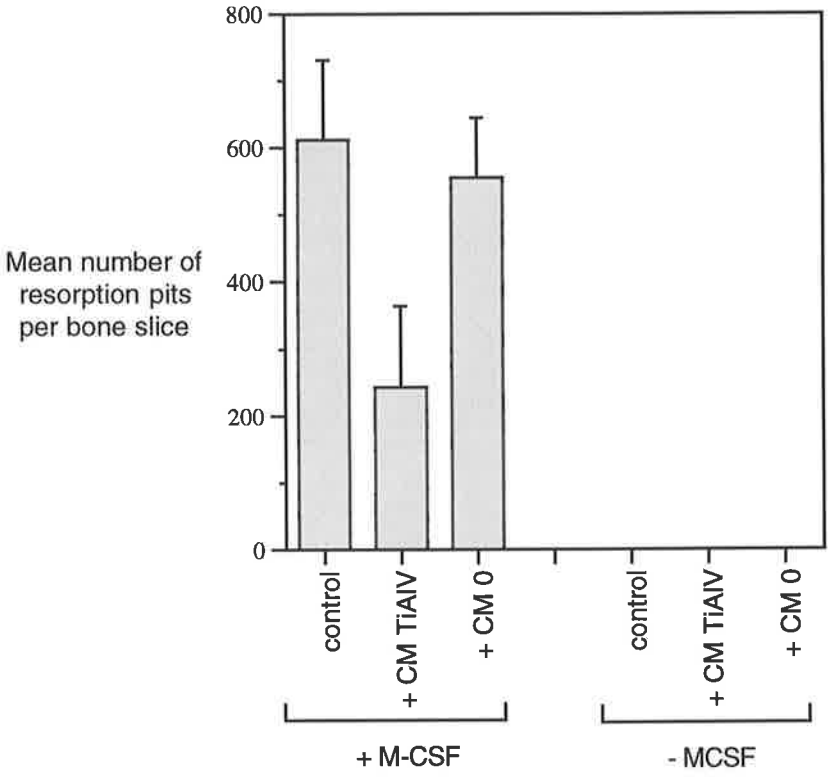

Figure 26. The effect of TiAlV particle stimulated human monocyte conditioned media in the presence and absence of M-CSF on lacunar bone resorption in human monocyte-UMR 106 cell cocultures after 21 days incubation.

The results are expressed as the mean number of resorption pits per bone slice ± SD (n=3).

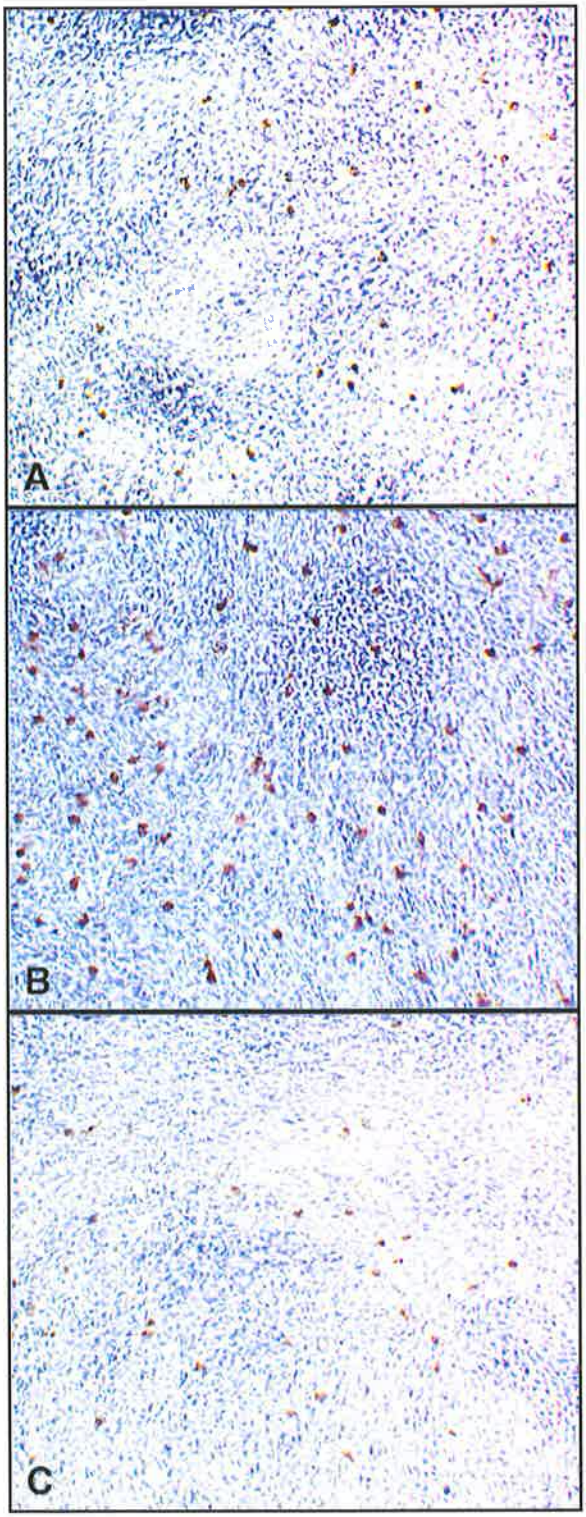

Figure 27. 14 day cocultures of human monocytes and UMR 106 cells incubated in the presence of $1,25(OH)_2D_3$, dexamethasone but in the absence of M-CSF. (A) CD14 expression in control cocultures (x100); (B) CD14 expression in cocultures incubated in the presence of TiAlV particle stimulated conditioned media (x100); and (C) CD14 expression in cocultures incubated in non stimulated conditioned media (x100). Counterstained with haematoxylin.

stimulated conditioned media did cause a reduction in the number of resorption pits formed (Figure 26). Bone resorption was reduced by 60.3% when TiAlV conditioned media was added. The addition of non-stimulated conditioned media had no effect on the number of resorption pits formed.

In the absence of M-CSF, very few CD14 positive mononuclear cells were seen in the control 14 day cocultures. However, the addition of TiAlV-stimulated conditioned media during the incubation period resulted in a substantial increase in the numbers of CD14 cells present (Figure 27). This increase in CD14 positive cells was not seen when non-stimulated conditioned media was added to the cocultures. Despite the presence of increased numbers of CD14 positive cells, no VNR positive cells and no bone resorption was seen when TiAlV-stimulated conditioned media was added to the cocultures in the absence of exogenous M-CSF (Figure 26). Similarly, no VNR positive cells and no bone resorption was seen when non-stimulated conditioned media was added in the absence of exogenous M-CSF.

Levels of M-CSF in the supernatants incubated in the absence of exogenous M-CSF were measured after four days coculture. The presence of M-CSF was not detected in the control cocultures but was detected in the four day supernatants from CM TiAlV treated and CM 0 treated cocultures. The levels of M-CSF were 66.5±7.8 and 11±15.6 pg/ml respectively (mean±SD, n=2).

5.6.4. Discussion

The results of this preliminary study have not supported the hypothesis that prosthesis wear particles increase osteoclast formation and bone resorption indirectly via the release of mediators that promote monocyte-osteoclast differentiation. In contrast, the addition of TiAlV-stimulated conditioned media, in the presence of M-CSF, caused a reduction in osteoclastic bone resorption which was not seen when non-stimulated conditioned media was added to the cocultures. TiAlV particles are known to induce the release of a number of potent bone resorbing mediators from human monocytes (Rogers et al 1997). One possible explanation is that PGE_2 or another mediator released by the activated

monocytes has an inhibitory effect on osteoclast formation and bone resorption in the human monocyte-UMR 106 cocultures.

In the absence of M-CSF, increased levels of soluble M-CSF were detected in the supernatants of four day cocultures incubated in the presence of TiAlV stimulated conditioned media. Increased numbers of CD14 positive macrophages were also seen after 14 days coculture in the presence of TiAlV stimulated conditioned media. This effect was not seen when non-stimulated conditioned media was added. These results suggest that TiAlV particles may stimulate human monocytes to release M-CSF thereby promoting proliferation and/or survival of osteoclast precursor cells. Despite this increase in macrophage numbers, however, TiAlV stimulated conditioned media in the absence of exogenous M-CSF did not induce osteoclast formation and bone resorption. These results are preliminary but suggest that whilst TiAlV particles may stimulate monocytes to release factors important for osteoclast formation such as M-CSF, other factors which inhibit osteoclast formation in this coculture system may also be released.

5.7. Summary

In summary, the results of these studies have shown that metal wear particles of phagocytosable sizes directly inhibit osteoclast formation from human monocytes resulting in a decrease in lacunar bone resorption *in vitro*. This inhibition was shown to be partly due to the toxic nature of these particles. TiAlV wear particles were also shown to stimulate M-CSF release from human monocytes and indirectly stimulated monocyte proliferation and long term survival. However, this increase in M-CSF release was not sufficient to stimulate osteoclast formation and bone resorption in long term human monocyte-UMR 106 cell cocultures.

6. The Ability of Human Bone-Derived Stromal Cells to Support Periprosthetic Macrophage-Osteoclast Differentiation and Bone Resorption *In Vitro*

6.1. Introduction

The previous studies in this thesis have shown that osteoblastic cells are an essential requirement for osteoclast differentiation from both periprosthetic macrophages and human peripheral blood monocytes. The use of a cell line for these *in vitro* studies has certain advantages in that it is a homogeneous population which offers consistency and ease of use. However, these cannot be described as normal cells in that they are immortalised cell lines which have been halted at a certain stage of differentiation. Thus, the effects of these stromal cells at different stages of their differentiation from early progenitor to fully differentiated osteoblasts can not determined. The UMR 106 cell line was originally established from a rat osteosarcoma and the cells have an osteoblast-like phenotype (Partridge et al 1993). This cell line has been used in a large number of studies on osteoclast formation, and is one of only a few cell lines capable of supporting osteoclast differentiation and bone resorption. Using UMR 106 cell cocultures, the importance of $1,25(OH)_2D_3$, M-CSF and IL-6 in macrophage-osteoclast differentiation has be determined (Chapter 3). The mechanism of action of M-CSF in human monocyte-osteoclast differentiation has also been investigated (Chapter 5).

