MEDIATORS OF LOCALISED PATHOLOGICAL BONE LOSS

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"….so long as we avoid accepting as true what is not so, and always preserve the right order for deduction of one thing from another, there can be nothing too remote to be reached in the end, or too well hidden to be discovered."

Descartes

DECLARATION OF ORIGINALITY

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.

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1 INTRODUCTION

1.1 BONE REMODELLING

Healthy bone is in a dynamic state, continually being removed and replaced through the process of remodelling. Remodelling of bone relies on the integrated activity of the osteoblast (bone forming) and osteoclast (bone resorbing) cells to maintain the balance of bone metabolism (Suda et al., 1995). An imbalance in bone metabolism due to either excessive resorption or decreased bone formation can result in bone loss. While we have known for well over a decade about the factors, such as the bone morphogenic proteins, which regulate bone formation by osteoblasts, it is only relatively recently that we understand how osteoclasts form and resorb bone. While this review of osteolysis will focus on the mediators that regulate osteoclasts, it is important to recognise that bone formation by osteoblasts may also be disrupted in bone loss pathologies.

Localised bone loss is seen in several pathological states, such as adjacent to prosthetic joints, in periodontal disease, in rheumatoid arthritis (RA), Paget's disease, and cancers such as giant cell tumours and myeloma. The focus of the work described in this thesis is on bone loss around prosthetic joints, in periodontal disease and RA. These three pathologies are similar in that the localised bone loss is associated with a chronic inflammatory response in the surrounding soft tissues. The bone loss in each disease appears to be initiated in response to foreign material, such as wear debris, in the case of prosthetic loosening, bacteria in the case of periodontitis, or an autoimmune response (as suggested) in the case of rheumatoid arthritis.

Osteolysis is normally is carried out osteoclasts that resorb bone, under the control of cytokines and other mediators. Factors that regulate physiologic bone resorption may also regulate pathologic bone loss. This thesis explores the possibility that bone resorption that is not balanced by bone formation is caused by an abnormal expression of factors that regulate osteoclast formation and activity in the tissue adjacent to the site of pathological bone loss (Martin and Ng, 1994). This thesis seeks not only to identify these factors in human tissues *in situ* but also to elucidate a possible mechanism by which osteolytic mediators induce bone osteolysis in several bone pathologies associated with bone loss.

1.2 DISEASES ASSOCIATED WITH LOCALISED BONE LOSS

1.2.1 Failure of Prosthetic Joint Implants

Artificial joint prostheses are now widely used and it is estimated that approximately one million prostheses are implanted worldwide each year. Despite ongoing research and the overall success of prosthetic implants failure of these implants is a significant problem with

10-15% of joints requiring replacement within 15-20 years (Malchau et al., 1993, Johnsson et al., 1994). Despite the undoubted benefit of joint replacement, premature failure of implants remains a considerable clinical problem. The cost to the community, resulting from premature failure of hip and other arthroplasties, is of increasing concern, as the number of revisions will continue to increase as the world population ages. Compounding this problem is the increase in incidence of joint replacement surgery at a younger age, which increases the likelihood that an individual will outlive the prosthesis (Malchau et al., 1993, Johnsson et al., 1994).

Aseptic prosthetic implant failure, may be due to a number of reasons such as poor surgical technique, but small particles of prosthetic material are often associated with failed implants, which have become loose. These particles are produced by wear at the articulating surfaces of the prosthetic joint or micromovement of the prosthesis against the surrounding bone and cement. The presence of a third body (prosthetic material or bone fragment) between articulating surfaces can accelerate the production of wear particles. These particles, produced by wear at the articulating surface of the prosthesis (Vernon-Roberts and Freeman, 1977, Willert and Semlitsch, 1977, Jacobs et al., 1994), are ingested by tissue macrophages. This is thought to initiate a cascade of events leading to the loss of peri-prosthetic bone, which is characteristic of this pathology. The effect particles of different alloys have on these cytokines is discussed bellow in more detail.

Maintaining healthy bone at the surface of prosthetic implants is vital for the long- term stability of artificial joints. Previous reports have investigated the effect of particles of different prosthetic alloys on various inflammatory cytokines (Haynes et al., 1993, Shanbhag et al., 1995a, Rogers et al., 1997, Catelas et al., 1999a, Ingham et al., 2000) but not the primary mediators of bone loss, which are discussed in detail below. One of the important concepts this thesis seeks to address is whether particles of prosthetic material induced expression of primary osteoclast mediators by human monocyte/macrophage cells *in vitro*. The presence of pre-osteoclasts and osteoclasts in the soft tissue of the bone/implant interface is also assessed. In addition to this, levels of these mediators are investigated at the level of both mRNA expression and protein production in diseased compared to control tissues *in vivo*. The levels of mRNA are then compared to the ability of cells extracted from the peri-prosthetic tissue to form osteoclasts *ex vivo*.

1.2.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common disease characterised by chronic synovial inflammation and hyperplasia resulting in joint destruction (Mulherin et al., 1996, Harris, 1990). Histopathological changes observed in RA include villus formation, hyperplasia of the cells lining the synovial membrane, fibrin deposition and incorporation, and cellular infiltration. Similar features may be seen in inflammatory arthropathies of ankylosing spondylitis, psoriatic arthropathy, Reiter's disease and other seronegative diseases (Vernon-Roberts, 1978). The diagnostic criteria for classifying RA includes four or more of the following features; 1) morning stiffness, 2) soft tissue swelling, 3) swelling in the proximal interphalangeal, metacarpophalangeal or wrist joints, 4) symmetric swelling, 5) rheumatoid nodules, 6) the presence of rheumatoid factor, 7) radiographic erosions and/or peri-articular osteopenia in hand and/or wrist joints (Arnett et al., 1988).

The inflammatory changes in RA are associated with the breakdown of both cartilage and bone in the rheumatoid joint. The synovial membrane in RA becomes thickened with villous folds. The chronic inflammatory tissue, defined as the pannus, then extends over and into the articular surface from the joint margin. Extension of the chronically inflamed synovial tissue leads to destruction of the articular cartilage and underlying subchondral bone causing juxta-articular erosions and subchondral cysts. Extensive bone erosion is often observed radiographically as joint erosions, and is predictive of a poorer prognosis (Van Zeben et al., 1993). This indicates the importance of studying bone resorption in the region of erosions and subchondral cysts where there is osteoclastic resorption of trabecular bone. It is important to note that the peri-articular bone shows marked juxta articular osteopaenia due to active inflammation and resorption of subchondral bone. The bone erosion is not only localised to the inflamed joint (Gravallese et al., 2000), but can be systemic, with secondary osteoporosis often being associated with RA (Gough et al., 1998).

The area below the lining of the synovium (sublining) in RA is infiltrated with lymphocytes, plasma cells, macrophages and fibroblasts (Ishikawa and Ziff, 1976, Rooney et al., 1989, Yanni et al., 1992, Tak et al., 1997). Peri-vascular cellular infiltrates are either diffuse or form focal accumulations of variable composition of the infiltrating cells (Ishikawa and Ziff, 1976, Tak et al., 1997). The inflammation and tissue destruction in RA is thought to involve interactions between lymphocytes, monocytes/macrophages as well as type A and B synoviocytes. These cell interactions result in the production of matrix metalloproteinases, cathepsins and mast cell proteinases that cause cartilage and bone destruction (Woolley et al., 1977, Trabandt et al., 1991, Tetlow and Woolley, 1995). Recently it has been reported that,

under certain conditions, human osteoclasts can be derived from cells present in or near to the tissues of arthritic joints (Fujikawa et al., 1996b, Toritsuka et al., 1997). Formation of bone resorbing cells (osteoclasts) from cells of the macrophage/monocyte lineage at the cartilage-pannus junction is associated with the destruction of bone matrix in RA patients (Gravallese et al., 2000, Takayanagi et al., 2000, Romas et al., 2000).

Despite the widespread prevalence of rheumatoid arthritis, we are yet to have a complete understanding of the processes of this chronic systemic disease. Most studies have investigated the inflammation that occurs in the soft tissues. However recent advances in our understanding of bone metabolism allow us now to better investigate the mechanisms of the bone loss in RA. This thesis seeks to verify whether precursors or osteoclasts are present within the tissue and to assess the ability of cells isolated from RA tissues to form osteoclasts. Importantly studies will also assess the levels of primary mediators of bone resorption in soft tissue taken from human arthritic joints and compare this with the osteoclastic activity shown in *ex vivo* studies.

1.2.3 Periodontal Disease

Periodontitis is one of the most prevalent human diseases associated with destruction of bone. Destructive periodontal disease, periodontitis, often involves loss of attachment of the periodontal ligament as well as the loss of the adjacent alveolar bone. Gingivitis is a non-specific inflammatory reaction of the gingival tissues that localises to the surface gingival tissues. Interestingly, both diseases are characterised by an inflammatory reaction in the gingival tissues and can occur concurrently. While most periodontitis patients have had gingivitis, not all gingivitis cases develop adult periodontitis (Jeffcoat, 1994). By definition only periodontitis is associated with alveolar bone destruction, where as gingivitis is localised to the soft tissue. The progression from gingivitis to periodontitis has been suggested to be associated with the movement of an inflammatory infiltrate towards alveolar bone (Graves et al., 1998). The susceptibility of an individual to these diseases may be dependent on many risk factors. However, it is clear that certain types of oral bacteria are involved in the initiation and progression of these diseases (Clarke and Hirsch, 1995, Jeffcoat, 1994, Schwartz et al., 2000).

The inflammation and tissue destruction seen in periodontitis is thought to be regulated by infiltrating inflammatory cells, such as T and B lymphocytes, plasma cells and monocytes/macrophages, as well as inflammatory cytokines (Page and Schroeder, 1976, Moskow and Polson, 1991, Iwasaki et al., 1998, Assuma et al., 1998, Graves et al., 1998,

Schenkein, 1999). Gingival crevicular fluid from patients with periodontitis is reported to stimulate bone resorption *in vitro* (Lerner et al., 1998), indicating that the cytokines in the crevicular fluid can promote osteoclast formation and bone resorption (Chu et al., 1992, Deleuran et al., 1992, Chu et al., 1991, Suda et al., 1995, Kotake et al., 1996, Kobayashi et al., 2000a).

Until recently very little work has been carried out on human tissues to identify whether osteoclasts are present in the soft tissue adjacent to localised bone loss in periodontal disease. There are also few reports on the role of primary osteoclast mediators in periodontitis and these have been in animal models (Kong et al., 1999b, Teng et al., 2000). The chapter on periodontitis in this thesis aims to investigate whether precursors or osteoclasts cells are present in the periodontitis tissue. In addition, studies seek to determine whether primary mediators of bone resorption are expressed in tissues from patients with periodontitis. Levels of these mediators are then compared to those seen in tissues obtained from patients without periodontitis.

1.3 THE ORIGIN OF OSTEOCLASTS

1.3.1 The Phenotype of the Osteoclast

The work in this thesis focuses on the formation of osteoclasts in the soft tissue adjacent to sites of localised bone loss in three different diseases. It is therefore important to address what phenotypic features constitute a mature osteoclast. Osteoclasts are defined as being multinucleated giant cells with the ability to resorb mineralised tissue (Athanasou, 1996). It is generally believed that they arise from haematopoietic stem cells of the monocyte-macrophage lineage that, in the process of osteoclast differentiation, lose macrophage markers (such as CD11a, CD11b, CD14, HLA-DR and CD68) and acquire markers specific to osteoclasts (Takahashi et al., 1994, Conner et al., 1995, Fujikawa et al., 1996a). Osteoclasts do not express these markers but possess tartrate resistant acid phosphatase (TRAP) with calcitonin receptors (CTR) (Mundy, 1987) and abundant vitronectin receptors (VNR) (Horton et al., 1985). The function of osteoclasts is associated with the active production of acids and the definitive ability to resorb pits on mineralised tissue (Mundy, 1987). Additional osteoclast markers include carbonic anhydrase II (CAII), cathepsin K (cath K), matrix metalloproteinase MMP9, osteopontin (OPN) and β3 Integrin (Conner et al., 1995, Takeshita et al., 2000). More recently, the receptor activator of necrosis factor κB (RANK) has been identified as a differential marker of pre-osteoclasts and osteoclasts (Nakagawa et al., 1998, Hsu et al., 1999, Burgess et al., 1999, Myers et al., 1999, Arai et al., 1999, Dougall et al., 1999, Li et al., 2000).

Many studies of osteoclast activity have used the expression of TRAP to identify individual osteoclasts. However, caution needs to be taken when using TRAP as the sole marker for mature osteoclasts as positive staining has not always been a reliable marker for osteoclast phenotype in bone (Hattersley and Chambers, 1989b). It is important to note that TRAP has been identified as an enzyme present on human alveolar macrophages (Efstratiadis and Moss, 1985) and develops in macrophages activated *in vivo* (Bianco et al., 1987). In addition to this, pre-osteoclasts that have not yet acquired the ability to resorb bone also stain for TRAP. It is interesting to note that TRAP has been detected on foreign body giant cells in peri-prosthetic tissues (Kadoya et al., 1994), although because the ability to resorb bone was not assessed these cells may actually have been functional osteoclasts. Other markers, such as CTR expression, may better identify mature osteoclasts in tissues near pathological bone resorption, and more than one marker may be needed to conclusively identify mature osteoclasts (Hattersley and Chambers, 1989a).

Earlier it was proposed that the osteoclast is initially mononuclear but becomes multinucleate as it matures (Hattersley and Chambers, 1989b). It was later proposed that multinucleated cells are in fact osteoclasts that have not yet fully differentiated and therefore are unable to resorb bone. The occurrence of mononuclear $23c6^+$ cells before the formation of multinucleate forms was thought to support the belief that osteoclasts are formed by fusion of mononucleate precursors (Sarma and Flanagan, 1996). Fujikawa *et al* proposed that human mononuclear osteoclast precursors circulate in the monocyte fraction, and under the right conditions can differentiate into mature osteoclasts by acquiring osteoclast phenotypic features (Fujikawa et al., 1996a).

It is now well established that both immature cells and mature cells of the monocyte-macrophage lineage can develop into osteoclasts when a suitable microenvironment is provided by bone marrow-derived stromal cells (Tsurakai et al., 1998, Fujikawa et al., 1996a, Udagawa et al., 1990, Quinn et al., 1996). In addition to the monocyte fraction of peripheral blood, precursors of osteoclasts are also present in the marrow compartment and macrophage compartment of extraskeletal tissue (Quinn et al., 1998c). These osteoclast precursors are shown to express phenotypic characteristics of monocyte/macrophage, not osteoclasts (Quinn et al., 1998c). The mononuclear phagocytes may represent a subpopulation of relatively immature monocytes and macrophages capable of proliferating and differentiating into the tissue specific mononuclear phagocyte system cell and in this case become bone-resorbing osteoclasts. This supports the theory that osteoclasts originate in the human mononuclear phagocyte system and may form by fusion of mononuclear phagocytes (Quinn et al., 1998c).

1.3.2 Osteoclast Formation

Recent research has made major advances in our understanding of the process of osteoclast formation. Rodan and Martin (Rodan and Martin, 1982) were the first to hypothesize that osteoblasts played a significant role in the regulation of osteolysis. The early work of Martin *et al* (Martin and Ng, 1994) supported this hypothesis and took it further by proposing the mechanism by which cells of the osteoblast lineage controlled osteoclast formation and activity. Multiple studies have now established the requirement for co-culture with a stromal element in order for osteoclast differentiation to occur. Most of this research has involved using human and animal physiologic models of osteoclast formation *in vitro* and relatively few studies have been carried out in human pathologies thus far.

Models of osteoclast formation have been based on simple co-cultures of cells from the monocyte lineage and osteoblastic cells. It appears cells of the macrophage/monocyte lineage at all stages of differentiation may have the potential to become osteoclasts and that only relatively immature haemopoietic cells, derived from the bone marrow itself, are capable of resorbing bone on bone slices in the absence of added bone stromal cells (Quinn et al., 1998c). When mouse or rat monocytes or spleen cells are cultured with 1,25 dihydroxy vitamin D_3 and murine osteoblasts, TRAP positive cells form within 8 days of culture (Takahashi et al., 1988b, Quinn et al., 1996). In studies with human cells, blood-derived monocytes (positive for CD11a, CD11b, CD14 and HLA-DR) expressed TRAP, vitronectin and calcitonin receptors when incubated with UMR 106 cells in the presence of Macrophage Colony Stimulating Factor (M-CSF) and 1,25 dihydroxy vitamin D_3, and were able to resorb bone by day 14 (Quinn et al., 1997a).

More mature monocytes from the peripheral blood can also become osteoclasts (Fujikawa et al., 1996a, Quinn et al., 1998c, Haynes et al., 1999). Even cells considered to be terminally differentiated, such as murine alveolar macrophages, have been shown to develop into active osteoclasts (Udagawa et al., 1990). The precursors of osteoclasts can be derived from a variety of tissues. Quinn *et al* demonstrated that human monocytes, peritoneal macrophages and bone marrow cells (expressing monocyte/macrophage specific antigens), are capable of differentiating into mature osteoclasts when cultured with a stromal element (UMR 106 cells), M-CSF and dexamethasone (Quinn et al., 1998c). These studies show that cells of the monocyte/macrophage lineage, at various stages of differentiation, present at various sites throughout the body, are capable of becoming bone-resorbing cells. These findings are very relevant to this study as osteoclasts may arise from several sources near orthopaedic implants,

rheumatoid joints and periodontal disease. These can include immature cells present in the bone marrow, recruited monocytes from the peripheral blood and mature tissue macrophages (Takahashi et al., 1988b, Udagawa et al., 1990, Kodama et al., 1991a, Martin and Ng, 1994, Suda et al., 1995, Fujikawa et al., 1996a, Tsurakai et al., 1998, Quinn et al., 1998c).

Not only is the presence of a stromal element required to stimulate osteoclast differentiation and activation but actual cell-cell contact between osteoclast precursors and stromal cells in the microenvironment of the bone must also occur. Separation of monocytes from stromal cells via a membrane filter, even in the presence of 1,25 dihydroxy vitamin D_3 and M-CSF, prevents osteoclasts from forming (Fujikawa et al., 1996a, Takahashi et al., 1988b, Takayanagi et al., 2000). In addition, replacing stromal cells with conditioned media in the presence of 1,25 dihydroxy vitamin D_3 and M-CSF (Fujikawa et al., 1996a, Quinn et al., 1997a, Quinn et al., 1998c) does not support osteoclast formation. The inability of mature osteoclasts to develop under these conditions demonstrates that cell-cell contact is required for differentiation.

It was proposed that the signals that promote osteoclast differentiation from precursor cells act via membrane bound receptors and ligands on both the stromal and osteoclast cells rather than soluble factors (Martin and Ng, 1994). *In situ*, localised activation of these cells may therefore lead to site directed osteoclast formation. Signalling probably acts via membrane bound receptors and ligands on both the stromal and osteoclast cells rather than soluble mediators. These factors are important regulators of osteoclast formation in the peri-prosthetic tissues and will be discussed in detail later in this review.

1.3.3 Osteoclast Activity and Localised Bone Loss

The localised osteoporosis seen in peri-prosthetic loosening, RA and periodontitis may be due to excessive formation of osteoclasts in the tissues in addition to an upregulation in bone resorption, which may not be matched by bone formation. The large numbers of cells of the macrophage/monocyte lineage in the soft tissues adjacent to bone in these diseases are likely to be important in determining the extent of osteolysis by releasing cytokines to upregulate osteolysis or by differentiating into osteoclasts themselves. Evidence that the interface tissue obtained during revision of failed implants, adjacent to areas with osteolysis, actually contains significantly more cells positive for macrophage marker CD11b than areas without lysis (Kadoya et al., 1996) indicates that these cells have an important role in osteoclast formation in these diseases.

There is now strong evidence for the presence of pre-osteoclastic and osteoclastic cells in the peri-prosthetic tissues (Chun et al., 1999) and rheumatoid arthritic tissues (Bromley and Woolley, 1984, Fujikawa et al., 1996c, Gravallese et al., 1998, Hummel et al., 1998). Immunohistochemical studies have been carried out using monoclonal antibodies recognising cytokine receptors found on osteoclastic cells. It has been shown that macrophages and foreign body giant cells, which are seen in the pseudomembrane surrounding loose prostheses, express similar markers to that found on mature osteoclasts (Neale and Athanasou, 1999, Kadoya et al., 1994). This supports the contention that the macrophages present in the peri-prosthetic tissues are a population of cells with the potential to become bone-resorbing osteoclasts, which would significantly contribute to peri-prosthetic osteolysis and implant failure.

Several *in vitro* studies show that mononuclear cells from the peri-prosthetic tissues of patients with prosthetic joint loosening are capable of differentiating into bone resorbing osteoclasts when cultured under appropriate conditions (Sabokbar et al., 1997, Sabokbar et al., 1998, Neale et al., 1999b, Itonaga et al., 2000a). Macrophages from the inflammatory cell infiltrates in the pseudocapsules and pseudomembranes around failed joints that form osteoclasts *ex vivo* usually require co-culture with osteoblastic cells (Sabokbar et al., 1997, Neale et al., 1999b). Support for osteoclast formation can be provided by culture with either rat UMR 106 cells, murine ST2 cells (Sabokbar et al., 1997, Udagawa et al., 1989, Quinn et al., 1997a) or human bone-derived cells (Neale et al., 1999b, Quinn et al., 1998b). In addition, 1,25 dihydroxy vitamin D3 and M-CSF are required for up to 3 weeks before precursor cells differentiate into osteoclasts that resorb bone (Quinn et al., 1998a).

In RA there is an infiltrate of chronic inflammatory cells, which includes lymphocytes, plasma cells and macrophages, in the inflamed synovium from which pannus develops (Fujikawa et al., 1996b). It is important to note that in rheumatoid patients the degree of joint erosion significantly correlates with the number of synovial macrophages in rheumatoid tissue (Yanni et al., 1994). This is a similar finding to that reported in studies with peri-prosthetic tissues and these macrophages are likely to regulate osteoclast formation or differentiate into osteoclasts themselves.

Histopathological analysis of bone erosions in human and experimental RA strongly implicate osteoclasts as the main cell type effecting bone at the site of marginal erosions and bone resorption (Bromley and Woolley, 1984, Gardner, 1992). Osteoclast-like cells have been identified in tissue from the bone-pannus interface of RA patients (Bromley and Woolley,

1984). *In situ* hybridisation has shown these cells to be positive for expression of osteoclast markers such as TRAP, Cathepsin K and CTR (Hummel et al., 1998, Gravallese et al., 1998, Gravallese et al., 2000). TRAP staining in frozen sections of RA synovium shows large numbers of TRAP positive cells in the synovial lining layer and to a lesser degree in the sublining (Suzuki et al., 2001). Studies, similar to those carried out with cells isolated from peri-prosthetic tissue, have shown that osteoclasts can be derived from cells isolated from the joints of patients with rheumatoid arthritis (Fujikawa et al., 1996c, Fujikawa et al., 1996b, Itonaga et al., 2000b, Suzuki et al., 2001). RA synovial macrophages can develop into TRAP, VNR and CTR positive cells capable of resorbing bone by day 14 in the presence of UMR 106 cells, M-CSF and 1,25 dihydroxy vitamin D3 (Fujikawa et al., 1996b).

In addition to monocytes and macrophages in the peri-prosthetic tissues, many large multinucleated cells that resemble mature osteoclasts are also present. Multinucleated giant cells are uncommon in the rheumatoid synovium (Kotake et al., 1996) but in peri-prosthetic tissue, these cells, which often contain particles of prosthetic material, are similar to giant cells seen in granulomatous tissue during the immune reaction to foreign bodies. It is possible that these are mature osteoclasts, which have phagocytosed particles. Previously it was believed that osteoclasts do not phagocytose wear particles at the bone-implant interface (Athanasou, 1996). However, *in vitro* studies have shown that human osteoclasts derived from giant-cell tumours have the ability to phagocytose small particles of latex, polymethymethacrylate (PMMA) and titanium in culture and retain the ability to resorb bone (Wang et al., 1997). It is also possible that the multinucleated cells containing wear particles are in the process of differentiating from macrophages to mature osteoclasts (Kadoya et al., 1996). This is supported by *in vitro* cultures where murine macrophages having phagocytosed PMMA, polyethylene (PE), titanium or cobalt chromium particles, are also able to differentiate into TRAP positive cells that are able to resorb bone when cultured with UMR 106 cells and 1,25 dihydroxy vitamin D3 (Sabokbar et al., 1998). It is therefore possible that many of these multinucleated cells are mature osteoclasts that either phagocytose particles or have derived from macrophages in the peri-prosthetic tissues that have phagocytosed particles previously.

In the context of inflammatory disease where there is a large macrophage infiltrate, osteoclast differentiation from monocytes and macrophages could determine pathological bone destruction in these regions.

1.4 RECRUITMENT OF OSTEOCLAST PRECURSORS

1.4.1 Chemokines

The accumulation of large numbers of cells of the macrophage/monocyte lineage, contributing to granuloma formation, is a characteristic of several pathologies. The accumulation of macrophages in the tissues adjacent to bone is likely to be a significant initial event in pathological bone loss as cells of the monocyte/macrophage lineage can differentiate into mature osteoclasts (Fujikawa et al., 1996a, Quinn et al., 1998c, Quinn et al., 1996). This granulomatous response is often initiated by pathogens or foreign materials, which are not easily removed or degraded.

Over the past decade we have begun to understand the role chemokines play in the recruitment of macrophages in the formation of granulomas. Numerous chemokines have been identified that are likely to be involved in the recruitment of macrophages and other cells that form granulomas. In addition, chemokines are involved in the activation of fibroblast-like synoviocytes by enhancing production of interleukin (IL)-6 and IL-8 (Nanki et al., 2001). These chemotactic molecules have been classified into four distinct groups according to the arrangement of cystine amino acid groups in their structure and several recent papers review the classification of both chemokines and their receptors (Murphy et al., 2000, Lloyd, 2002, Ajuebor and Swain, 2002).

Chemokines can be involved in the recruitment of many types of cells but it is those that are involved in the recruitment of monocytes/macrophages and lymphocytes that are likely to be most important in peri-prosthetic, RA and periodontal osteolysis. Chemokines belong to a family of molecules that are different from the more classical chemoattractants such as fragments C3a and C5a of complement, platelet activating factor, other metabolites of arachidonic acid and bacterial products. It is those that belong to group of chemokines known as the CC chemokines that are most likely to be involved in granuloma formation in the peri-prosthetic tissues. Three important CC chemokines are chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and RANTES and these are likely to be most important as they are reported to be chemotactic for both monocytes and activated T cells (Lloyd, 2002).

There is strong evidence that MCP-1 is important in granulomatous formation as abnormalities in monocyte recruitment and granuloma formation are seen MCP-1 deficient mice (Lu et al., 1997) as well as in mice with knockout of the chemokine receptor (CCR)2 gene, the cell surface receptor for MCP-1 (Gao et al., 1997, Boring et al., 1997, Warmington

et al., 1999). Several other chemotactic molecules have been implicated in studies on experimental and human granulomatous diseases showing strong expression of MIP-1α (Hashimoto et al., 1998) and RANTES (Chensue et al., 1999, Petrek et al., 1997, Hogaboam et al., 1998) as well as MCP-1 (Hashimoto et al., 1998, Friedland et al., 1993, Desai et al., 1999, Conti et al., 1997, Conti et al., 1999). MCP-1 and MIP-1α are implicated in the recruitment of osteoclasts (Kukita et al., 1997, Volejnikova et al., 1997, Zheng et al., 1998) and MIP-1α may also mediate osteoclast differentiation (Scheven et al., 1999).

There are reports that chemokines are present in the interface tissue around loosening implants (Ishiguro et al., 1997), and that prosthetic particles stimulate production of molecules chemotactic for monocytes (Ishiguro et al., 1997, Rhodes et al., 1997, Nakashima et al., 1999). Three members of the C-C group of chemokines, MCP-1, MIP-1α and RANTES, were stimulated by prosthetic particles *in vitro* (Nakashima et al., 1999). The *in vitro* studies indicate that different types of prosthetic particles may induce different patterns of chemokines.

The granulomatous response is also observed in RA and periodontal disease. In RA it may occur as an autoimmune reaction but this is still being debated. In periodontal disease the granulomatous reaction occurs as a host response to bacteria present in the periodontal tissue. The accumulation of macrophages in these tissues is likely to be a significant initial event in osteolysis.

1.4.2 Interleukin (IL)-8

Interleukin (IL)-8 is a chemokine that functions as a mediator in diverse inflammatory disorders by promoting the recruitment, proliferation, and activation of vascular and immune cells. The synovial-like interface membrane and pseudocapsular tissues from peri-prosthetic samples obtained at total hip replacement have significantly higher levels of IL-8 compared to normal knee synovium (Shanbhag et al., 1995a). IL-8 is also elevated in similar bone loss pathologies such as RA (Rothe et al., 1998). Dual immunostaining identified fibroblastic cells as the cell type producing IL-8 in the peri-prosthetic tissue (Lassus et al., 2000). Human osteoclasts isolated from osteoporotic femoral heads have also been shown to synthesise mRNA for IL-8 and release IL-8 protein in culture (Rothe et al., 1998). IL-8 is also present at high levels in periodontitis lesions, where it is associated with the junctional epithelium and macrophages (Tonetti et al., 1994, Fitzgerald and Kruetzer, 1995).

Inflammatory stimuli such as LPS, IL-1α and tumour necrosis factor (TNF)-α significantly increase IL-8 mRNA expression and IL-8 protein released from osteoclasts. In contrast non-inflammatory cytokines and systemic hormones such as IL-6, TGF-β1 and TGF-β3 do not stimulate IL-8 release (Rothe et al., 1998). Rothe (Rothe et al., 1998) suggested human osteoclast derived IL-8 may be an important autocrine/paracrine mediator of bone cell physiology and immunoregulation involved in normal or pathological bone remodelling.

It is likely that a variety of chemokines play a key role in the bone loss seen in implant loosening, RA and periodontal disease. As yet we do not know which particular chemokines are the most important in the formation of osteoclasts in peri-prosthetic, RA and periodontal osteolysis. This information may identify targets for therapy in the future as new drugs become available which inhibit the activity of individual chemokines.

1.5 LOCAL AND SYSTEMIC REGULATORS OF OSTEOCLAST FORMATION ACTIVITY

Osteoclast formation occurs in an environment driven by a balance between positive and negative influences. Normal bone metabolism is regulated by a plethora of cytokines and hormones whose negative and positive influences are in balance. In pathological states, where there is excessive bone loss, there is likely to be an excess of factors that stimulate bone resorption relative to the levels in those factors that inhibit bone resorption.

1.5.1 Parathyroid Hormone (PTH)

Parathyroid hormone is a systemic hormone involved in bone remodelling. PTH has a critical role in calcium metabolism and its effects are often systemic. PTH was considered a bone catabolic agent, however current evidence demonstrates differing effects on the bone dependent on how PTH is administered. A chronic continuous excess of PTH increases bone resorption (Fujita, 2001) and at high levels has been shown to inhibit osteoblast synthesis of collagen and other matrix proteins (Athanasou, 2001). Conversely, when delivered in low doses intermittently, PTH causes an increase in bone mass (Fujita, 2001). This may be because PTH causes an increase in the number of osteoblasts in bone (Athanasou, 2001). The main stimulus of PTH is a fall in serum ionised calcium levels. PTH levels are also affected by the systemic levels of magnesium, vitamin D metabolites, prostaglandins and hormones. PTH has a direct stimulatory effect on osteoclast formation from mononuclear precursors and an indirect effect on stimulating osteoclast bone resorbing activity via osteoblasts.

1.5.2 Prostaglandins (PGE_2)

Prostaglandins are non-peptide local factors that affect both osteoblastic cells and cells of the mononuclear phagocyte lineage. The action of different prostanoids in the regulation of bone turnover is quite complex and they have been shown to have opposing effects. The differing effects of prostanoids may lie in the timing and location of their release and the fact that they have a relatively short half-life *in situ.*

PGEs may have different effects on osteoclast development at different stages of osteoclast differentiation. PGE_2 was shown to stimulate osteoclastic bone resorption in bone organ culture systems and stimulate osteoclast formation in murine marrow cultures (Takahashi et al., 1988a). PGE_2 has been identified in the peri-prosthetic psuedosynovial membrane (Perry et al., 1997). Arthroplasty derived cells, cultured with human bone cells, form bone resorbing osteoclasts after 14 days incubation (Neale et al., 1999b). The addition of exogenous PGE_2 in this cell culture system caused a dose-dependent increase in lacunar bone resorption of two–three times greater that untreated controls after 14 days incubation(Neale et al., 1999b). Conversely, while it appears to be a requirement for normal osteoclast formation, excessive PGEs may inhibit osteoclast formation (Quinn et al., 1997b, Quinn et al., 1994). Other arachidonate metabolites may also be involved in osteoclast formation in the peri-prosthetic tissues. Anderson *et al* have developed a culture system that uses particles to stimulate osteoclast formation (Anderson et al., 2001). Using this model they showed inhibition of osteoclast formation by specific inhibitors of leukotriene synthesis. This indicates other metabolites of Arachidonic acids besides PGEs may regulate osteoclast formation in disease.