It is difficult, however, to be certain of the effect of PGE_2 on osteoclast formation and bone resorption in periprosthetic tissues surrounding loose implant components using this *in vitro* coculture technique which employs UMR 106 cells. Quinn et al (1997) showed that the effects of PGE_2 on murine monocyte-osteoclast differentiation are highly dependent on the type of supporting stromal cell present in the cocultures. Whilst PGE_2 has been shown to have an inhibitory effect in murine monocyte-UMR 106 cell cocultures, the effect of PGE_2 on human macrophage-osteoclast differentiation cocultured with human bone stromal cells is not known. This is particularly important in the context of periprosthetic bone resorption where macrophages and osteoblasts are present in the same location. In addition, PGE_2 is known to be released from wear particle-stimulated macrophages, and increased levels have been found at the bone-implant interface

of failed prostheses (Goldring et al 1983). PGE_2 is also known to inhibit directly, and transiently, mature osteoclast motility and bone resorption (Chambers et al 1985) but is also known to be a potent stimulator of bone resorption in organ cultures (Klein and Raisz 1970). PGE_2 has been reported to stimulate osteoclast differentiation in murine marrow cultures and murine spleen haematopoietic cell and bone stromal cell cocultures (Akatsu et al 1989; Collins and Chambers 1991), and has also been reported to both stimulate and inhibit osteoclast differentiation in long term human marrow cultures (Chenu et al 1990; Flannagan et al 1995).

In order to develop an *in vitro* system of human macrophage-osteoclast differentiation which more closely parallels what occurs *in vivo*, a human macrophage-human osteoblastic cell coculture system of osteoclast formation was devised. Specifically, it was determined whether human bone-derived stromal cells are capable of supporting osteoclast differentiation from mononuclear precursors isolated from periprosthetic tissue surrounding loose implants. The cellular and humoral conditions required for this to occur *in vitro* were investigated. In addition, the effect of PGE_2 on macrophage-osteoclast differentiation and bone resorption was also determined. The presence of endogenous levels of PGE_2 and M-CSF in periprosthetic macrophage-bone stromal cell cocultures was measured, and the effect on periprosthetic macrophage-osteoclast differentiation of PGE_2 added at various time points during the coculture period was also determined.

6.2. Methods

6.2.1. Primary human bone-derived stromal cell culture

Human trabecular bone was obtained at the time of preparation of the site for insertion of the femoral prosthesis in patients undergoing primary total hip replacement surgery for OA. The medullary bone was collected from the region of the greater trochanter, a site which is remote from the joint surface and grossly and microscopically consists of normal bone. Clinical details of the patients (HB1-HB6) from whom bone was collected are shown in Appendix 3.

The bone fragments were cut into small pieces and washed vigorously in sterile PBS to remove blood, fat and marrow. Equal amounts of bone were transferred into $25cm^2$ tissue culture flasks containing 5 mls of media. The media used was Dulbecco's Modification of Eagle's Minimal Essential Medium (DMEM; Cat. no. D-6546, Sigma, UK) supplemented with 10% heat treated FCS (Cat no. 10106-169, Gibco, UK), 100U/ml Penicillin and 100µg/ml Streptomycin sulphate (Cat no. 15140-114, Gibco, UK), 2mM L-glutamine (Cat no. 25030-024, Gibco, UK), 100µM L-ascorbic acid 2-phosphate (Asc-2-P; Cat. no. 013-12061, Wako Pure Chemical Industries Ltd, Japan), 2% Hepes buffer (Cat. no. H-0887, Sigma, UK), and 10^{-8}M dexamethasone (Cat. no. D-4902, Sigma, UK) (DMEM standard media). The cultures were incubated at 37°C in 5% CO_2 with media changed after seven days, and every 3-4 days thereafter. The cultures were grown to confluence (4-6 weeks), and were then passaged for experimentation.

To passage the cells, the cell layer was washed twice with DMEM followed by collagenase treatment (25U/ml in DMEM only; type VII, Cat. no. C-0773, Sigma, UK) for two hours at 37°C in 5% CO_2. The cells were then washed twice in sterile PBS and incubated for approximately 2 minutes in trypsin to detach and separate the cells. The cell suspension was passed through a 100µm cell strainer to remove clumps of matrix, and centrifuged at 1500 rpm for 5 minutes to pellet the cells. The supernatant was removed and the pellet resuspended in DMEM standard media. A concentration of $2x10^4$ cells/100µl was added to 7mm wells of a 96 well Multiwell plate. These wells contained either glass coverslips or pre-wetted human cortical bone slices. The human bone stromal cells were incubated at 37°C in 5% CO_2 for 24 hours. After 24 hours, the media was changed and the cells were incubated in αMEM+10% FCS media. These bone-derived cells were denoted as passage 1 (P1). The majority of cells used in the following experiments were P1 cells although occasionally P2 cells were used and this was noted in the text. Explant cultures of human trabecular bone give rise to a number of cell types including osteoblastic, fibroblastic and adipocytic cells (Gallagher et al 1982; Beresford et al 1983a; Beresford et al 1983b). The presence of fibroblast-like stromal cells that are capable of rapid proliferation in these cultures means that primary cultured cells of low passage number must be used (Chavassieux et al 1990).

6.2.2. Characterisation of human bone-derived stromal cells for osteoblastic phenotype

With the last three bone cultures passaged (HB4-HB6), $2x10^4$ cells/ml were also added to 16mm wells of two 24 well plates for characterisation of these bone-derived stromal cells for the osteoblast phenotype. The cells were cultured in DMEM standard media for 12 days with media changes twice a week and then the cells were characterised for alkaline phosphatase expression and their ability to mineralise. The cultured cells were characterised histochemically for the expression of alkaline phosphatase using a commercially available kit (Cat no. 86-R, Alkaline Phosphatase kit, Sigma, UK). A von Kossa reaction for mineral was used to demonstrate the presence of insoluble phosphate and carbonate in the cultures which signifies the presence of calcium and mineralisation (Bancroft and Stevens 1977).

6.2.3. Preparation of periprosthetic macrophage-human bone stromal cell cocultures on coverslips and bone slices

Adherent cells were isolated from tissue specimens obtained from patients undergoing revision arthroplasty surgery, as described previously. A concentration of $1x10^5$ cells/ml of the cell suspension was added to the 7mm wells which contained the coverslips and bone slices seeded 48 hours earlier with human bone-derived stromal cells. The cells were allowed to adhere for one hour at 37°C in 5% CO_2, before the coverslips and bone slices were removed from the wells, washed vigorously in αMEM to remove the non-adherent cells, and placed in larger 16mm wells containing 1ml of αMEM+10% FCS media.

6.2.4 Cellular and humoral requirements for osteoclast differentiation from periprosthetic macrophages

Adherent cells were isolated from tissue specimens obtained from six patients undergoing revision arthroplasty surgery (ADM4-ADM6, ADM16-ADM18; Appendix 1). These isolated cells were cocultured with human bone-derived stromal cells from six different patients (HB2, HB4-HB6 all P1 and HB1, HB3 P2; Appendix 3) in the presence and absence of 10^{-7}M $1,25(OH)_2D_3$ and 10^{-8}M dexamethasone. Cultures were also included which consisted of coverslips and bone slices that had periprosthetic macrophages but no bone stromal cells added, and coverslips and bone slices that had bone stromal cells but no periprosthetic macrophages added. The coverslips were removed after ten days incubation and characterised histochemically for the expression of TRAP, and immunohistochemically for VNR expression and expression of the macrophage-associated antigens, CD11b and CD14. The cortical bone slices were removed after 14 days incubation and bone resorption was measured as the number of resorption pits per bone slice. Each treatment was studied in triplicate for each tissue specimen. Additional control coverslips and bone slices were also included which consisted of cocultures of periprosthetic macrophage and human bone stromal cells. These were removed after 24 hours incubation and the coverslips were assessed for CD11b, CD14, TRAP and VNR expression. The bone slices were assessed for resorption pit formation.

6.2.5. Mediator release during periprosthetic macrophage-human bone stromal cell coculture

To measure the levels of mediators released, supernatants were collected during media changes from four periprosthetic macrophage-bone stromal cell cocultures incubated in the absence of $1,25(OH)_2D_3$ and dexamethasone. Supernatants were collected from days 1, 4, 7 and 11. The supernatants were centrifuged to remove wear particles and cell debris, and stored at -20°C until ready for testing. Levels of human M-CSF and PGE_2 were measured by enzyme linked immunoassays (ELISAs) purchased from R&D Systems, UK and Amersham International, UK.

6.2.6. Effect of PGE_2 on osteoclast formation and bone resorption in periprosthetic macrophage-human bone stromal cell cocultures

Adherent cells were isolated from tissue specimens obtained from six patients undergoing revision arthroplasty surgery (ADM5, ADM7, ADM8, ADM15, ADM19, ADM20; Appendix 1). These cells were cocultured with human bone-derived stromal cells from six different patients (HB1, HB2, HB4-HB6 all P1 and HB3 P2; Appendix 3), in the presence and absence of 10^{-8}M dexamethasone. The effect of PGE_2 was determined by the addition of various concentrations of PGE_2 ranging from 10^{-8} to 10^{-6}M at the beginning of each experiment and every media change. PGE_2 (Cat. no. 5640, Sigma, UK) was dissolved in absolute ethanol and the 10^{-2}M stock stored in aliquots at -20°C until use. The coverslips were removed after ten days incubation and characterised for the expression of TRAP and VNR activity. The cortical bone slices were removed after 14 days incubation and bone resorption was measured as the number of resorption pits per bone slice. Each treatment was studied in triplicate for each tissue specimen.

Additional wells were also set up whereby 10^{-6}M PGE_2 was added to the cocultures, in the absence of dexamethasone, for the first time either at day four or day seven of the incubation period. The extent of resorption pit formation was compared firstly to cocultures which had no PGE_2 added (control), and secondly to cocultures which had PGE_2 added at the beginning of the experiment (Day 0) and every media change.