In periodontal disease a significant amount of PGE_2 can be produced in the inflamed tissues (Leibur et al., 1999). Levels of PGE_2 in the gingival crevicular fluid are reduced in patients with improved clinical parameters following treatment (Leibur et al., 1999). This suggests PGE_2 may be involved in the pathogenesis of dental disease.

While prostaglandins and leukotrienes are important mediators of osteoclast formation, it is not known if they have a direct effect on the process or if their effects are mediated via regulation of other important factors involved in oseoclastogenesis.

1.5.3 Interleukin (IL)-1

Interleukin (IL)-1 is a pro-inflammatory multifunctional cytokine, which upregulate bone resorption and is involved in recruitment of inflammatory cells, stimulation of eicosanoid (specifically PGE_2) release by monocytes and fibroblasts and stimulation of matrix

metalloproteinases release. The ability of IL-1β to stimulate bone loss in disease is well-recognised (Gowen et al., 1983). IL-1β acts directly on pre-osteoclasts and osteoclasts via functional IL-1R and appears to be important in the coordinated signalling between pre-osteoclasts and osteoblastic cells during osteoclast formation (Haynes et al., 1999). The addition of IL-1β to mononuclear osteoclast like cells *in vitro* prolongs the survival of these cells and induces multinucleation (Jimi et al., 1999). IL-1β may act by inducing actin ring formation by multinucleated cells in cultures of osteoclast like cells, in the absence of stromal cells, as well as bone resorption on dentine. In addition, IL-1β may also prevent the death of osteoclasts by preventing apoptosis (Jimi et al., 1999). It has also recently been suggested that IL-1β may even induce osteoclastic activity in the absence of osteoblast/stromal cells (Jimi et al., 1999).

Levels of IL-1 are raised in peri-prosthetic tissue from revision samples (Konttinen et al., 1996) as well as in the synovial fluid of RA patients (Kahle et al., 1992, Arend and Dayer, 1995, Kotake et al., 1996). In general IL-1 is also elevated in the periodonatal tissues (Jandinski et al., 1991, Stashenko et al., 1991) as well as the gingival crevicular fluid (Preiss and Meyle, 1994) from inflamed periodontal tissues compared to normal healthier sites. The inflammatory response and bone loss in experimental periodontitis can be inhibited by antagonists to IL-1 and TNF, demonstrating the role IL-1 may have in bone loss in this disease (Assuma et al., 1998).

In vitro both murine ST-2 stromal cells and human peripheral blood mononuclear cells (PBMC) express mRNA encoding IL-1 and IL-1 receptor (Atkins et al., 2000a). In the peri-prosthetic tissues macrophages containing wear particles express both IL-1β and TNF-α protein (Jiranek et al., 1993, Appel et al., 1990, Chiba et al., 1994, al-Saffar and Revell, 1994, Perry et al., 1997). Macrophages, foreign body giant cells and osteoclasts isolated from peri-prosthetic tissues also express IL-1β receptor (Neale and Athanasou, 1999). The detection of IL-1β on fibroblasts, without concurrent mRNA expression, suggests macrophages release IL-1β that then binds to fibroblasts and possibly macrophages (Jiranek et al., 1993).

It is possible that osteoclast formation may be mediated by the release of IL-1 in peri-prosthetic, RA and periodontal osteolysis diseases. This is quite important in the light of new data that IL-1 may act as a co-factor for and may be synergistic with receptor activator of nuclear factor (NF)-κB ligand (RANKL) (as discussed below), or induce osteoclast formation without a stromal population (Jimi et al., 1999).

1.5.4 Tumour necrosis factor (TNF)

In inflamed joints, tumour necrosis factor (TNF)–α is primarily produced by activated monocytes/macrophages (Goodman et al., 1998), while TNF-β (lymphotoxin) is produced by activated T lymphocytes. TNF-α mediates leukocyte recruitment and activation, synovial macrophage and fibroblast cell proliferation, increased prostaglandin and matrix degrading MMP activity, as well as bone and cartilage destruction. The production of TNF-α in inflammation has long been recognised as an inducer of bone resorption (Bertolini et al., 1986). In support of this, a correlation has been identified between the presence of TNF and the severity of osteolysis (Stea, 2000).

TNF-α has been observed on macrophages in areas containing wear debris as well as on fibroblasts and some endothelial cells (Xu et al., 1996) and there are numerous reports of how many types of prosthetic wear particles stimulate macrophages to release TNF-α *in vitro* (Haynes et al., 1993, Merkel et al., 1999). TNF-α and its receptors are also upregulated in the synovial fluid and membranes from patients with active rheumatoid arthritis (Chu et al., 1991, Arend and Dayer, 1995). The administration of TNF antagonists to patients with RA has been shown to reduce symptoms (Elliott et al., 1993, Moreland et al., 1997) and is now used as an alternative treatment in this disease.

In addition to having a direct effect on osteoclast formation, TNF can act indirectly to induce factors expressed by osteoblasts that promote osteoclast formation (Haynes et al., 1997). TNF is also reported to suppress osteoblast activation involved in bone formation (Canalis, 1986, Smith et al., 1987). It is important to note that recent studies have suggested that TNF-α may stimulate osteoclastogenesis directly, in the absence of RANKL (Kobayashi et al., 2000a). However this may be dependent on the exposure of macrophages to low levels of RANK ligand (Lam et al., 2000).

1.5.5 Interleukin (IL)-6

Interleukin (IL)-6 stimulates plasma cell proliferation and antibody production and is produced by lymphocytes, monocytes and fibroblasts. In addition, murine ST-2 stromal cells, which support osteoclast formation *in vitro*, have also been shown to express mRNA encoding IL-6 and IL-6R (Atkins et al., 2000a). The importance of IL-6 in osteoclast formation is demonstrated by experiments using an IL-6 receptor antagonist (Sant 5) in human marrow cultures. Sant 5 blocks IL-6, IL-1 and TNF induced osteoclast formation, whereas the addition of recombinant IL-6 stimulates osteoclast formation (Devlin et al.,

1998). This indicates that IL-6 mediates the effects of IL-1 and TNF during human osteoclast formation further highlighting its role in bone metabolism (Devlin et al., 1998).

IL-6 and soluble IL-6 receptors are elevated in the synovial fluids of RA patients (Kotake et al., 1996) and are produced by macrophages and other cells present in the peri-prosthetic tissues during osteoclast formation (Goodman et al., 1998, Neale et al., 1999a). IL-6 is also elevated in inflamed periodontal tissues with notably higher levels in periodontitis tissue than in gingivitis tissue (Yamazaki et al., 1994, Geivelis et al., 1993).

IL-6 is thought to have a role in regulating the inflammatory response within the bone implant interface as well as having an adverse effect on the process of bone remodelling (Devlin et al., 1998). It is important to note that IL-6 has been positively related to the severity of osteolysis around prostheses, although not to the same extent as TNF (Stea, 2000). Furthermore, the IL-6 receptor is present on the macrophages and giant cells, possible precursors of osteoclasts, which are present in these peri-prosthetic tissues (Neale and Athanasou, 1999).

Together these findings show that IL-6 produced in soft tissue adjacent to the bone interface may regulate differentiation of osteoclast precursors and the activity of osteoclasts at the interface of bone and soft tissue.

1.5.6 Transforming Growth Factor (TGF)-β

Molecules belonging to the Transforming Growth Factor (TGF)-β superfamily are known to regulate cell proliferation, differentiation and apoptosis (Moses et al., 1996). TGF-β itself has been shown to stimulate the differentiation of osteoblast cells (Linkhart et al., 1996). However, the effect of TGF-β on osteoclast cells is still controversial. TGF-β is clearly important in modulating the healing of bone and soft tissue but may have opposing effects in bone loss pathologies as it has been reported to stimulate (Kaneda et al., 2000) and inhibit (Chenu et al., 1988) osteoclast formation. In the bone matrix TGF-β was found to strongly inhibit the proliferation and fusion of human osteoclast precursors (Chenu et al., 1988). However, more recently, the addition of TGF-β to cultures of haematopoietic cells, treated with RANKL and M-CSF, increased TRAP- and CTR-positive multinucleated cell formation capable of resorption (Galvin et al., 1999). In addition, TGF-β1 pathways have been shown to stimulate osteoclast-like cell formation in isolated bone marrow macrophages and mouse monocytic cell line RAW264.7 (Koseki et al., 2002).

TGF-β is not only produced by osteoblast cells but has been shown in peri-prosthetic tissues near loose hip implants where it may modulate bone metabolism (Konttinen et al., 1997). In peri-prosthetic tissue TGF-β has been associated macrophages (Goodman et al., 1998). *In vitro* both murine ST-2 stromal cells and PBMC express mRNA encoding TGF-β (Atkins et al., 2000a). The actions of TGF-β in pathological bone loss are uncertain but its activities may depend on the types of cells and cytokines present in the soft tissues adjacent to the bone.

1.5.7 Macrophage Colony Stimulating Factor (M-CSF)

Macrophage colony stimulating factor (M-CSF) is an important, if not essential, cytokine regulating bone metabolism. There is good evidence suggesting M-CSF is an essential factor for both proliferation of osteoclast progenitors, and their differentiation into mature osteoclasts, in mouse and human *in vitro* and *in vivo* models (Fujikawa et al., 1996b, Quinn et al., 1997a, Quinn et al., 1998a, Suda et al., 1992, Sarma and Flanagan, 1996, Suda et al., 1999). M-CSF is thought to work in concert with other factors derived from osteoblastic cells.

The osteopetrotic mouse of the type *op* has been used in investigating CSF-1. In these mice daily injection of CSF-1 reverses the osteopetrotic phenotype. This provided evidence that osteoclast formation depends on this cytokine (Kodama et al., 1991b).

M-CSF is known to enhance osteoclast formation at two stages of osteoclast formation. Firstly, during the early stages of *in vitro* osteoclast formation, it is thought to induce proliferation of pre-osteoclasts and, secondly, during the final differentiation phase of osteoclast formation (Tanaka et al., 1993). M-CSF may also prolong the survival of mature osteoclasts, as well as modulate their osteoclastic activity *in vitro* (Fuller et al., 1993, Edwards et al., 1998). These cultures were of human mononuclear cells and rodent stromal cells and required the addition of recombinant hM-CSF. This suggests that the M-CSF produced by rodent stromal cells does not bind to the human receptors (Fujikawa et al., 1996a).

M-CSF production by activated cells in the soft tissue adjacent to localised bone loss is likely to be an important factor in osteoclast formation. It is therefore important that, in addition to bone marrow stromal cells and osteoblasts, M-CSF has been associated with a variety of cell types, such as monocyte/macrophages, fibroblasts, and vascular endothelial cells (Takei et al., 2000). M-CSF is present in the synovial fluid and in the synovial-like membrane of peri-prosthetic tissues taken from patients with aseptic loosening (Xu et al., 1997, Takei et al., 2000). M-CSF is also present in RA synovial tissue (Takei et al., 2000) and periodontal tissue

(Rani and MacDougall, 2000) where its release may be involved in the perpetuation of bone erosion in these inflammatory diseases. Higher levels of M-CSF have been identified in the peri-prosthetic tissues from adjacent to failed prostheses than in the synovial membrane of patients undergoing primary hip replacement (Xu et al., 1997). It is important to note that M-CSF receptor is present on the surface of macrophages and foreign body giant cells in the peri-prosthetic tissues (Neale et al., 1999a). These findings support the contention that M-CSF plays an important role in the regulation of osteoclastogenesis in disease.

Studies of osteoclast formation from cells isolated from the peri-prosthetic tissues illustrate the importance of M-CSF and its receptor in peri-prosthetic osteolysis. High levels of M-CSF are produced during osteoclast formation *in vitro* in co-cultures of arthroplasty derived mononuclear cells and human osteoblastic cells (Neale et al., 1999a). The addition of antibodies to block endogenous human M-CSF binding to its receptor markedly reduced the numbers of osteoclasts that formed in these co-cultures. However, the addition of exogenous M-CSF or IL-6 to these cultures only slightly increased the numbers of osteoclasts that formed. It is significant to note that, unlike other models of *in vitro* osteoclast formation, additional M-CSF was not required for osteoclast formation from these cells *in vitro*. This suggests that there are cells in the peri-prosthetic tissue that are releasing enough M-CSF required to mediate osteoclast formation (Neale et al., 1999a).

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a growth factor related to M-CSF, which is also present in the bone implant interface tissues (al-Saffar et al., 1996). Like M-CSF it regulates the growth and maturation of cells of the macrophage monocyte lineage and is reported to stimulate osteoclast formation in much the same way as M-CSF. However, GM-CSF is weaker stimulator than M-CSF of osteoclast formation so it may not be as important as M-CSF in mediating osteolysis associated with peri-prosthetic loosening, RA and periodontitis.

1.5.8 Interleukin (IL)-11

IL-11 is a multifunctional cytokine, closely related to IL-6 with many of its properties overlapping with those of IL-6 (Blaumann and Schendel, 1991). IL-11 has been implicated in the regulation of bone metabolism in normal physiology and disease (Yang, 1993). It can act by inducing osteoclastogenesis (Girasole et al., 1994) and osteoblast mediated osteoid degradation (Hill et al., 1998). IL-11 is not produced by T cells or monocytes but is probably produced by cells of the mesenchymal lineage (Lisignoli et al., 2000). Cells of the murine osteoblast cell line, ST-2, express mRNA encoding IL-11 *in vitro* (Atkins et al., 1999).

Immunohistochemical staining has identified IL-11 in the interface and pseudocapsular tissues obtained during THR for aseptic loosening (Xu et al., 1998). Cells expressing IL-11 are more numerous at the bone-implant interface and pseudocapsular tissues from patients with aseptic loosening than in control synovial tissues from patients undergoing primary hip replacement. The presence of IL-11 in peri-prosthetic tissue suggests it may be another factor involved in stimulating peri-prosthetic osteolysis. IL-11 has also been shown to have a role in regulating the inflammatory process in the RA synovium (Walmsley et al., 1998). In rheumatoid arthritis IL-11 is produced predominantly by the synovial cells at both the RNA and protein level (Taki et al., 1998) and is detected in both the serum and synovial fluid (Trontzas et al., 1998). It is likely that IL-11 production in the RA joint is involved in bone destruction via osteoclasts (Taki et al., 1998), however this needs to be verified by further studies.

1.5.9 Martix metalloproteinases (MMP)

Matrix metalloproteinases (MMP) are believed to play an important role in joint destruction in a range of arthritides and osteolytic diseases, by contributing to degradation of cartilage extracellular matrix. Fibroblasts from the interface membrane can be stimulated by wear particles to produce metalloproteinases in culture (Yao et al., 1995). Elevated levels of matrix MMP, reported near loose artificial hip joints (Takagi et al., 1998) and in the synovial fluids from RA patients (compared with osteoarthritis (OA)) (Yoshihara et al., 2000), can cause extracellular matrix degradation associated with osteolysis (Takagi et al., 1998). The MMP-specific degrading activity of RA synovial fluid may contribute to cartilage destruction and eventual bone loss. Tissue inhibitors of metalloproteinases (TIMPs) specifically regulate the enzymatic activity of MMPs. The balance between MMPs and TIMPs is thought to be particularly important in determining resultant cartilage damage. A recent study found the molar ratio of MMPs to TIMPs was 5.2-fold higher in RA patients compared with OA patients (Yoshihara et al., 2000).

1.5.10 Interleukin (IL)-17

IL-17 is a T cell derived cytokine involved in inflammation(Kotake et al., 1999). IL-17 is present in the synovium of rheumatoid patients (Chabaud et al., 1999) and is significantly higher in the synovial fluid of RA patients compared with OA patients (Kotake et al., 1999). IL-17 is reported to stimulate osteoclast activity and bone lysis in rheumatoid arthritis (Kotake et al., 1999). In fact, the addition of anti-IL-17 in culture significantly inhibits osteoclast formation induced by the culture media of RA synovial tissues (Kotake et al., 1999). Recent

studies using a murine model also suggest a role for IL-17 in cartilage and bone destruction. The intra-articular administration of IL-17 into a normal mouse induced cartilage destruction. Conversely, blocking IL-17 with specific inhibitors protected the bone by inhibiting bone destruction (Chabaud et al., 2001). The effect of IL-17 on bone and cartilage may be explained by its ability to stimulate the production of the pro-inflammatory cytokines (IL-1, TNF-α) from macrophages as well as IL-6, IL-8, PGE_2 and RANKL expression by stromal/osteoblast lineage cells (Kotake et al., 2001).

Whether IL-17 is involved in peri-prosthetic osteolysis is yet to be demonstrated. As IL-17 is a T-cell derived cytokine, its role in peri-prosthetic osteolysis may not be as important as the factors mentioned above because T cells are in low numbers in this tissue (Vernon-Roberts and Freeman, 1977, Jiranek et al., 1993, Jacobs et al., 1994). However a recent study involving a two-species model of osteoclast formation with co-culture of human PBMC and ST-2 cells (murine derived stromal cells) found mRNA encoding IL-17 to be expressed by both cell lineages. The production of IL-17 by stromal cells may therefore be involved in physiological and pathological bone loss in various diseases (Atkins et al., 2000a).

1.5.11 Other Factors Involved in Osteolysis

Other factors may be involved in osteolysis and it has been disputed that the acid- and cathepsin-K- driven pathological mechanism of bone resorption in aseptic loosening of total hip replacements is mediated by macrophages rather than by osteoclasts in the subosteoclastic space (Konttinen et al., 2001). The proposed mechanism is based on acidification and bone mineral dissolution. However the localisation of the enzyme found in the interface tissue macrophage/giant cells, pseudosynovial fluid and tissue extracts indicates that this occurs in the peri-prosthetic soft tissue-bone interface rather than the subosteoclastic space (Konttinen et al., 2001).

1.5.12 Inhibitors of Osteolysis

IL-4, IL-10, IL-12 and IL-18 are cytokines that may inhibit osteoclast formation (Riancho et al., 1993, Owens et al., 1996, Horwood et al., 2001). There is a negative correlation between IL-10 production and radiographic joint damage and progression of joint damage (Verhoef et al., 2001) indicating a protective role in RA. IL-18 mRNA is expressed by both stromal and human PBMC when cultured (Atkins et al., 2000a). Recently a number of important co-activators of RANK and other inhibitors of RANKL activity have been identified (Zhou et al., 2001) and there are several reports that other cytokines may induce osteoclast formation in the absence of RANK stimulation. While it is possible that they may have a role to play in peri-

prosthetic, RA and periodontal osteolysis, the relative levels of these cytokines have not been widely investigated in these pathological tissues. Understanding the molecular mechanisms of bone loss in these pathologies can lead to strategies for therapy.

1.6 KEY MEDIATORS OF OSTEOCLASTOGENESIS

1.6.1 Receptor Activator NF Kappa B Ligand (RANKL)

Although the factors described above influence osteoclast formation it was suggested that there was a previously unidentified factor essential for the differentiation of osteoclasts from their precursors. The requirement for cell-to-cell contact between osteoclast precursors, such as monocytes, and osteoblastic cells led to the concept that an essential signal promoting osteoclast differentiation was mediated via ligands or receptors expressed by stromal or osteoblast cells (Rodan and Martin, 1982, Martin and Ng, 1994). The membrane bound factor regulating osteoclast differentiation on the surface of stromal and/or osteoblast cells was the focus of much investigation (Suda et al., 1995). This factor was eventually cloned simultaneously by two groups, Snow Brand Milk Co in Japan (Yasuda et al., 1998b) and AMGEN in the USA (Lacey et al., 1998), and named osteoclast differentiating factor and OPG-ligand respectively. Using an expression library of the murine myelomoncytic cell line 32D, a ligand for OPG, the soluble inhibitor of osteoclastogenesis discussed below, was cloned and found to be identical to ODF. OPGL was also found to be identical to TRANCE (TNF-related activation induced cytokine) (Wong et al., 1997a) and RANKL (receptor activator of nuclear factor (NF)-κB ligand) (Anderson et al., 1997), previously identified by independent groups as a novel member of the TNF ligand family. TRANCE was identified in murine T cell hybridomas (Wong et al., 1997b) while RANKL was cloned from a cDNA library of murine thymoma EL4-5 cells (Anderson et al., 1997). Soon after identifying the elusive osteoclast differentiating factor, many studies demonstrated that it is an essential factor in human osteoclastogenesis (Quinn et al., 1998a, Yasuda et al., 1998b, Matsuzaki et al., 1998).

The nomenclature of this molecule has recently been resolved. It has been decided that RANKL (or RANK ligand) will be the term used for the molecule known as OPGL/TRANCE/ODF (Nomenclature, 2000) and this term will be used throughout this thesis.

RANKL is a type 2 transmembrane glycoprotein with a long extracellular stalk region followed by a receptor-binding core domain (Suda et al., 1999, Lacey et al., 1998). This transmembrane protein is synthesised as a membrane-bound protein but can be cleaved into a

soluble form by metalloproteinase and TNF-α converting enzyme (TACE) (Lum et al., 1999). However, membrane-bound RANKL is significantly more efficient than soluble RANKL in the generation of osteoclasts (Nakashima et al., 2000).

Analysis of cultures lacking in osteoblast/stromal cells have shown that RANKL is not expressed by osteoclast lineage cells (Hakeda et al., 1998) but is in fact on the membrane of osteoblasts (Yasuda et al., 1998b). The binding of {^{125}I} sRANKL onto C7 cells provides evidence that RANKL has a direct effect on osteoclast progenitors (Yasuda et al., 1998b). RANKL is now known to be essential for osteoclastogenesis and acts on osteoclast precursors via a membrane receptor, RANK (receptor activator of NF-κB) (Nakagawa et al., 1998, Hsu et al., 1999). RANKL acts through three distinct intracellular pathways, VD receptor, protein kinase A and gp130 (Yasuda et al., 1998b).

It is important to note that in addition to RANK, RANKL can also bind to osteoprotegerin (OPG), a secreted receptor (Yasuda et al., 1998b, Lacey et al., 1998, Simonet et al., 1997). OPG can function as a decoy receptor, preventing RANKL from interacting with RANK, thereby inhibiting osteoclastogenesis (Tsuda et al., 1997). OPG is discussed in detail below.

In the absence of stromal cells, the addition of soluble forms of RANKL and M-CSF can stimulate formation of TRAP positive, VNR positive osteoclasts capable of resorbing bone when added to adherent human PBMC (Matsuzaki et al., 1998). More specifically, highly purified CD14+ cells selected from a human PBMC population are able to differentiate into osteoclasts (Nicholson et al., 2000). The generation of osteoclasts in the absence of a stromal element *in vitro* has demonstrated the key role RANKL has in osteoclastogenesis (Matsuzaki et al., 1998, Nicholson et al., 2000, Tsurukai et al., 2000). The generation of osteoclasts from purified $CD14^+$ cells characterizes the phenotype of circulating osteoclast precursors (Nicholson et al., 2000)

Not only does RANKL stimulate osteoclast formation but it is also a necessary stimulus for the hormone-mediated activation of mature osteoclasts (Fuller et al., 1998). RANKL affects mature osteoclasts by signalling through RANK to induce rearrangement of the actin cytoskeleton into actin rings and to induce formation of the specialised extracellular bone resorbing compartments (Fuller et al., 1998, Burgess et al., 1999). Activation of the mature osteoclasts can occur in the absence of M-CSF (Burgess et al., 1999). Although RANKL does not increase the number of multinucleate osteoclasts, it increases the surface area of bone resorbed by each cell, causing multiple, spatially associated cycles of resorption *in vitro*

(Burgess et al., 1999). The administration of RANKL causes an immediate rise in serum calcium levels in mice indicating that it has a rapid and direct effect on osteoclast activity**.** Further to this, the effects of RANKL on both actin ring formation and bone resorption could be blocked by OPG in these animals (Burgess et al., 1999).

RANKL knockout mice exhibit osteopetrosis and defective tooth eruption associated with a complete absence of osteoclasts (Kong et al., 1999b). Irregular bone surfaces due to the defect in remodelling, abnormal growth plates with club-shaped long bones, as well as growth retardation at several skeletal sites, are also observed (Kim et al., 2000b). Conversely, administration of soluble RANKL in normal mice induces hypercalcemia (Lacey et al., 1998). Knockout mice also demonstrate defects in T-cell and B-cell maturation, as well as an absence of lymph nodes (Kong et al., 1999b, Kim et al., 2000a). These findings also suggest a role in immune cell differentiation.

RANKL plays a key role in several bone pathologies. RANKL protein and mRNA are produced by synovial fibroblasts and activated T cells in the arthritic joints of rats with adjuvant arthritis (Kong et al., 1999a). In rheumatoid arthritis (Romas et al., 2000, Gravallese et al., 2000) and periodontal disease (Teng et al., 2000) T cells have been suggested as the major source of RANKL. Production of RANKL by activated T cells may also enhance dendritic cell survival by interacting with high levels of its receptor present on dendritic cells (Wong et al., 1997a).

While the production of RANKL by T cells may stimulate osteoclast formation particularly in rheumatoid arthritis and periodontal disease, this is yet to be proven in osteolysis around implants. The relatively low numbers of T cells present near peri-prosthetic osteolysis make it unlikely that T cells are the major source of RANKL in peri-prosthetic osteolysis (Vernon-Roberts and Freeman, 1977, Jiranek et al., 1993, Jacobs et al., 1994). The partial rescue of osteoclast formation in RANKL-deficient mice, through selective expression of RANKL in T cells by adoptive transfer, indicates that T cells may contribute to osteoclast function under non-pathological conditions as well (Kim et al., 2000b).

Fibroblasts have also been suggested as a major source of RANKL in RA tissue (Quinn et al., 2000, Gravallese et al., 2000). Murine fibroblastic stromal cells from various tissues express RANKL mRNA and are able to support osteoclast formation from spleen cells in culture in the presence of dexamethasone (Quinn et al., 2000). Synovial fibroblasts from sites of bone lysis in RA are also reported to support osteoclast formation by producing RANKL

(Takayanagi et al., 2000). These data suggest the possibility that fibroblasts associated with bone loss around implants may directly stimulate osteoclast formation by presenting RANKL to monocyte/macrophages in these tissues.

While the presence of soluble RANKL has yet to be reported in the synovial fluid of patients with joint loosening, it has been identified in the synovial fluid from patients with rheumatoid arthritis (Kotake et al., 2001). Interestingly, elevated levels were also associated with active osteolysis in this disease (Kotake et al., 2001). While it is possible that there are elevated levels of soluble RANKL in the joint fluids of patients with peri-prosthetic osteolysis the significance is yet to be determined, as membrane-bound RANKL is reported to work more efficiently than soluble RANKL (Nakashima et al., 2000). The studies in this thesis were designed to investigate the cells types producing RANKL in peri-prosthetic tissues, RA and periodontitis in human tissues. In addition, the levels of RANKL mRNA and protein were investigated.

There are claims that RANKL alone is sufficient for osteoclast formation from precursor cells (Quinn et al., 1998a, Itonaga et al., 2000b) but this needs to be investigated further. It appears that RANKL is only active in the presence of M-CSF (Lacey et al., 1998) a cytokine likely to act as a survival factor for both mature osteoclasts and their precursors. Other studies have shown that, under certain conditions, RANKL may not be an essential factor for osteoclast formation. TNF-α and IL-1β stimulation, independent of RANKL, has been reported to induce osteoclast formation *in vitro* (Jimi et al., 1999, Kobayashi et al., 2000a). TNF-α and IL-1β are present in the tissues near loose implants so it is possible that osteoclasts could form in the absence of RANKL if these levels of these cytokines were high enough.

1.6.2 Receptor Activator NF Kappa B (RANK)

If RANKL is a significant, if not essential, mediator of osteoclast formation in the peri-prosthetic, RA and periodontal tissues, then expression of its sole cell surface receptor RANK (Li et al., 2000) is also important. The receptor activator of NFκB (RANK) is a new member of the TNF receptor family that was first cloned by Anderson *et al* from a cDNA library of human dendritic cells (Anderson et al., 1997). Northern blot analysis on mRNA from human tissues found ubiquitous expression of RANK with highest levels in skeletal muscles and the thymus. RANKL acts by direct binding to RANK on the cell surface of osteoclast precursor cells (Anderson et al., 1997). Detection of mRNA encoding RANK, in total RNA extracted from co-cultures of human PBMC and osteoblast-like cells, shows RANK expression exclusive to cells of the haematopoietic lineage (Atkins et al., 2000a). This is consistent with

findings that RANK is present on pre-osteoclasts (Nakagawa et al., 1998) as well as mature osteoclasts (Myers et al., 1999).

Intracellular signalling involves RANK interacting with tumour necrosis factor receptor associated factors (TRAF) 1, 2, 3, 5 and 6 *in vitro*. There are multiple TRAF binding sites clustered in two domains in the RANK cytoplasmic tail. These sites have selective binding for different TRAF proteins and are important for RANK dependent induction of NFκB and cJun NH2 terminal kinase activities (Galibert et al., 1998). Cytoskeletal rearrangement and integrin engagement after osteoclast attachment to substrate is enhanced by the stimulation of RANK (Burgess et al., 1999).

In vitro and *in vivo* models demonstrate that RANK is essential for osteoclastogenesis. The addition of genetically engineered soluble RANK blocks the binding of RANKL to RANK and inhibits osteoclastogenesis, indicating that RANK is essential for signal transduction in RANKL-mediated osteoclastogenesis (Lacey et al., 1998, Nakagawa et al., 1998, Akatsu et al., 1998a). In addition, polyclonal antibodies against the extracellular domain of RANK induced osteoclast formation in spleen cell cultures, in the presence of M-CSF (Nakagawa et al., 1998, Hsu et al., 1999).

RANK knockout -/- murine models conclusively demonstrate the requirement for RANK on osteoclast for osteoclastogenesis to occur (Li et al., 2000). The lack of osteoclasts and bone resorption in these mice (Li et al., 2000, Dougall et al., 1999) is also restored on transfer of RANK cDNA back into haematopoietic precursors as RANK expression is restored (Li et al., 2000).

There is some speculation that the presence of mRNA may not necessarily prove the expression of RANK protein and this is addressed in these studies.

1.6.3 Osteoprotegerin (OPG)

While this thesis tends to focus on the activity of factors stimulating osteolysis, those factors that inhibit osteolysis are just as important. Bone levels may be maintained by the balance of factors stimulating and inhibiting osteolysis. Elevated osteolysis by osteoclasts in bone pathologies might be due to a reduction in the levels of factors that inhibit osteoclast activity. Numerous inhibitors of osteoclast formation and activity have been identified. The relevance of each individual factor to peri-prosthetic, rheumatoid and periodontal osteolysis is yet to be determined. However, the identification of the major factors stimulating osteolysis discussed

above gives us some insight into those inhibitory factors that are likely to be most important in pathogenic osteolysis.

There is increasing evidence that the interaction of RANK and RANKL is fundamental to the formation of osteoclasts in health and disease. The natural inhibitor of RANKL, osteoprotegerin (OPG), is therefore an important factor to consider in peri-prosthetic, rheumatoid and periodontal osteolysis. The identification of an osteoclastogenesis-inhibitory factor, OPG, preceded the discovery of RANKL (Simonet et al., 1997). Within the same year an independent group isolated osteoclastogenesis inhibitory factor (OCIF) from human fibroblast cultures and found its cDNA sequence to be identical to OPG (Tsuda et al., 1997). OPG is a member of the TNF receptor family, however unlike other members of this family, it lacks a transmembrane domain. It was, therefore, predicted to be membrane bound and a secreted protein similar to other members of the soluble TNF receptor family (Simonet et al., 1997).

OPG is present as both a monomer with an apparent molecular mass of 60kDa and a homodimer of 120kDa (Tsuda et al., 1997). OPG contains four cysteine-rich domains (D1-D4) (Yasuda et al., 1998a) with two death domain homologous regions (D5, D6) (Yamaguchi et al., 1998). *In vitro* studies have shown that osteoclast formation, from cells in co-culture with stromal cells, can be inhibited by the N-terminal portion of OPG containing D1-D4. OPG can exist as a monomer or dimer as a result of formation of a disulphide bond using Cys^{400} present in D7, although dimerisation is not a requirement for its inhibitory osteoclastogenic activity (Yamaguchi et al., 1998).

OPG is ubiquitously expressed in many tissues (Yasuda et al., 1998a, Simonet et al., 1997). Various human osteoblast lineage cells have been shown to express OPG, including marrow stromal cells (Vidal et al., 1998a), normal trabecular osteoblasts, and immortalised foetal osteoblasts with expression possibly related to the stage of differentiation (Hofbauer et al., 1998). In addition, there are several reports of endothelial cells expressing OPG by other investigators (Collin-Osdoby et al., 2001, Malyankar et al., 2000).

The OPG molecule is a heparin binding basic glycoprotein (Tsuda et al., 1997) that acts as a decoy receptor, specifically inhibiting osteoclastogenesis both *in vivo* and *in vitro* (Simonet et al., 1997, Akatsu et al., 1998a, Hakeda et al., 1998, Yasuda et al., 1998b, Yasuda et al., 1998a, Yamaguchi et al., 1998, Tsurukai et al., 2000). OPG can abolish osteoclast formation elicited through three pathways, vitamin D receptor, protein kinase A and gp130 (Yasuda et al.,

1998b). The addition of osteoblasts from OPG-deficient mice to cultures of OPG-deficient bone marrow cells significantly increases osteoclast formation and this bone resorption can be inhibited by the addition of OPG, without affecting levels of RANKL mRNA (Udagawa et al., 2000).

It has been suggested that, although OPG may play a role in regulating osteoclast formation and survival, it does not effect proliferation of the osteoclast progenitors (Akatsu et al., 1998a). In the study carried out by Akatsu *et al* 1998, the addition of OPG to mouse marrow cultures between days 0-3 did not affect the final peak of osteoclast formation whereas the addition of OPG from day 5 caused a reduction in osteoclast number (Akatsu et al., 1998a). In the same study the concentration of OPG required to inhibit OC formation and survival were similar, indicating that OPG disrupted the interaction of stromal cells with OC thereby inhibiting survival of OC. In support of this, the numbers of OC, in cultures of crude populations, decreased following OPG addition, whereas in stromal depleted populations, although the OC population spontaneously decreased, OPG did not enhance the decrease in cell numbers (Akatsu et al., 1998a).

Originally OPG was found not to have a role in regulating the function of mature osteoclasts (Tsuda et al., 1997) but this has been disputed (Lacey et al., 1998, Hakeda et al., 1998). Hakeda *et al* found that, apart from blocking the generation of osteoclasts, OPG may directly inhibit osteoclast function when added to isolated mature rabbit osteoclasts (Hakeda et al., 1998). It was suggested that OPG could decrease bone resorption, independently of the blockage against RANKL, possibly through an alternative OPG-binding protein that inhibits osteoclastic bone resorbing activity by disrupting the F-actin ring surrounding the cells (not by apoptosis), even in the absence of osteoclast-osteoblast/stromal cell interaction (Hakeda et al., 1998).

The hypocalcaemia effect of OPG is clearly demonstrated by the administration of OPG and the generation of transgenic animal models. Over expression of OPG in transgenic mice results in severe osteopetrosis with a marked decrease in trabecular osteoclasts (Simonet et al., 1997). The decrease in trabecular osteoclast number without a deficiency in precursors suggests its role in the later stages of osteoclast differentiation (Simonet et al., 1997). Systemic administration of OPG in normal rats acts specifically on bone tissue to increase bone mineral density and volume. This is also associated with a decrease in active osteoclast numbers (Simonet et al., 1997, Yasuda et al., 1998a, Yamaguchi et al., 1998). Intraperitoneal injection of OPG in normal mice also reduces serum Ca levels and serum phosphate levels,

which is suggestive of its inhibitory effect on bone resorption (Akatsu et al., 1998b). In hypercalcemic tumour-bearing nude mice this reduction in serum Ca levels is dramatic (Akatsu et al., 1998b). Administration of a chimeric Fc fusion form of OPG has been shown to inhibit the bone resorptive and hypercalcemic effects of IL-1β, TNF-α, PTH, PTHrT and vitamin D3 (Morony et al., 1999). Bone loss following ovariectomy in mice is also prevented by administration of OPG (Hakeda et al., 1998).

In contrast to the effects of OPG or OPG over expression in transgenic mice, OPG knockout mice have enhanced osteoclastogenesis and severe osteoporosis (Bucay et al., 1998)(Mizuno et al., 1998). OPG deficient mice have decreased trabeculae and cancellous bone postnatally (Bucay et al., 1998). These mice are still viable and fertile, which reinforces the specificity of OPG as a negative regulator of osteoclastogenesis *in vivo* (Mizuno et al., 1998). It is important to note that studies on OPG deficient mice also found a medial calcification of the aorta and renal arteries (Bucay et al., 1998). This is interesting considering the association between osteoporosis and a higher incidence of arterial calcification in humans.

Studies in humans have found an increase in the serum concentration of OPG in postmenopausal women with a high rate of bone turnover and an age-dependent increase in OPG serum levels, which may be a compensatory mechanism against age-dependent bone loss (Yano et al., 1999). Although serum OPG may not effect bone metabolism a recent study has shown that circulating OPG may have some biological effects on bone. Evidence for this has recently been shown in postmenopausal women where administration of a single dose of OPG was able to reduce bone turnover (Becker et al., 2001).

Relevant to the pathologies investigated in this thesis was the demonstration that OPG inhibits the formation of osteoclasts from macrophages isolated from the peri-prosthetic tissues of patients retrieved at revision, co-cultured with UMR 106 cells, into bone resorbing osteoclasts (Itonaga et al., 2000a). In addition, OPG inhibits the ability of joint fluid from patients with failed total hip arthroplasty to induce osteoclast formation *in vitro* (Kim et al., 2001). The rat adjuvant arthritis and serum induced arthritis models provide additional evidence for the role of RANKL in the pathogenesis of bone erosion in inflammatory arthritis (Kong et al., 1999a, Pettit et al., 2001). Arthritic rats treated with OPG early in the course of erosion demonstrate only minimal erosion of the cortical and trabecular bone compared with untreated (Kong et al., 1999a). These studies indicate that low levels of OPG combined with elevated levels of bone resorbing cytokines may contribute to peri-prosthetic, rheumatoid and periodontitis osteolysis.

The ability of OPG to bind to RANKL and inhibit its interaction with RANK suggests the ratios of the level of RANKL to OPG are likely to be important in regulating osteoclast formation in bone loss pathologies (Nagai and Sato, 1999, Hofbauer et al., 1999, Kotake et al., 2001). The ratio of the concentration of soluble RANKL to that of OPG was significantly higher in synovial fluid of RA patients than in synovial fluid of patients with OA or gout (Kotake et al., 2001). Thus far, the relative level of RANKL and OPG has not been investigated in the tissues adjacent to sites of osteolysis in human pathologies. In this thesis, *ex vivo* and *in situ* studies explore the significance of RANKL and OPG levels in peri-prosthetic loosening, RA, and periodontitis in humans.

In addition to RANKL, TRAIL is a ligand for OPG (Emery et al., 1998). It is important to note that the osteoclastogenic inhibitory action of OPG can be blocked by TRAIL in culture (Emery et al., 1998). TRAIL is a TNF-related ligand that, on binding to its death domain containing receptors DR4 and DR5, induces apoptosis (Emery et al., 1998). TRAIL may have anti-inflammatory activities (Song et al., 2000) and may be able to modulate osteoclast activity through its interaction with OPG. TRAIL has not yet been identified in the inflamed tissues adjacent to localised bone loss.

1.7 REGULATION OF KEY MEDIATORS OF OSTEOCLAST DIFFERENTIATION

While most studies have shown that RANK/RANKL interactions are the essential factors for osteoclast formation in health and disease, it is becoming clear that the cytokine environment can modulate the activity of RANKL, RANK and OPG. The local concentrations of RANKL will be more or less important depending on other factors that may alter RANK expression and/or signalling, as well as altering the expression and secretion of OPG.

Factors present in the peri-prosthetic tissues are also involved in the cross talk between the pre-osteoclasts and the stromal cells expressing RANKL (Haynes et al., 1999, Yasuda et al., 1998b, Wani et al., 1999). There seem to be two things happening; agents that stimulate RANKL expression on cell surface and those that collaborate with RANKL to stimulate RANK or that affect the system in some other way. PTH, IL-1 and PGE_2, IL-1β, IL-6, IL-11, IL-17 and TNF-α increase the expression of membrane bound RANKL, as well as induce the release of soluble RANKL in osteoblastic cells (Kotake et al., 1999, Nakashima et al., 2000). Many of these cytokines are known to be present in the peri-prosthetic, RA and periodontal tissues and are probably involved in the cross talk between pre-osteoclasts and stromal cells

expressing RANKL that is required for osteoclast formation (Haynes et al., 1999, Yasuda et al., 1998b, Wani et al., 1999). In addition TNF-α, IL-6, IL-11, IL-17 (Nakashima et al., 2000) and PGE_2 have been shown to down regulate OPG mRNA expression in murine osteoblast cultures (Murakami et al., 1998).

1.7.1 Regulation of Osteoclast Mediators by Parathyroid Hormones (PTH)

PTH has been shown to mediate RANKL expression and induce bone resorption in a foetal mouse long bone culture system (Tsukii et al., 1998). The PTH induced bone resorption could be abolished by the addition of anti-RANKL neutralising antibody and OPG. PTH has also been shown to cause an increase in the RANKL:OPG ratio in osteoblastic cell lines (Horwood et al., 1998). In concordance with this, PTH not only dose-dependently increased RANKL mRNA expression while decreasing OPG expression in cultured murine bone marrow, calvaria and osteoblasts but also correlated with increased osteoclast cell formation (Lee and Lorenzo, 1999).

1.7.2 Regulation of Osteoclast Mediators by Prostaglandins (PGEs)

PGE_2 has been shown to regulate both RANKL and OPG. RANKL expression, mediated by PGE_2, induces bone resorption in foetal mouse long bone culture systems (Tsukii et al., 1998). The osteolysis induced by PGE_2 can be abolished by the addition of anti-RANKL neutralising antibody or OPG (Tsukii et al., 1998). In support of its osteoclastic role PGE_2 has been shown to cause a dose dependent increase in the RANKL:OPG ratio in an osteoblastic cell line (Nagai and Sato, 1999). The addition of PGE_2 in the presence of RANKL and M-CSF further stimulates osteoclast formation and resorption from haemopoietic precursors demonstrating, that PGE_2 cooperates with RANKL in osteoclast induction causing synergistic activation of differentiation, cell spreading, and fusion (Wani et al., 1999). However, a dose-dependent inhibition of osteoclast formation and bone resorption occurred when Itonaga *et al* added PGE_2 to human PBMC in the presence of RANKL and M-CSF (Itonaga et al., 1999). PGE_2 was also demonstrated by Hofbauer *et al* to stimulate OPG production (Hofbauer et al., 1999), which would explain the decrease in osteoclast formation reported by Itonaga (Itonaga et al., 1999). Due to conflicting findings regarding the role of PGE_2 it is clear that further studies are required to elucidate the precise role of PGE_2.

1.7.3 Regulation of Osteoclast Mediators by Interleukin (IL)-1

It is likely that IL-1β increases osteoclast formation by stimulating RANKL expression and protein release (Hofbauer et al., 1999, Nakashima et al., 2000). However, while most studies have shown that RANK/RANKL interactions are the essential factors for osteoclast formation

in health and disease there are several reports that cytokines such as IL-1 may induce osteoclast formation in the absence of RANK stimulation. This is supported by findings that IL-1 can induce multinucleation and the bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells and RANKL (Jimi et al., 1999).

The natural inhibitor of RANKL, OPG, is also regulated by several cytokines present in the peri-prosthetic tissues. IL-1α has been reported to stimulate OPG mRNA expression when added to human osteosarcoma cells, human osteoblast-like cells (Vidal et al., 1998b) or human bone marrow cells (Brandstrom et al., 1998b, Brandstrom et al., 2001, Brandstrom et al., 1998a) in culture. In contrast, in studies with murine cells, Nakashima found IL-1β suppressed OPG expression while at the same time stimulating RANKL expression (Nakashima et al., 2000). Murakami also reported a decrease in expression of OPG mRNA (Murakami et al., 1998).

In summary IL-1 appears to be a potent stimulator of osteoclast formation and bone resorption. It is also possible IL-1 and M-CSF enable osteoclasts to maintain their bone resorption activity by preventing apoptosis.

1.7.4 Regulation of Osteoclast Mediators by Tumour Necrosis Factor (TNF)

TNF-α is an osteoclastogenic factor thought to act indirectly through specific receptors on stromal/oteoblast-like cells to enhance RANKL expression (Horwood et al., 1998) and therefore increase osteoclast formation and activity.

There has been a recent resurgence in research into the importance of TNF-α as a key mediator of bone resorption and there are reports that TNF-α [Kobayashi, 2000 #194] can simulate osteoclastogenesis in the absence of RANKL. The injection of recombinant TNF-α over the calvaria of RANK-deficient mice induces small numbers of osteoclasts (Li et al., 2000). In addition, in cultures of mouse bone marrow cells, OPG or anti-RANK antibody failed to inhibit osteoclast formation in the presence of M-CSF and TNF-α and further bone resorption only occurred in the presence of IL-1α (Kobayashi et al., 2000a). However, the notion that TNF-α may stimulate osteoclast differentiation by a mechanism independent of RANK/RANKL interaction (Kobayashi et al., 2000a) is still controversial. For instance, in stromal-free cultures of macrophage-lineage myeloid cells, TNF-α alone fails to induce differentiation of osteoclasts (Lam et al., 2000). However, it is possible that TNF-α acts in synergy with RANKL to generate osteoclasts (Komine et al., 2001) and it is suggested that

TNF-α may permit RANKL at levels normally too low to stimulate osteoclast production (Lam et al., 2000).

Interestingly, TNF-α and TNF-β have also have been shown to time and dose-dependently upregulate mRNA levels of OPG in the human osteosarcoma cell line MG-63 (Brandstrom et al., 1998a). This upregulation of OPG mRNA by TNF-α has also been noted in foetal osteoblastic and human osteoblastic cells (Hofbauer et al., 1998).

The relevance of these *in vitro* studies to *in vivo* situations need to be assessed as concentrations of the cytokines may be significantly higher than found in the tissues adjacent to loosened implants, RA joints and alveolar bone. As both IL-1β and TNF-α are seen in peri-prosthetic, RA and periodontal tissues, osteoclast formation in the absence of RANKL remains a possibility. However, what may be more important in these pathologies is the fact that these cytokines act in synergy with RANKL during osteoclast formation as well as also stimulating RANKL expression.

1.7.5 Regulation of Osteoclast Mediators by Transforming growth factor (TGF)-β

Galvin (1999) demonstrated an increase in osteoclast differentiation induced by TGF-β in cultures of haematopoietic cells stimulated with RANKL and M-CSF (Galvin et al., 1999). More recently TGF-β has been shown to enhance RANKL-induced TRAP activity in RAW264.7 cells (Koseki et al., 2002). When in a lymphocyte-rich microenvironment, TGF-β1 stimulates the osteoclast formation from peripheral blood haematopoietic precursors (Massey et al., 2001).

However it seems that, at the level of osteoblasts, TGF inhibits RANKL expression and has an effect on increasing extra cellular matrix proteins. TGF-β has been shown to stimulate OPG expression (Thirunavukkarasu et al., 2001) and can suppresses the mRNA expression of RANKL in bone marrow stromal cells and osteoblast cells while increasing the OPG mRNA level dose-dependently (Murakami et al., 1998). Anti-OPG IgG can partially prevent a TGF-β induced decrease in the number of osteoclasts, which supports the view that TGF effects are mediated, at least in part, by OPG (Murakami et al., 1998).

1.7.6 Regulation of RANK

In comparison to RANKL and OPG far less is known about the regulation of RANK expression. Stimulation and timing of RANK expression on the surface of cells of the

monocyte/macrophage lineage is critical for osteoclast formation (Arai et al., 1999). It is significant to note that M-CSF stimulates RANK expression on cells of the monocyte/macrophage lineage (Arai et al., 1999). Although this is yet to be shown in human cells, this finding is consistent with the consensus that M-CSF is important in osteoclast formation in the peri-prosthetic tissues. This may explain why blocking of endogenous M-CSF production during the formation of osteoclasts from cells obtained from the peri-prosthetic tissues of failed implants markedly reduces the numbers of differentiated osteoclasts (Neale et al., 1999a).

1.7.7 TNF-Related Apoptosis-Inducing Ligand (TRAIL)

TRAIL is a member of the tumour necrosis factor (TNF) family that shares homology with RANK and RANKL (Degli-Esposti et al., 1997). The identification of TRAIL has generated a lot of interest in recent years as TRAIL is able to induce death of cancer cells without causing apoptosis in normal cells (Wiley et al., 1995, Pitti et al., 1996). TRAIL has also been shown to inhibit autoimmune inflammation and cell cycle progression (Song et al., 2000).

The apoptotic activity of TRAIL is mediated through its cell-surface death-domain receptors, DR4 and DR5 (TRAIL-R2) (Golstein, 1997, Pan et al., 1997b, Pan et al., 1997a, Sheridan et al., 1997, MacFarlane et al., 1997). The deletion of the death domain in the receptors abolishes the ability of TRAIL to induce apoptosis (MacFarlane et al., 1997). TRAIL also interacts with decoy receptors, DcR1 and DcR2 (TRAIL-R3) (Emery et al., 1998, Sheridan et al., 1997, Degli-Esposti et al., 1997, MacFarlane et al., 1997). These receptors lack functional domains and therefore do not induce apoptosis (Emery et al., 1998, Sheridan et al., 1997, Degli-Esposti et al., 1997, MacFarlane et al., 1997).

It is important to note that OPG is also a receptor for TRAIL (Emery et al., 1998, Holen et al., 2002) but, like the decoy receptors, OPG does not induce apoptosis as it lacks the death domain (Emery et al., 1998, Holen et al., 2002). *In vitro* studies have shown that OPG can inhibit TRAIL-induced apoptosis of Jurkat cells dose-dependently by competing with DR4 and DR5 for TRAIL (Emery et al., 1998). The capacity of TRAIL to induce apoptosis cultures of human prostate cancer cells is inversely proportional to the endogenous production of OPG in the medium by the prostate cells (Holen et al., 2002). OPG may therefore be a soluble antagonist for TRAIL thereby decreasing its activity.

Of relevance to this study of bone loss pathologies is the finding that TRAIL can block the anti-resorptive activity of OPG by preventing OPG from interacting with RANKL (Emery et

al., 1998). TRAIL may therefore affect the amount of OPG available to block RANKL from interacting with RANK. This suggests soluble TRAIL may regulate the anti-resorptive activity of OPG and may be a determinate in bone resorption. Secreted TRAIL may antagonise OPG activity or prevent signalling by membrane-anchored TRAIL through the death receptors DR4 and DR5. These studies imply cross regulation between TRAIL and OPG (Emery et al., 1998)

TRAIL expression can be induced in human monocytes/macrophages (Halaas et al., 2000) and only recently has abundant expression of TRAIL been identified in human osteoblast-derived cultures (Atkins *et al* unpublished). If TRAIL is present in inflamed soft tissue, adjacent to areas of osteolysis, it could modulate the inflammation and the availability of OPG and therefore affect the RANKL:OPG ratio. The studies carried out here seek to investigate TRAIL mRNA expression in peri-prosthetic tissue, periodontal disease and RA.

The regulation of OPG, RANKL and RANK by cytokines is clearly complex and may be dependent on the type of cells, the species and the tissues investigated. Although it is still not clear how expression of these mediators is controlled, their regulation in the peri-prosthetic tissues, periodontitis and rheumatoid tissue is likely to be very important. Although beyond the scope of this work, it is important to consider the effects of these cytokines on proliferation, differentiation and production of bone by osteoblasts. Bone loss results from an imbalance between resorption and bone formation, which may be due not only to excessive osteoclast activity but also the inability of osteoblasts to replace the resorbed bone.

1.8 ADDITIONAL FACTORS INFLUENCING BONE RESORPTION IN BONE LOSS PATHOLOGIES

1.8.1 The Effects Wear Particles from Joint Implants on Osteoclastogenic Mediators

The weight of pathological and experimental evidence now supports the hypothesis that particles of prosthetic material found in surrounding soft tissues, produced by wear of joint components, are a major factor in osteolysis. *In vivo* studies have shown that these wear particles stimulate a chronic inflammatory-like response dominated by macrophages (Jiranek et al., 1993, Howie and Vernon-Roberts, 1988, Howie, 1990, Lerouge et al., 1997, al-Saffar et al., 1995b, al-Saffar and Revell, 1994). Monocytes and macrophages are recruited to, and may proliferate within, the implant bed in response to prosthetic particles (Jiranek et al., 1993). Macrophages, including those that have phagocytosed particles, are stimulated to release pro-inflammatory products and mediators that stimulate osteoclastic differentiation and bone resorption (Glant et al., 1983, Jiranek et al., 1993, al-Saffar et al., 1995b, al-Saffar

and Revell, 1994). In support of this, conditioned media from the pseudomembrane of OA undergoing revision has been shown to induce 45Ca release from bone rudiments (Appel et al., 1990).

While few lymphocytes are usually seen in the peri-prosthetic tissues (Vernon-Roberts and Freeman, 1977, Jiranek et al., 1993, Jacobs et al., 1994) T lymphocyte modulation of macrophage function has been suggested to be important in peri-prosthetic osteolysis (Goodman et al., 1998). Increased numbers of macrophages, T lymphocyte subgroups and increased TNF-α, IL-1 and IL-6 expression have been reported in the tissue around cemented prostheses (Goodman et al., 1998).

Wear of early types of metal-on-metal implants was sometimes associated with peri-prosthetic osteolysis, and metal particles have been reported to stimulate pro-inflammatory responses in cells in culture (Rogers et al., 1997). However, it is generally acknowledged that wear of PE components of metal on polyethylene articulations and the associated osteolysis are also a major problem (Vernon-Roberts and Freeman, 1977, Willert et al., 1989). Submicron PE particles have been identified as the dominant type of wear particle in peri-prosthetic tissues associated with uncemented implants (Jacobs et al., 1994). Analyses of retrieved metal-on-PE implants and surrounding peri-prosthetic tissues have shown a strong correlation between PE wear and peri-prosthetic osteolysis (Kadoya et al., 1998, Wan and Dorr, 1996, Oparaugo et al., 2001). Moreover, correlations found between peri-prosthetic osteolysis and the number of PE particles in the surrounding tissues, but not other particles (Kadoya et al., 1998, Wan and Dorr, 1996), are consistent with the more mild tissue responses that have been observed with metal particles in tissues retrieved from around metal-on-metal prostheses than with PE particles (McGee et al., 2000).

Various pro-inflammatory products and mediators have been detected in peri-prosthetic tissue adjacent to sites of osteolysis (Jiranek et al., 1993, Shanbhag et al., 1995a). *In vivo* studies, using canine (Dowd et al., 1995) and rat pouch (Gelb et al., 1994) models have investigated the differences between prosthetic wear particles of differing size and biochemistry in their effects on cells in peri-prosthetic tissue in terms of osteolytic cytokine release and cellular response.

In vitro studies have shown that particles of different composition can elicit different biological responses when placed on macrophages. Many of these *in vitro* studies assessed particles in terms of toxicity and the ability to stimulate osteolytic cytokine release by

incubating particles, such as titanium 6-aluminium 4-vanadium alloy (TiAlV), PE and cobalt chromium (CoCr) particles, with rat peritoneal macrophages or human monocytes (Haynes et al., 1993, Haynes et al., 1998, Rogers et al., 1997, Shanbhag et al., 1994a, Shanbhag et al., 1995b, Kubo et al., 1999, Horowitz and Purdon, 1995, Glant et al., 1996, Chiba et al., 2001).

Human monocytes have been used to study differences in toxicity and release of mediators in response to wear particles of different metal alloys of the same size and shape (Rogers et al., 1997, Haynes et al., 1998). Stainless steel (316 L S/S) and cobalt-chromium-molybdenum particles were toxic while TiAlV particles did not affect cell viability (Haynes et al., 1998). Stainless steel particles were the strongest stimulator of IL- 1β while TiAlV particles were the strongest stimulator of IL-6 and PGE_2. This is consistent with the study on effects on rat peritoneal cells (Haynes et al., 1993). TiAlV particles have also been shown to stimulate higher levels of IL-1α, IL-1β and PGE_2 when compared to PE (Shanbhag et al., 1995b).

Only slight differences in the chemical composition of prosthetic particles can cause differences in the biological response. For example, TiAlV particles are a stronger stimulator of osteolytic factors, PGE_2, IL-1, TNF and IL-6, than titanium 6-aluminium 7-niobium (TiAlNb) particles at similar concentrations (Rogers et al., 1997). It is suggested that particles that are less toxic may be more detrimental in the long term as they could continually stimulate the release of mediators implicated in bone resorption (Haynes et al., 1993). The findings of studies like these may influence the choice of biomaterials used in articulating implants where wear particles may be produced.

Studies of particles isolated from peri-prosthetic revision tissues show that most particles are less than 1 μm in diameter (Willert and Semlitsch, 1977, Howie, 1990, Shanbhag et al., 1994b, Milosev et al., 2000, Lee et al., 1992). This size of particle, approximately the same size as pathogenic bacteria, is easily phagocytosed by macrophages and can induce the release of many osteolytic cytokines. *In situ*, particles of PE have been shown to elicit a difference in the cellular response depending on the particle size (Howie et al., 1993). *In vitro* studies support this and show that the size of prosthetic particles may influence the levels of osteoclastogenic mediators released from monocytes and macrophages (Gelb et al., 1994, Shanbhag et al., 1994a, Green et al., 1998). These effects may also depend on the shape and surface area of particles encountered (Shanbhag et al., 1994a).

Previous reports have shown that wear particles may also stimulate macrophages to indirectly influence fibroblast proliferation (Shanbhag et al., 1997) and osteoblasts activity(Haynes et al., 1997) via the release of cytokines but this was not addressed in these studies.

The direct effect of various prosthetic particles on the expression of primary mediators of osteoclast formation has not yet been investigated. The studies in this thesis addressed the mRNA expression of RANKL, RANK, OPG and M-CSF in response to different types of metal particles using reverse transcription polymerase chain reaction (RT PCR).

1.8.2 The Role of the Osteoblast in Maintaining Bone

Although the activity of osteoblasts was not investigated in this thesis, the affect of various stimuli on the activity of osteoblasts is important to consider. Resorption itself is not a problem if the bone is repaired and restored to a normal physiological state. Therefore osteoblasts near sites of peri-prosthetic osteolysis may have an important role to play in regulating bone loss. Osteoblasts not only produce the surrounding bone matrix but they provide support essential for osteoclast formation. A recent report has shown that there is high bone turnover around loose-cemented total hip joints (Takagi et al., 2001). However, the bone that formed at these sites, frequently adjacent to macrophages/monocytes in granulomatous tissue, was described as immature and brittle due to poor materialisation. Weak bone near implants may be unable to support the implant and result in joint failure. The presence of poor quality bone at this site was thought to show that factors released from macrophages not only stimulated osteoclast activity but also adversely affected osteoblast bone formation.

A recent study found bone-lining cells (osteoblasts) could resorb fragments of the bone in resorption sites left by osteoclasts in Howship's lacunae (Everts et al., 2002). Several studies report that osteoblast activity may be affected directly through contact with or phagocytosis of wear particles (Dean et al., 1999b, Rodrigo et al., 2000, Lohmann et al., 2000, Shida et al., 2000, Vermes et al., 2001). Heinemann *et al* (Heinemann et al., 2000) suggested that human osteoblastic cells may change in culture to become cells that can phagocytose prosthetic particles and may express markers found on both osteoblasts and macrophages. Long-term cultures of human bone derived osteoblasts express bone-specific alkaline phosphatase as well as the macrophage marker CD68. Transmission EM on cultures of these human osteoblasts cultured with less than 3μm in diameter cpTi, TiAlV, CoCr or UHMWPE particles demonstrated particles intracellularly (Lohmann et al., 2000). It is important to establish

whether phagocytosis of wear particles by osteoblasts is a major cause of prosthetic loosening.

Although osteoblasts may phagocytose particles, numerous *in vivo* studies of peri-prosthetic tissues obtained at revision show that monocytes and macrophages are the main cells involved in phagocytosis of wear particles. It may be more likely that wear particles affect osteoblasts indirectly by first stimulating macrophages to release factors which then modulate the osteoclastogenic activity of osteoblasts (Haynes et al., 1997, Pollice et al., 1995, Horowitz et al., 1994, Horowitz and Gonzales, 1996). Two studies using particles of PMMA (Pollice et al., 1995, Horowitz et al., 1994) have demonstrated that osteoblasts may mediate osteoclastic bone resorption in the peri-prosthetic tissues. Studies using human osteoblastic cells support this concept and show that IL-6, TNF-α and PGE_2 can be released from human osteoblastic cells stimulated by particle-activated macrophages (Haynes et al., 1997). IL-1-β and TNF-α were identified as the major mediators released by the particle-activated macrophages that then stimulate the release of IL-6 and PGE_2 from human osteoblastic cells (Haynes et al., 1997). These studies address the signalling that occurs between osteoblasts and macrophages in a controlled situation that is reflective of the cross modulation that occurs *in situ*.

There is evidence that factors released from particle-activated macrophages may control osteoblast proliferation and differentiation (Haynes et al., 1997). It was found that TNF-α, released by mononuclear phagocytes in response to TiAlV particles, inhibited osteoblastic cell proliferation and differentiation. Inhibition of TNF-α by blocking antibodies restores proliferation of bone cells to normal levels. IL-1-β stimulates cell proliferation and differentiation of bone cells (Haynes et al., 1997).

Particles may also directly affect the differentiation and bone forming ability of osteoblasts (Glant et al., 1996, Dean et al., 1999a, Vermes et al., 2000). Studies using ultra high molecular weight polyethylene particles and MG63 osteoblast-like cells have shown that particles reduced alkaline phosphatase activity and $\{^{35}S\}$ sulphate (Dean et al., 1999a). The polyethylene particles also increased PGE_2 release while there was a decrease in TGF-β. The authors concluded that polyethylene particles might therefore affect bone formation through inhibition of cell differentiation, a reduction of TGF-β production and matrix synthesis. It has been suggested that wear particles may effect bone formation by osteoblasts by altering gene expression following phagocytosis of wear particles by osteoblasts (Vermes et al., 2000). Studies using osteoblastic cell lines show that particulate wear debris induced NF-κB

activation and IL-6 release while decreasing collagen synthesis. Particles of titanium, titanium alloy, chromium orthophosphate and PE, suppressed levels of pro-collagen α {I} and α {II} accompanied by reduced type I collagen synthesis (Vermes et al., 2000). Osteoblast specific genes such as osteonectin and alkaline phosphatase were not altered. Phagocytosis of the particles by the osteoblast cells was shown to be crucial as inhibition of phagocytosis by cytochalasin D markedly reversed the particle-induced suppression of pro-collagen α1 {I} gene expression (Vermes et al., 2000).

Particles of different size and chemical composition were shown to have different effects on macrophage responses and the same may be true for osteoblasts. The affect of particles too large to be phagocytosed on human bone cells from mature trabecular bone has also been investigated (Martinez et al., 1998b, Martinez et al., 1998a). Particles of UHMWPE of diameters up to 160μm decreased cell growth while alumina particles of the same size did not. While both types of particle of less than 80μm in diameter induced an inhibition in osteoblastic cell growth the inhibition induced by PE particles was greater.

The effect of particles on RANKL and OPG expression by osteoblasts is yet to be investigated. However, a recent study has attempted to determine whether wear particles can affect the ability of osteoblasts to support osteoclast formation. Wear particles of CoCr, 316L SS and TiAlV were cultured with human PBMC and osteoblastic cells to determine their effect on osteoclast formation and bone resorption *in vitro* (Neale et al., 1999b, Neale et al., 1999a). Cells were characterised with macrophage-associated markers (CD11b and CD14 positive and vitronectin {VNR}-negative), osteoclast-associated markers (VNR) and lacunar bone resorption. Osteoclast formation and lacunar resorption was inhibited dose dependently following phagocytosis of metal particles by monocytes *in vitro.* This effect occurred with all particles but the degree of response and the degree of cell toxicity varied depending on the type of alloys (Neale et al., 1999a).

1.8.3 Infection

It is important to note that osteolysis adjacent to joint prostheses and in response to periodontitis may result from infection. Joint failure due to infection may involve similar mechanisms to those seen in aseptic loosening. Histological diagnosis is usually based on the degree of acute inflammatory infiltrate, such as the presence of neutrophil polymorphs per high power field (Pandey et al., 2000). There is a strong correlation between the presence of acute inflammatory cells in peri-prosthetic tissue and the degree of bone loss in septic loosening. Infected cases usually involve inflamed granulation tissue and an inflammatory

exudate covering arthroplasty tissues (Pandey et al., 2000). Infection usually causes extensive and rapid osteolysis. Inflammatory cells are recruited into the area engulf the bacteria and release chemokines and cytokines, such as IL-1-β and TNF-α. These cytokines can then stimulate osteolysis as discussed in previous sections of this review. It is probable that it is endotoxin (lipopolysaccharide) released from bacteria activates cells, including tissue macrophages, which causes the release of TNF-α, IL-1-β and other osteolytic cytokines.

It has also been suggested that endotoxin may be involved in aseptic loosening due to wear particles. Endotoxin is known to adhere strongly to many materials and can readily adhere to the surface of wear particles. This would greatly affect the macrophage response to these particles and may contribute to the release of osteolytic cytokines from macrophages that have phagocytosed particles. It is not clear how wear particles that form *in vivo* would come into contact with endotoxin in the absence of infection. However, it does demonstrate that experiments with particles isolated from peri-prosthetic tissues or with particles produced artificially need to be carefully analysed for endotoxin contamination (Hitchins and Merritt, 1999).