6.3. Results

6.3.1. Characterisation of human bone-derived stromal cells for osteoblastic phenotype

The passaged cells from all three bone cultures studied were shown to express the characteristics of osteoblasts. They were positive for alkaline phosphatase activity and were capable of mineralisation as evidenced by von Kossa staining (Figure 28).

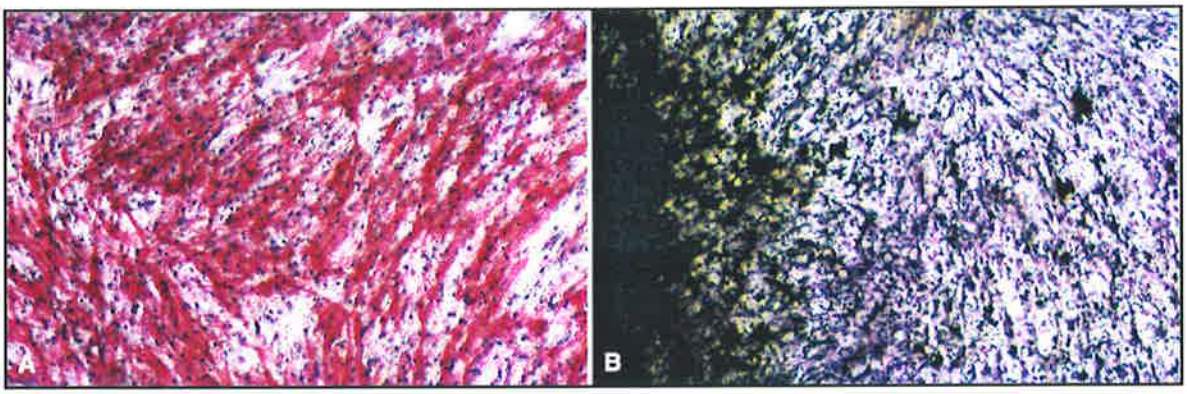

Figure 28. Cultures of human bone derived stromal cells (P1) showing (A) alkaline phosphatase activity (x40; counterstained with haematoxylin); and (B) von Kossa reaction for mineralisation after the addition of 10mM phosphate (x40; counterstained with toluidine blue).

6.3.2. Cellular and humoral requirements for osteoclast differentiation from periprosthetic macrophages

After 24 hours incubation, numerous adherent cells in the periprosthetic macrophage-human bone stromal cell cocultures on glass coverslips were found to express strongly the macrophage cell surface antigens, CD11b and CD14. These 24 hour cultures were largely negative for TRAP and VNR positive multinucleated cells although scattered TRAP and VNR positive mononuclear cells were present in most preparations (Figure 29). In three of the six arthroplasty tissue specimens, a few TRAP and VNR positive multinucleated cells were also noted (<5 cells per coverslip). Very few resorption pits were seen on the bone slices after 24 hours coculture. The mean number of total resorption pits in each experiment (n=6 experiments; 3 bone slices per experiment) ranged from 0 to 12 pits per bone slice.

In the ten day cocultures of periprosthetic macrophages and human bone stromal cells on glass coverslips, in the absence of $1,25(OH)_2D_3$ and dexamethasone, numerous large TRAP positive cells and clusters of smaller TRAP positive mononuclear cells were seen (Figure 29). Numerous large VNR positive multinucleated cells were also present (>30 per coverslip). After 14 days coculture on bone slices, extensive lacunar bone resorption was evident on all the bone slices studied. All six human bone derived stromal cell preparations were found to support osteoclast formation and bone resorption.

It was found that $1,25(OH)_2D_3$ was not an essential requirement for osteoclast formation and bone resorption in periprosthetic macrophages-human bone stromal cell cocultures. Although accurate quantitation of the number of TRAP positive cells was not possible, there appeared to be slightly fewer TRAP cells in the ten day cocultures incubated in the presence of $1,25(OH)_2D_3$ than in those cocultures incubated in the absence of $1,25(OH)_2D_3$ (Figure 30). The addition of $1,25(OH)_2D_3$ had no effect on the numbers of resorption pits formed after 14 days incubation (Figure 31). It was also found that dexamethasone was not an essential requirement for osteoclast formation and bone resorption in periprosthetic macrophage-human bone stromal cell cocultures. As for $1,25(OH)_2D_3$, the presence of dexamethasone was associated with a decrease in the number of

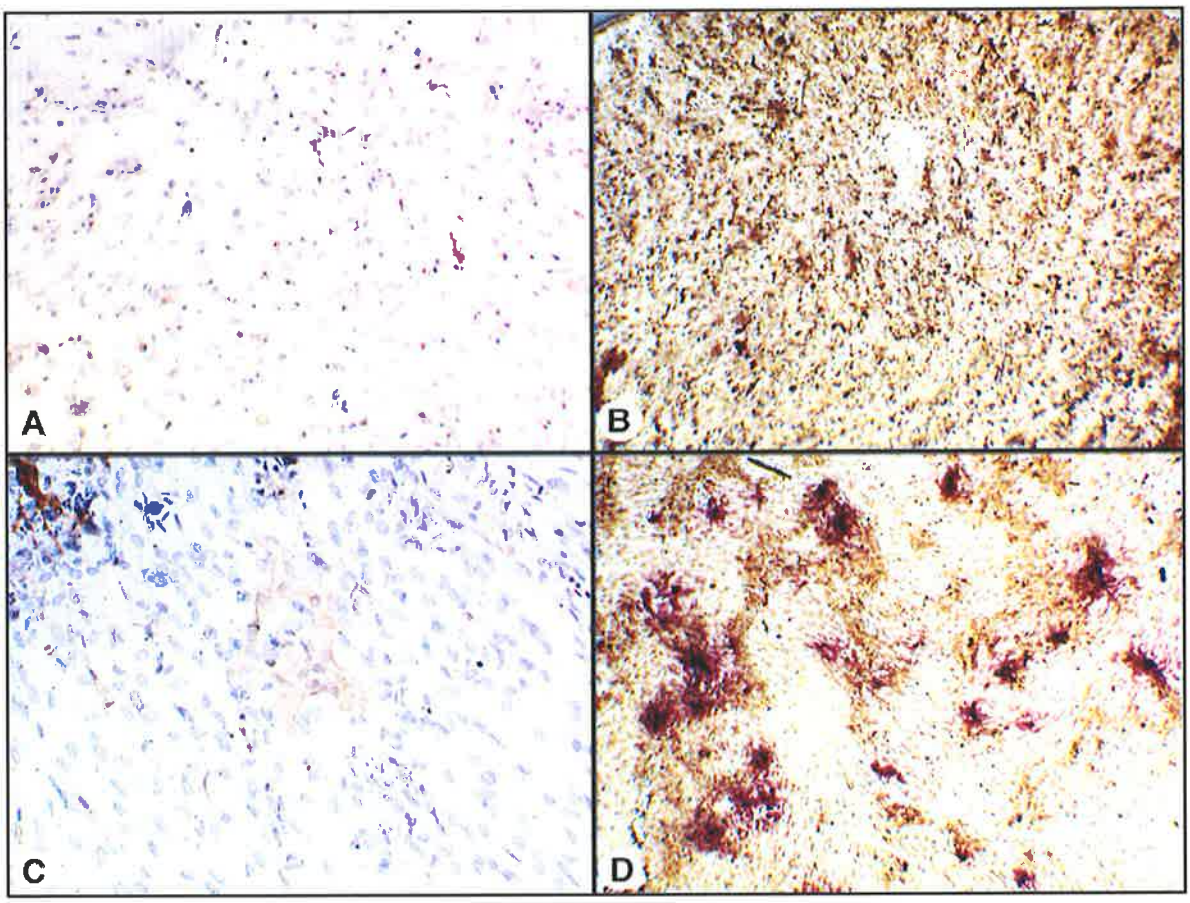

Figure 29. 24 hour and ten day cocultures of arthroplasty derived macrophages and human bone stromal cells showing (A) scattered VNR positive mononuclear cells (x100) and (B) scattered TRAP positive mononuclear cells (x40) after 24 hours coculture; (C) large VNR positive multinucleated cells (x100) and large TRAP positive cells as well as clusters of TRAP positive mononuclear cells (x40) after ten days coculture. Counterstained with haematoxylin.

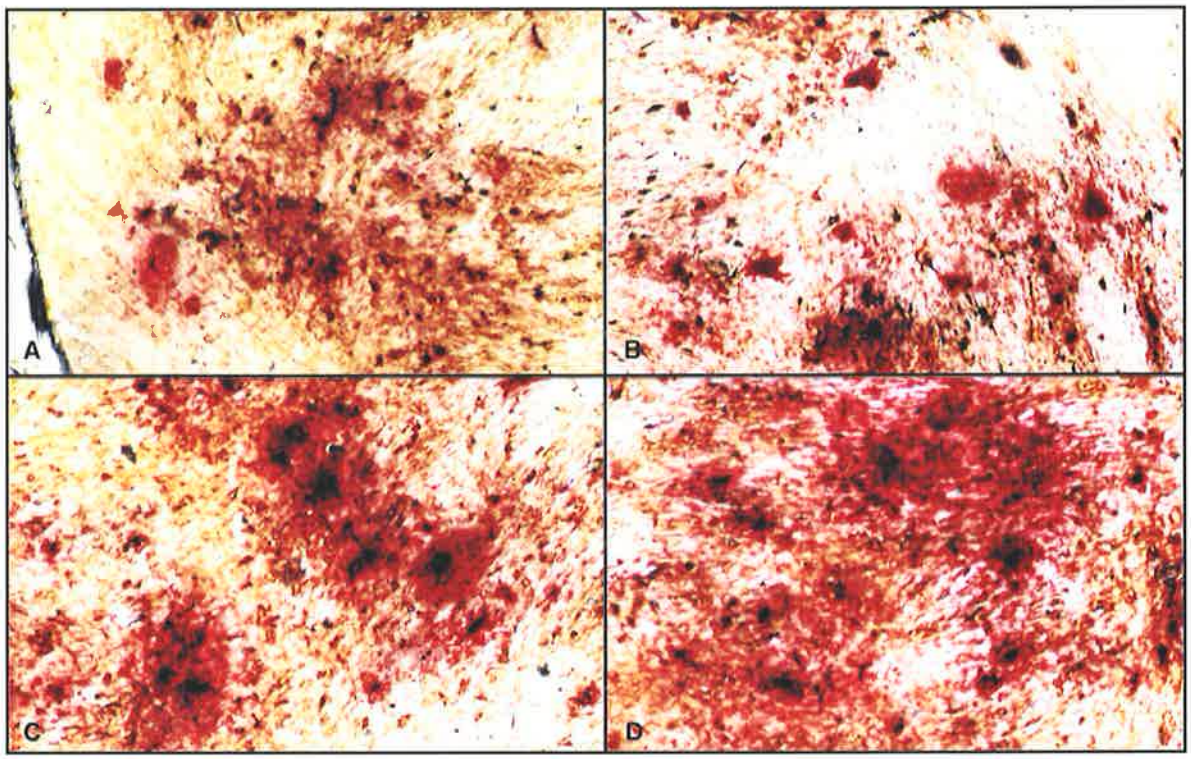

Figure 30. Ten day cocultures of arthroplasty derived macrophages and human bone stromal cells showing (A) TRAP activity in the presence of both $1,25(OH)_2D_3$ and dexamethasone (x40); (B) TRAP activity in the presence of $1,25(OH)_2D_3$ but in the absence of dexamethasone (x40); (C) TRAP activity in the absence of $1,25(OH)_2D_3$ but in the presence of dexamethasone (x40); (D) TRAP activity in the absence of both $1,25(OH)_2D_3$ and dexamethasone (x40).

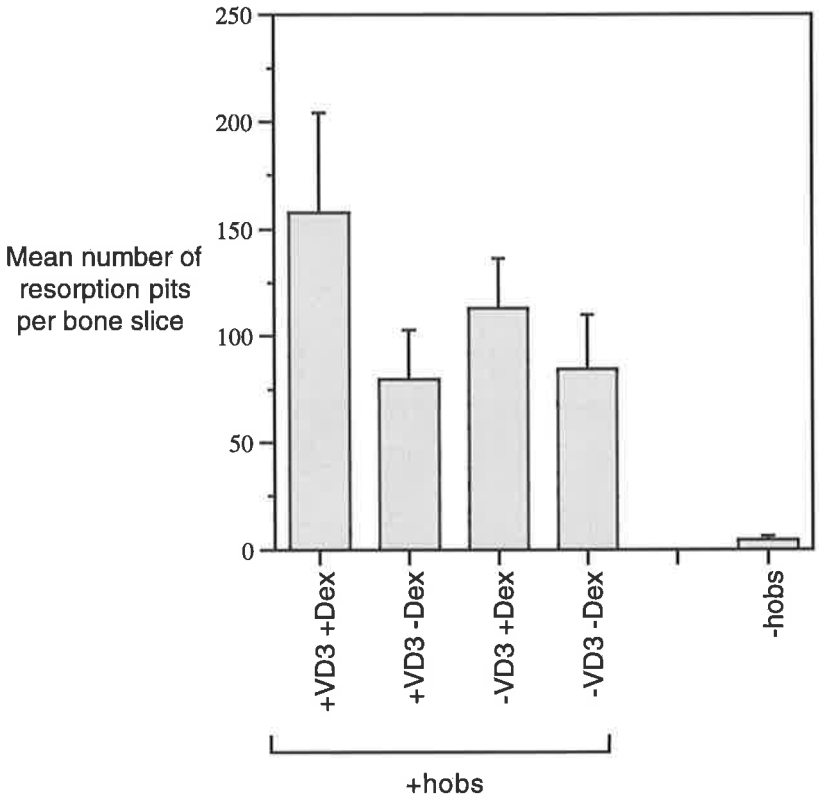

Figure 31. Cellular and hormonal requirements for osteoclast differentiation and bone resorption in arthroplasty derived macrophage-human bone stromal cell cocultures after 14 days incubation.

The results are expressed as the mean number of resorption pits per bone slice ± SEM. Human bone stromal cells (hobs) from six different patients were studied in triplicate (n=18). Statistical analyses were performed using a two factor with replication ANOVA test.

TRAP positive cells present in the ten day cocultures (Figure 30). However, a reduction in the number of resorption pits formed after 14 days coculture was not seen (Figure 31).

Less bone resorption was seen when human bone stromal cells were used to support macrophage-osteoclast differentiation compared with UMR 106 cells. The mean number of resorption pits (mean±SEM) was 76.1 ± 17.7 pits per bone slice for the six experiments using human bone stromal cells and 186.9 ± 39.9 pits per bone slice for the UMR 106 coculture controls in the same experiments. This may reflect the heterogenous nature of the human bone stromal cell population derived from trabecular bone fragments unlike UMR 106 cells which are all of osteoblastic phenotype. These human cells consist of a mixed population of osteoblastic, fibroblastic and adipocytic cells at different stages of differentiation.

In the control cultures where prosthetic macrophages were incubated for ten days on glass coverslips in the absence of human bone stromal cells, scattered TRAP and VNR positive mononuclear cells were seen. In three of the six experiments, a few TRAP and VNR positive multinucleated cells were also seen. As described earlier (section 3.4.3.), these multinucleated cells are most likely derived from pieces of bone which are attached to or embedded in the arthroplasty pseudomembrane. Extensive lacunar bone resorption was not seen on the bone slices after 14 days incubation (Figure 31). The mean number of total resorption pits for each experiment (n=6 experiments; 3 bone slices per experiment) ranged from 0 to 16 pits per bone slice.

In the absence of periprosthetic macrophages, the bone stromal cell cultures were completely negative for TRAP and VNR activity and no lacunar resorption pits formed. Alkaline phosphatase activity was found.

6.3.3. Mediator release during periprosthetic macrophage-human bone stromal cell coculture

Levels of M-CSF in the supernatants were substantially increased after four and seven days coculture (Table 7). The levels of M-CSF measured in the periprosthetic macrophage-human bone stromal cell cocultures were twice those

measured in the periprosthetic macrophage-UMR 106 cell cocultures (see section 3.6.2.). This result would suggest that both the cells isolated from arthroplasty tissue and the human bone stromal cells are important sources of M-CSF in these cocultures. Strikingly high levels of PGE_2 were detected in the supernatants after 24 hours and four days incubation of periprosthetic macrophage-human bone stromal cell cocultures (Table 7). Although still present, the levels of PGE_2 were greatly decreased in the supernatants from the seven and ten day cocultures. The levels of PGE_2 in the 24 hour and four day periprosthetic macrophage-human bone stromal cell supernatants were sixty and sixteen times higher respectively than those measured in the corresponding periprosthetic macrophage-UMR 106 cell supernatants. As the PGE_2 immunoassay measures both human and rodent PGE_2, this result suggests that human bone derived stromal cells are capable of producing substantial amounts of PGE_2 after stimulation. This is in contrast to PGE_2 release from the UMR 106 stromal cells.

Table 7. Release of mediators during periprosthetic macrophage-human bone stromal cell coculture.

Incubation period (Days)	Day 1	Day 4	Day 7	Day 11
M-CSF (pg/ml)	77.8 (range 43-125)	313.3 (range 185-400)	445.0 (range 230-660)	ND
PGE_2 (pg/ml)	1500.0 (range 625-2500)	2780.0 (range 450-5000)	185.0 (range 55-340)	26.9 (range 17.6-35)

Periprosthetic macrophages were isolated from tissue specimens from patients ADM5-ADM7, ADM15 (Appendix 1).

Results are expressed as the mean level of mediator release.

ND=not determined

6.3.4. Effect of PGE_2 on osteoclast formation and bone resorption in periprosthetic macrophage-human bone stromal cell cocultures

In the ten days cocultures of periprosthetic macrophages and human bone stromal cells incubated in the absence of dexamethasone, numerous large TRAP and VNR positive cells were seen on the coverslips. After 14 days incubation, extensive lacunar bone resorption was seen on the bone slices cocultured with human bone stromal cells, in the absence of dexamethasone. The mean number of total resorption pits for each experiment (n=6 experiments; 3 bone slices per experiment) ranged from 46.7 to 187.0 pits per bone slice. In the absence of human bone stromal cells, occasional TRAP and VNR positive cells were seen on the coverslips after ten days incubation but few if any resorption pits formed after 14 days incubation. The mean number of total resorption pits for each experiment (n=6 experiments; 3 bone slices per experiment) ranged from 0 to 6.7 pits per bone slice.

The addition of exogenous PGE_2 caused a significant dose dependent increase in lacunar bone resorption (Figure 32). At concentrations of 10^{-6}M and 10^{-7}M, the number of resorption pits formed after 14 days incubation were approximately three and two times greater than the untreated control ($p=0.0012$ and $p=0.0008$ respectively). 10^{-8}M PGE_2, the lowest concentration studied, had no effect on resorption pit formation ($p=0.413$). Cocultures on bone slices incubated in the presence of 10^{-6}M PGE_2 showed a significant increase in bone resorption compared to controls as assessed by counting pit numbers. This amount of bone resorption is likely to have been underestimated, however, as it was noted that, whereas lacunar bone resorption on control bone slices appeared as single pits or clusters of small pits, with generally five individual pits per cluster, the bone resorption seen on bone slices incubated in the presence of 10^{-6}M PGE_2 was characterised by the presence of large areas of lacunar resorption with often greater than 20 pits in each resorption area. Moreover, each individual pit in these resorption areas was often extensive and convoluted (Figure 33). Although a significant increase in bone resorption was seen when the cocultures were incubated in the presence of 10^{-6}M PGE_2, no increase in osteoclast formation was

seen. The numbers of TRAP and VNR positive cells in the ten day cocultures appeared to be similar to the numbers present in the untreated controls.