There is compelling evidence that T cells are involved in the development of RA in the joint and the resultant bone erosion. Isolated T cell clones show features that suggest that they may recognise antigens in the synovium (Sioud et al., 1991). It has been suggested that the progression of RA is due to the continuing presence of antigens in the joint (Gaston, 1998). Viruses have been shown to induce the proliferation of lymphocytes derived from the synovial tissue of RA joints (Shadidi et al., 2001).

1.8.4 Physical Effects on Bone Resorption

An increase in mechanical stress or fluid flow may upregulate osteoclastogenic factors leading to bone loss in pathologies. Although this has not been investigated in relation to RA joints these mechanical influences can cause bone loss adjacent to prosthetic joints and in alveolar bone. The release of the osteolytic cytokines, IL-6 and TNF-α, IL-β, from monocyte-derived-macrophages is significantly increased when exposed to cyclic pressure regimes of different frequencies in culture (Ferrier et al., 2000). Mechanical strain or fluid sheer stress has also been reported to regulate PGE_2, TGF-β, OPG and IL-11 expression (Ogasawara et al., 2001, Kurata et al., 2001, Kobayashi et al., 2000b, Sakai et al., 1999, Farber et al., 2001). In addition, fluid sheer stress is reported to stimulate bone-resorbing activity of osteoclasts in culture (Kurata et al., 2001, Kobayashi et al., 2000b, Sakai et al., 1999).

Mechanical factors have been suggested as a factor inducing peri-prosthetic osteolysis prior to the production of wear particles (Van Der Vis et al., 1998a). Pressure changes or fluid flow have been suggested as possible causes of peri-prosthetic bone resorption and loosening (Van Der Vis et al., 1998a, Aspenberg and Van Der Vis, 1998, Van Der Vis et al., 1998b, Skripitz and Aspenberg, 2000). Co-cultures of human PBMC and periodontal ligament cells, as the stromal support, have demonstrated an increase in osteoclastogenesis when cells were placed under pressure (Kanzaki et al., 2002). In support of this, when periodontal ligament cells were placed under a compressive force there was an increase in expression of RANKL mRNA protein (Kanzaki et al., 2002).

These studies indicate that mechanical factors and fluid pressure may regulate peri-prosthetic and alveolar osteolysis through cytokine-mediated activation of osteoclasts.

1.8.5 Immune Reactions

The involvement of specific immune responses directed against the prosthetic materials in peri-prosthetic osteolysis is controversial (Farber et al., 2001, Hallab et al., 2001b) and has been reviewed recently (Hallab et al., 2001a). In the tissues the foreign body reaction to prosthetic materials resembles that seen in the type IV hypersensitivity reaction in which T cells are thought to regulate macrophage functions. While few lymphocytes are usually seen in the peri-prosthetic tissues, T lymphocyte modulation of macrophage function has been suggested to be important in peri-prosthetic osteolysis (Goodman et al., 1998). Activated T cells are known to directly induce osteoclastogenesis from human monocytes and have a role in bone destruction in other bone pathologies (Kotake et al., 2001, Taubman and Kawai, 2001). The activated T cells seen in these pathologies may express RANKL and may directly stimulate osteoclast formation (Romas et al., 2000, Teng et al., 2000). However, the degree of infiltration of lymphocytes seen in these other pathologies is rarely seen in aseptic loosening. Significantly, those lymphocytes that are present do not express markers normally found on activated lymphocytes (Li et al., 2001).

1.9 ADDITIONAL DISEASES ASSOCIATED WITH LOCALISED BONE LOSS

1.9.1 Paget's Disease

An interesting bone disorder resulting in bone lesions is Paget’s disease, a common disorder of bone remodelling affecting up to 3% of the population over 60. Paget’s disease involves the formation of abundant new woven bone, effecting either single or multiple skeletal lesions (Singer and Roodman, 1996, Graver and Siris, 1999). The pathology is most commonly

found in the pelvis, lumbar spine, femur, tibia and skull. In the early stages Paget's is associated with marked osteolysis, which is visible radiographically. The lesions may be due to local stimulation of osteoclast formation as well as resorption (Athanasou, 2001).

Generally, the histopathological changes indicate abnormal bone remodelling (Revell, 1986, Bartl and Frisch, 1993, Milgram, 1977). The early stage involves disorganised bone remodelling and abundant woven bone formation, which are later replaced with lamellar bone. Osteoclasts are larger with a greater number of nuclei than normal osteoclasts and contain viral-like nuclear inclusions (Roodman, 1995). The pre-osteoclasts and osteoclasts appear hyperresponsive to 1,25 dihydroxy vitamin D3 and RANKL (Reddy et al., 2001).

In Paget's disease there is an increase in IL-6 levels, which is associated with increased osteoclast formation (Singer and Roodman, 1996, Graver and Siris, 1999). IL-6 is produced by the osteoclasts which express IL-6 mRNA as well as IL-6 receptors (Roodman, 1995). Usually there is a marked increase in serum alkaline phosphatase, while serum calcium and phosphate remain normal. There is also an increase in osteocalcin. In immobilized patients hypercalcemia may be observed. In a review on osteoclasts, Roodman (2001) pointed out similarities between Paget's disease and bone metastases. The increased osteoclastogenesis are both mediated by IL-6 and RANKL (Roodman, 2001). The marrow spaces are abundant and well vascularised with cellular fibrous tissue. Later in the course of the disease, cellular activity is diminished and few or no osteoclasts are present.

1.9.2 Giant Cell Tumour

Cancers of bone can cause removal of surrounding bone. These include primary cancers such as GCT and multiple myelomas as well as metastatic disease from cancers such as lung and prostate. The giant cell tumour occurs in 4-8% of primary bone tumours and rarely metastasises. It is classified as a benign, locally aggressive and lytic tumour that is generally non-metastatic (Sanerkin, 1980).

The giant cell tumour occurs most often in the distal femur and proximal tibia but the end of any tubular bone may be involved. Lesions are also found in the skull and vertebrae. The giant cell tumour is characterised morphologically by numerous osteoclast-like giant cells lying within a well-vascularised stroma. The osteoclast-like giant cells are uniformly scattered amongst round or spindle shaped mononuclear cells. Numerous osteoclastic giant cells within the giant cell tumour of the bone are thought to be formed from mononuclear, macrophage-like osteoclast precursors present in the mononuclear component of the tumour

(Joyner et al., 1992). The giant cells have vesicular nuclei with prominent nucleoli. The cytoplasm is abundant and eosinophilic and often these cells are much larger than normal osteoclasts. They express phenotypic features of osteoclasts such as the vitronectin receptor (CD51/CD61) and a restricted range of macrophage associated antigens. The giant cells are CD68 positive and CD11/18-, CD14- and HLA-DR-negative. The tumour stroma is well vascularised and consists of bands of cellular or collagenous fibrous tissue (Athanasou, 2001).

1.9.3 Myeloma

Myeloma is a common bone tumour occurring in 43% of bone malignant tumours in the Mayo Clinic Series (Unni, 1996). The lesion is most commonly detected in the spine, pelvis, ribs and proximal long bones. Myeloma is a plasma cell dyscrasia involving expansion of a single clone of immunoglobulin secreting cells. As in Paget's disease, proliferation and differentiation of myeloma cells is associated with high serum levels of IL-6. Osteoclast formation and activity is stimulated by production of cytokines and growth factors such as IL-1, IL-6, TNF-β and M-CSF. The actual tumour cells express CD38, a plasma cell-associated antigen. They may express epithelial membrane antigen and are negative for leukocyte common antigen and CD22, a mature B cell marker (Athanasou, 2001).

1.10 THE FOLLOWING THREE CHAPTERS AIM TO:

- identify oseoclast-like cells in soft tissue adjacent to bone loss near failed prosthetic joints, in RA and in periodontitis
- verify that cells extracted from peri-prosthetic and RA tissues can differentiate into bone resorbing osteoclasts *ex vivo*
- investigate mRNA expression of osteoclastogenic mediators in soft tissue adjacent to bone loss near failed prosthetic joints, in RA and in periodontitis
- compare levels of mRNA expression in cells extracted from sites of osteolysis, and their ability to become functional osteoclasts *ex vivo*
- investigate whether RANKL and OPG protein are produced in tissues adjacent to osteolytic regions
- compare RANKL and OPG protein to levels in relevant control tissues using semi-quantitative and digital image analysis
- identify the cell lineages associated with RANK, RANKL and OPG production in peri-prosthetic tissue, RA and periodontal tissue

1.11 ABBREVIATIONS

AEC	aminoethylcarbazole
Al_2O_3	alumina
APAAP	alkaline phosphatase anti-alkaline phosphatase
APTS	aminopropyltriethoxysilane
BABA	beta-adrenergic blocking agent
BCIP	5bromo-5chloro-3indolyl-Phosphate
cDNA	complementary deoxyribonucleic acid
CoCr	cobalt chrome alloy
CpTi	commercially pure titanium
CTR	calcitonin receptor
DE AEC	double enhancement aminoethylcarbazole
DEPC	diethylpyrocarbonate
DIG	digoxygenin
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunoabsorbant assay
gm	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HBDC	human bone derived cells
HPF	high power field
HRP	horseradish peroxidase
HRT	hormone replacement therapy
IL	interleukin
IOD	integrated optical density
LPS	lipopolysaccharide
Mab	monoclonal antibody
M-CSF	macrophage colony stimulating factor
mg	milligram
ml	millilitre
MOD	mean optical density
mRNA	messenger ribonucleic acid
N	normal
NBT	nitro blue tetrazolium
NSAIDs	non-steroidal anti-inflammatory drugs
NSS	normal sheep serum
OA	osteoarthritis

OPG	osteoprotegerin
OSP	osteoporosis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	polyethylene
PP	peri-prosthetic
RA	rheumatoid arthritis
RANK	receptor activator NF kappa beta
RANKL	receptor activator of NF kappa beta ligand
RF	rheumatoid factor
RT PCR	reverse transcription polymerase chain reaction
SpA	spondyloarthropathy
SS	stainless steel
SSC	standard-saline solution
TGF	tumour growth facor
TNF	tumour necrosis factor
TiAlNb	titanium aluminium niobium
TiAlV	titanium aluminium vanadium alloy
TRAP	tartrate resistant acid phosphatase
TRAIL	TNF-related apoptosis-inducing ligand

2 PERI-PROSTHETIC OSTEOLYSIS

2.1 INTRODUCTION

Despite the undoubted benefit of joint replacement, premature failure of joint implants remains a considerable clinical problem (Malchau et al., 1993, Johnsson et al., 1994). Maintaining healthy bone around the surface of prosthetic joint implants is vital for the long-term stability of cemented and uncemented artificial joints. Aseptic failure of prosthetic joints in the short term may be due to a number of reasons, such as poor surgical technique, device failure or massive loss of bone due to stress shielding. However, loosening of joint prostheses in the medium to long term is characterised by large numbers of wear particles and the accumulation of mononuclear phagocytic cells in peri-prosthetic tissues (Vernon-Roberts and Freeman, 1977, Willert and Semlitsch, 1977). The accumulation of macrophages and foreign body giant cells in the peri-prosthetic implant bed is believed to be important in aseptic peri-prosthetic bone loss.

In peri-prosthetic bone lysis, large numbers of macrophages are seen to contain wear particles in the surrounding tissues (Vernon-Roberts and Freeman, 1977, Willert and Semlitsch, 1977). The ingestion of particles by tissue macrophages is thought to initiate a cascade of events, resulting in the loss of peri-prosthetic bone that is characteristic of this pathology. *In vivo* studies have shown that these wear particles stimulate a chronic inflammatory-like response dominated by macrophages and the release of the cytokines, growth factors and prostaglandins known to stimulate bone resorption (Jiranek et al., 1993, Howie and Vernon-Roberts, 1988, Howie, 1990, Lerouge et al., 1997, al-Saffar et al., 1995b, Shanbhag et al., 1995a, al-Saffar and Revell, 1994, Shanbhag et al., 1995b, Konttinen et al., 1997). In support of this, macrophages containing wear particles in the peri-prosthetic tissues express interleukin (IL)-1β and tumour necrosis factor (TNF)-α protein (Jiranek et al., 1993, Appel et al., 1990, Chiba et al., 1994, Perry et al., 1997). *In vitro* studies have shown that particles of different size and composition can elicit different biological responses when placed on macrophages releasing cytokines such as prostaglandin (PG)E_2, IL-1, TNF and IL-6 (Haynes et al., 1993, Shanbhag et al., 1994a, Gelb et al., 1994, Glant et al., 1996, Haynes et al., 1998, Rogers et al., 1997).

Osteoclasts are thought to be the cells responsible for resorption of peri-prosthetic bone that causes implant loosening. Cells isolated from granulomatous tissues adjacent to areas of peri-prosthetic bone loss can develop into functional osteoclasts under appropriate conditions *in vitro* (Sabokbar et al., 1997, Neale et al., 1999a, Itonaga et al., 2000a). The studies described

in this chapter provide further evidence that precursors of osteoclasts reside in peri-prosthetic tissues.

Newly identified molecules, RANKL (also known as osteoprotegerin ligand, osteoclast differentiating factor or TRANCE) and osteoprotegerin (OPG) (also known as osteoclastogenesis inhibitory factor) have been identified as two central players in the development and activation of osteoclasts (Lacey et al., 1998, Hakeda et al., 1998, Yasuda et al., 1998a, Yasuda et al., 1998b, Kong et al., 1999b). A recent report has shown that OPG can inhibit the formation of osteoclasts from cells derived from tissues around failed prostheses, suggesting that RANKL may be important in determining whether osteoclasts form in these tissues (Itonaga et al., 2000a). However, as yet, there is no direct evidence for the presence of RANKL and OPG protein in the peri-prosthetic tissues adjacent to osteolysis.

RANK, a TNF receptor family member present on pre-osteoclasts and osteoclasts, is the receptor for RANKL (Lacey et al., 1998, Nakagawa et al., 1998, Myers et al., 1999). RANK mediates osteoclast differentiation and activation induced by RANKL (Hsu et al., 1999). The expression of RANK mRNA and protein in the peri-prosthetic tissue may reflect the presence of cells of the osteoclast lineage in the tissue adjacent to lysis.

This chapter also investigates the expression of another member of the TNF family, TNF-related apoptosis-inducing ligand (TRAIL) (Wiley et al., 1995, Pitti et al., 1996, Walczak and Krammer, 2000). OPG can bind to TRAIL and prevent apoptosis *in vitro* (Emery et al., 1998). Conversely TRAIL blocks OPG mediated inhibition of osteoclast formation (Emery et al., 1998). The presence of TRAIL in peri-prosthetic tissue may play a role in mediating the OPG available for inhibition of osteoclast formation. By modulating the ratio of RANKL to OPG in the joint, TRAIL would also modulate osteolysis.

The accumulated evidence suggests that monocytes and macrophages are present *in situ* and are recruited to the tissue from the peripheral blood in response to wear particles. These cells are stimulated to release cytokines that are able to increase osteoclastogenesis. Initial *in vitro* experiments were designed to determine whether human adherent peripheral blood mononuclear cells (PBMC) express mRNA corresponding to cytokines and primary mediators of bone resorption (RANKL, OPG and RANK), when exposed to prosthetic particles. Different types of metal alloys that are commonly used in prosthesis manufacture were tested. A semi-quantitative reverse transcription polymerase chain reaction (RT PCR) technique was used to determine levels of mRNA. The effects of particles of different prosthetic material on

mRNA expression were then compared. The ability of particles to stimulate the release of the cytokine M-CSF was also assessed.

Although abundant polyethylene (PE) shards are noted in the tissue retrieved from sites of osteolysis in failed joints during routine histological assessment these particles were not included in the study. This was because the required number of endotoxin free PE particles, within the same size range, could not be produced using the same methods employed for the production of the metal particles.

Studies were also carried out to explore the mRNA expression of osteoclastogenic mediators in peri-prosthetic tissue adjacent to bone loss that is commonly seen near failed prosthetic joints. The *in vivo* levels of mRNA corresponding to mediators of osteolysis (RANKL, OPG and M-CSF) were investigated in the total cell population isolated from soft tissue around failed implants in the hip, knee, shoulder and wrist joints, using RT PCR. Levels of mRNA corresponding to the apoptotic mediator, TRAIL, were also investigated. In addition, RT PCR was used to see if the cells isolated from the peri-prosthetic tissue expressed mRNA corresponding to the osteoclast markers tartrate resistant acid phosphatase (TRAP), RANK and calcitonin receptor (CTR). The levels of mRNA expression in cells extracted from sites of osteolysis, which contained particles of different materials, were compared.

Where possible, cells were isolated from soft tissue around failed implants in the hip, knee, shoulder and wrist to determine whether this tissue contains cells that are capable of differentiating into bone-resorbing osteoclasts. Cells were cultured with or without human bone-derived osteoblast-like cells, to determine whether contact with human osteoblast-like cells was required for osteoclast formation. The levels of RANKL mRNA, and of RANKL relative to OPG mRNA, in tissues adjacent to areas of osteolysis, were determined and these mRNA parameters were then compared with the ability of the adherent cell population, isolated from these tissues, to develop into osteoclasts *ex vivo*.

The expression of RANKL and OPG protein produced in the peri-prosthetic tissues, adjacent to osteolytic regions, were determined using a three-step immunoperoxidase immunohistochemical staining technique. The levels of OPG and RANKL in the peri-prosthetic tissue were compared with normal and osteoarthritic (OA) tissue samples by using established semi-quantitative assessment (SQA) and quantitative digital image analysis techniques. To identify cell types associated with these proteins, dual labelling with OPG or

RANKL antibodies was followed by an alkaline phosphatase anti-alkaline phosphatase staining using antibodies to cell type- specific markers.

In the absence of commercially available antibodies to RANK suitable for immunohistochemistry, *in situ* hybridisation was carried out using a riboprobe specific for RANK to identify the pre-osteoclasts and osteoclasts in peri-prosthetic tissue. In addition, TRAP staining was carried out to further identify osteoclastic cells *in situ***.**

The studies were designed to test the hypothesis that prosthetic particles stimulate macrophages to differentiate into osteoclasts by stimulating the expression of osteoclastogenic mediators.

2.2 MATERIALS AND METHODS

2.2.1 Generation of Wear Particles

Metal particles were generated from a range of prosthetic materials, using a method mimicking *in situ* wear, for use *in vitro* to determine their effect on human monocytes. Particles were produced from a range of alloys commonly used in prosthesis manufacture; Titanium aluminium vanadium (TiAlV) (Protasul 64, Sulzer Orthopaedics, Zurich Switzerland), Cobalt-chrome alloy (CoCr) (Vitallium, Howmedica, Rutherford, N.J. USA) and 316L stainless steel (SS) (DePuy Orthopaedics, Warsaw, IN). Particles of PE were not included in these studies because the required number of endotoxin free PE particles, within the same size range, could not be produced using the same methods employed for the production of the metal particles.

The method of particle production was the same as previously described (Rogers et al., 1993). Briefly, small metal blocks and milling solution, consisting of endotoxin-free Dulbucco's phosphate buffered saline (PBS) (Gibco BRL; Life Technologies, Melbourne, Australia), 10% foetal calf serum (Gibco, BRL), 5μg/ml of penicillin and 50U/ml of streptomycin (Gibco, BRL), were placed inside hemispherical cups of the same alloy and bound together. Cups were then shaken for 72 hour cycles and particles were collected and stored at –20^{o}C until ready for sizing. Washing and preparation steps were performed in clean, heat-sterilised glassware to produce particles free from endotoxin contamination. Prior to being shaken, materials were washed in 0.01% E-Toxa-Clean (Sigma-Aldrich, St. Louis, MO, USA) and rinsed three times in endotoxin-free deionised water. The metal components were then washed in 20% nitric acid for 20 minutes, in accordance with ASTM F86-76 standard recommended practice for the surface preparation and marking of metallic surgical implants (Materials, 1980). Components were washed thoroughly in distilled water then sterilised by dry heating at 180^{o}C overnight.

All preparations of particles and media used for these studies contained less than 10pg/ml of endotoxin (measured using the E-toxate kit from Sigma-Aldrich, St. Louis, MO, USA). This is well below levels known to affect the production of mediators by monocytes (Haynes et al., 1998), indicating that endotoxin contamination was not responsible for the effects described in this study.

Particles generated from each alloy were sized by differential centrifugation to isolate those within the range 0.5-3μm (Rogers et al., 1993), according to Stoke's law. Stoke's law relates particle size, viscosity, density, speed, and time as shown:

Stokes Law:

$$r^2 = \frac{9\eta h}{2t\,(r - r_0)\,w^2 n} \qquad w = \frac{rpm^2}{60}$$

r = radius
η = viscosity of medium
h = height of sample
t = time
r = density of particle
r_o = density of the fluid
w = angular velocity
n = distance of sample from axis of rotation

The size of the particles was confirmed using scanning electron microscopic analysis and a Coulter counter (Rogers et al., 1993). Particles within this range have been reported to be present in tissues surrounding failed prostheses (Lee et al., 1992, Howie, 1990). The shape of the particles of different alloys produced using this method is similar to those found *in vivo* (Rogers et al., 1993). Particles were used at a concentration of 4×10^7 particles/ml, which is representative of the concentrations of particles found in tissue surrounding prosthetic joints.

2.2.2 Isolation and Culture of Peripheral Blood Mononuclear Cells (PBMC)

In vivo cells of the monocyte/macrophage lineage are recruited to peri-prosthetic tissue in response to wear particles. Therefore, monocytes were used in the *in vitro* studies, designed to determine the effect of particles of different metal alloys on the expression of mediators of osteoclast formation this cell type.

Human blood buffy coats from healthy donors were obtained from donors to the Red Cross Transfusion Service (Adelaide, South Australia). Buffy coats were diluted in a ratio of 1:3 in Hank's balanced salt solution (HBSS) (Gibco, BRL), human mononuclear cells, from three donors, were then isolated using Ficoll hypaque separation gradient (Pharmacia Biotech, Uppsala, Sweden). After centrifugation at 300 x g for 30 minutes, the mononuclear cell layer was removed and cells were washed three times in HBSS. Previous studies have shown that >95% of these cells that are adherent to glass coverslips stained positive for non-specific esterase, a marker for cells of the macrophage/monocyte lineage (Haynes et al., 1993). The

blood mononuclear cells were resuspended at 4 x 10^6 cells in 1ml of RPMI-1640 medium (Gibco, BRL), containing 25mM HEPES and including buffer supplemented with 10% foetal calf serum (Gibco, BRL), 5μg/ml of penicillin and 50U/ml of streptomycin (Gibco, BRL). 1 ml of the cell suspension was placed into individual wells of 16mm flat-bottomed 24-well trays (Falcon, Becton Dickinson Labware, New Jersey, USA). Cells were left to adhere for one hour at 37^{o}C in a 5% CO_2 incubator and the non-adherent cells were then removed by rinsing with HBSS. The adherent cells were incubated in 1ml of the RPMI medium containing particles of TiAlV, CoCr or 316L stainless steel at a concentration of 4 x 10^7 particles/ml. Lipopolysaccharide (LPS) (*Eschericia coli* 0111:B4; Sigma-Aldrich, St. Louis, MOUSA) at 5μg/ml was used at a positive control for monocyte activation. Monocytes cultured with media alone were used as a negative control.

2.2.3 *In vitro* Treatments

Monocytes isolated from buffy coats from the three donors were treated as described above. The supernatants were aspirated off after 6, 12, 24 or 48 hours culture and total cellular RNA was then extracted from the monocytes using Trizol reagent (Gibco, BRL) (this is described below in detail).

Complementary DNA (cDNA) was transcribed from the RNA and semi-quantitative RT PCR was used to assess the pattern of expression of mRNA corresponding to RANKL, RANK, M-CSF and OPG. The RT PCR method is described in detail below. Cells from all donors were tested individually and the results were expressed as the average of these values.

2.2.4 RNA Extraction

Suspensions from each well, containing 250μl of Trizol and cell lysates, were centrifuged briefly to pellet cell membranes. The suspension was then transferred to 1.5ml RNAse free tubes. To each sample, chloroform was added, at a 1:5 ratio of the original volume of Trizol. The 1.5ml tubes were vortexed for 15 seconds and then allowed to sit on ice for 2 minutes. Samples were then centrifuged at 4^{o}C for 15 minutes at 12, 000 rpm.

Following centrifugation and separation into two phases, the upper colourless aqueous phase was collected and transferred to a fresh 1.5ml RNAse free tubes. An equal volume of ice cold isopropanol was added to the tube, mixed vertically and stored at –20oC for 30 minutes. Samples were centrifuged at 4^{o}C for 15 minutes at 12, 000 rpm. The supernatant was carefully removed and discarded leaving a pellet of RNA precipitate in the tube. The pellet

was washed with 500 µl of 75% ice-cold ethanol, vortexed and centrifuged at 4^{O}C for 8 minutes at 7, 500 rpm. The supernatant was removed carefully and the pellet briefly air-dried.

Pellets were resuspended in diethylpyrocarbonate (DEPC) treated Milli-Q water. The quantity and quality of RNA was assessed by measuring the UV absorbance at 260 and 280nm and by visualisation on a 1x MOPS/formaldehyde agarose gel.

2.2.5 Preparation of RNA and Reverse Transcription Polymerase Chain Reaction (RT PCR)

First strand cDNA was synthesized from total RNA extracted from each sample using an AMV reverse transcriptase cDNA kit, as per manufacturers instructions (Promega, Madison, WI, USA). 1µg of total RNA was reverse transcribed for each sample. cDNA was then amplified by PCR in a thermal cycler (Eppendorf, Hamburg, Germany) to generate products corresponding to mRNA encoding the gene products for RANKL, OPG, RANK and M-CSF, which are listed in Table 1. Each 20µl amplification mixture contained 1µl of the cDNA sample or water control, 0.2mM dNTPs and 1U of Platinum *Taq* DNA polymerase (Life Technologies), 100ng each of forward and reverse primers, 1.5mM $MgCl_2$, 1x PCR reaction buffer, and sterile DEPC H_2O to a final volume of 20µl. Twenty-two cycles of PCR were performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 24-36 cycles for the other primer pairs**.** The initial step involved activation of the polymerase at 95^{o}C for 2 minutes, each cycle consisted of 15 seconds denaturation at 94^{o}C, 15 seconds annealing, at temperatures indicated in Table 1**,** and 1 minute extension at 72^{o}C.

Amplification products were resolved by electrophoresis on a 2% w/v agarose gel at 120V for approximately 1.5 hours and post-stained with SYBRgold nucleic acid stain for 20 minutes (Molecular Probes, Eugene, OR, USA). The intensity of the PCR products was visualised on a FluorImager (Molecular Dynamics, Sunnyvale, California, USA) and the optical density of each band was measured using Quantity One software (Bio-Rad, Hercules, California, USA). Amplified products were normalised to GAPDH products for each sample, and the data was expressed as a ratio of the respective GAPDH PCR product.

To control for false positive results, PCR reactions were carried out using non-reverse transcribed RNA and on reaction mixtures, in which no RNA was added. Human tissue and cell line-derived cDNAs were used as positive controls to confirm the activity of each primer pair under the conditions used. The specificity of the PCR reaction was confirmed by Southern-blot transfer onto a nylon membrane (Hybond-N; Amersham Life Science, Inc.,

Arlington Heights, IL) as well as hybridisation with digoxigenin (DIG)-labelled internal oligonucleotide probes.

Preliminary RT PCR experiments were performed using a range of PCR cycles for each of the primers corresponding to the mRNA sequences to be investigated. Values derived from the quantitation of the amplified products were plotted on a graph against the number of PCR cycles used. The amplification curves were then used to determine at which cycle number the mRNA expression, corresponding to each primer, fell within in the linear range. This was done to ensure that the number of PCR cycles was within the exponential phase of the amplification curve, enabling semi-quantitative comparisons to be made between the levels of expression of the various RNA species in the samples.

Table 1. RT PCR Primers and Conditions for the Specific Amplification of Human mRNA Targets

Target gene	*Sense*[a]	*Primer sequence (3'-5')*	*Annealing temperature*[b] (^{o}C)	*Expected product size* (BP)
GAPDH	S	CACTGACACGTTGGCAGTGG	60	414
	AS	CATGGAGAAGGCTGGGGCTC		
CTR	S	GCAATGCTTTCACTCCTGAGAAA	62	782
	AS	AGTGCATCACGTAATCATATATC		
IL-1β	S	AGGAAGATGCTGGTTCCCTGC	60	151
	AS	CAGTTCAGTGATCGTACAGGTGC		
IL-6	S	ATGAACTCCTTCTCCACAAG	60	546
	AS	GTGCCTGCAGCTTCGTCAGCA		
sIL-6R	S	CCCATGCAGGCACTTACTAC	60	350/256
	AS	GTGGCTCGAGGTATTGTCAG		
M-CSF	S	CAGTTGTCAAGGACAGCAC	60	670
	AS	GCTGGAGGATCCCTCGGACTG		
RANKL	S	AATAGAATATCAGAAGATGGCACTC	62	668
	AS	TAAGGAGGGGTTGGAGACCTCG		
OPG	S	TGCTGTTCCTSCAAAGTTTACG	62	435
	AS	CTTTGAGTGCTTTCGTGCGTG		
RANK	S	CCTACGCACAAGGCGAAGATGC	62	704
	AS	CGTAGACCACGATGATGTCGCC		
TNF-α	S	TCAGATCATCTTCTCGAACC	60	361
	AS	CAGATAGATGGGCTCATACC		
TRAIL	S	ACAGCAGTCAGACTCTGACAGG	62	838
	AS	CATGGTCCATGTCTATCAAGTGC		
TRAP	S	CTGGCTGATGGTGCCACCCCTG	65	469
	AS	CTCTCAGGCTGCAGGCTGAGG		

2.2.6 Immunoassay Measurement of Supernatant Cytokine Levels.

Monocytes from eight donors were incubated with metal particles for 48 hours (as described above). Supernatants were removed and centrifuged at 4000g for ten minutes to remove any cells or particles. Supernatants were then stored at -20ºC prior to testing with enzyme-linked immunosorbent assays (ELISA). Human M-CSF (R&D Systems, Minneapolis, MN, USA) was measured using ELISA kits that were commercially available, following the manufacturer's instructions. Levels of RANKL, RANK and OPG protein were not determined as appropriate techniques were not available at the time of the *in vitro* studies.

2.2.7 Statistical Analysis of *In Vitro* Data

The PCR results were analysed by integrating the area beneath the response curve from zero to 48 hours, using image analysis software (NIH, Image version 1.61 PPC; National Institutes of Health, Bethesda, USA). This integration step was included to evaluate the cumulative effects of mRNA expression over time. Repeat measures of variance with a within-donor factor of treatment for unstimulated, LPS, TiAlV, CoCr- and 316L S/S treated cells were assessed using program 5V of BMDP statistical software (BMDP Statistical Software Incorporated, Los Angeles, California). A Wald statistic was used to test for differences between treatments. If a statistical difference was found, pair-wise treatment differences were calculated from their respective means and standard errors. Z-scores and corresponding p values were then calculated and a value of less than 0.01 was accepted as significant to account for multiple-testing.

2.2.8 Tissue Processing for *In vivo* and *Ex vivo* Studies

Isolation of cells and mRNA from Peri-Prosthetic Tissue Adjacent to Sites of Osteolysis

Tissue samples were taken at operation from patients undergoing surgery for the removal or replacement of hip, knee, wrist (Figures 1A, B C and D), or elbow prosthetic joints. Tissue was taken from adjacent to sites of osteolysis as indicated on the X-rays in Figure 1. In all patients failure was due to osteolysis. Peri-prosthetic tissue was immediately placed in HBSS (Gibco BRL) and where possible the tissue was divided into three pieces: 1) fixation with 10% formalin and paraffin embedding for routine histology and *in situ* hybridisation 2) freezing in Tissue Tek OCT medium (Miles Diagnostics, Elkhart, IN, USA) for immunohistochemistry and 3) digestion for cell culture and to assess mRNA expression. General histology was routinely carried out on the peri-prosthetic tissue. Consistent with previous reports, large numbers of macrophages and wear particles were evident within the tissue adjacent to zones of osteolysis(Vernon-Roberts and Freeman, 1977, Willert and

Semlitsch, 1977). The study protocol was approved by The University of Adelaide Human Ethics Committee, in accordance with the regulations of the National Health and Medical Research Council of Australia.

To release cells from the tissues for RT PCR and cell culture studies, the tissue was digested in a solution of calcium- and magnesium-free HBSS containing 1mg of collagenase (Sigma-Aldrich, St. Louis, MO, USA) and 1mg/ml of dispase (Sigma-Aldrich Chemical Co., Castle Hill, Australia) and incubated at 37^{o}C, 5% CO_2. After 60 minutes, 0.5mg/ml of trypsin (Sigma-Aldrich, St. Louis, MO, USA) in HBSS solution was added and the tissue incubated for a further 30 minutes at 37^{o}C, CO_2. Large clumps of undigested connective tissue were removed by filtering the cells through a 70μm cell sieve (Falcon; Becton Dickinson Labware, New Jersey). The yield of cells varied depending on the amount of tissue digested (approximately 1cm^3) and the cellularity of the tissue. Between 5 x 10^5 and 25 x 10^6 cells were isolated from each tissue sample, with a mean of 5 x 10^5 cells/mg wet weight of tissue. The cell suspension was then washed in HBSS and cells were resuspended in RPMI 1640 media at a concentration of 1 x 10^6cells per ml.