The addition of 10^{-6}M PGE_2 for the first time at Day 4 of the incubation period caused a significant increase in bone resorption compared to controls which did not have 10^{-6}M PGE_2 added ($p=0.011$) (Figure 32). No significant difference in the amount of bone resorption seen after 14 days incubation was found between cocultures which had 10^{-6}M PGE_2 added at Day 4 and those which had 10^{-6}M PGE_2 added from the beginning of the experiments ($p=0.882$). A slight but not significant increase in bone resorption compared to the controls was also seen when 10^{-6}M PGE_2 was added for the first time at Day 7 of the incubation period ($p=0.323$). This increase was not as great as the increase seen when 10^{-6}M PGE_2 was added at earlier time points (i.e. Day 0 or Day 4). This was most probably due to the fact that until Day 7 resorption pit formation by osteoclastic cells was at a reduced level in the absence of exogenous PGE_2.

When 10^{-6}M PGE_2 was also added to the cocultures, dexamethasone induced a marked increase in lacunar bone resorption (Figure 34). The number of pits formed after 14 days incubation in the presence of 10^{-6}M PGE_2 was more than two times greater when dexamethasone was added than when dexamethasone was omitted ($p=0.0001$). Dexamethasone alone did not stimulate bone resorption. The mean number of resorption pits was 125.3 pits per bone slice for the dexamethasone treated cocultures compared to 84.9 pits per bone slice for the untreated controls ($p=0.086$). An increase in the numbers of TRAP and VNR positive cells was not seen when PGE_2 and dexamethasone were added to the cocultures.

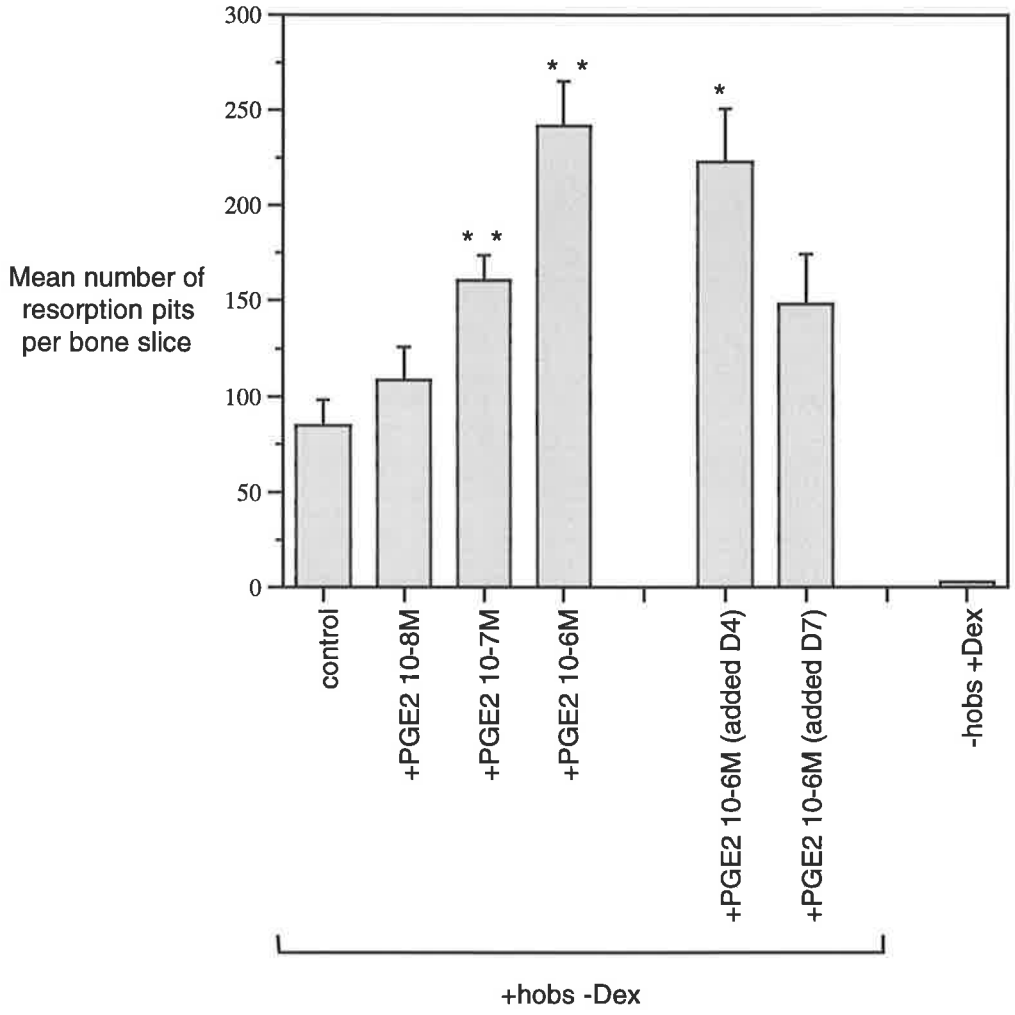

Figure 32. Effects of PGE_2 on lacunar bone resorption in arthroplasty derived macrophage-human bone stromal cell cocultures after 14 days incubation.

The results are expressed as the mean number of resorption pits per bone slice ± SEM. Human bone stromal cells from six different patients were studied in triplicate (n=18). Statistical analyses were performed using a two factor with replication ANOVA test.
**p<0.005, *p<0.05 compared to control

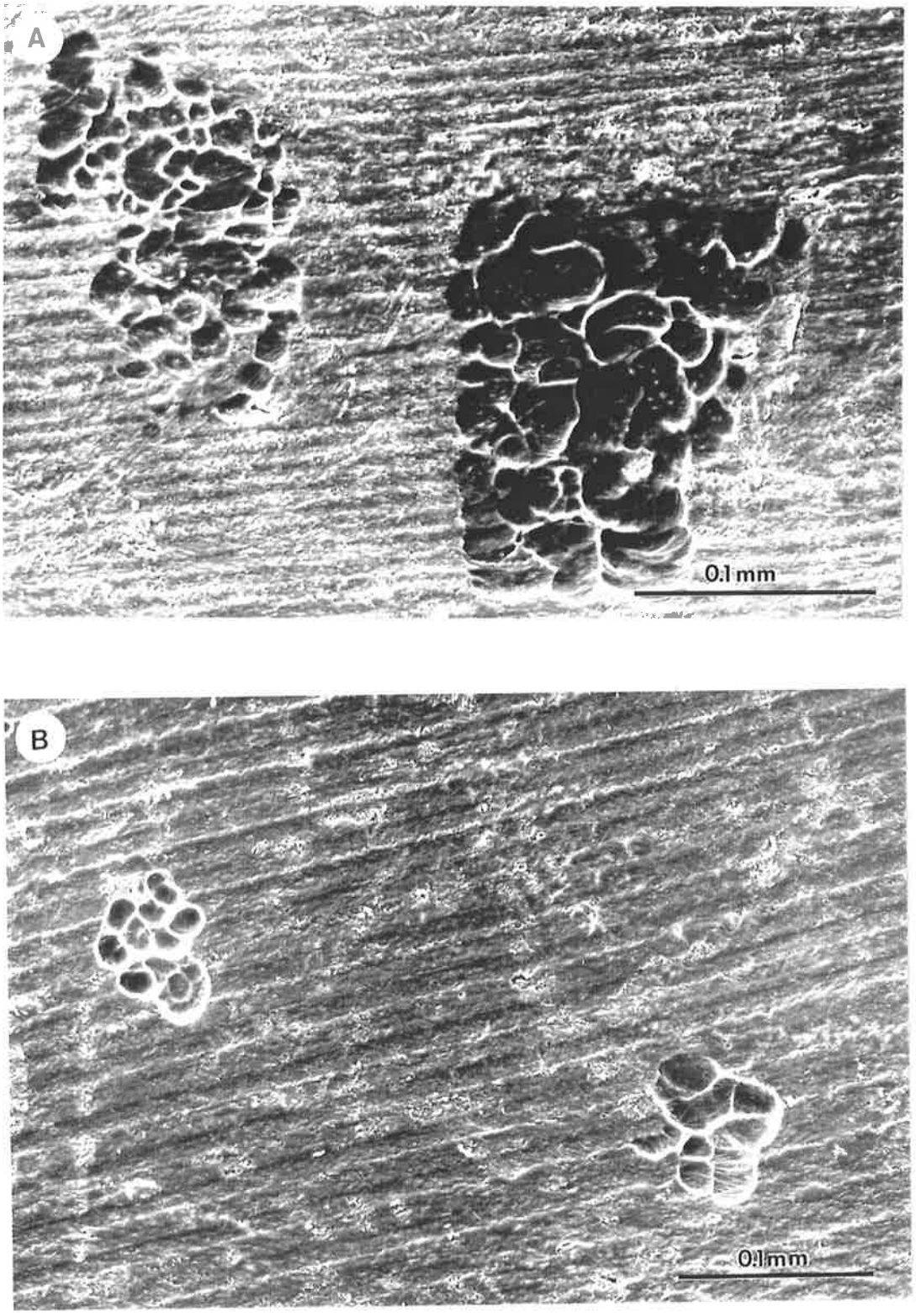

Figure 33. (A) Fourteen day coculture of arthroplasty derived macrophages and human bone stromal cells incubated in the presence of 10^{-6}M PGE_2 on human bone slices. (B) Fourteen day coculture of arthroplasty derived macrophages and human bone stromal cells incubated in the absence of exogenous PGE_2 on human bone slices. The cells have been removed to reveal evidence of lacunar bone resorption.

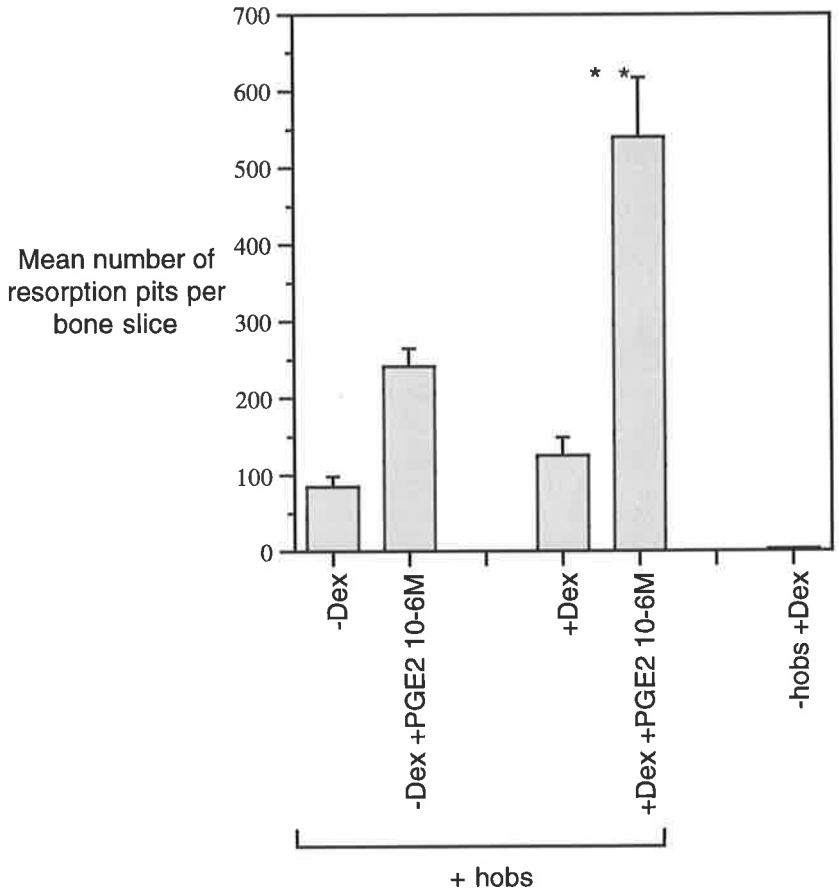

Figure 34. Effects of dexamethasone on lacunar bone resorption in arthroplasty derived macrophage-human bone stromal cell cocultures after 14 days incubation.

The results are expressed as the mean number of resorption pits per bone slice ± SEM. Human bone stromal cells from six different patients were studied in triplicate (n=18). Statistical analyses were performed using a two factor with replication ANOVA test.
**$p<0.005$ compared to PGE_2 - Dex control

6.4. Discussion

The results of this study have shown that human bone derived stromal cells are capable of supporting osteoclast formation from cells present in the periprosthetic tissues surrounding a loose implant. Adherent cells isolated from the arthroplasty tissue largely expressed a macrophage phenotype. However, after long term coculture with human bone stromal cells, numerous large osteoclastic TRAP and VNR positive cells appeared on the coverslips, and extensive lacunar bone resorption was seen on all the bone slices. Human bone stromal cells derived from six different patients were all found to support human osteoclast formation. It was also found that the requirements for osteoclast formation in periprosthetic macrophage-human bone stromal cell cocultures were markedly different to those required for osteoclast formation when periprosthetic macrophages were cocultured with UMR 106 cells. Notably, the addition of $1,25(OH)_2D_3$ was not found to be essential for human macrophage-osteoclast differentiation.

$1,25(OH)_2D_3$ has been consistently shown to be essential for osteoclast formation in *in vitro* coculture systems. In a study by Collins and Chambers et al (1992), however, it was shown that PGE_2 could substitute for $1,25(OH)_2D_3$ in murine marrow-osteoclast differentiation. Measurement of PGE_2 levels in the periprosthetic macrophage-human bone stromal cell coculture supernatants demonstrated that high levels of PGE_2 were released in the first four days of coculture. Thus, human bone stromal cells may be capable of releasing sufficient levels of PGE_2 to promote osteoclast differentiation in this coculture system. This was not found in the UMR 106 cell cocultures, and hence these cocultures required the presence of $1,25(OH)_2D_3$. This explanation for the finding that $1,25(OH)_2D_3$ is not required for macrophage-osteoclast differentiation is supported by the findings of one experiment where the effect of indomethacin on periprosthetic macrophage-osteoclast differentiation was determined. In the 14 day cocultures of periprosthetic macrophages and human bone stromal cells incubated in the presence of 10^{-6}M indomethacin, resorption pit formation was substantially reduced compared to the untreated controls. The mean numbers of resorption pits per bone slice were 10.5 and 120.7 pits per bone slice respectively (n=3). This inhibitory effect of indomethacin needs to be confirmed in further

experiments. Substantial levels of M-CSF were also found to be released endogenously by cells isolated from arthroplasty tissue and by human bone derived stromal cells. These levels appeared to be sufficient to support osteoclast precursor proliferation, maturation and differentiation into osteoclastic bone resorbing cells.

The addition of PGE_2 exogenously was found to strongly stimulate osteoclastic bone resorption in this coculture system but did not appear to affect osteoclast precursor proliferation and differentiation. One explanation is that there was sufficient PGE_2 released by the human bone stromal cells in the first four days for the addition of exogenous PGE_2 to have no effect. The levels of PGE_2 detected in the coculture supernatants at day seven, however, were substantially reduced. Thus, the addition of exogenous PGE_2 now had an effect. As osteoclasts were already formed by this stage, this effect was only seen on bone resorption. PGE_2 increased bone resorption in a dose dependent manner and this effect was enhanced synergistically by the presence of dexamethasone. The synergistic effect of dexamethasone and PGE_2 on osteoclastic bone resorption was significant. Dexamethasone alone has been shown to stimulate M-CSF release and inhibit the release of bone resorbing mediators, including TNFα, IL-6 and PGE_2 (Russell 1993). In combination with cytokines such as IL-1β and TNFα, however, dexamethasone has been found to enhance the bone resorbing effects of these cytokines (Haynesworth 1997). The mechanism of action of dexamethasone in periprosthetic macrophage-osteoclast differentiation after coculture with human bone stromal cells remains unknown.

In summary, the results of this study have shown that human bone derived stromal cells are capable of supporting osteoclast formation from cells present in the macrophage-rich periprosthetic tissues surrounding a loose implant. This human macrophage-human osteoblastic cell coculture system shows striking differences in the requirements for osteoclast formation and should prove useful in analysing more accurately cellular and humoral influences on human osteoclast formation. M-CSF, which has been shown in previous studies to be essential for osteoclast precursor proliferation and differentiation, was found to

be released from cells isolated from periprosthetic tissue and human bone stromal cells. Substantial amounts of PGE_2 were also found to be released early by the human bone-derived stromal cells in coculture. These mediators may have profound effects on the formation of new osteoclasts from mononuclear osteoclast precursors as well as affecting mature osteoclastic activity at sites of pathological bone resorption associated with aseptic loosening of total joint replacements.

7. Characterisation of Cells Isolated from Pigmented Villonodular Synovitis (PVNS) Tissue

7.1. Introduction

Multinucleated giant cells similar to those seen in giant cell tumours of bone (see Chapter 4) have been found in a wide variety of giant cell lesions of bone and extraskeletal soft tissues. The giant cells from these various lesions have all been shown to be capable of lacunar bone resorption, a characteristic feature of osteoclasts. In skeletal giant cell containing lesions such as giant cell tumour of bone and giant cell reparative granuloma of jaw, the giant cells exhibit all the defining characteristics of the osteoclast phenotype (Flanagan and Chambers 1985; Joyner et al 1992). In contrast, giant cells derived from extraskeletal giant cell lesions, such as breast carcinoma with giant cells, pilar tumour of scalp, and giant cell tumour of tendon sheath, do not exhibit the antigenic phenotype of osteoclasts and are unresponsive to calcitonin (Athanasou et al 1989; Athanasou et al 1991; Athanasou and Quinn 1992).

Pigmented villonodular synovitis (PVNS) is a diffuse villous or nodular tumour-like synovial lesion that affects joints, tendons and bursae (Jaffe et al 1941; Flandry and Hughston 1987). In about one third of cases PVNS behaves aggressively, causing para-articular osteolysis with the formation of multiple cysts in bone. Histologically, PVNS is characterised by the proliferation of synovial lining cells and a heavy diffuse subintimal infiltrate of mononuclear cells, largely macrophages, amongst which are scattered multinucleated giant cells. Ultrastructural studies of both villous and nodular forms of PVNS have shown the presence of synovial type A macrophage-like cells and synovial type B fibroblast-cells (Alguacil-Garcia et al 1978). The multinucleated cells show similar features to the type A mononuclear cells, suggesting that they are formed by fusion of type A cells.

During the course of my research, a case of PVNS presented at the hospital and tissue was made available for investigation. The aim of this study was to determine whether the giant cells present in PVNS express the phenotypic

characteristics of osteoclasts or macrophage polykaryons in an attempt to determine the nature of this potentially osteolytic synovial lesion.

7.2. Methods

7.2.1. Clinical details

Tissue was obtained fresh from an eighteen year old girl undergoing a synovectomy for spontaneous haemarthrosis of the knee. Prior to surgery, a MRI examination showed changes typical of PVNS with thickened synovial fronds and abundant haemosiderin. There was no radiological evidence of bone involvement. During surgery it was also noted that there was no evidence of bone involvement or damage to the articular surface, ligaments or menisci.

7.2.2. Isolation and culture of cells

A sample of the PVNS synovial tissue was washed thoroughly with sterile PBS before being cut into small pieces and digested in αMEM containing 1mg/ml collagenase type 1 (Cat no. C-0130, Sigma, UK) for 30 minutes at 37°C, and 5 mls of trypsin (Cat no. T-4424, Sigma, UK) for 1 hour. The digested tissue was then filtered with a 100μm cell strainer, and the filtrate centrifuged at 1500rpm for 5 minutes. After two washes in αMEM only, the pellet was resuspended in αMEM+10%FCS media. The cell suspension was finally counted in a haemocytometer after lysis of the red blood cells using a 5% (v/v) acetic acid solution.

The cell suspension at a concentration of $1x10^5$ cells/100μl was added to 7mm wells of a 96 well tissue culture plate containing either pre-wetted dentine slices, human cortical bone slices or 6mm glass coverslips. The cell suspension was allowed to adhere for 1 hour at 37°C in 5% CO_2 before being washed in αMEM media and transferred to fresh wells containing αMEM+FCS. Half the cultures were incubated for 24 hours and the remaining half for 7 days. The media was removed and fresh media added after 3 days to the 7 day cultures.

7.2.3. Characterisation of isolated and cultured cells for osteoclast phenotype

Specimens of PVNS synovial tissue were snap-frozen in liquid nitrogen and stored at -20°C for cryostat sectioning. In addition, imprints were made directly from small samples of pigmented synovial tissue. Glass coverslips containing 24 hour and 7 day cultures were also fixed in cold acetone for immunohistochemistry.