Initially, RT PCR was performed on total RNA extracted from cells isolated from a total of nine patients. Six were undergoing revision of hip arthroplasty, one revision of knee arthroplasty, one removal of a silastic lunate wrist prosthesis and one removal of a silastic elbow prosthesis. In some cases tissue was taken from more than one site adjacent to areas of osteolysis. RNA was extracted from between 5 x 10^5 and 1 x 10^6 cells for mRNA analysis by RT PCR (this method is described above in detail). In addition to OPG, RANKL, M-CSF and RANK, the expression of various inflammatory cytokines was also investigated. PCR products corresponding to the mRNA encoding the gene products listed in Table 1 were generated. When there were sufficient cells remaining, studies for the generation of osteoclasts were carried out with the help of Mrs Maria Capone, Technical Assistant, at The University of Adelaide. The details of the patients and medication at time of surgery for patients used in this part of the study are listed in Table 2.

Table 2. Demographic and Clinical Details of Patients Included in the RT PCR and Cell Culture Studies

Patient	Age (years)	Sex (M/F)	Site	Tissue Type	Prosthetic Alloy	Current medication
1	56	F	Hip	Acetabular membrane	CoCr[1], PE[2]	Premarin, Ca^{++} mineral
1	56	F	Hip	Joint capsule	CoCr[1], PE[2]	Premarin, Ca^{++} mineral
2	72	F	Hip	Acetabular membrane	CoCr[1]	None
3	46	M	Hip	Joint capsule	CoCr[1]	None
4	60	M	Hip	Fem membrane	CoCr[1], PE[2]	NSAID[3]
4	60	M	Hip	Joint capsule	CoCr[1], PE[2]	NSAID[3]
4	60	M	Hip	Acetabular membrane	CoCr[1], PE[2]	NSAID[3]
5	75	M	Hip	Acetabular membrane	CoCr[1], PE[2]	None
5	75	M	Hip	Joint capsule	CoCr[1], PE[2]	None
6	79	F	Hip	Acetabular membrane	CoCr[1], PE[2]	calcium, calcitrol (affects calcium and bone metabolism)
6	79	F	Hip	Joint capsule	CoCr[1], PE[2]	calcium, calcitrol (affects calcium and bone metabolism)
7	70	F	Knee	Joint capsule	PE[2], zimmer	NSAID[3]
8	46	F	Elbow	Joint capsule	Silicone	HRT[4]
8	46	F	Elbow	Membrane	Silicone	HRT[4]
9	50	F	Wrist	Joint capsule	Silicone	None

[1] Cobalt Chromium
[2] Polyethylene,
[3] Non-steroidal anti-inflammatory drugs
[4] Hormone replacement therapy

To investigate additional mediators and markers of osteoclast activity in the tissue adjacent to osteolytic regions, an additional 13 peri-prosthetic samples, from a group of eleven patients, were processed for RT PCR. Additional cell culture studies could not be carried out on these samples as the cell yields were too low. Details of the patients and medication at time of surgery used in these RT PCR experiments are listed in Table 3.

Table 3. Demographic and Clinical Details for Patient Tissues Investigated for TRAIL, CTR and TRAP mRNA Expression using RT PCR

Patient No.	**Age (years)**	**Sex (M/F)**	**Site**	**Tissue Type**	**Prosthetic Alloy**	**Current medication**
1	74	F	Hip	Femoral membrane	SS[1], PE[2]	None
2	67	M	Hip	Femoral membrane	SS[1], PE[2]	BABA[4]
3	75	F	Hip	Femoral membrane	zimmerloy, PE[2]	None
4	28	F	Hip	Femoral membrane	PE[2], CoCr[3], vitallium	BABA[4]
5	75	M	Hip	Joint capsule	zimmerloy, PE[2]	BABA[4]
6	33	M	Knee	Joint capsule	CoCr[3]	NSAID[5]
7	73	F	Hip	Tibial membrane	titanium alloy, PE[2], CoCr[3] (zimaloy)	NSAID[5]
8	70	F	Hip	Acetabular membrane	SS[1], PE[2]	None
9	71	F	Knee	Joint capsule	Titanium, PE[2], CoCr	None
10	74	M	Hip	Joint capsule	PE, titanium, CoCr	None
11	60	M	Knee	Joint capsule	Tivanium, carbon	NSAID[5]
12	73	M	Knee	Joint capsule	PE[2], vitallium	None
12	73	M	Knee	Synovium	PE[2], vitallium	None
13	62	M	Knee	Joint capsule	PE[2], titanium	None

[1] Stainless steel
[2] Polyethylene
[3] Cobalt Chromium
[4] Beta-adrenergic blocking agent
[5] Non-steroidal anti-inflammatory drugs

2.2.9 Isolation and Culture of Human Bone-Derived Osteoblast-Like Cells (HBDC)

Human bone derived osteoblast-like cells (HBDC) were used for co-culture studies with the cells extracted from peri-prosthetic tissues retrieved during revision surgery. Trabecular bone chips were obtained from normal patients during primary hip replacement as described previously (Haynes et al., 1997). Bone chips were cultured in 75cm^2 flasks (Falcon, Becton Dickinson Labware, New Jersey, USA) with Minimal Essential Media (alpha) supplemented with 10% foetal calf serum, 5μg/ml of penicillin and 50U/ml of streptomycin, 1% ascorbate-2-phosphate and 1% L-glutamine. Once cells became confluent they were detached from the flasks using collagenase (Sigma-Aldrich, St. Louis, MO, USA) and dispase (Sigma-Aldrich, St. Louis, MO, USA).

HBDCs were resuspended at 3 x 10^4 cells/ml in medium and seeded onto either 13-mm diameter sterile glass coverslips in 24 well trays or 3.0 x 0.1mm thick discs of sperm whale dentine (a gift from the Australian Customs Service, Canberra, Australia) in 96 well trays. HBDC cells were left to adhere for 24hr before addition of cells isolated from the peri-prosthetic tissues. The medium was removed from the overnight cultures of HBDCs and 4 x 10^5 (coverslip) or 2 x 10^5 (dentine) peri-prosthetic derived cells were added. In some experiments, as indicated, cells from peri-prosthetic tissues were added to dentine in the absence of osteoblast-like cells. After one hour of incubation at 37ºC, 5% CO_2, non-adherent cells were removed by washing in HBSS.

The individual coverslips and pairs of dentine slices were placed in 16-mm diameter wells with 1ml of α-MEM medium containing 10^{-8}M 1α,25 dihydroxy D_3, 10^{-8}M dexamethasone (Fauldings, Adelaide, Australia) and 25ng/ml recombinant human M-CSF (a kind gift from the Genetics Institute, Cambridge, MA, USA). Medium was replenished every 3 days for the duration of the experiment. All the cell-culture experiments were carried out in duplicate for each peri-prosthetic sample.

2.2.10 Tartrate Resistant Acid Phosphatase (TRAP) *Ex vivo*

To identify cells of the osteoclast-lineage in peri-prosthetic tissue cells were stained for the presence of TRAP, using a commercial staining kit (Sigma-Aldrich, St. Louis, MO, USA) as recommended. Cytospins of freshly isolated peri-prosthetic cells and cultures of cells seeded onto the coverslips were stained for TRAP at 1, 4, 7, 14 and 21 days. Cells were then briefly counterstained with the nuclear stain Methyl Green. Immunostaining to detect TRAP was carried out with the help of Mrs Maria Capone (Technical Assistant, Department of Pathology, The University of Adelaide).

2.2.11 Resorption Pit Formation

The ability of a cell to form lacunae by the resorption of bone is considered to be confirmation that it is a functional osteoclast. Cells isolated from the peri-prosthetic tissue were seeded onto whale dentine slices with and without the previous addition of HBDC cells for 7 or 14 days to see if they could resorb the dentine.

At these time points cells were removed by treating the dentine with 25% ammonia solution for 15 minutes, ultrasonicated for 5 minutes followed by 15 minutes in trypsin at 37^{o}C and a final sonication for 5 minutes to remove any remaining adherent cells. The dentine discs were then dehydrated by passage through graded alcohol solutions from 70% to 100%, followed by drying under vacuum in a desicator overnight before being mounted on stubs and carbon-coated. Discs were then visualised using a Philips XL-20 scanning electron microscope to look for and count lacunae. Mrs Maria Capone, (Technical Assistant, Department of Pathology, The University of Adelaide) helped assess the number of pits formed on the dentine slices.

Comparisons were then made between the RT PCR analysis on RNA extracted from the total cell population and cell culture results using adherent cells isolated from the same peri-prosthetic samples. In particular, the ratio of RANKL and OPG mRNA was compared with osteoclast formation in culture.

2.2.12 *In situ* Hybridisation with Digoxigenin (DIG)-labelled Riboprobes

RANK, the membrane bound receptor for RANKL, is expressed by cells of the osteoclast-lineage (Nakagawa et al., 1998, Myers et al., 1999). In order to identify the location and type of cells expressing mRNA corresponding to RANK, *in situ* hybridisation was performed on peri-prosthetic tissue from twelve patients. The non-radioactive method of *in situ* hybridisation was based on the methods of Smith *et al*(Smith et al., 1997) using riboprobes labelled with digoxigenin (DIG).

Generation of the Riboprobe

To produce sense and anti-sense riboprobes to hybridise with RANK mRNA, PCR products of less than 800bp were amplified using specific sequence primers. PCR products were electrophoresed on a 1.5% DNA grade agarose gel, excised and extracted using QIAEX II agarose gel extraction (QIAGEN, Germany). Restriction endonucleases (*Nco 1* and *Sal 1*) were chosen so that the PCR product fragments had different restriction endonuclease sites at

the 5' and 3' ends of the PCR product. This allowed insertion in a known orientation within the polycloning site of an appropriate plasmid (pGEM T easy vector, Promega, Madison, Wisconson), so that the fragment was flanked by the SP6 and T7 RNA polymerase promoters. Following transformation of competent bacteria (JM109 cells, Invitrogen) with the RANK plasmid, successful transformants were isolated using ampicillin selection on L-Agar plates. Single colonies were picked and used to innoculate 10ml L-Broth containing 100μg/ml Amplicillin to generate enough plasmid for use. Plasmids containing inserts were then extracted from the cells using a Concert Rapid Plasmid mini-prep kit (Gibco, BRL).

Purified recombinant plasmids were electrophoresed on a 1% DNA grade gel, next to a negative control consisting of the plasmid minus the insert, to ensure the selected plasmids contained the RANK insert. Using a cycle sequencing ready reaction kit (containing labelled dNTPs and ampli taq DNA polymerase) the insert was sequenced by an ABI PRISM Dye Terminator to confirm that the full and correct RANK sequence was inserted into the plasmid.

Plasmid vectors were linearised using *Nco 1* and *Sal 1* restriction endonucleases so that the PCR product insert could be transcribed into an RNA copy from either the SP6 or T7 RNA polymerase promoters without including any vector sequence. Linearised plasmids containing the insert were again electrophoresed on a 1% DNA grade gel to check complete linearisation of the vector and then extracted using Concert Rapid Gel extraction system (Life Technologies, BRL). A small sample was then electrophoresed on a 1% DNA agarose gel to ensure efficient extraction of linearised plasmid plus insert.

RNA copies were transcribed using a commercial riboprobe generation kit, which included digoxigenin-labelled UTP (Boehringer Mannheim, Mannheim GmbH, Germany) as part of the nucleotide mix. Copies of the sense or antisense strand of the product were transcribed with T7 or SP6 RNA polymerase, respectively, to generate digoxigenin-labelled UTP riboprobes. Template DNA was removed by incubation with an RNase free DNase (Boehringer Mannheim, Mannheim GmbH, Germany). Riboprobes were precipitated and resuspended in DEPC treated water. Labelling of the appropriate sized fragment was confirmed by Northern blot gel transfer followed by detection with anti-digoxigenin antibodies then viewing following a colourimetric reaction after addition of the colour substrate, 5bromo-5chloro-3indolyl-Phosphate, Boehringer Mannheim BCIP/Nitro Blue Tetrazolium (BCIP/NBT, Boehringer Mannheim, Mannheim GmbH, Germany). Aliquots were stored at –70^{o}C for up to 12 months. The same concentration of sense and antisense probes were used for *in situ* hybridisation.

In situ Hybridisation Method

Two paraffin sections from each sample were processed concurrently. Sections were dewaxed and rehydrated in histolene and graded concentrations of ethanol. The *in situ* hybridisation method is based on a published Biotin labelled non-radioactive method (Smith et al., 1997). Sections were circled with a PAP pen and slides were then rinsed in autoclaved milli Q water and placed in 0.2N HCL for 20 minutes at room temperature. The sections were again washed in autoclaved water for 2 minutes. To increase access of the probe, the tissue sections were digested with 50μg/ml proteinase K (Boehringer Mannheim, Mannheim GmbH, Germany) for 20 minutes at 37^{o}C. After rinsing with autoclaved water, sections were treated with 0.1M Tris-HCL 0.2M glycine solution (pH 7.2) for 10 minutes at room temperature. Sections were rinsed in autoclaved water and then 2X SSC for 5 minutes.

Prehybridisation buffer, (10% Dextran Sulphate, 0.05% Polyvinylpyrrolidone (PVP), 2X SSC, 50% Formamide, deionized, 0.05% Triton X-100), containing denatured Herring Sperm, was added to the sections, covered with parafilm and incubated for 2 hours at 55^{o}C. The solution was then drained off and prehybridisation buffer containing the sense (negative) or antisense (positive) riboprobe (denatured at 80^{o}C and set on ice) was added to each section. Sections were covered with acid-cleaned coverslips, sealed with a rubber cement and hybridised overnight at 60^{o}C. After hybridisation overnight, the sections were washed at 45^{o}C in 2X SSC solution for 10 minutes, 1X SSC for 5 minutes, 0.1X SSC for 15 minutes then 0.1X SSC for 5 minutes at room temperature.

Digoxigenin Detection

Sections were washed with hybridisation buffer 1 (0.1M Tris, 0.015M Sodium chloride pH 7.5) for 5 minutes. To block non-specific binding, 10% normal sheep serum (NSS) (Jackson Immuno Research) diluted in hybridisation buffer was added to sections, incubated for 45 minutes at room temperature then drained off. Anti-digoxigenin alkaline phosphatase-labelled antibody (anti-DIG AP, Boehringer Mannheim) diluted 1:500 in hybridisation buffer containing 10% NSS was then added for 60 minutes. Sections were washed in hybridisation buffer I (3 x 10minutes) to remove excess antibody and then equilibrated in hybridisation buffer II for 2 minutes. The colour substrate, BCIP/NBT was then added and the colour developed in the dark. The reaction was stopped with milli Q water and sections were mounted using aquamount or left unmounted for dual labelling.

2.2.13 Dual Labelling: *In situ* Hybridisation and Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP)

To identify the lineage expressing RANK mRNA a selection of eight sections were dual labelled with an antibody to the monocyte/macrophage marker, CD68. Dual labelling on paraffin sections involved *in situ* hybridisation for detection of RANK mRNA followed by immunohistochemistry (Wikaningrum et al., 1998, Parker and Smith, 1999).

Following hybridisation and detection of the probe the development of the digoxigenin colour substrate reaction with BCIP/NBT was carried out. Sections were then washed and Tris glycine solution was added for 1 hour to prevent cross reactivity between the primary antibody and the linking antibody. The CD68, an IgG1, kappa class antibody (as listed in Table 4) was added and sections were incubated overnight at room temperature. Standard APAAP immunohistochemistry was carried out (as described in detail below) and detected with fast red as the dye (Tak et al., 1995, Parker and Smith, 1999).

Table 4. Details of Antibodies used in ELISAs and Immunohistochemical Staining

Antibody/ CLONE	**Antigen**	**Raised in/Isotype**	**Cell Lineage**	**Dilution used**	**Source**
OPG Mab 805	OPG	Mouse IgG1	To be determined	1/400 (DE AEC)	R & D Systems Inc
OPG BAF805	OPG	Goat IgG	Not applicable	1/500 ELISA	R & D Systems Inc
OPG Mab 8051	OPG	Mouse IgG2a	To be determined	1/1000 (DE AEC)	R & D Systems Inc
RANKL Mab626	RANKL	Mouse IgG2b	To be determined	1/400 (DE AEC)	R & D Systems Inc
CD3 Leu-4	CD3	Mouse IgG1	Pan T lymphocytes	1/10 (APAAP)	Becton Dickinson, CA, USA
CD68 EMB11	CD68	Mouse IgG1kappa	Monocytes, macrophages	1/400 (APAAP)	DAKO, Denmark
CD68 KP1	CD68	Mouse IgG1	Monocytes, macrophages		Novo Castra
Mab 67	CD55	Mouse IgG1	Endothelial cells, type B synoviocytes	1/7000 (APAAP)	Serotec, UK
Von Willebrand F8/86	Von Willebrand Factor	Mouse IgG1kappa	Endothelial cells	1/50 (APAAP)	DAKO, Denmark
CD22 6B11	CD22	Mouse IgG1	B lymphocytes	1/300 (APAAP)	CLB

2.2.14 Immunohistochemical Experiments

Materials

Homodimeric Recombinant human OPG/Fc (amino acids 22-401 conjugated to Fc, glycosylated, 805-OS, R&D Systems, Inc.), recombinant human OPG-Fc (dimeric, amino acids 22-194 conjugated to human Fc, non-glycosylated) and RANK (prepared against amino acids 140-317) were generous gifts from Amgen Inc. (Thousand Oaks, CA., USA). Recombinant human TRAIL was a gift from Immunex (Seattle, Washington, USA). Human umbilical vein endothelial (HUVE) cells were obtained from the laboratories of Dr Jenny Gamble (Institute of Medical and Veterinary Science, Australia).

Patients

Details of the patients included in this part of the study are displayed in Table 5**.** Three types of tissue samples from adjacent to joints were included in the study; peri-prosthetic tissue, OA tissue and normal tissue. Tissue samples from around prostheses were taken from sites adjacent to osteolysis (as indicated in Figure 1) in patients undergoing surgery for the removal or replacement of hip, knee, wrist or elbow prosthetic joints. Control samples consisted of OA tissue from patients undergoing primary knee replacements as well as normal joint tissue. OA synovial tissue (ST) was obtained at the time of primary joint replacement surgery while normal synovial tissue was obtained during arthroscopy for unexplained knee pain. Samples from patients with OA of the knee fulfilled published criteria for the classification of OA (Altman et al., 1986). Samples taken from twelve patients undergoing joint replacement surgery, along with samples from five patients with OA of the knee joint, and samples from five normal subjects, were investigated with immununohistochemical staining. The study protocol was approved by the University of Adelaide's Human Ethics Committee, in accordance with the regulations of the National Health and Medical Research Council of Australia.

Table 4 . Demographic and Clinical Details of Patients from which Samples were included in the OPG and RANKL Immunohistochemical Studies

Patient	**Sex (M/F)**	**Age (years)**	**Site**	**Tissue Type**	**Prosthetic Alloy**	**Current Medication**
PP1	F	48	Elbow	Synovium	Silicone	None
PP2	M	73	Wrist	Synovium	Silicone	Unknown
PP3	F	81	Knee	Joint capsule	PE[1], TiAlV[2], CoCr[3]	None
PP4	F	71	hip	Acetabular membrane	SS[4]	Prednisolone, NSAID[5], Premarin
PP5	F	62	hip	Acetabular membrane	Cobalt chrome, PE[1]	Ca^{++} mineral, multivitamins
PP6	F	53	Knee	Synovium	PE[1]	HRT[6]
PP7	F	57	hip	Joint capsule	CoCr[3], titanium zimmerloy	HRT[6], Ca^{++} mineral
PP8	M	77	Knee	Joint capsule	Vitallium alloy, (CoCr), PE[1]	Thyroxine

OA	**Sex (M/F)**	**Age (years)**	**Site**	**Current Medication**
OA1	M	73	Knee	NSAID[5]
OA2	M	56	Knee	BABA[7]
OA3	F	73	Knee	BABA[7]
OA4	M	69	Knee	None
OA5	M	71	Knee	None
N	**Sex (M/F)**	**Age (years)**	**Site**	**Current Medication**
N1	M	46	Knee	None
N2	M	29	Knee	None
N3	M	54	Knee	NSAID[5]
N4	F	59	Knee	None
N5	F	23	Knee	None

PP-Peri-prosthetic, OA-osteoarthritis, N-Normal

[1] Polyethylene
[2] Titanium alloy
[3] Cobalt Chromium
[4] Stainless Steel
[5] Non-Steroidal Anti-Inflammatory Drugs
[6] Hormone Replacement Therapy
[7] Beta-adrenergic blocking agent

Representative tissue samples were separately snap-frozen in Tissue-Tek OCT (Miles Diagnostics, Elkhart, IN, USA) and stored at -80^{o}C until used. Five-micrometer sections were cut on a cryostat and mounted on APTS (Sigma-Aldrich, ST Louis MO) coated glass slides, fixed for 4 minutes in cold acetone and dried at room temperature. The glass slides were boxed and stored at -20°C until immunohistologic analysis.

All immunohistochemical staining was carried out on acetone fixed frozen sections at room temperature, unless otherwise stated.

Immunohistochemistry

Serial sections were stained with mouse monoclonal antibodies (Mab) from commercial sources with known antigen specificities (details are listed in Table 4). For semi-quantitative and quantitative analysis, to eliminate variability in immunohistochemical staining, all sections were processed at the same time using identical procedures. Immunohistochemical staining was carried out using appropriate controls to study the antibodies. For the positive control RA synovial tissue, with a previously defined staining pattern, lymph node or tonsil was stained. The negative controls consisted of omission of the primary antibody and substituting with an irrelevant Mab similar immunoglobulin isotype and subclass for the specific antibodies. The absence of staining was identical to that obtained when a non-specific primary antibody was used (Smith et al., 1992).

2.2.15 Double Enhancement Immunohistochemistry Aminoethylcarbazole (DE AEC)

RANKL or OPG protein was detected using a double enhancement method. Sections were removed from the freezer and allowed to reach room temperature then washed with PBS to remove OCT medium.

Endogenous peroxidase activity was inhibited using 0.1% sodium azide and 1% hydrogen peroxide (BDH, Poole, England) in Tris PBS buffer. Following incubation with each of the primary antibodies to RANKL or OPG (diluted in PBS with 1% BSA) for 60 minutes, bound antibody was detected according to a three-step immunoperoxidase method (Tak et al., 1995, Parker and Smith, 1999, Kraan et al., 2000).

A secondary HRP conjugated-antibody (goat anti-mouse) (diluted 1/100 in PBS containing 1% BSA and 10% normal human serum) was added for 30 minutes and then sections were washed in PBS. A tertiary HRP conjugated-antibody, (swine anti-goat) (diluted 1/60 in PBS containing 1% BSA with 10% normal human serum) was then added for a further 30 minutes

and washed in PBS. Horseradish peroxidase (HRP) activity was detected using hydrogen peroxide as the substrate and aminoethylcarbazole (AEC) (Sigma-Aldrich, St Louis, MO, USA) as the dye. Stained sections were counterstained briefly with haematoxylin solution and mounted in Gurr Aquamount (BDH, Poole, UK). Sections to be dual labelled were left unmounted and processed as described below. Affinity-purified HRP-conjugated goat anti-mouse antibody was obtained from DAKO, (Denmark) affinity purified HRP-conjugated swine anti-goat Ig from Biosource International (CA, USA) and AEC from Sigma-Aldrich (St. Louis, MO, USA).

2.2.16 Dual Labelling: DE AEC and Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP)

Dual immunohistochemical labelling was performed, as previously published, on all sections stained with the Mab OPG 805 and on a selection of samples from each of the groups stained with Mab RANKL 626 and Mab OPG 8051 (Parker and Smith, 1999, Wikaningrum et al., 1998). To identify cells producing OPG, sections were dual labelled with either Mab OPG 805 and anti-Factor VIII or Mab OPG 8051 and anti-CD55 or CD68. Sections labelled with Mab RANKL were dual labelled with anti-CD68, anti-CD55, anti-CD3 or anti-CD22 antibodies. Details of these antibodies are included in Table 4.

In brief, the synovial tissue was incubated with the primary antibody to RANKL (anti-RANKL Mab 626) or OPG (Mab 805 or 8051) and subsequent steps, in a standard three-step immunoperoxidase method, were performed. The final colour product was developed with AEC. Immunoperoxidase staining, developed with AEC, was followed by detection of cell markers.

APAAP

Between the first and second immunohistochemical reactions the tissue was washed in Tris PBS and blocked with Tris-glycine to prevent cross-reaction between the primary and the second linking antibody. After washing in Tris PBS, 20% Normal Donkey serum (Jackson Immuno Research Laboratories Inc, PA) was added for 60 minutes, to block non-specific binding. The excess blocking solution was removed and primary antibodies for cell markers were added.

Antibodies to the cell markers were diluted in Tris PBS with 1% bovine serum albumin and incubated with sections overnight at 4^{o}C. Following washing in Tris PBS a secondary donkey anti-mouse antibody (Jackson Immuno Research Laboratories Inc, PA), diluted in Tris PBS

with 1% BSA (Sigma, St Louis, MO, USA) was added for 40 minutes at room temperature. Sections were washed again in Tris PBS, and then incubated with alkaline phosphatase anti-alkaline phosphatase complex (APAAP-complex) (DAKO, Denmark) at 1/50 in Tris PBS with 1% BSA, for 45 minutes at room temperature. After rinsing in Tris PBS, a chromogenic substrate for alkaline phosphatase, with Fast Blue BB salt (Sigma-Aldrich, St Louis, USA), was applied to the sections for 15 minutes. The colour reaction was stopped with milli Q water and sections were mounted in Aquamount (BDH, Poole, UK).

2.2.17 Determining the Specific Activity of the Mabs 805 and 8051 by OPG Absorption

Two different staining patterns were found during preliminary studies when OPG was detected with Mab 8051 and Mab OPG 805. Therefore experiments were done to test the activity and specificity of the two antibodies. Absorption studies with recombinant OPG were carried out to determine the specific activity of the antibodies directed against OPG. Western blots and ELISA assays were used to investigate the form of OPG detected by these two antibodies and their affinity for binding to TRAIL and RANKL. The antibody to RANKL had been previously characterised by R & D Systems and our group(Atkins et al., 2001). In preliminary studies it was found that blocking the activity of the RANKL antibody by pre-incubation for 24 hours with an excess of human recombinant RANKL (a gift from AMGEN Inc. Thousand Oaks, Ca) eliminated nearly all staining (not shown here).

For the absorption studies synovial tissue from patients with inactive Rheumatoid Arthritis (RA) was used because these tissues were found to strongly expressed OPG. Mab 805 bound only to endothelial cells lining the blood vessels (Figure 2A), while Mab 8051 predominantly bound to cells in the lining layer of the synovial membrane (Figure 2C). At higher concentrations of Mab 8051, weak staining of endothelial cells was also seen. Antibody activity was blocked by incubation of Mab 805 and Mab 8051 at 0.01 μg/ml with human recombinant human OPG at 0.1μg/ml. The diluent containing either OPG antibody with recombinant OPG was left overnight at room temperature to absorb. A control batch of each antibody was incubated with antibody diluent containing an irrelevant protein (bovine serum albumin) at a similar concentration to the recombinant OPG. The following day, both the absorbed and non-absorbed antibody solutions were centrifuged at 12,000rpm in a refrigerated centrifuge to remove any precipitate. The solutions were then diluted in antibody diluent to the usual working dilution and placed on sections of synovial tissue from inactive RA joints. The standard three-step immunoperoxidase method was performed and developed with AEC.

Staining of both the vascular structures (detected with Mab 805) and the synovial lining (detected with Mab 8051) in the inactive rheumatoid synovial tissue could be blocked with excess of recombinant OPG (Figure 2B and 2D). These data show for the first time two different forms of OPG in human tissue adjacent to bone. Differential staining by the two antibodies indicates that they may bind to OPG that is bound to RANKL or free OPG (this is discussed in the ELISA methods below).

2.2.18 Characterising the OPG Antibodies using Western Blots

The immuno-specificity of the two monoclonal antibodies against OPG was investigated using a Western blot. Western blot analysis was carried out using unreduced (dimeric) and reduced (monomeric) recombinant human OPG. Two forms of OPG were used, dimeric OPG (110Kd) and dimeric OPG-Fc (90Kd). Reduced (dithiothreitol) and unreduced OPG were run on gels and immunoprecipitated with Mab 805 and Mab 8051 antibodies using standard methods. Mab OPG 805 was able to recognise only the unreduced (dimeric) form of OPG molecules, while Mab OPG 8051 was able to detect both the unreduced (dimeric) and reduced (monomeric) form of OPG and OPG-Fc (Figure 3).

Results from the Western blots suggest that the OPG present on endothelial cells, as detected with OPG Mab 805, is dimeric. When used at high concentrations, Mab 8051 is able to detect OPG on blood vessels. Therefore as the Mab 8051 is localised to OPG on the lining of the synovium, this suggest that the synovium may be associated with the monomeric and dimeric form of OPG. Western blots were therefore used to verify the form of OPG in the lining of the synovium, by examining the molecular forms of OPG present in the supernatants and cell lysates from synovial tissue cell cultures. The supernatants and cell lysates were absorbed to Mab 8051 linked to magnetic beads (Dynabeads M-450, Dynal Biotech, Oslo, Norway) and the bound protein was eluted to concentrate the antigens recognised by Mab 8051. The eluates were then run on a western blot and probed with Mab 8051. The supernatants and cell lysates of primary cultures of synovial cells demonstrated that Mab 8051 specifically detected two bands of protein that corresponded in molecular weights to the native monomeric and dimeric OPG (Dr Andrew Zannettino, personal communications).

Western blots were carried out with the help of Mr Chris Holding, Dr A. Zannettino and Dr Gerald Atkins (The University of Adelaide and The Hanson Centre, IMVS, South Australia).

2.2.19 Characterising the OPG Antibodies using ELISAs

An ELISA system was used to further investigate the specificity of binding of the two OPG monoclonal antibodies. ELISA assays were carried out using the OPG antibodies, as recommended by the commercial supplier, R&D Systems Inc. A standard dilution curve was used for measurement of recombinant OPG detected with Mab 805 and Mab 8051. Briefly, the primary (capture) antibody Mab 805 or Mab 8051 was diluted to a concentration of 2μg/ml in PBS and added to each well of a 96-well plate (MaxiSorp; Nalge Nunc). After an over night incubation at room temperature plates were washed with Wash Buffer (PBS containing 0.05% (v/v) Tween 20) and then incubated with Blocking Buffer (PBS containing 1% BSA). Following another washing step, 100μl of the rhOPG standard (805-OS, R7D Systems, Inc.) or test sample, diluted in complete medium, was added to each well. After two hours incubation, plates were washed in buffer and Biotinylated goat polyclonal antibody (BAF805, R&D Systems, Inc) diluted to a concentration of 0.1μg/ml in Detection Antibody Diluent (Tris Buffered Saline containing 0.1% BSA) was used as the secondary antibody. Bound antibody was detected with Streptavidin conjugated HRP (Genzyme, Cambridge, MA, USA) and detected with TMB substrate reagent (Sigma-Aldrich).

As it is reported that Mab 805 can block the binding of TRAIL to OPG (R&D Systems, Inc, Mab 805 specification sheet), the effect of RANKL and TRAIL on the binding of OPG to both antibodies was compared. OPG, at various concentrations (0.005 – 3.33 ng/ml), was incubated with recombinant human RANKL or TRAIL for 1 hour at 37^{o}C then tested in the ELISA assay using either Mab 805 or Mab 8051 as primary antibodies.

Binding of Mab 805 to OPG was blocked by both RANKL (Figure 4A) and TRAIL (Figure 4B). RANKL was more than 10 fold more effective at blocking OPG binding when compared to TRAIL. This may indicate that RANKL bound to OPG with higher affinity than TRAIL. Binding of Mab 8051 to recombinant OPG was only affected slightly by RANKL and TRAIL, even at a concentration of 100 ng/ml (Figure 4C). These results show that Mab 805 recognises an epitope associated with RANKL and TRAIL binding and that Mab 8051 recognises an epitope that is not closely associated with RANKL and TRAIL binding. ELISA assays were carried out with the help of Mr Chris Holding and Mr Martin Hutchens (The Department of Pathology, The University of Adelaide).

2.2.20 TRAP Staining in Tissue Sections

TRAP enzyme was detected to identify cells of the osteoclast lineage in serial frozen tissue sections from the same peri-prosthetic, OA and normal patients included in the RANKL and OPG immunohistochemical staining studies. A commercial acid phosphatase leukocyte kit (Sigma-Aldrich, St Louis MO) was used and sections were counterstained with Methyl Green, air-dried and mounted in DPX medium.