The above preparations were stained histochemically for TRAP, and immunohistochemically for the presence of macrophage (CD11b and CD14) and osteoclast (VNR) associated antigens with the monoclonal antibodies MO 1, 10G 3.3 and 23C6 respectively.

Cells isolated from PVNS synovial tissue and cultured on glass coverslips for 3 days were also assessed for the presence of CT receptors by autoradiography using ^{125}I-labelled salmon CT ligand binding. The coverslips were incubated for 2 hours at 37°C in 5% CO_2 in αMEM+FCS containing 0.125μCi ^{125}I-salmon CT (Cat. no. IM250, Amersham International, UK). The labelled CT was left to bind, then the cells were washed with sterile PBS, fixed in 2% glutaraldehyde-10% formalin solution for 10 minutes, and air dried. Non-specific binding was assessed in the presence of an excess amount of unlabelled CT (300mM). The coverslips were then processed for autoradiography.

To assess the functional ability of cultured cells to carry out lacunar bone resorption, cells isolated from the PVNS synovial tissue and cultured on the dentine slices were fixed in glutaraldehyde, and critical point dried before being sputtered with gold. Dentine slices were used as these surfaces were smooth and devoid of any naturally occurring pits. The slices were examined using SEM for osteoclast-like cells associated with lacunar resorption pits. The remaining human cortical bone slices had the cells removed from the bone surface and the number of resorption pits per bone slice counted to assess accurately the extent of lacunar resorption by these cultured cells.

7.3. Results

7.3.1. Histology of PVNS tissue

Grossly, the synovial membrane and capsule were tan/brown in colour and showed focal areas of nodular thickening. Histologically, villous hypertrophy, thickening of the synovial membrane and intimal hyperplasia was seen. A prolific subintimal macrophage and giant cell infiltrate was noted with many cells containing phagocytosed haemosiderin or lipid. Scattered lymphocytes and very occasional plasma cells were also noted.

7.3.2. Expression of osteoclast markers by multinucleated cells in PVNS

Giant cells in frozen sections of the PVNS tissue and imprint preparations were strongly TRAP positive and showed positive staining for VNR. The giant cells were negative for CD11b and CD14 in frozen sections, but a small number in imprint preparations weakly expressed these antigens. These imprint preparations contained over one hundred giant cells of which fewer than five, mostly giant cells with fewer than three nuclei, showed positive staining for CD11b and CD14. In contrast, mononuclear cells were positive for CD11b and CD14 but negative for VNR in frozen sections. As previously noted, synovial lining cells in the hyperplastic intima were found to express VNR.

Giant cells isolated from PVNS tissue and cultured on glass coverslips for 24 hours were similarly found to be TRAP and VNR positive and negative for CD11b and CD14 (Figure 37). In addition, cultured giant cells expressed numerous CT receptors when assessed for their ability to bind ^{125}I-labelled CT by autoradiography (Figure 37). A few giant cells, however, were found to be negative for CT receptors.

In the SEM bone resorption assay, there was functional evidence of bone resorption with giant cells lying directly over or beside resorption pits on dentine slices (Figure 38). Extensive lacunar resorption was also seen on cortical bone slices where the cultured cells had been removed. All twelve bone slices on which cells had been cultured for 24 hours and 7 days showed evidence of lacunar resorption. The average number of lacunar bone resorption pits seen after cells were cultured for 24 hours on the six bone slices on which cells had

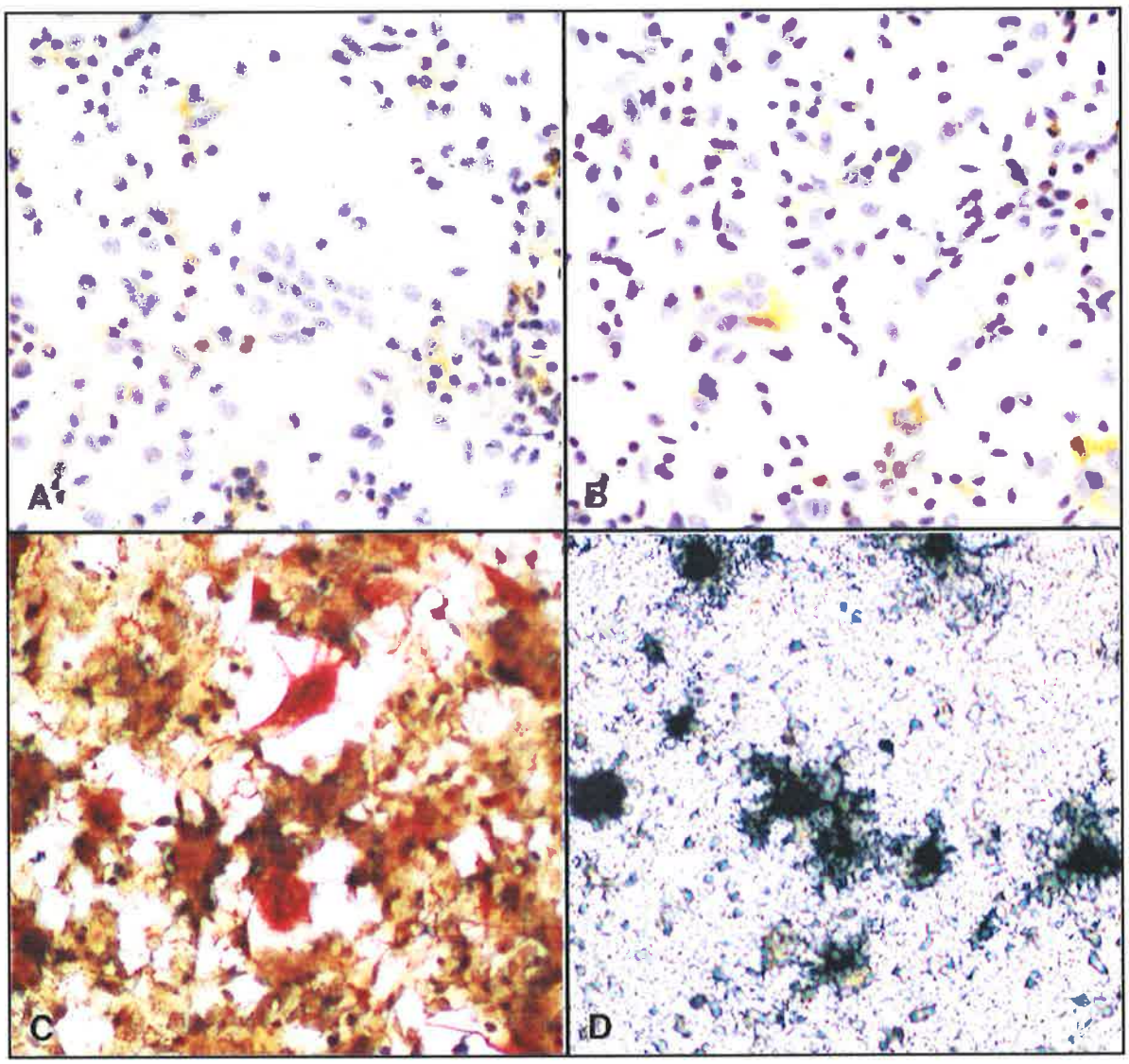

Figure 37. 24 hour cultures of PVNS cells on glass coverslips showing (A) CD14 positive mononucleated cells and CD14 negative multinucleated cells (x200); (B) VNR positive mononuclear and multinucleated cells (x200); (C) TRAP positive multinucleated cells (x100); and (D) binding of ^{125}I labelled calcitonin (x100).

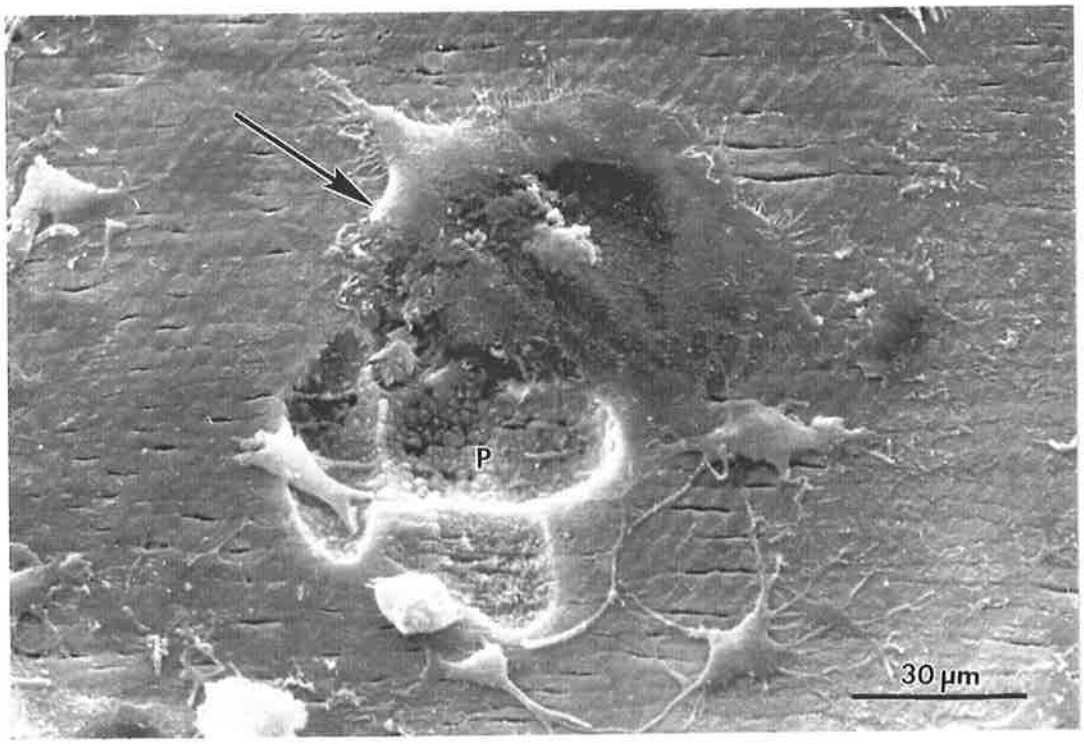

Figure 38. PVNS giant cell (arrowed) overlying a lacunar resorption pit (P) on the dentine surface.

been removed was 43 (range 35 - 59), and on the same number of bone slices after 7 days the average number of lacunar bone resorption pits was 149 (range 124 - 173).