2.2.21 Quantitation of Immunohistochemical Staining

The peri-prosthetic and control tissue sections were stained for RANKL using Mab 626 and OPG (using Mab 805 dual labelled with anti-Factor VIII antibody). TRAP staining was carried out with a commercial staining kit. The staining was scored by a semi-quantitative assessment (SQA) method. SQA was graded on a 5 point scale (grades 0-4, with 0 for the absence of any stain) by two independent observers in a random order, as described previously (Tak et al., 1995, Kraan et al., 2000). The SQA of RANKL and TRAP staining gave information regarding the numbers of cells that produce the RANKL protein or TRAP enzyme. The SQA of OPG gives information regarding the proportion of Factor VIII positive blood vessels with any staining for OPG.

Sections dual stained for OPG and Factor VIII were also analysed in a random order by computer-assisted image analysis, as previously published (Kraan et al., 2000, Wikaningrum et al., 1998, Youssef et al., 1997a, Youssef et al., 1997b). Two parameters were measured by digital image analysis; these included the mean optical density (MOD) field and the integrated optical density (IOD). The MOD is a measure of the average density of staining, which is proportional to the concentration of protein per cell. The IOD, which is measured in pixel units, is calculated as the MOD multiplied by the area of AEC staining and is proportional to the total amount of OPG staining. The IOD of OPG staining was quantitated in each high power field (HPF). In addition, the blood vessel count per HPF and the IOD of OPG per blood vessel per HPF were assessed.

2.2.22 Statistical Analysis of Immunohistochemical Staining

Non-parametric tests were used to analyse the mean ranks of RANKL, TRAP and OPG semi-quantitative scores. The SQA for three groups of patients was compared using a Kruskal-Wallis test. The non-parametric data was not normally distributed therefore the median and interquartile ranges were determined and this was carried out using SPSS 10 software. The quantitative data for OPG was tested for variability and non-parametric methods were then

used. A Kruskal-Wallis test was used to compare the quantitative data for the three groups of patients. Significance was accepted if $p < 0.05$.

2.3 RESULTS

2.3.1 The Effects of Metal Particles on Human Monocytes

Adherent peripheral blood mononuclear cells, from three separate donors, were incubated with particles of TiAlV, CoCr or SS and the effect of these metal particles on mRNA expression of RANKL, RANK OPG and M-CSF were compared. Figure 5 shows the results obtained from one donor, and is similar to the results obtained with cells from the other two donors. The combined semi-quantitative data obtained for the three donors tested are shown graphically in Figure 6. Monocytes expressed RANKL, RANK, OPG and M-CSF mRNA at very low or undetectable levels when cultured in the absence of particles but this was increased when cells were stimulated by metal particles. The results show that mRNA expression, calculated as a ratio of GAPDH mRNA expression, was at its highest between 4 and 12 hours for all mediators tested.

The responses to LPS, which was used as the positive control of stimulation of monocytes, were different in the magnitude and timing when compared to those induced by particles. There were also clear differences in the effect of various particles on the expression of the mRNA species of the mediators investigated. It is important to note that the RANKL:GAPDH and RANK:GAPDH mRNA ratios were significantly higher in monocytes incubated with TiAlV and SS particles compared with monocytes incubated with media alone ($p< 0.01$). The expression of RANK mRNA was highest at the 6 hour time point when stimulated with LPS, TiAlV and SS, whereas the response to CoCr peaked at 12 hours.

The OPG:GAPDH ratio was significantly increased following treatment with TiAlV particles and LPS ($p< 0.01$). While TiAlV particles stimulated OPG levels to peak briefly at 6 hours, stimulation of OPG by LPS was maintained at high levels for up to 12 hours. M-CSF mRNA expression was stimulated by all types of wear particles. A statistically significant difference was seen when cells stimulated with TiAlV particles were compared with cells incubated with medium alone.

Studies were carried out to compare mRNA expression in response to metal particles with the levels of protein stimulated by these particles. Monocytes were incubated with metal particles for 48 hours and the level of M-CSF protein released into the media was assessed by ELISA. Both TiAlV and SS particles stimulated the release of M-CSF.

Earlier studies had shown that the highest levels of protein were produced between 24 and 48 hours. mRNA levels were therefore assessed after 6 hours treatment and the corresponding

M-CSF protein was assessed after 48 hours treatment. In most cases, elevated mRNA expression coincided with an increase in the release of the corresponding protein. However, TiAlV particles stimulated higher levels of expression of M-CSF mRNA than SS particles but SS particles stimulated the release of higher levels of M-CSF protein than TiAlV particles Table 6. This may indicate that post-transcriptional events are also involved in the induction of these factors by metal particles. Often LPS stimulated similar levels of mRNA expression as the particles, however, the levels of protein released were generally more than 10 fold higher than those stimulated by metal particles. This supports the concept that cytokine stimulation by LPS occurs by mechanisms distinct from stimulation by metal particles.

Table 6. Comparison of M-CSF Protein and mRNA Expression Following Stimulation with LPS or 4 x 10^7 Particles/ml TiAlV, CoCr or SS

	No. of particles	**LPS (5pg/ml)**	**TiAlV**	**CoCr**	**SS**
Protein (pg/ml)	36[1]	2800	790	81	1127
mRNA (RATIO TO GAPDH)	0.36[2]	0.69	3.22	0.66	0.83

TiAlV- Titanium Aluminium Vanadium, CoCr- Cobalt Chromium, SS- Stainless Steel

[1] Human monocytes (n=5) were also incubated with particles and the release of M-CSF into the media over 48 hours was measured using ELISA. Mean values are expressed.

[2] Human monocytes (n=3) were incubated with particles and after 6 hours mRNA levels of M-CSF were expressed as a ratio to GAPDH, determined using semi-quantitative RT-PCR as described in the text. Mean values are expressed.

The results from these *in vitro* studies show that cells on the monocyte/macrophage lineage have the capacity to express mRNA encoding factors of osteoclastogenesis RANKL, RANK, M-CSF and OPG. The stimulation in M-CSF mRNA expression was generally reflected by an increase in protein released from the cells.

2.3.2 mRNA Expression of Mediators of Osteoclast Formation in Peri-Prosthetic Tissues

Peri-prosthetic tissues were obtained from patients undergoing revision of failed prosthetic joints. Radiographs of the joint of all patients showed extensive bone loss adjacent to the prosthesis in all patients, which was confirmed during revision surgery. Where possible peri-prosthetic tissue was obtained from around several osteolytic sites from each patient, and this is indicated in Table 2 and Figure 7. Six of the patients (cases 1 to 6) were operated on for the removal of a hip prosthetic joint, in which the acetabular component was made of PE and the femoral component of cobalt chrome alloy. Microscopic analysis of peri-prosthetic tissue revealed large numbers of particles of both PE and cobalt chrome present in these tissues. Tissue samples were taken from one patient (case 7) undergoing the removal of a knee prosthesis that had loosened and this tissue contained large numbers of PE particles only. In the remaining two patients (cases 8 and 9) silastic implants were removed from the elbow and wrist, respectively, and the tissue contained large numbers of silastic particles.

RT PCR was used to determine the levels of mRNA corresponding to RANKL, RANK and OPG as shown in Figure 7. In addition, mRNA corresponding to inflammatory cytokines was analysed in the cell extracts from tissues described in Table 2. RANKL mRNA was expressed by cells isolated from all patients, with the highest levels in cells isolated from tissues containing silastic particles. A great deal of variation was observed in the expression of RANK mRNA in peri-prosthetic tissue samples, with high expression seen in the tissues obtained from the two patients with silastic implants. M-CSF and OPG mRNA were expressed in tissues from all the patients. High levels of mRNA corresponding to IL-6 and sIL-6r species were detected in most tissue samples. The mRNA expression for IL-1β and TNFα, however, was relatively low in all samples tested.

Eight of the samples described in Table 2 yielded sufficient cell numbers for cell culture studies. As there is evidence that the ratio of RANKL:OPG may be important in regulating osteoclast formation in disease (Nagai and Sato, 1999, Atkins et al., 2000b, Hofbauer et al., 2000) the ratio of RANKL to OPG mRNA was determined. The ratios of mRNA expression were then compared to the ability of cells isolated from the same tissues to form osteoclasts in culture. Table 7 shows that the ratio of RANKL to OPG mRNA was markedly higher in tissues adjacent to silastic implants, compared with other peri-prosthetic revision tissues.

Table 7. Comparison of the RANKL and OPG mRNA Expression (mean, range) with the Formation of Resorption Lacunae in Osteoclasts by Cells from Hip Revision Tissues Containing Wear Particles of Polyethylene and Cobalt-Chrome Alloy and From Tissues Containing Wear Particles of Silicone.

Peri-Prosthetic Tissue	**RANKL:GAPDH**	**RANKL:OPG**	**Resorption Pits day 7**	**Resorption Pits day 14**
Hip (n=6)	0.2 (0.11 to 0.83)	0.034 (0.028 to 0.042)	0/6*	0/6
Hip + HBDC[#] (n=6)	As above	As above	0/6	5/6
Silastic (n=2)	12.0 (11.3 to 12.8)	0.88 (0.84 to 0.91)	2/2	2/2
Silastic + HBDC (n=2)	As above	As above	2/2	2/2

[*] number of samples tested which resulted in pit formation (>20 pits per dentine slice)

[#] human bone-derived osteoblast-like cells

To investigate the mRNA expression of osteoclast markers and compare this to mRNA expression of osteoclast mediators, RT PCR analysis was carried out on cells isolated from peri-prosthetic tissue from sites adjacent to osteolysis in an additional group of twelve patients. These patients were also undergoing revision surgery for joint failure due to osteolysis and their details are in Table 3. These results verified the expression of RANKL, RANK, OPG and M-CSF mRNA in cells isolated from the peri-prosthetic tissues (Figure 8). In addition, CTR, TRAP and TRAIL mRNA were expressed by many of the peri-prosthetic tissues**.** TRAP and TRAIL mRNA were expressed in all samples. CTR mRNA expression varied between samples, with five of the thirteen samples expressing undetectable levels. Due to the low number of cells isolated from these additional samples, it was not possible to carry out cell culture studies in addition to RT PCR. It was therefore not possible to compare the mRNA expression of CTR, TRAP and TRAIL with the ability of cells extracted from these samples to form functional osteoclasts

2.3.3 Identifying Osteoclast Precursors in Peri-Prosthetic Tissues

Ex vivo experiments were performed to investigate whether osteoclast precursors accumulate in tissue adjacent to osteolytic zones around joint prostheses. TRAP expression was used to indicate the presence of osteoclastic cells. The proportion of TRAP-positive cells isolated from the peri-prosthetic tissue samples varied widely from 4% to 54%. Where there were sufficient cells, the morphology of the TRAP positive cells was observed after the cells were allowed to adhere to glass coverslips and then cultured for 24 hours (Figure 9A). Adherent cells on glass had the typical morphology of osteoclasts, being large, multinucleate and expressing TRAP. It was interesting to note that there were fewer large TRAP-positive cells among adherent cells isolated from the tissues containing CoCr and PE particles (Figure 9B) when compared to cells isolated from tissue containing silastic particles (Figure 9C).

The numbers of TRAP positive cells in cultures of cells isolated from peri-prosthetic tissue containing CoCr and PE particles reduced with time when cultured without HBDC compared with those cultured with. However, the numbers of TRAP positive cells derived from peri-prosthetic tissue containing silastic particles were maintained even in the absence of a stromal element. When human bone derived osteoblast-like cells were co-cultured with the peri-prosthetic cells isolated from tissue with CoCr and PE particles, many large multinucleated TRAP positive cells were seen even after fourteen days in culture (Figure 9D).

The hallmark of osteoclasts is their ability to resorb bone. Therefore, cells derived from the peri-prosthetic tissues were cultured on dentine slices alone or in the presence of human osteoblastic cells in order to assess their ability to resorb mineralised tissue. Cells isolated from peri-prosthetic tissue, containing metal or PE particles, were able to differentiate into cells capable of resorbing pits only when cultured in the presence of added osteoblast-like cells. This was notably different from cells isolated from the two patients with silastic implants. Cells extracted from these tissues were able to form large numbers of pits (>200 from 2 x 10^5 cells) in the absence of added osteoblast-like cells. Cells isolated from sites containing other prosthetic materials formed resorption pits only after having been co-cultured with human osteoblast-like cells for 14 days (Figure 9E). Cells isolated from the tissue containing silastic particles formed resorption lacunae within five days with up to several hundred pits seen after 7 days culture (Figure 9F).

It may be significant to note that the ability of cells, extracted from tissues containing silastic wear particles, to become bone-resorbing osteoclasts without the need for bone cells, corresponded with the high levels of RANKL mRNA expression. Importantly, the ratio of

RANKL:OPG mRNA expressed by cells extracted from these tissues was also high (Table 7). It would be worth comparing the ability of cells extracted from tissues containing different metal alloy particles to differentiate into osteoclasts.

2.3.4 Expression of RANK mRNA in Peri-Prosthetic Tissues

In situ hybridisation using DIG-labelled riboprobes was carried out to detect RANK mRNA in paraffin embedded peri-prosthetic tissue (Figures 10A, C, D and E). Riboprobes corresponding to the RANK mRNA sequence (sense probes) were labelled and used as a negative control (Figure 10B). The antisense riboprobes, complementary to RANK mRNA, detected RANK expression in peri-prosthetic tissue adjacent to sites of osteolysis (Figure 10A, C, D and E). Cells expressing RANK mRNA were generally large multinucleated cells present within and around cell aggregates in these tissues. Many of the cells expressing RANK mRNA contained particles of metal or large PE shards. When tissue sections were viewed under polarized light, multiple biorefringent PE particles were clearly seen adjacent to, or encased by, large multinucleated cells expressing RANK mRNA (Figure 10C and E). In eight of the samples labelling for RANK mRNA was followed by immunohistochemistry. An antibody to the monocyte/macrophage cell marker, CD68, confirmed that these cells were of this cell linage (Figures 10D and E). There were also many smaller, CD68 positive, cells in aggregates in the surrounding tissue that did express RANK mRNA indicating that they are monocytes.

2.3.5 TRAP Positive Cells in Peri-Prosthetic Tissue

Although not a definitive marker for mature osteoclasts, TRAP staining was used to identify pre-osteoclasts and osteoclasts in the peri-prosthetic, OA and normal tissues. Serial sections of tissue samples used in the immunohistochemistry study (Table 4) were stained for TRAP expression. These cells were detected in all the peri-prosthetic tissue sections (Figures 10F and G) but were only seen in tissues from one of the OA and normal patients (Figure 10H). Statistical analysis of the semi-quantitative assessment (SQA) scores for TRAP staining supported this observation as TRAP expression was found to be significantly greater in the peri-prosthetic tissues ($p= 0.004$) (as shown in Table 8).

TRAP staining was associated with small mononuclear cells as well as large multinucleated cells that often contained particles of different prosthetic materials. The large TRAP positive cells were more often associated with large PE particles (Figure 10F) than smaller metal particles (Figure 10G). The appearance of large multinucleated TRAP positive cells is consistent with a foreign body response to large particles of PE (Figure 10F). There has been

a recent resurgence in interest regarding the effect of PE particles on osteolysis, however the effect of metal particles on expression of osteoclast mediators was the focus of this work.

2.3.6 RANKL Protein in Peri-Prosthetic Tissues

Immunohistochemical staining was used to detect RANKL protein in the peri-prosthetic (Figures 11A, C, E and G), OA (Figures 11B, D, F and H) and normal tissues (normal groups are shown in the results section of Chapter 3). Staining for RANKL protein was graded by SQA and the results are shown in Table 8. It must be noted that histological assessment of the H&E stained peri-prosthetic sections showed that many sections contained areas of cellular infiltrate as well as large acellular fibrous tissue. Video-image analysis to quantitate RANKL was therefore not carried out as the morphology of the peri-prosthetic tissue was too variable and the tissues had undefined margins. In addition to this, the prosthetic particles in the regions of staining interfered with digital image analysis.

RANKL protein was predominantly found in areas of inflammatory cell infiltrates and often associated with cells that had phagocytosed particles (Figure 11A, C, E and G). RANKL protein was absent in the cells of the vasculature in all groups. In comparison to the peri-prosthetic tissue samples, RANKL protein was significantly lower in tissues from OA patients without prosthetic implants (Figure 11B, D, F and H) and normal samples (similar to OA results) ($p=0.006$).

Dual staining for the presence of RANKL protein and expression of CD3, a cell marker on pan T lymphocytes, revealed few CD3 positive cells present in the inflammatory infiltrates in the peri-prosthetic tissues. The few CD3 positive T lymphocytes that were present in the peri-prosthetic and OA tissue rarely expressed RANKL protein (Figure 11C and 11D respectively).

Dual staining with anti-CD22, a marker for B cells, also revealed that the few B lymphocytes were present in these tissues were also negative for RANKL protein. In contrast, dual staining for the presence of RANKL and CD68 expression revealed that approximately 50% of the CD68 positive mononuclear and multinuclear cells present throughout the peri-prosthetic tissues expressed RANKL protein (Figure 11E). The OA tissue contained few cells expressing CD68 and these did not express RANKL (Figure 11F).

RANKL also appeared to be detected in fibroblast-like cells beneath the “synovial-like” lining in the peri-prosthetic tissue. However, the monoclonal antibody to CD55, a marker of

fibroblasts, detects CD55 on fibroblasts in the lining of the synovium alone (which was not always present) and not the fibroblast-like cells evident throughout the tissue (Figures 11G and 11H). Therefore, it was not possible to verify that RANKL corresponded to fibroblast cells throughout the synovial tissue.

Table 8. The Semi-Quantitative Assessment of TRAP and RANKL in Peri-prosthetic Tissues

	TRAP	**RANKL**
OA **N=5**		
Median	0	1.00
IR[1]	0.5	1.00
MR[2]	6.7	7.7
N **n=5**		
Median	0.0	0.00
IR	0.0	0.5
MR	5.5	4.9
PP **n=8**		
Median	2.0	1.5
IR	1.5	1.00
MR	13.75#	13.5*

OA-Osteoarthritis, N-Normal synovium, PP-peri-prosthetic

[1] Interquartile range
[2] Mean rank

# $p < 0.005$ compared with the control tissues
* $p < 0.05$ compared with the control tissues

2.3.7 OPG Protein in Peri-Prosthetic Tissues

Experiments were also performed to detect OPG protein in peri-prosthetic tissue from sites adjacent to osteolysis, as well as in OA and normal tissues. Immunohistochemistry was used to determine if OPG was present in the soft tissue adjacent to bone and if there was a reduction in OPG in the soft tissue from sites adjacent to regions of osteolysis. OPG was detected with both Mab 805 (detecting its dimeric form) (Figures 12A and C) and 8051 (monomeric and dimeric forms of OPG) (Figures 13A, C and E) in peri-prosthetic tissues adjacent to osteolytic zones. In addition, tissues obtained from OA patients undergoing knee surgery (Figure 12B, D and 13B, D and F) and normals (micrographs shown in Chapter 3) were investigated as the control tissues.

OPG was detected with Mab 805 in cells of the vasculature in both the peri-prosthetic and control tissues. The levels of OPG protein detected in the blood vessels of the soft tissue near failed implants was often reduced (Figure 12A) when compared with the protein levels detected on the blood vessels in the control tissues (Figure 12B). However, neither the SQA values, nor the IOD values for OPG detected in blood vessels per high power field were statistically different between the groups. Although not significant, the IOD per HPF for OPG was higher in the peri-prosthetic tissues and may be attributed to the trend towards a higher number of blood vessels per HPF in areas of the peri-prosthetic tissues. The IOD of OPG per blood vessel per high power field not statistically different between any of the groups. This is surprising, as the staining appeared less in the blood vessels of the peri-prosthetic tissues. A failure to find a significant difference may be due to a type II error and therefore a greater number of samples may need to be tested to obtain more meaningful results.

Dual labelling with Mab 805 (DE AEC method) to detect OPG was followed by detection of endothelial cells using antibodies to Factor VIII (APAAP method with fast blue substrate) (Figure 12C and 12D). This identified the endothelial cells in the tissue sections and confirmed that OPG, detected by Mab 805, was confined to the endothelial cells of the vasculature. OPG expression by endothelial cells was then graded by SQA as well as DIA. The SQA analysis and computed assisted image analysis results for Mab 805 are displayed in Table 9.

Histological assessment of the H&E stained peri-prosthetic sections indicated that not all sections contained a clearly defined synovial lining. Preliminary staining showed that detection of OPG with Mab 8051 was localised mainly to the lining layer of the synovial

membrane in OA (13B), inactive RA tissue (Figure 2C) and normal samples (shown in the chapter on RA), therefore only a selection of peri-prosthetic samples were stained with this antibody for quantitation rather than all samples. Staining with Mab 8051 in peri-prosthetic samples appeared to be reduced (13A, C and E) compared with control groups (13B, D and F), although due to the absence of a defined lining layer of synovium in the peri-prosthetic samples, SQA was not carried out to verify the reduction. OPG appeared to correspond with intimal macrophage/monocyte cells in peri-prosthetic (Figure 13A), OA (Figure 13B) and normal samples (as shown in the results section of Chapter 3). When sections were dual labelled, with the Mab 8051 to OPG and an antibody to the monocyte/macrophage marker, CD68, cells were dual stained (Figure 13C and 13D). However, OPG detected by Mab 8051 on cells in the synovial-like lining did not correspond with fibroblast-like synovial cells, as identified by CD55 antibodies (Figure 13E and 13F).

Table 9. The Semi-Quantitative and Quantitative Assessment of OPG in Peri-prosthetic Tissues

	OPG SQA	**OPG IOD/HPF**	**BV count/HPF**	**OPG IOD/BV COUNT/HPF**
OA n=5				
Median	3.0	918.0	1.8	815
IR[1]	1.0	3219.0	1.4	1363.5
MR[2]	8.1	8.6	8.4	9.0
N n=5				
Median	3.0	952.0	0.8	1095
IR	1.5	1723.0	1.9	335.0
MR	9.2	8.6	8.2	11.2
PP n=8				
Median	3.5	1765.0	2.3	779.5
IR	2.0	1456.7	4.45	672.5
MR	10.56	10.63	11.00	8.75

OA-Osteoarthritis, N-Normal synovium, PP-Peri-Prosthetic
$F8^-$ Factor VIII positive blood vessels, IOD- Integrated Optical Density, HPF- High Power Field, BV- Blood Vessels

[1] Interquartile Range
[2] Mean Rank
*$p < 0.05$ compared with the control tissues

2.4 DISCUSSION

Failure of prosthetic implants can be due to a variety of reasons, however, small particles of prosthetic material are often associated with osteolysis, leading to the loosening and subsequent failure of joint implants. These particles, produced by wear at the articulating surface of prostheses, are likely to cause the accumulation of macrophages and macrophage polykaryons in the prosthetic implant bed, which is believed to be an important event in aseptic peri-prosthetic bone loss (Vernon-Roberts and Freeman, 1977, Willert and Semlitsch, 1977). *In vivo* studies have shown that prosthetic wear particles stimulate a chronic inflammatory response dominated by macrophages (Jiranek et al., 1993, Howie and Vernon-Roberts, 1988, Howie, 1990, Lerouge et al., 1997, al-Saffar et al., 1995b, al-Saffar and Revell, 1994). Particles are ingested by tissue macrophages, and this in turn is thought to cause the release of mediators of bone resorption (Haynes et al., 1998, Jiranek et al., 1993). The cells of the monocyte/macrophage lineage that accumulate in the implant bed may also directly cause osteolysis by differentiating into functional osteoclasts themselves (Fujikawa et al., 1996a, Quinn et al., 1996, Yasuda et al., 1998a, Quinn et al., 1998b).

Recent studies have shown that the macrophage-rich tissues adjacent peri-prosthetic osteolysis, contain cells capable of becoming functional osteoclasts when cultured under appropriate conditions (Sabokbar et al., 1997, Neale et al., 1999a, Itonaga et al., 2000a). This indicates that pre-osteoclasts as well as possible mature osteoclasts may be present in the peri-prosthetic tissues. RANKL has been identified as the key factor stimulating osteoclast formation and acts on osteoclast precursors and mature osteoclasts via a membrane receptor, RANK (Nakagawa et al., 1998, Hsu et al., 1999, Myers et al., 1999). Therefore, in order for these cells to differentiate into osteoclasts under the influence of RANKL, pre-osteoclasts must express RANK (Nakagawa et al., 1998).

The expression of RANK mRNA by cells isolated from the peri-prosthetic tissues in the studies described here is consistent with the presence of pre-osteoclasts, and possibly osteoclasts, in tissue adjacent to sites of osteolysis. This concept is further supported by the i*n situ* hybridisation studies, which for the first time showed that many cells in the peri-prosthetic tissue expressed RANK mRNA**.** It is, therefore, not surprising that many large multinucleated cells resembling osteoclasts were seen in peri-prosthetic tissues. Many of these cells not only expressed RANK mRNA but also contained wear particles. Dual labelling with a monoclonal antibody directed against the macrophage cell marker, CD68, showed that the majority of the cells expressing RANK were of the monocyte/macrophage lineage.

The expression of RANK mRNA by cells of the monocyte/macrophage lineage observed *in situ* is consistent with the *in vitro* results, where RANK mRNA was expressed by monocytes treated with wear particles. These findings suggest that monocyte/macrophage cells may be able to differentiate into osteoclasts when stimulated by wear particles present in the peri-prosthetic tissue. These results are in agreement with studies demonstrating the ability of mature monocytes and macrophages to differentiate into osteoclasts (Udagawa et al., 1990, Quinn et al., 1998c). While there is some speculation that the presence of mRNA may not necessarily correlate with the expression of RANK protein, it is significant to note that there were high levels of RANK mRNA in tissues from which large numbers of osteoclast formed *ex vivo*. The expression of RANK by particle stimulated monocytes *in vitro*, together with the expression *in vivo* by cells in the macrophage rich revision tissue, is further evidence that RANK may have a key role in osteoclast generation in the peri-prosthetic tissues.

TRAP expression is another marker that is widely used to identify osteoclasts. TRAP mRNA was found to be expressed by cells isolated from the majority of peri-prosthetic tissues. It is important to note that TRAP positive cells were significantly more abundant in peri-prosthetic tissue, whereas there was a notable absence of TRAP staining in OA and normal tissues. These observations were consistent with a previous report demonstrating large, multinucleated, TRAP positive cells in peri-prosthetic tissues (Kadoya et al., 1994). TRAP positive cells often contained particles of prosthetic material and had the appearance of giant cells that form in response to foreign bodies. However, TRAP is not a unique marker of functional osteoclasts as it is also expressed by macrophages *in vitro* and pre-osteoclasts not yet able to resorb bone (Efstratiadis and Moss, 1985, Hattersley and Chambers, 1989a, Takeshita et al., 2000). Therefore, although TRAP staining, RANK mRNA expression and multinucleation are consistent with the osteoclast phenotype, the question remains as to whether these cells in the peri-prosthetic tissue were fully differentiated osteoclasts or required further stimulation to fully differentiate into bone resorbing cells.

Cells isolated from the peri-prosthetic tissue could develop into functional osteoclasts under appropriate conditions *in vitro*, which is in agreement with previous reports (Sabokbar et al., 1997, Neale et al., 1999a, Itonaga et al., 2000a). Consistent with TRAP staining results *in vivo*, TRAP positive cells were evident within 24 hours when cultured. However, cells from peri-prosthetic tissue samples containing metal and PE particles only formed resorption lacunae when incubated with HBDC for up to 14 days. It is therefore likely that macrophages that have phagocytosed particles may acquire several osteoclast phenotypic features in the

process of differentiation. This is supported by evidence that murine macrophages, having phagocytosed polymethylmethacrylate (PMMA), PE, titanium or CoCr particles are only able differentiate into TRAP positive cells that are able to resorb bone when cultured with UMR 106 cells and VD3 (Sabokbar et al., 1998). Previously, it was believed that osteoclasts do not phagocytose wear particles at the bone-implant interface (Athanasou, 1996). However, it may be possible that many of the cells positive for TRAP and RANK *in vivo* are mature osteoclasts that can phagocytose particles, as human osteoclasts derived from giant-cell tumours can retain the ability to resorb bone in culture after phagocytosis of small alloy particles (Wang et al., 1997).

As discussed above, TRAP and RANK might be expressed by cells that have not yet fully differentiated into osteoclasts and possibly cells of other lineages (Hattersley and Chambers, 1989a, Haynes et al., 2001). It has been suggested that CTR might be a more definitive marker of mature bone resorbing osteoclasts (Hattersley and Chambers, 1989a, Takeshita et al., 2000). In this study RT PCR on total RNA extracted from cells isolated from peri-prosthetic tissues showed mRNA expression of TRAP and RANK in the majority of samples, suggesting the presence of pre-osteoclasts or osteoclasts in the tissue. However, it is important to note that CTR mRNA expression was absent in six of these fourteen samples. It is likely that the tissues expressing CTR contain more mature osteoclasts. Additional cell culture studies would need to be carried out to determine if CTR expression correlated with osteoclast activity *ex vivo*.

In the context of peri-prosthetic inflammation, where there is a large macrophage infiltrate, osteoclast differentiation from monocytes and macrophages could cause pathological bone destruction adjacent to the prostheses, ultimately leading to joint failure. Overall the findings suggest that it is likely that most of the TRAP and RANK positive cells identified in culture and *in situ* have not yet fully developed into mature osteoclasts and may require further stimulation to resorb mineralised tissue. It is also possible that cells positive for these as well as CTR may be mature osteoclasts but not necessarily activated cells. It is likely that factors such as RANKL are needed to activate the pre-osteoclasts and osteoclasts (Burgess et al., 1999) and maintain their resorptive activity in culture (Hakeda et al., 1998).

Many studies have identified RANKL as a key factor in the differentiation and activation of osteoclasts (Quinn et al., 1998a, Fuller et al., 1998, Burgess et al., 1999, Yasuda et al., 1998b, Matsuzaki et al., 1998). The addition of soluble recombinant RANKL, along with M-CSF, to monocytes in culture can replace the requirement for contact with a stromal element in order

for cells of the monocyte/macrophage lineage to differentiate into mature osteoclasts (Matsuzaki et al., 1998). OPG is a decoy receptor, which binds to RANKL, thereby inhibiting interaction with RANK causing inhibition of osteoclast formation (Lacey et al., 1998, Yasuda et al., 1998b). This study identified the expression of RANKL and OPG mRNA in the peri-prosthetic tissues, which is consistent with these factors being the main proteins modulating osteoclast formation not only in normal bone physiology but also in the tissue adjacent to sites of pathological bone loss. Based on the finding that RANKL mRNA is expressed by cells isolated from the soft tissue, it would not be unexpected for non-osteoblastic cells to provide RANKL-mediated support for osteoclast formation. Cell types, such as fibroblasts (Quinn et al., 2000, Sakai et al., 2002) and lymphocytes (Wong et al., 1997a, Kong et al., 1999a, Horwood et al., 1999, Kotake et al., 2001), have been shown to be capable of expressing RANKL and could therefore stimulate osteoclast formation in the soft tissue through RANKL/RANK interactions. This would suggest that pre-osteoclasts would not need to be in contact with bone to differentiate into mature osteoclasts.

It is possible that osteoclast formation in the soft tissues in response to particles occurs as a consequence of the tissue's attempts to eliminate the foreign body particles. The production of pro-inflammatory molecules is part of the body's normal response to foreign bodies intended to recruit and activate macrophages. However, as similar molecules are involved in bone turnover, these cytokines cause the local disregulation of bone turnover, resulting in osteolysis. Many inflammatory cytokines have been associated with osteolysis (al-Saffar and Revell, 1994, Neale et al., 1999b, Xu et al., 1996, Merkel et al., 1999) and the results from RT PCR showed expression of a number of skeletally active cytokines, IL-6, sIL-6r, IL-1β and TNFα, in most of the peri-prosthetic tissue samples. Recent studies have shown that many of these cytokines may affect osteoclast formation by modulating RANK-RANKL interactions by regulating the expression of RANKL and OPG (Kotake et al., 1999, Nakashima et al., 2000, Brandstrom et al., 1998a, Hofbauer et al., 1998). This is significant considering pre-osteoclasts and osteoclasts may also be present in the tissues adjacent to pathological bone loss.

The expression of M-CSF mRNA was demonstrated in all of the peri-prosthetic tissues. This is significant as M-CSF production is also an important requirement for osteoclastogenesis (Quinn et al., 1997a, Quinn et al., 1998a, Tsurukai et al., 2000). In mouse and human *in vitro* and *in vivo* models, M-CSF has been shown to induce proliferation of pre-osteoclasts (Tanaka et al., 1993) and promote differentiation into osteoclasts (Fujikawa et al., 1996b, Quinn et al., 1997a, Quinn et al., 1998a, Suda et al., 1992, Sarma and Flanagan, 1996, Suda et al., 1999).

Consistent with this, M-CSF has been shown to induce RANK expression in monocytes (Arai et al., 1999). In addition, M-CSF has also been shown to prolong the survival of mature osteoclasts as well as stimulate their osteoclastic activity *in vitro* (Fuller et al., 1993). It is also important that M-CSF has been shown to increase in the synovial-like membrane of the prosthetic tissues in the aseptic loosening of total hip replacements (Xu et al., 1997). The ability of prosthetic wear particles to stimulate M-CSF mRNA expression by monocytes *in vitro*, as shown in the present study, may indicate that macrophages are a major source of M-CSF in the peri-prosthetic tissues. These results from this suggest that the formation of osteoclasts in the peri-prosthetic tissue may be influenced by expression of M-CSF by monocyte/macrophages in response to wear particles.

In addition to cytokines, RANKL and OPG, TRAIL mRNA was expressed in peri-prosthetic tissues. TRAIL is an apoptosis inducing ligand (Wiley et al., 1995, Walczak and Krammer, 2000) that can also inhibit autoimmune inflammation and cell cycle progression (Song et al., 2000). Soluble active TRAIL is generated by deletion of the transmembrane and intracellular domains (Pitti et al., 1996). OPG is able to bind to TRAIL and inhibit TRAIL induced cyotoxicity *in vitro* (Emery et al., 1998). Although TRAIL acting directly on osteoclasts has no effect on osteoclast formation, it may be of significant that soluble TRAIL can block OPG from binding to RANKL, and prevent OPG from inhibiting osteoclast formation (Emery et al., 1998). The expression of TRAIL mRNA by cells isolated from the peri-prosthetic samples suggests that TRAIL may play a role in regulating osteoclast formation by influencing the interaction of OPG and RANKL. However, results from ELISAs showed that TRAIL was 10 fold less effective at blocking OPG binding when compared to RANKL. This indicates that TRAIL binds to OPG with less affinity than RANKL. However, its efficacy in regulating osteoclast formation *in vivo* in peri-prosthetic tissue is yet to be determined. Furthermore, binding in solid phase may be different from that seen in solution, as OPG is effective in blocking TRAIL-induced apoptosis at equimolar concentrations in *in vitro* assays (Dr Andreas Evdokiou, personal communication). TRAIL has recently been shown to be abundant in osteoblasts that have been grown from human bone (Atkins *et al* unpublished). Further studies investigating the role of TRAIL in this and other pathologies may provide interesting results.

The levels of RANKL and OPG in the peri-prosthetic joint may be affected either directly by particles or indirectly by cytokines released in response to particles. Results from earlier *in vitro* experiments using human or murine monocytes and macrophages have shown that particles of different prosthetic materials can not only affect the monocyte/macrophage cell

viability, morphology and phagocytic activity but also stimulate the cells to release a wide variety of cytokines (Garrett et al., 1983, Gelb et al., 1994, Shanbhag et al., 1994a, Haynes et al., 1993, Catelas et al., 1999b, Rogers et al., 1997, Catelas et al., 1999a, Shanbhag et al., 1995b). The *in vitro* studies carried out here assessed the direct effect of particles of metal alloys, generated from prosthetic joints, on human adherent PBMC. This study was unique in that previous studies by others have not investigated the direct effect particles have on expression of the key mediators of bone resorption (RANKL, RANK and OPG).

In *in vitro* experiments discussed here TiAlV, CoCr or SS were able to stimulate RANKL, RANK, OPG and M-CSF mRNA expression by human adherent monocytes/macrophages. TiAlV and SS particles, but not CoCr particles, were able to stimulate monocytes/macrophages to express significantly higher levels of RANKL and RANK mRNA when compared with mRNA expression in untreated cells. These observations are consistent with previous *in vitro* studies that demonstrated a difference in cytokine response depending on the different types of particles investigated (Haynes et al., 1993, Rogers et al., 1997, Haynes et al., 1998, Catelas et al., 1999a). It is, therefore, possible that different types of particles induce a different osteoclastic response. It is important to determine the effects of wear particles, as this is an important consideration when assessing which alloys are best to use in the construction of prosthetic devices.

Analyses of retrieved metal-on-PE implants and surrounding peri-prosthetic tissues have shown a strong correlation between PE wear and peri-prosthetic osteolysis (Kadoya et al., 1998, Wan and Dorr, 1996, Oparaugo et al., 2001). *In vivo* studies, using a murine air pouch model, have shown that ultra high molecular weight PE particles stimulate high numbers of cells to infiltrate the pouch/synovial membrane. This results in the highest increase in the density of cell in the membrane when compared with TiAlV, CoCr and PMMA particles (Wooley et al., 2002). In the study described here, the large multinucleated TRAP positive and RANK expressing cells often contained shards of PE. It is important to note that recent *in vitro* studies have shown that ultra high molecular weight PE particles are able to stimulate production of inflammatory cytokines by macrophages (Chiba et al., 2001, Catelas et al., 1999a). Further *in vitro* studies like those reported here should be carried out with PE particles of different sizes in light of the findings of the *in vivo* studies reported here. It must also be noted that particles of different alloys may also act synergistically to increase cell recruitment and cytokine release and this should be investigated as there are often more than one type of wear particle present in tissues near peri-prosthetic osteolysis. In addition, it is

also important that the indirect effect of particles on osteoblastic and possibly fibroblastic cells via cytokine release is considered.

Several studies have shown that cells isolated from tissues containing PE and metal particles were able to form mature osteoclasts in culture. These cells required co-culture and contact with a stromal element, in order for functional osteoclasts to form (Sabokbar et al., 1997, Neale et al., 1999b). Although the *ex vivo* studies described here are consistent with these findings, previous studies do not address the likelihood that the different types of wear particles present may influence osteoclast differentiation and activity in these tissues. It may, therefore, be important to note that in the present study osteoclasts formed more readily from the adherent cell population isolated from the tissues containing large numbers of silastic wear particles, than from tissues containing metal and/or PE wear particles. Furthermore, in some cases cells isolated from these tissues could form mature osteoclasts rapidly within several days of culture, even in the absence stromal cells of the osteoblast lineage. Not only do these findings indicate that pre-osteoclasts are present in the peri-prosthetic tissues but that particles of different alloys may be more likely to promote osteoclast formation. These findings also suggest that in tissues containing silastic particles, osteoclasts precursors may have almost, or may have already, fully differentiated into osteoclasts *in vivo*. A greater number of samples containing silastic particles need to be compared to tissues containing other particles to give more meaningful results.

As indicated by the *in vitro* studies the ability of different types of prosthetic particles to stimulate osteoclast formation *in situ* may vary depending on how they affect the levels of RANKL and RANK. It is important to note that levels of RANKL and RANK mRNA were highest in the total cell population isolated from the tissues containing silastic particles. The ability of cells isolated from tissue containing silastic particles to become mature osteoclasts without an additional stromal element and the corresponding high RANK and RANKL mRNA levels in the tissue support a role for these mediators in osteoclast formation.

The relative levels of RANKL to OPG have also been suggested as an important factor in upregulating osteolysis (Horwood et al., 1998, Nagai and Sato, 1999). It is significant that the ratio of RANKL:OPG mRNA was higher in cells isolated from the samples containing silastic particles compared with the expression in cells isolated from tissue containing PE and CoCr particles. As mentioned previously, osteoclast formation from cells isolated from these tissues was more rapid and did not require osteoblast-like cells for stromal support. Results from the cell culture studies described here support the contention that there is a relationship

between the RANKL:OPG ratio and the osteoclastogenic potential of cells isolated from the different revision tissues. Studies with larger numbers of patients will be required to further explore the relationship between the expression of particular osteoclastogenic molecules and different prosthetic particulate materials *in vivo*. *In vitro* studies comparing silicone particles with other types of particles would be useful and may indicate that this type of material is particularly dangerous when compared to other material, in particulate form. The relationship between RANKL to OPG and osteoclast formation in disease is further explored in detail in Chapter 3.

The differentiation of cells of the monocyte/macrophage lineage into functional osteoclasts *in situ* is likely to be quite complex, with a number of cell types and mediators present in the soft tissue and bone matrix capable of promoting osteoclast formation. However, the production of RANKL is considered to be an important, if not the most important, factor regulating osteoclast formation. The results of immunostaining verified the initial RNA analyses and demonstrated production of RANKL protein in the peri-prosthetic tissues adjacent to osteolytic zones. It is important to note that the production of RANKL in peri-prosthetic tissues was observed to be higher in peri-prosthetic tissues when compared with OA and normal tissues.

It was significant to note that the majority of RANKL staining was associated with inflammatory infiltrates of mononuclear and multinuclear cells around wear particles. RANKL was often associated with cells in the peri-prosthetic tissue that had phagocytosed wear particles. The production of RANKL by cells of the monocyte/macrophage lineage, as indicated by immunostaining, is consistent with the *in vitro* data from these studies showing expression of RANKL mRNA by monocyte/macrophage cells in response to activation by wear particles. In addition, although it is possible that contaminating cells, such as lymphocytes, might also produce RANKL (Kong et al., 1999a, Kotake et al., 2001), very few cells that were not of the monocyte/macrophage lineage were seen in the *in vitro* monocyte enriched cultures (Haynes et al., 1993).

It is difficult to accurately identify cell types based on morphology alone. Therefore dual labelling with cell specific markers was used to identify the cell lineages associated with RANKL production. As expected, many cells positive for CD68, a macrophage lineage cell marker, were present in cell aggregates around wear particles in the peri-prosthetic tissues. Dual labelling with monoclonal antibodies directed against CD68 and RANKL verified that many of the cells associated with RANKL in the peri-prosthetic tissue were of the

monocyte/macrophage lineage. It has been suggested that RANKL is not expressed by cells of the osteoclast lineage (Hakeda et al., 1998) but is bound to the membrane of osteoblastic cells (Yasuda et al., 1998b). It is therefore possible that the RANKL detected with Mab 626 is not actually produced by CD68 positive cells but may be soluble RANKL bound to these cells via a receptor such as RANK. Although the protein seemed to be located in the cytoplasm, further studies need to be carried out to prove that this is RANKL produced by other cells. To confirm the source of the RANKL in these sections, *in situ* hybridisation would need to be carried out. Unfortunately, in the preliminary work, *in situ* hybridisation using riboprobes to detect RANKL mRNA did not provide clear or reproducible results. However, results here show that human monocytes can express RANKL mRNA when stimulated with metal particles *in vitro*. In addition to this, further studies have been carried out by our group since completion of this study and have identified macrophages as expressing RANKL mRNA *in situ*.

It has been suggested that fibroblasts in peri-prosthetic tissue may contribute to osteolysis adjacent to prosthetic implants (Yao et al., 1998, Santavirta et al., 1998). Synovial fibroblasts from peri-prosthetic granulomatous tissues (Sakai et al., 2002) have been shown to be able to support osteoclast formation directly *via* RANKL expression. In this study, detection of RANKL protein on fibroblast-like cells was occasionally noted in peri-prosthetic sections, concurring with recent immunohistochemical studies in RA synovium (Kotake et al., 2001) and granulomatous tissue from the pseudocapsule of revision arthroplasties (Sakai et al., 2002). These data indicate the possibility of fibroblasts in tissues around peri-prosthetic bone loss stimulating osteoclast formation through the production of RANKL. However, few cells expressing the fibroblastic cell marker, CD55, labelled with the antibody detecting RANKL protein, were observed in the peri-prosthetic tissues. CD55 is only associated with fibroblasts in the synovial lining and does not identify all fibroblasts present in the tissues. The low numbers of cells expressing CD55 may be due to the nature of the tissue, as few samples had synovial lining present as the lining may be lost during implantation of the implant. It is therefore possible that some tissue fibroblasts, other than these synovial fibroblasts, are producing RANKL. However, overall the findings suggest that the major cell type in these tissues that is producing RANKL is macrophage.

The involvement of immunological responses directed against the prosthetic materials in peri-prosthetic osteolysis, remains controversial (Farber et al., 2001). In the peri-prosthetic tissues, the foreign body reaction to prosthetic materials resembles that seen in the type IV hypersensitivity reaction, in which T cells are thought to regulate macrophage functions. T

lymphocyte modulation of macrophage function has also been suggested to be important in peri-prosthetic osteolysis (Goodman et al., 1998). Production of RANKL by activated T cells may also enhance dendritic cell survival by interacting with high levels of its receptor present on dendritic cells (Wong et al., 1997a). It is possible that T cells in the peri-prosthetic tissue may be activated by particles in a similar way to cultures of PBMC derived T cells, which have been shown to express RANKL when activated (Horwood et al., 1999). However in contrast to RA and periodontal disease (as discussed in Chapters 3 and 4), relatively low numbers of lymphocytes were observed near peri-prosthetic osteolysis in this and other studies (Vernon-Roberts and Freeman, 1977, Jiranek et al., 1993, Jacobs et al., 1994). Dual labelling with monoclonal antibodies directed against the pan T cell marker, CD3, and RANKL detected few T cells positive for CD3 in the peri-prosthetic tissue. In addition, the few CD3 positive T cells that were present rarely expressed RANKL protein. This means it is unlikely that T cells are the major source of RANKL in peri-prosthetic osteolysis. T cells probably need to become activated before they express RANKL (Kotake et al., 2001). Therefore, the small numbers of T lymphocytes that are usually present in the peri-prosthetic tissue are not likely to be activated (Li et al., 2001).

In addition to macrophages and T lymphocytes, B lymphocytes were also seen in peri-prosthetic inflammatory infiltrates. The numbers of B cells were, however, less than CD68 and CD3 positive cells. Few studies have investigated the role of B cells in the production of RANKL. It has been suggested recently that B lymphoid lineage cells may be able to support osteoclastogenesis through production of RANKL (Manabe et al., 2001). However, in immunohistochemical studies using human RA tissue, B cells were not associated with the production of RANKL protein (Kotake et al., 2001). Results from dual labelling experiments in this study were consistent with the findings of Kotake *et al* as the lymphoid cells detected with monoclonal antibodies directed against the B cell marker, CD22, did not express RANKL protein.

While RANKL protein in the peri-prosthetic tissues may be critical in the formation and activation of osteoclasts, levels of OPG are also likely to be of great consequence as OPG competes for binding of RANKL to its receptor (RANK) on pre-osteoclasts (Lacey et al., 1998, Yasuda et al., 1998b). It is important to note that OPG can inhibit the formation of osteoclasts from cells derived from tissues around failed prostheses *ex vivo* (Itonaga et al., 2000a). This demonstrates that RANKL and OPG may be crucial in determining whether osteoclasts form in these tissues. Clearly, if high levels of OPG were stimulated by particles in the revision tissues then the pro-osteoclastic effects of RANKL would be reduced.

The expression of OPG mRNA by the total cell population isolated from peri-prosthetic tissue samples adjacent to sites of osteolysis suggests that cells other than osteoblast cells produce OPG in the tissue adjacent to pathological bone loss. The *in vitro* data here also suggests that cells of the monocyte/macrophage lineage are capable of producing OPG when activated by wear particles. Once again, the immunostaining results verified the RT PCR findings and demonstrated production of OPG protein in the peri-prosthetic tissue adjacent to osteolytic zones. It is important to note that OPG staining often appeared to be reduced in the peri-prosthetic tissues when compared with the OA and normal tissues.

OPG protein production by cells in the peri-prosthetic tissue was investigated using two commercially available antibodies. Preliminary staining of the test tissue (inactive RA) samples demonstrated two different staining patterns for OPG. One Mab (8051) predominantly identified OPG in cells in the synovial lining with weak staining of OPG in blood vessels, while the other Mab (805) exclusively identified OPG in cells in blood vessels. It was surprising to see two separate staining patterns for OPG in the same tissue with two different monoclonal antibodies, particularly as immuno-absorbance studies demonstrated that both staining patterns could be blocked with excess recombinant OPG. However, subsequent studies described here showed that this paradox is likely to be due to different forms of OPG being recognised by the two monoclonal antibodies.

These results may be important as the different forms of OPG may have different roles in the tissues. The failure of Mab 805 to detect OPG on the synovial lining layer may be due to the fact that RANKL is bound to the OPG, which would block Mab 805 binding to the epitope. Mab 8051 is able to detect OPG on cells in the lining layer of the synovial membrane therefore it is possible that RANKL:OPG complexes are bound to macrophage cells present in the synovial lining layer. This latter explanation appears unlikely as RANKL was not detected either at the mRNA (Fujikawa et al., 1996b) or protein level on CD68 positive synoviocytes, and RANKL expression in RA synovial membranes is seen in T cell rich areas of the synovial membrane and not on the synovial lining layer (Kotake et al., 2001).

Dual immunohistochemical labelling on selected samples in the same patient groups was used to verify the cell lineages associated with OPG production *in situ*. Labelling with monoclonal antibodies to OPG (Mab 805) and the lineage specific Mab for endothelial cells, Factor VIII, verified OPG protein on endothelial cells in the small blood vessels in peri-prosthetic, OA and normal tissues. This is in agreement with several reports by other investigators showing

endothelial cells expressing OPG (Collin-Osdoby et al., 2001, Malyankar et al., 2000). It is interesting to note that OPG is reported to be a survival factor for endothelial cells and may be involved in angiogenesis and tissue ingrowth (Malyankar et al., 2000). Vascular health and blood vessel formation may be important requirements for the formation of granulomatous tissue that is often seen in peri-prosthetic tissues near failed joints (al-Saffar et al., 1995a).

Dual labelling was also carried out with monoclonal antibodies to OPG, detected with the Mab 8051 antibody, and monoclonal antibodies to the monocyte/macrophage marker, CD68, and the fibroblast-like synoviocyte marker, CD55. OPG was associated with staining for the monocyte/macrophages cell marker, CD68, in the synovial-like lining but not the fibroblast-like synoviocyte marker, CD55. This was more evident in the normal and OA tissues, where there was a clear synovial lining present. In these tissues OPG protein was clearly associated with type A (macrophage-like synoviocytes) cells.

The RT PCR results showed how expression of OPG and RANKL mRNA in peri-prosthetic tissues related to osteoclast formations *ex vivo*. Further to this it was important to investigate whether RANKL and OPG protein levels differed between peri-prosthetic and control tissues (OA and normal tissue) using immunohistochemical staining. These immunohistochemical studies were unique in that it was possible to assess whether the levels of RANKL and OPG protein were different between the diseased and control tissues using semi-quantitative (SQA) and quantitative analysis.

RANKL was expressed at very low levels or remarkably absent in tissues from patients with OA of the knee and normal synovium. It is important to note that there was a statistically significant increase in the SQA scores for RANKL protein expression in peri-prosthetic tissues when compared to the SQA scores for RANKL in tissues from OA and normal patients. SQA scores may be underestimating the amount of RANKL in this osteolytic disease as the SQA grading reflects the proportion of cells staining positive through the section and not the amount of staining. There were often large fibrotic acellular areas observed in between inflammatory infiltrates, therefore in future studies it would be worth comparing the levels of RANKL in peri-prosthetic tissue that is cellular and actively producing cytokines with collagenous samples where the reaction is no longer active and particles are surrounded by collagen.

OPG, as detected on blood vessels by Mab 805, was seen in all patient groups. A general observation was that the amount of OPG (intensity of the stain) produced by the blood vessels

was much lower in the peri-prosthetic tissue from areas adjacent to sites of osteolysis when compared with the OA and normal tissues. The semi-quantitative grading of OPG was not significantly different between the groups. Unfortunately the SQA values only reflect the proportion of blood vessels with any positive staining rather than the amount of OPG on the blood vessels. Therefore, the SQA of OPG detected by Mab 805 is not sensitive to changes in the amount of OPG produced by the blood vessels.

Digital image analysis was carried out on OPG staining but due to the variable nature of the tissue and interference of the wear particles this could not be carried out on the RANKL staining. The IOD of OPG detected per blood vessel per high power field was reduced in the peri-prosthetic tissues, although again, the values were not significantly different. It is of interest to note that the blood vessel count per HPF was greater in the peri-prosthetic group and this may explain why even though the IOD of OPG per blood vessel was less in the peri-prosthetic tissue the overall IOD of OPG per HPF was not significantly reduced. Granulomatous tissue is highly vascular as a result of angiogenesis that occurs as part of the inflammatory response. Therefore, although there are more blood vessels in the peri-prosthetic tissue it appears the blood vessels express lower levels of OPG individually in areas where there are infiltrations of inflammatory cells.

Previously, OPG was reported to be associated with cells only in the bone shaft but these findings suggest OPG is produced within the soft tissue adjacent to bone. This work has focused on the local levels of OPG in areas of osteolysis. The production of OPG by endothelial cells in this region may have a major role in regulating bone metabolism. OPG production in the tissue may play a role in reducing osteoclast formation from precursors resident in the tissue or recruited to the joint in response to inflammation.

OPG released into the blood by endothelial cells may also significantly contribute to serum OPG. Serum OPG levels increase with age in men and women (Yano et al., 1999, Browner et al., 2001, Szulc et al., 2001) suggesting that OPG may play a role in Ca homeostasis. Serum OPG concentrations are significantly higher in postmenopausal women than age matched controls (Yano et al., 1999). Within osteoporotic groups serum OPG is higher in patients with low bone mass (Yano et al., 1999). Serum OPG concentrations are also higher in women with a high bone turnover rate (Yano et al., 1999). No significant correlation exists between serum OPG levels and bone mineral density (Browner et al., 2001). However, the source, form (monomer or dimer) and significance of circulating OPG are not well understood. In

addition, serum OPG may not reflect the OPG present in the bone microenvironment, where bone loss is associated with prosthetic joint failure (Ueland et al., 2001).

Consistent with previous reports, the *in vitro* and *in vivo* data from these studies show that particles of prosthetic material readily stimulate osteoclastic mediators and that the levels of each mediator can vary depending upon the type of prosthetic particles (Haynes et al., 1998, Howie, 1990). The data from the studies here support the findings that cells extracted from macrophage rich tissues adjacent to failed prosthetic implants are capable of forming osteoclasts (Sabokbar et al., 1997, Neale et al., 1999a, Itonaga et al., 2000a). This study goes further to demonstrate that the formation of osteoclasts from these tissues is associated with the expression of the osteoclastogenic mediators RANK and RANKL and that osteoclasts may form more readily from tissues containing certain types of wear particles. In addition to this, cells from tissue with silastic particles were able to differentiate even in the absence of a stromal element which may be due to the a high RANKL:OPG mRNA ratio expressed by cells isolated from these tissues.

These studies were unique in that, immunohistochemical staining verified the protein production of RANKL and OPG in tissue adjacent to peri-prosthetic bone loss. Semi-quantitative and quantitative analysis enabled comparison of the levels of these proteins in peri-prosthetic tissue adjacent to sites of osteolysis to levels in control tissues. Dual labelling identified monocytes and macrophages as the major cell type involved in RANKL production in peri-prosthetic tissue while CD68 positive synoviocytes and endothelial cells were identified as the source of OPG. These findings highlight the importance of the RANKL, RANK and OPG mediators in peri-prosthetic osteolysis may offer new ways of treatment in preventing this type of pathological bone loss.

These findings provide good evidence that treatment with inhibitors of osteoclast formation, such as OPG or “OPG-like” molecules, might be useful in preventing or delaying prosthetic implant failure. Therapies targeted to RANKL, by its natural inhibitor OPG (Kim et al., 2001, Itonaga et al., 2000a), have been been used *in vivo* and *in vitro* in experimental models. Recent studies have investigated the efficacy of RANK blockade in the treatment of titanium particle induced localised bone loss using a murine model. RANK-Fc treatment effectively reduced particle induced bone resorption without affecting bone formation, which was observed as similar to that seen in mice with deletion of the RANK gene (Childs et al., 2002). In addition, exogenous OPG or OPG gene therapy were also effective in preventing titanium-induced osteolysis in the mouse calvarial model (Goater et al., 2002). The results of these

studies have shown that these approaches to the treatment of peri-prosthetic lysis are promising. Our understanding of the cytokines and mediators that cause peri-prosthetic osteolysis is still evolving. However, therapies based on regulating the mediators and cytokines of osteolysis may soon be used to enhance the long-term survival of implants.

3 RHEUMATOID ARTHRITIS

3.1 INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease, characterized by articular cartilage and bone destruction, following growth of the inflamed synovial tissue (referred to as pannus) over and into the articular surface. The bone erosion can be localised to the inflamed joint (Gravallese et al., 2000), as well as being generalised, with secondary osteoporosis often being associated with RA (Gough et al., 1998). Extensive bone erosion is often observed as marginal joint erosions radiographically, and is predictive of a poorer prognosis (Van Zeben et al., 1993).

The inflammation and tissue destruction in RA is thought to involve cell-cell interactions between lymphocytes, subintimal monocytes/macrophages and type A (macrophage-like) synoviocytes and type B (fibroblast-like) synoviocytes. These cell interactions can result in the production of matrix metalloproteinases, cathepsins and mast cell proteinases that cause cartilage and bone destruction (Woolley et al., 1977, Trabandt et al., 1991, Tetlow and Woolley, 1995, Fujikawa et al., 1996c). Despite the widespread prevalence of rheumatoid arthritis, we still have an incomplete understanding of the processes of this chronic systemic disease.

Osteoclast formation at the cartilage-pannus junction has been associated with the destruction of bone matrix in RA patients (Fujikawa et al., 1996b, Gravallese et al., 1998, Gravallese et al., 2000, Takayanagi et al., 2000, Romas et al., 2000). Inflammatory cytokines that have been reported to promote osteoclast formation and bone resorption are found in the RA synovial fluid and tissue. These cytokines include interleukin (IL)-1α and β, IL-6, tumour necrosis factor (TNF)-α, IL-17 and macrophage colony stimulating factor (M-CSF) (Chu et al., 1992, Deleuran et al., 1992, Chu et al., 1991, Kotake et al., 1996, Kotake et al., 1999, Kobayashi et al., 2000a). Inflammatory chemokines in the RA joint may also contribute to osteoclast formation (Zheng et al., 1998).

The chemokines and cytokines may up regulate bone resorption by attracting monocyte derived osteoclast precursors to the soft tissue of the joint (Fujikawa et al., 1996b, Quinn et al., 1998c). Studies have shown that mature osteoclasts can also be derived from cells of the macrophage/monocyte lineage present within the soft tissues of arthritic joints (Fujikawa et al., 1996b, Toritsuka et al., 1997, Itonaga et al., 2000b, Suzuki et al., 2001). Cytokines may also act by increasing production of receptor activator of NFκB ligand (RANKL), a recently identified key mediator of osteoclastogenesis (Hofbauer et al., 1999, Nakashima et al., 2000).

Abundant *in vitro* experimental evidence suggests that (Myers et al., 1999)L and M-CSF are required for osteoclast formation. RANKL binds directly to RANK (receptor activator of NFκB) on pre-osteoclasts and osteoclasts, enabling signal transduction for the differentiation of osteoclast progenitors, as well as activation of mature osteoclasts (Yasuda et al., 1998b, Nakagawa et al., 1998, Lacey et al., 1998, Hsu et al., 1999, Kong et al., 1999b, Burgess et al., 1999). Differentiation of cells of the monocyte/macrophage lineage into mature osteoclast in the rheumatoid joint has been shown to be dependent on RANKL expression within the synovial tissue (Itonaga et al., 2000b).

Osteoblasts/stromal cells (Yasuda et al., 1998b, Fuller et al., 1998), synovial fibroblasts (Gravallese et al., 2000) and activated T lymphocytes (Gravallese et al., 2000, Kotake et al., 2001) have been identified as sources of RANKL in the RA joint. Fibroblasts and activated T lymphocytes in the soft tissue may be able to stimulate osteoclast differentiation directly through expression of RANKL. In support of this, fibroblasts (Quinn et al., 2000) and activated T lymphocytes (Horwood et al., 1999) are able to stimulate the differentiation of monocyte/macrophage cells into mature osteoclasts in culture.

In addition to their pivotal role in bone resorption, RANKL and RANK also have a role in the regulation of the immune system (Kong et al., 1999b). RANKL has been shown to influence immune cell differentiation and T cell/dendritic cell interactions (Wong et al., 1997a, Anderson et al., 1997, Wong et al., 1997b, Kong et al., 1999b). This may be important in regulating RA, which is believed to be an autoimmune reaction.

As described in detail previously, osteoprotegerin (OPG) acts as a soluble decoy receptor, preventing osteoclastogenesis by binding to RANKL with high affinity, essentially blocking RANKL from interacting with RANK both *in vivo* and *in vitro* (Lacey et al., 1998, Simonet et al., 1997, Yasuda et al., 1998a). OPG is a member of the TNF receptor family but lacks a transmembrane domain and is a secreted protein that is structurally distinct from RANK (Yasuda et al., 1998a, Gravallese et al., 2001).

The biological relevance of OPG as a regulator of RANKL/RANK interaction, initiating osteoclast formation and activation, is clearly demonstrated by the development of osteopetrosis in OPG transgenic mice and severe osteoporosis in OPG knockout mice (Simonet et al., 1997, Bucay et al., 1998, Mizuno et al., 1998). It is relevant that, in a rat adjuvant model, administration of OPG prevented the accumulation of TRAP positive

osteoclast-like cells and the bony erosions that often accompany joint inflammation in RA (Kong et al., 1999a). The relative levels of RANKL and OPG are likely to be important in determining whether osteoclasts will form (Hofbauer et al., 2000) in RA tissues.

TRAIL (TNF-related apoptosis-inducing ligand) is a member of the TNF family and shares homology with RANKL (Wiley et al., 1995, Walczak and Krammer, 2000). TRAIL has also been shown to bind to OPG (Emery et al., 1998), preventing it from interacting with RANKL. Recently, the abundant expression of TRAIL has been identified in human osteoblast-like cells (Atkins *et al* Bone, in press). The presence of TRAIL in soft tissue adjacent to areas of osteolysis may modulate the availability of OPG and therefore affect the RANKL:OPG ratio in RA.

Until recently, most studies have investigated the process of inflammation in the soft tissues adjacent to the bone in RA. Recent advances in our understanding of bone metabolism allow us now to better investigate the mechanisms of the bone loss in RA. The initial objective of this study was to determine whether cells of the osteoclast lineage and mediators of bone resorption were within RA tissue and to test the concept that their expression within the human RA pannus and synovial membrane was associated with osteoclastic bone resorption. *Ex vivo* tissue studies first sought to identify mRNA expression of mediators of osteoclast formation and osteoclast markers in the soft tissues using a semi-quantitative reverse transcription polymerase chain reaction (RT PCR). The expression of mediators of osteoclast formation and osteoclast markers, in cells isolated from either the pannus or synovial tissue, were also compared.

Cells, isolated from the pannus and synovial membrane of the RA joint, were cultured to determine their ability to differentiate into bone resorbing osteoclasts. Cells were cultured with and without human osteoblast-like cells to assess whether a stromal element was required for osteoclast differentiation. Results from the cell culture studies were compared with mRNA expression corresponding to various pro-resorptive cytokines and the ratio of RANKL to OPG mRNA, to assess whether there was a correlation between osteoclast formation and relative expression of RANKL and OPG in the tissues. Cells of the osteoclast lineage were also identified *in situ* by tartrate resistant acid phosphatase (TRAP) staining and *in situ* hybridisation detection of RANK mRNA.

Further studies were then carried out using immunohistochemical staining to detect OPG and RANKL protein levels in the synovial tissue from active RA patients and compare them with

that seen in patients with inactive RA, spondyloarthropathies, osteoarthritis (OA) and normal subjects. Dual labelling on synovial tissues from patients in each group identified the cell lineages associated with the production of RANKL and OPG. This study was novel in that established semi-quantitative and quantitative video image analysis was used to assess the levels of RANKL and OPG in synovial tissues from different patient groups in order to compare protein levels in a range of arthritides.

Using this data we aimed to test the hypothesis that RANKL protein is expressed at higher levels in RA patients with active disease than in synovial tissue from patients with inactive RA or other arthritides and, conversely, whether OPG is reduced in active disease compared to the other groups. Due to the availability of a large tissue bank it was also possible to follow the level of RANKL and OPG in selected patients during treatment over time.

3.2 METHODS

3.2.1 Tissue Processing for RT PCR, Cell Culture and *In situ* Hybridisation

Tissue samples were taken at surgery from patients who had been diagnosed as suffering from RA. Each of the patients had suffered from the disease for the duration of 6-25 yrs at the time of tissue retrieval. In each of the patients advanced erosion of the bone was evident on X-rays taken of the diseased joints. Tissues samples were taken from joints with active disease, that is, the disease had not burnt out, as seen in the later stages of the disease. Details of the patients, the medication at the time of surgery and the sites from which samples were taken from for these studies, are shown in Table 1.

In all cases, the classification of the tissue was made at the time of surgery by the surgeon (Dr Greg Bain). The tissue samples were classified as corresponding to either the synovial membrane or the pannus region (Figures 1A and B respectively). The pannus region was adjacent to the bone where erosion was occurring and was considered to be that part of the synovial membrane that had infiltrated the bone and cartilage (Figures 1C and D). The tissue that was classified as synovial membrane was not adjacent to the bone but rather capsular synovial tissue separate from the bone-cartilage infiltrate (as indicated on Figure D).