7.4. Discussion

PVNS is an enlarging, locally aggressive synovial tumour which commonly causes erosion of bone adjacent to the joint synovium and is characterised by the presence of numerous macrophages and giant cells. In the PVNS case studied, the macrophages and giant cells were confined to the synovium and there was no evidence of osteolysis. Despite this, the giant cells isolated from the specimen were shown to express all the phenotypic characteristics of osteoclasts. Unlike macrophage polykaryons, PVNS giant cells were strongly positive for VNR and negative for CD11b and CD14. The PVNS giant cells also expressed CT receptors, as previously noted by Gravellese et al (1993), and were capable of extensive lacunar bone resorption.

A prominent feature of both skeletal and extraskeletal giant cell-containing lesions is the presence of a heavy macrophage infiltrate. As well as containing numerous macrophages which are capable of fusing to form macrophage polykaryons, these lesions also contain a number which are primed and activated for osteoclast differentiation. This may explain why a variable number of the giant cells in these lesions express the antigenic phenotype of macrophage polykaryons rather than osteoclasts (Athanasou et al 1989; Athanasou et al 1991; Athanasou and Quinn 1992). It could also account for the marked variation in the amount of lacunar bone resorption by giant cells isolated from different extraskeletal giant cell containing lesions. In a similar previous study, giant cells isolated from giant cell tumour of tendon sheath, a lesion which closely resembles joint PVNS histologically, formed fewer than 10 resorption pits over seven days culture on cortical bone slices (Athanasou et al 1991). In the PVNS case studied, over 100 pits formed. This marked difference in lacunar resorption may be accounted for by a difference in the number of primed mononuclear osteoclast precursors present in these two morphologically similar extraskeletal giant cell containing lesions.

The origin and nature of both the diffuse and localised forms of joint PVNS is controversial. It is not certain whether PVNS represents a true neoplasm of synovial tissue or an exuberant proliferative response of the synovium, possibly related to trauma or inflammation. Cytogenetic studies and flow cytometry have shown evidence of clonality in PVNS tissues, suggesting that PVNS may represent a true synovial neoplasm (Bridge et al 1990; Ray et al 1991). A similar controversy surrounds the nature of giant cell tumour of bone which has also been found to exhibit consistent chromosomal abnormalities (Choong et al 1995). PVNS, giant cell tumour of bone and giant cell reparative granuloma of the jaw are distinguished from other skeletal and extraskeletal giant cell lesions by the fact that the giant cells express the phenotype of osteoclasts rather than macrophage polykaryons. These three lesions also exhibit a similar pattern of osteolysis, behaving inherently as locally aggressive tumours with tumour growth or expansion being accompanied by bone destruction. On the basis that the giant cells are osteoclastic in nature, calcitonin administration has been used successfully to treat the growth of giant cell reparative granulomas of the jaw (Harris 1993), and potentially a similar approach could be used to control the osteolysis of PVNS.

8. Summary

The majority of studies described in this thesis have examined macrophages in human periprosthetic tissues surrounding loose prostheses and osteoclast differentiation and pathological bone resorption associated with aseptic loosening of total joint replacements.

The results presented suggest that one cellular mechanism whereby bone resorption and implant loosening may occur is by osteoclastic differentiation from mononuclear phagocyte precursor cells present in periprosthetic tissue. The requirements for this to occur *in vitro* were found to be dependent on the osteoblast-like cells used to support osteoclast formation. Both the osteoblast-like rat cell line, UMR 106, and human bone-derived stromal cells were found to support osteoclast formation from periprosthetic cells.

It was found that mediators implicated in osteoclast formation and osteoclastic bone resorption, namely M-CSF, IL-6 and PGE_2, are released from cells isolated from periprosthetic tissues retrieved from around loose implants. Both M-CSF and IL-6 were found to be important for the proliferation and differentiation of precursor cells into osteoclastic bone resorbing cells. Receptors for these cytokines were found to be present on periprosthetic cells. In cocultures of periprosthetic cells and human osteoblastic cells, PGE_2 appeared to stimulate osteoclast formation and osteoclastic bone resorbing activity.

The results of these studies suggested that not only do the mediators contribute to osteoclastic activity, but they are also important for the formation of new osteoclasts at sites of pathological bone resorption. In the context of the prolific foreign body response to wear particles at the bone-implant interface, osteoclast differentiation may represent an important cellular mechanism whereby bone resorption leads to prosthesis loosening.

Future studies should involve the use of the *in vitro* models of osteoclast formation examined in this thesis to characterise further the mechanism of osteoclast formation from mononuclear precursor cells. Using fluorescence-

activated cell sorting, the role of different cell types in periprosthetic tissue in mediator release and the promotion of osteoclast formation *in vitro* can be determined. Specific antibodies and inhibitors can be used to determine the importance of mediators such as M-CSF, IL-6 and PGE_2 in osteoclast formation from human periprosthetic cells cocultured with human bone-derived stromal cells. In addition, the human peripheral blood monocyte-human bone stromal cell coculture system should be further developed and used to study the effects of conditioned media from wear particle-stimulated monocytes on osteoclast formation.

APPENDIX 1. Clinical details of patients from whom revision arthroplasty specimens were obtained.

Case number	Age (years)	Sex	Implant materials	Duration of implant (years)	Type of retrieved tissue
ADM 1	76	M	cemented UHMWPE, SS	8	femoral membrane
ADM 2	61	F	cemented UHMWPE, SS	9	femoral membrane
ADM 3	68	M	cemented, UHMWPE, SS	5	acetabular membrane
ADM 4	60	M	cemented, UHMWPE, SS	7	femoral membrane
ADM 5	81	F	cemented UHMWPE, SS	18	acetabular membrane
ADM 6	79	M	cemented, UHMWPE, ?	6	femoral membrane
ADM 7	76	F	cemented UHMWPE, SS	10	capsule
ADM 8	78	M	cemented UHMWPE, SS	13	femoral membrane
ADM 9	80	F	cemented UHMWPE, SS	9	acetabular and femoral membrane
ADM 10	88	M	cemented UHMWPE, SS	11	femoral membrane
ADM 11	52	F	uncemented, UHMWPE, CoCr	3	femoral membrane
ADM 12	72	F	cemented UHMWPE, SS	6	acetabular membrane
ADM 13	50	F	uncemented, UHMWPE, CoCr, TiAlV	5	femoral membrane
ADM 14	77	F	cemented, UHMWPE, CoCr	12	acetabular membrane
ADM 15	64	M	cemented, UHMWPE, SS	8	femoral membrane
ADM 16	80	F	cemented UHMWPE, CoCr	15	acetabular membrane
ADM 17	26	F	cemented UHMWPE, CoCr, TiAlV	5	acetabular membrane
ADM 18	62	F	uncemented, UHMWPE, TiAlV	4	acetabular membrane
ADM 19	67	M	cemented UHMWPE, cpTi, CoCr	9	acetabular membrane
ADM 20	69	M	cemented UHMWPE, SS	20	femoral membrane

Clinical details of patients from whom OA synovium specimens were obtained.

Case number	Age (years)	Sex
OA 1	82	F
OA 2	62	F

APPENDIX 2. Cytokine Receptor McAbs used for immunohistochemical staining.

McAb code[1]	Clone name	Donor	Reactivity	Isotype
C-79	hIL-1R-M1	Armitage	IL-1R Type1	G1
C-25	7G7B6	Nelson	IL-2Rα	G2a
C-53	MEM-145	Horejsi	IL-2Rα	M
C-54	MEM-140	Horejsi	IL-2Rα	M
C-90	H-31	Sugamura	IL-2Rα	G1
C-89	TUGh4	Sugamura	IL-2Rγ	G2b
C-67	9F5	Lopez	IL-3Rα	G1
C-4	S456C9	Agthoven	IL-4R	G1
C-81	hIL-4R-M57	Armitage	IL-4R	G1
C-74	B-N12	Wijdenes	IL-6R	G1
C-75	B-F19	Wijdenes	IL-6R	G1
C-63	M5	Brochier	IL-6R	
C-88	M195	Brochier	IL-6R	G2a
C-5	R34.34	Agthoven	IL-7R	G
C-77	B-G20	Wijdenes	IL-8R	M
C-94	htr9	Lesslauer	TNFR/55kD	G1
C-95	utr1	Lesslauer	TNFR/75kD	G1
C-13	B-R9	Clement/Wijdenes	gp130	G1
C-16	B-T12	Clement/Wijdenes	gp130	G1
C-18	B-T9	Clement/Wijdenes	gp130	G1
C-24	B-S8	Clement/Wijdenes	gp130	G2a
C-34	B1	Brochier	gp130	G1
CD115[#]	7-7A3		M-CSFR	G1
C-29	57A5	Buhring	SCFR	G1
C-45	NU-C-KIT	Nakamura	SCFR	G1
C-46	L15	Nakamura	SCFR	G1
C-48	NU-SCF1	Nakamura	SCFR	G1
C-68	MTK1	Morita	SCFR	G1
C-69	MTK2	Morita	SCFR	G1
C-72	17F11	Agthoven	SCFR	M
C-3	SC06	Agthoven	GM-CSF-R	G1
C-57	SC04	Agthoven	GM-CSF-R	G1

[1]The monoclonal antibodies (McAbs) used were derived from the Cytokine Panel of McAbs analysed in the 6th International Workshop on Human Leukocyte Differentiation Antigens.
[#]An antibody specific for the human CSF-1 receptor (M-CSFR) was purchased from Cambridge Bioscience, UK (Cat no. 13-2800).

APPENDIX 3. Clinical details of patients from whom human trabecular bone was obtained.

Case number	Age (years)	Sex
HB 1	36	M
HB 2	54	M
HB 3	59	M
HB 4	72	F
HB 5	56	M
HB 6	84	F

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