On retrieval, tissue was immediately placed in Hank's balanced salt solution (HBSS). Where possible the tissue was divided in to two representative portions, to be either fixed in 10% formalin and paraffin embedded to carry out routine histology and *in situ* hybridisation, or digested for cell culture and RT PCR studies. H & E staining was routinely carried out to enable general histological assessment of the samples and examples are shown in Figure 1. Generally the pannus and synovial membrane samples were similar and contained large numbers of infiltrating mononuclear cells. The only major difference was that the pannus tissues did not have a layer of cells lining the surface of the synovial membrane. The study protocol was approved by the University of Adelaide Human Ethics Committee, in accordance with the regulations of the National Health and Medical Research Council of Australia.

For RT PCR and cell culture studies the tissue was digested, as described in the methods section of Chapter 2. Cells were isolated from a total of 11 patients with RA. In two of these patients tissue was taken from two separate sites. The yield of cells varied depending on the amount of tissue digested (approximately $1cm^3$) and the cellularity of the tissue. Between 5×10^5 and 25×10^6 cells were isolated from each tissue sample with a mean of 5×10^5 cells/mg

wet weight of tissue. For the purpose of cell culture, cells were resuspended in RPMI 1640 medium at a concentration of 1 x 10^6 cells per ml.

RNA was extracted from between 5 x 10^5 and 1 x 10^6 cells to investigate mRNA expression using RT PCR. A sufficient cell number, for the studies on osteoclasts generation, was obtained from samples from eight of the patients. Cell culture studies were carried out with the help of Mrs Maria Capone, Technical Assistant, at The University of Adelaide.

Table 1. Demographic and Clinical Details of Patients Included in the RT PCR and Cell Culture Experiments.

Patient sample	**Age (years)**	**Sex (M/F)**	**Disease duration months**	**Site**	**Region**	**Medications**
1	58	M	17	Wrist	Pannus	Gold, Prednisolone
2	71	F	19	Knee	Pannus	NSAID[1]
3a	37	M	15	Elbow	Pannus	NSAID
3b	37	M	15	Elbow	Membrane	NSAID
4	48	F	20	Wrist	Pannus	Gold
5	52	F	30	Wrist	Membrane	Methotrexate
6	44	F	6	Wrist	Pannus	Herbal Therapies only
7a	51	M	17	Foot	Membrane	Methotrexate
7b	51	M	17	Foot	Pannus	Methotrexate
8	57	F	25	Wrist	Pannus	NSAID
9	52	F	22	Wrist	Membrane	Methotrexate
10	50	F	9	Elbow	Membrane	Methotrexate
11	57	F	15	Wrist	Pannus	Gold, Prednisolone

[1] non-steroidal anti-inflammatory drugs.

3.2.2 RNA Extraction

The total cell population isolated from the pannus or synovial membrane was lysed by the addition of Trizol reagent (Gibco BRL, Life Technologies). Total RNA was prepared according to the manufacturer's instructions. The methods are described in detail in the methods section of Chapter 2.

3.2.3 Preparation of RNA and Reverse Transcription Polymerase Chain Reaction

cDNA was synthesized using an AMV RT cDNA kit (Promega, Madison, WI, USA). cDNA was then amplified by PCR in a thermal cycler (Eppendorf, Hamburg, Germany), to generate products corresponding to mRNA encoding the gene products listed in Table 2. Twenty-two cycles of PCR were performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 29-36 cycles for the other primer pairs**.** Primer sequences and predicted PCR product sizes are also listed in Table 2.

Amplification products were resolved by electrophoresis on a 2% w/v agarose gel and post-stained with SYBR Gold for 20 minutes (Molecular Probes, Eugene, OR, USA). The PCR products were scanned using a Molecular Imager Fx fluorescent scanner and the density of the bands was quantified using Quantity-One software (Bio-Rad RAD, Hercules, California, USA). Amplified products were expressed as a ratio of the respective GAPDH PCR product.

Preliminary RT PCR experiments were performed on several RNA samples derived from the rheumatoid tissues to ensure that the number of PCR cycles was within the exponential phase of the amplification curve. The methods for RT PCR are described in the methods section of Chapter 2.

Table 2. RT PCR Primers and Conditions for the Specific Amplification of Human mRNA

Target gene	*Sense*[a]	*Primer sequence (3'-5')*	*Annealing temperature*[b] (^{o}C)	*Expected product size* (BP**)**
GAPDH	S	CACTGACACGTTGGCAGTGG	60	414
	AS	CATGGAGAAGGCTGGGGCTC		
CTR	S	GCAATGCTTTCACTCCTGAGAAA	62	782
	AS	AGTGCATCACGTAATCATATATC		
M-CSF	S	CAGTTGTCAAGGACAGCAC	60	670
	AS	GCTGGAGGATCCCTCGGACTG		
RANKL	S	AATAGAATATCAGAAGATGGCACTC	62	668
	AS	TAAGGAGGGGTTGGAGACCTCG		
OPG	S	TGCTGTTCCTSCAAAGTTTACG	62	435
	AS	CTTTGAGTGCTTTCGTGCGTG		
RANK	S	CCTACGCACAAGGCGAAGATGC	62	704
	AS	CGTAGACCACGATGATGTCGCC		
TRAIL	S	ACAGCAGTCAGACTCTGACAGG	62	838
	AS	CATGGTCCATGTCTATCAAGTGC		
TRAP	S	CTGGCTGATGGTGCCACCCCTG	65	469
	AS	CTCTCAGGCTGCAGGCTGAGG		

3.2.4 Isolation and Culture of Human Bone-Derived Osteoblast-Like Cells (HBDCs)

Human bone-derived osteoblast-like cells (HBDCs) were used for co-culture studies with cells extracted from RA tissues retrieved during surgery. Trabecular bone samples were obtained from normal patients during primary hip replacement and cultured, as described in the methods section of Chapter 2.

3.2.5 Culture of Osteoclasts and Osteoclast Precursors

Cells isolated from the rheumatoid tissues were tested for osteoclast formation and activity. Details of the method used are described in the methods section of Chapter 2. Briefly, where HBDCs were used as the stromal population, 3×10^4 cells in 1ml of medium were seeded onto either 13-mm diameter sterile glass coverslips in 24 well trays or 3.0 x 0.1mm thick discs of sperm whale dentine in 96 well trays. HBDCs were left to adhere for 24 hours before the addition of cells isolated from the rheumatoid tissues. Rheumatoid cells were added at 4×10^5 (coverslip) or 2×10^5 (dentine) and left to adhere for 1 hour. After washing in HBSS, the individual coverslips and pairs of dentine slices were placed in 16-mm diameter wells with 1ml of α-MEM medium containing 10^{-8}M $1\alpha,25(OH),_2D_3$ (vitamin D3), 10^{-8}M dexamethasone (Fauldings, Adelaide, Australia) and 25ng/ml recombinant human M-CSF (a kind gift from Genetics Institute, Cambridge, MA, USA). Medium was replenished every 3 days throughout the experiment and all experiments were carried out in duplicate for each rheumatoid sample.

3.2.6 Assessment of Osteoclast Formation

Cells were stained for TRAP to identify the cell population of osteoclasts precursors and osteoclast-like cells. Cytospins of freshly isolated cells and cultures of cells seeded onto coverslips were stained for TRAP after 1 or 14 days of culture, using a commercial staining kit (Sigma, St. Louis, MO, USA). Cells were then briefly counterstained with the nuclear stain Methyl Green. Immunostaining to detect TRAP was carried out with the help of Mrs Maria Capone (Technical Assistant, Department of Pathology, The University of Adelaide).

3.2.7 Resorption Pit Formation

The ability of cells to form resorption lacunae in bone slices is confirmatory of functional osteoclasts. Dentine discs, seeded with cells isolated from rheumatoid tissues, were assessed for pit formation on day 14, as described in the methods section of Chapter 2. The numbers of resorption lacunae formed on dentine discs, seeded with co-cultures of RA tissue-derived cells and HBDCs, were assessed to determine the effect on bone resorption of contact with osteoblast-like cells.

3.2.8 *In situ* Hybridisation

In situ hybridisation with digoxigenin (DIG)-labelled riboprobes was used to investigate the presence of cells expressing mRNA, corresponding to RANK, in a selection of formalin fixed sections from 6 RA patients. The method used to generate DIG-labelled riboprobes is described in detail in the methods section of Chapter 2**.** The method of *in situ* hybridisation detection is based on a previously published method (Smith et al., 1997) and is described in the methods section of Chapter 2.

3.2.9 Immunohistochemistry

Patients

Thirty RA patients (21 with active synovitis in a knee joint and 9 with no evidence of any synovitis), twelve patients with spondyloarthropathy and active synovitis of a knee joint, seven patients with OA of the knee joint and eighteen normal subjects were investigated. All RA patients fulfilled the American College of Rheumatology criteria for RA (Arnett et al., 1988), while the spondyloarthropathy patients fulfilled the European Spondyloarthropathy Study Group (ESSG) criteria for the diagnosis of Ankylosing Spondylitis and Reiter's Disease (Dougados et al., 1991). Patients with Psoriatic Arthritis (Gladman and Farewell, 1999) and OA of the knee (Altman et al., 1986) fulfilled published criteria. All patients gave informed consent, and the study protocol was approved by the Repatriation General Hospital Medical Ethics Committee.

Clinical and demographic data of the active and inactive RA patients and the spondyloarthropathy patients included in this part of the study are presented in Table 3. The 7 OA patients included in the study were 5 males and 2 females, 4 of whom were taking NSAIDs at the time of synovial tissue removal at knee replacement surgery. The OA patients had a mean age of 67.4 years (range 56-74). The number of normal subjects included in the study was 18, which included 11 males and 7 females, with a mean age of 33.3 years (range 18-54).

Synovial Tissue Collection

A small-bore arthroscopy (2.7 mm arthroscope, Dyonics, Andover, Massachusetts, USA) was performed under local anaesthesia, as previously described (Smith et al., 1996). Synovial biopsies were obtained from all accessible regions of the knee joint, but mainly from the suprapatellar pouch. OA ST was obtained at the time of knee replacement surgery while

normal ST was obtained at the time of knee arthoscopy for unexplained knee pain. An experienced rheumatologist (Dr Malcolm D Smith) performed each of the biopsies.

The samples were separately snap-frozen in Tissue-Tek OCT (Miles Diagnostics, Elkhart, IN, USA) and stored at –80^{o}C until used. Cryostat sections (5μm) were mounted on glass slides (Superior Marienfeld, Germany), fixed for 4 minutes in cold acetone and dried at room temperature. The glass slides were boxed and stored at –20°C until immunohistologic analysis.

The first section of each tissue was stained with haematoxylin and eosin for routine histopathological assessment. Sections of synovial tissue with good morphology, lymphoid infiltration and synovial lining were included in the study.

Antibodies

Serial sections were stained with mouse monoclonal antibodies (Mab) from commercial sources with known antigen specificities and are listed in Table 4. To eliminate variability in immunohistochemical staining, all sections to be stained with a particular antibody were processed concurrently and the sections were incubated with the antibody for the same amount of time. Immunohistochemical staining was carried out using appropriate controls to study the antibodies. For the positive control RA synovial tissue, with a previously defined staining pattern, lymph node or tonsil was stained. The negative controls consisted of omission of the primary antibody and substituting with an irrelevant Mab similar immunoglobulin isotype and subclass for the specific antibodies.

Table 3. Demographic and Clinical Details of the RA and Spondyloarthropathy Patients Included in the RANKL Immunohistochemical Study

Active RA Patients

Patient No.	Age (years)	Sex (M/F)	Disease Duration (months)	DMARDs	CRP (mg/L)	RF (yes/no)	Erosions (yes/no)
RA1	76	M	2	NSAIDs	59	Yes	No
RA2	72	M	3	NSAIDs	120	Yes	No
RA3	65	M	9	Plaquenil	30	Yes	No
RA4	75	F	3	NSAIDs	47	Yes	Yes
RA5	47	M	3	NSAIDs	58	Yes	No
RA6	61	F	2	NSAIDs	84	No	No
RA7	75	M	2	NSAIDs	20	No	No
RA8	86	M	6	NSAIDs	76	No	No
RA9	78	F	3	NSAIDs	27	Yes	Yes
RA10	31	F	6	NSAIDs	32	Yes	No
RA11	67	F	2	Prednisolone	307	No	No
RA12	76	F	4	NSAIDs	89	No	No
RA13	77	M	3	NSAIDs	62	Yes	No
RA14	74	F	4	NSAIDs	46	Yes	Yes
RA15	70	M	2	NSAIDs	116	Yes	Yes
RA16	76	F	2	NSAIDs	32	No	No
RA17	59	F	4	Plaquenil	82	No	No
RA18	49	F	10	NSAIDs	35	No	Yes
RA19	28	F	2	SSZ	37	No	No
RA20	68	M	5	NSAIDs	34	Yes	Yes
RA21	62	M	2	NSAIDs	28	No	No

DMARDs- Disease Modifying Anti-Rheumatic Drugs, PsA – Psoriatic Arthritis, AS – Ankylosing Spondylitis, Reiter's – Reiter's Syndrome,
SSZ – Sulphasalazine, MTX – Methotrexate, IM Gold – IM Sodium Aurothiomalate, NSAIDs – non-steroidal anti-inflammatory drugs

Table 3 Continued

Inactive RA Patients

Patient No.	Age (years)	Sex (M/F)	Disease Duration (months)	DMARDs	CRP (mg/L)	RF (yes/no)	Erosions (yes/no)
RA1	78	M	30	MTX	< 1	No	No
RA2	79	M	24	IM Gold	5	Yes	No
RA3	75	F	16	MTX	< 1	Yes	No
RA4	73	M	34	IM Gold	< 1	Yes	No
RA5	60	F	7	IM Gold	< 1	No	No
RA6	63	M	8	Plaquenil	< 1	No	No
RA7	79	M	27	SSZ	2	Yes	No
RA8	76	F	14	IM Gold	5	No	Yes
RA9	68	M	36	IM Gold/ MTX	7	Yes	Yes

SpA Patients

Patient No.	Age (years)	Sex (M/F)	Disease Duration (months)	DMARDs	CRP (mg/L)	HLA B27 (yes/no)	Diagnosis
SpA1	26	F	36	NSAIDs	22	No	Psoriatic Arthritis
SpA2	60	F	96	SSZ	58	No	Psoriatic Arthritis
SpA3	28	F	24	SSZ	13	Yes	Reiter's
SpA4	43	F	192	NSAIDs	48	Yes	Reiter's
SpA5	33	M	168	NSAIDs	45	Yes	Reactive Arthritis
SpA6	40	M	24	NSAIDs	13	No	Psoriatic Arthritis
SpA7	49	F	60	NSAIDs	45	No	Psoriatic Arthritis
SpA8	65	M	56	NSAIDs	26	No	Psoriatic Arthritis
SpA9	75	M	8	NSAIDs	94	Yes	AS
SpA10	73	M	120	NSAIDs	73	Yes	AS
SpA11	63	F	6	SAS/ AZA/ Prednisolone	52	Yes	Colitic Arthritis
SpA12	30	M	72	SAS/ NSAIDs	34	Yes	AS

Table 4. Details of Antibodies used in the Immunohistochemical Studies

Antibody	**Antigen**	**Raised in/Isotype**	**Cell Lineage**	**Dilution Used**	**Source**
OPG Mab 805	OPG	Mouse IgG1	To be determined	1/400 (DE AEC)	R & D Systems Inc
OPG Mab 8051	OPG	Mouse IgG2a	To be determined	1/1000 (DE AEC)	R & D Systems Inc
RANKL Mab 626	RANKL	Mouse IgG2b	To be determined	1/400 (DE AEC)	R & D Systems Inc
CD3 Leu-4	CD3	Mouse IgG1	Pan T cells	1/10 (APAAP)	Becton Dickinson, CA, USA
CD68	CD68	Mouse IgG1kappa	Monocytes, macrophages	1/400 (APAAP)	DAKO, Denmark
Mab 67	CD55	Mouse IgG1	Endothelial cells, type B synoviocytes	1/7000 (APAAP)	Serotec, UK
Von Willebrand	Factor VIII	Mouse IgG1kappa	Endothelial cells	1/50 (APAAP)	DAKO, Denmark
CD22 6B11	CD22	Mouse IgG1	B cells	1/300 (APAAP)	CLB
CD45Ro UCHI	CD45Ro	Mouse IgG2a	Memory T cells	1/100 (DE AEC)	DAKO, Denmark

3.2.10 Double Enhancement Aminoethylcarbazole (DE AEC) Immunohistochemistry

To amplify the reactions a double enhancement method was used on sequential sections with the anti-RANKL antibody (Mab 626) as well as both OPG antibodies (Mab 805 and 8051). In addition, several sections from each group were stained with anti-CD45Ro to detect activated memory T cells.

Following a primary step of incubation with the Mabs to RANKL, OPG and CD45Ro, bound antibody was detected according to a three-step immunoperoxidase method (Kraan et al., 2000, Parker and Smith, 1999, Tak et al., 1995). Horseradish peroxidase (HRP) activity was detected using hydrogen peroxide as the substrate and aminoethylcarbazole (AEC) as the dye. Slides were counterstained briefly with haematoxylin solution and mounted in Gurr Aquamount (BDH, Poole, UK). The method of immunohistochemical staining is described in detail in the methods section of Chapter 2.

3.2.11 Dual Labelling: DE AEC and Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP)

Dual immunohistochemical labelling was performed, as previously published (Parker and Smith, 1999, Wikaningrum et al., 1998), to identify the cell lineages associated with RANKL and OPG in arthritic and normal tissues. In brief, the synovial tissue was incubated with the primary antibody to RANKL (anti-RANKL Mab 626) or OPG (Mab 805 or 8051) and subsequent steps were performed in a standard three-step immunoperoxidase method. The final colour product was developed with AEC. Immunoperoxidase staining, developed with AEC, was followed by detection of markers identifying the cell lineage.

APAAP

Cell markers were detected with monoclonal antibodies using an alkaline phosphatase anti-alkaline phosphatase staining (APAAP) method, developed with fast blue. Between the first and second immunohistochemical reactions the tissue was washed and blocked with Tris-glycine to prevent cross-reaction between the first primary and the second linking antibody.

The second primary antibody: anti-CD68, anti-CD55 (dual labelled with RANKL Mab 626 and OPG Mab 8051), anti-Factor VIII (dual labelled with OPG Mab 805), anti-CD3 or anti-CD22 (dual labelled with RANKL Mab 626), was placed on the sections overnight at 4^{o}C. A standard immunoalkaline phosphatase method was used to detect antibodies to the cell lineage markers (Tak et al., 1995, Parker and Smith, 1999), developing the colour reaction with Fast

Blue. No counterstain was used and the sections were mounted in an aqueous mounting medium. The dual labelling method is described in detail in the methods section of Chapter 2.

3.2.12 TRAP Staining in Tissue Sections

To identify osteoclastic cells in tissue sections TRAP was detected in several serial frozen sections from the same RA, OA and normal patients included in the RANKL and OPG immunostaining studies. A commercial acid phosphatase leukocyte kit (Sigma-Aldrich, St Louis MO) was used and sections were counterstained with Methyl Green. Sections were then air-dried and mounted in DPX medium.

3.2.13 Quantitation of Immunohistochemical Staining

After immunohistochemical staining, sections stained for OPG (Mabs 805 and 8051) and RANKL were analysed in a random order by computer-assisted image analysis, as previously published (Kraan et al., 2000, Wikaningrum et al., 1998, Youssef et al., 1997a, Youssef et al., 1997b). Two parameters were measured by digital image analysis; these included the mean optical density (MOD) field and the integrated optical density (IOD). The MOD is a measure of the average density of staining, equivalent to the concentration of protein per cell. The IOD, which is measured in pixel units, is calculated as the MOD multiplied by the area of AEC staining and is proportional to the total amount of OPG staining. In addition, staining of the sections was scored by semi-quantitative assessment (SQA) on a 5 point scale by two independent observers in random order, as described previously (Kraan et al., 2000, Tak et al., 1995).

3.2.14 Statistical Analysis

Non-parametric statistics were used to analyse the mean ranks of the semi-quantitative scores for RANKL and OPG staining in the five groups of patients. Statistical analysis was carried out using a Kruskal-Wallis one-way analysis of variance (ANOVA). The number of blood vessels, and the percentage of blood vessels staining positive for OPG, was also analysed using a Kruskal-Wallis ANOVA. As the data for area, MOD and IOD, measured by digital image analysis, were not normally distributed, the data was log transformed and analysed using a one-way analysis of variance. Post-hoc discrepancies between groups were analysed using Tukey HSD and Bonferroni tests. Statistical significance was accepted when $p < 0.05$.

3.2.15 Cell Culture with Human Umbilical Vein Endothelial Cells (HUVE cells)

To assess the effect of inflammatory cytokines on endothelial cells, rhTNF-α (R & D Systems) was added to human umbilical vascular endothelial (HUVE) cells in culture. HUVE

cells were cultured in M199 medium containing 20% FCS with Heparin and Growth Factor Supplement and then trypsinized as described previously (Gamble et al., 1985). Cells were then seeded onto 8-chamber slides (Nunc, Inc, Naperville, IL) at 5 x 10^4 cells/well in M199 medium. Confluent cells were supplemented with fresh medium prior to treatment with TNF-α at a final concentration of 5ng/ml. This concentration was found previously to activate HUVE cells without the induction of apoptosis (Molostov et al., 2001). Untreated HUVE cells served as a control.

After 24 hours supernatants from the HUVE cells were removed and centrifuged at 4000g to remove cellular debris. Supernatants were then stored at –20^{o}C until used in Enzyme-Linked Immunoabsorbance (ELISA) assays. Cells were fixed by adding methanol:acetone (1:1) to chamber wells for 4 minutes at 4^{o}C and then allowed to dry. Slides were stored at -80^{o}C until stained.

OPG protein was detected with Mab 805, followed by immunohistochemical staining using the DE AEC method and developed with AEC (as described in detail in Chapter 2) then counterstained with haematoxylin. Untreated cells were also stained with Mab 8051 and Mab RANKL 626 using the DE AEC method.

HUVE cells were cultured by Mr Chris Holding (Department of Pathology, The University of Adelaide) and ELISA assays were carried out with the help of Mr Chris Holding, as described in the methods section of Chapter 2.

3.2.16 RANKL and OPG Expression in RA Tissue During Patient Treatment

Biopsies were taken from eleven patients undergoing treatment for RA. Biopsies consisted of a sample taken at initial diagnosis and at each of up to four follow-up appointments. The minimum time between biopsies included in the studies was 3 months. Frozen sections from each of the samples were stained for RANKL and OPG using the DE AEC method as described in detail in the methods section of Chapter 2.

3.3 RESULTS

3.3.1 mRNA Expression of Regulators of Osteoclast Formation and Osteoclast Markers in Cells Extracted from Rheumatoid Tissues

PCR products were generated by RT PCR from total RNA extracted from cells isolated from rheumatoid tissues sampled from 11 patients (listed in Table 1). Figure 2 shows PCR products corresponding to the mRNA sequence for mediators of osteoclast formation, as well as pre-osteoclast and osteoclast markers.

RANKL, OPG, and M-CSF mRNA was detected in nearly all tissue samples. TRAIL mRNA, a TNF-α related molecule that has been shown to bind to and antagonize the inhibitory actions of OPG (Emery et al., 1998), was also expressed in all the samples tested. mRNA encoding the osteoclast markers RANK, TRAP and CTR were expressed in the majority of the rheumatoid tissues. TRAP and RANK were expressed in all but two tissue samples, while CTR was expressed in all eight of the pannus tissue samples but only one of the five synovial membrane samples. This may reflect the more specific expression of CTR by more mature osteoclasts.

3.3.2 The Generation of Osteoclast-Like cells From Cells Isolated from the RA Tissues

After culture for 24 hours, the population of cells digested from RA tissues, that were adherent to glass coverslips, contained many cells with the appearance of osteoclasts (Figure 3A). These osteoclast-like cells were large, TRAP-positive and contained many nuclei. This suggested that osteoclast-like cells are present in the arthritic tissues. Many more mononuclear and multinuclear cells expressing TRAP were seen after 14 days of culture, when cultured either alone (Figure 3B) or in the presence of human osteoblast-like cells.

When RA cells were cultured alone on dentine slices, large numbers of resorption pits were usually seen by day 14 (Figure 3C). More resorption pits were observed by day when RA cells were cultured with HBDCs (Figure 3D). Using inverted-phase microscopy, resorption pits could be observed during the culture period. No resorption pits were observed before day 7.

The numbers of resorption pits and TRAP-positive cells seen at day 14 are compared in Table 5. There was a slight increase in the mean numbers of cells expressing TRAP when cells from RA tissues were cultured with HBDCs compared with when cells from RA tissues were incubated alone. It is important to note there was a significant increase in the mean numbers resorption pits when RA cells were cultured with HBDCs.

Table 5. TRAP Expression and Resorption Pit Formation During 14 days Culture of Cells Isolated from RA Tissue. The Effect of Co-Culture with Human Bone-Derived Osteoblast-like Cells (HBDC) are Shown.

	RA Cells + HBDCs	**RA Cells alone**
Pits per dentine slice	88.8 ± 28.0*	45.1 ± 18.1
TRAP +ve cells per 10^6 RA cells	287 ± 56	196 ± 50

RA- Rheumatoid Arthritis
HBDCs -Human Bone-Derived Osteoblast-like Cells

*mean ± S.E.M. for n = 8

There is increasing evidence (Hofbauer et al., 2000, Atkins et al., 2000b, Nagai and Sato, 1999) that osteoclast formation and activation is dependent upon the relative concentration of RANKL and its inhibitor OPG. Therefore, the ratio of RANKL mRNA to OPG mRNA expressed by cells isolated from the RA tissue, as measured by RT PCR was compared with the number of resorption pits produced when cells isolated from the RA tissue were cultured without HBDCs (Figure 4). The mean number of resorption pits corresponded with an increase in the ratio of RANKL to OPG mRNA levels. Statistical analysis showed a strong positive correlation between these values (Spearman rank {non-parametric} 0.762; *P*=0.028). This is consistent with a role for RANKL in promoting resorption in human RA tissues.

A similar comparison of the numbers of resorption pits with the ratio of TRAIL mRNA to OPG mRNA was carried out but no significant correlation was noted ($p > 0.05$). This indicates that TRAIL may not regulate RANKL activity in RA to the same extent as OPG.

3.3.3 Detection of TRAP and RANK mRNA in Osteoclast-Like Cells in RA Tissue Sections

In situ hybridisation, using DIG-labelled riboprobes, was carried out on tissues from 6 active RA samples to identify the cells expressing RANK mRNA. Cells expressing RANK mRNA were detected with the antisense probe in all RA samples studies. The majority of these cells were mononuclear and were usually present in large numbers within aggregates of infiltrating

cells (Figure 5A). No staining was evident in serial sections incubated with the DIG-labelled sense riboprobe (the negative control corresponding to the RANK mRNA sequence) (Figure 5B). Dual labelling with antibodies directed against cell markers will be necessary to verify the type of cells expressing RANK.

TRAP staining was also used to identify osteoclast-like cells *in situ*. TRAP was expressed by mononuclear and multinuclear cells within large aggregates of cell infiltrates in the active rheumatoid group (Figures 5C and 5E). TRAP was not expressed in the normal tissues (Figure 5D) or OA tissues (Figure 5F), which contained very low or no inflammatory cell infiltrate.

3.3.4 Detection of RANKL in Different Arthritides

RANKL protein was detected by immunohistochemistry and then analysed by digital image analysis and SQA. RANKL protein was detected in all groups of patients, however, notably more extensive staining was seen in tissue samples from the patients with RA and spondyloarthropathy with active synovitis, at the time of synovial biopsy. RANKL protein was predominantly detected in areas of mononuclear cell aggregates in the synovial membranes from active RA (Figures 6A and 7A) and in the spondyloarthopathy patients (Figure 6C). It was noted that there was no expression of RANKL in regions of the RA synovial membranes that stained with Mabs 805 or 8051 for OPG.

Although all samples from active rheumatoid patients (Figures 6A and 7A) and spondyloarthopathy patients (Figures 6C and 7E) had active synovitis with lymphoid infiltration, the extent of staining varied. Many samples of active RA and spondyloarthropathy tissues did not show any RANKL staining. Levels of RANKL protein were consistently low or absent in synovial tissue from the inactive RA (Figures 6B, and 7C), OA (Figure 6D) and normal patients (Figure 7G). This suggests that aggregates of mononuclear or multinuclear cells are absent in tissues from patients with inactive RA disease patients.

The computer assisted image analysis results and analysis of the SQA scores for RANKL protein present in tissue from the various biopsies are displayed in Table 6. There was no significant difference in RANKL protein expression between samples from the active RA patients and other groups, as measured by SQA. However, there was a trend toward increased RANKL in the active RA and spondyloarthropathy groups with some samples graded as 3 or 4 using SQA, while RANKL in OA and normal patient samples was graded as 0 or 1 in all

samples. With a greater sample number this may have been significant (type II error). Assessment by the SQA method is also less sensitive to change than integrated optical density (IOD) (Kraan et al., 2000) as it is a grading of 5 points that reflects the overall staining in a section rather than quantifying the staining.

Digital image analysis of the total amount of RANKL staining was measured as the IOD. The IOD in inactive RA, normal and OA, was significantly less compared with active RA ($p < 0.005$). However, the digital image analysis of the mean optical density (MOD) of RANKL detected in each sample was not significantly different between the groups.

Table 6. Digital Image Analysis Results and Mean Semi-quantitative Scores for the Expression of RANKL using Mab 626 in Synovial Tissue from RA, OA, Spondyloarthropathy Patients and Normal Subjects.

	SQA *	**MOD ***	**IOD ***
Active RA	1 (1.1)	0.6267 (0.2176)	2426.8 (6524.2)
Inactive RA	0.2 (0.4)	0.6036 (0.1594)	35.7 (34.6)[§]
SpA	1.1 (1.2)	0.6363 (0.2153)	702.8 (903.3)
OA	0.4 (0.7).	0.6406 (0.2533)	52.9 (57.7)[§]
Normals	0.3 (0.5)	0.7653 (0.1650)	55.6 (73)[§]

RA- Rheumatoid Arthritis, SpA- Spondyloarthropathy, OA- Osteoarthritis
SQA -Semi-Quantitative Assessment
MOD -Mean Optical Density
IOD -Image Optical Density

* The data represent: mean (SD)

[§] $p < 0.005$ compared to active RA group

Dual immunohistochemical labelling with the RANKL antibody was carried out in combination with cell lineage specific Mabs for T cells (CD3), B cells (CD22), macrophages (CD68) and fibroblasts of the synovial lining (FLS) (Mab 67). The majority of cells within the cell infiltrates stained positive for CD3, a pan T cell marker. RANKL was present on a 30% of pan T cells as well as approximately 40% of infiltrating macrophages in tissues from active RA (Figures 6E and 6G respectively) and tissues from spondyloarthropathy patients. RANKL positivity did not co-localise with either B cells (CD22) (Figure 6F), or FLS (CD55) (Figure 6H) in active RA tissue samples.

Immunohistochemical staining with antibodies to RANKL and activated memory T lymphocytes (CD45Ro positive cells) was carried out in sequential sections from a selection of tissue samples from each of the control and diseased groups. CD45Ro positive cells were abundant in the lymphoid aggregates present in tissue from the active RA and spondyloarthropathy patients (Figures 7B and 7F respectively), while few were present in tissues from the inactive RA (Figure 7D) OA and normal (Figure 7H) patients. Aggregates of RANKL positive cells corresponded with both positive and negative CD45Ro cells in sequential tissue sections (Figures 7A and B). In tissue sections from the active RA patients, up to 70% of CD45Ro positive cells in the mononuclear aggregates corresponded with cells positive for RANKL. Aggregates of CD45Ro positive cells were also negative for RANKL staining in the corresponding areas of sequentially stained sections (Figure 7).

3.3.5 Detection of OPG in Different Arthritides

During preliminary staining two distinct patterns of staining for OPG were observed in synovial tissue (shown in the methods section of Chapter 2). Consistent with these results, Mab 805 detected OPG exclusively on endothelial cells, while Mab 8051 detected OPG mainly on the lining layer of the synovial membrane (Figures 8 and 9 of this chapter). Preliminary staining experiments had shown that endothelial cells were also weakly stained when Mab 8051 was used at higher concentrations. Studies to verify the specificity and efficiency of these antibodies were carried out and this was discussed in detail in Chapter 2.

Both forms of OPG expression were seen in tissue samples from the psoriatic arthritis, OA and normal groups (Figures 8 and 9 (panels B, C and D respectively)) with a notable reduction of staining in tissue from the RA patients with active synovitis at the time of synovial biopsy (Figures 8A and 9A). The staining in each group was therefore compared using semi-quantitative and quantitative assessment methods.

The results of the computer assisted image analysis and semi-quantitative scores for the detection of OPG, with Mabs 805 and 8051, are displayed in Table 7. When tissue sections were analysed by SQA, OPG detected with either 805 or 8051 antibodies was significantly reduced in active RA compared with samples from all other groups ($p < 0.0005$). There was also a significant reduction in the detection of OPG with Mab 805 in tissue from the active RA patients compared with samples from all other groups when image analysis values were analysed ($p< 0.005$) for MOD. The IOD values of OPG were significantly reduced in the active RA group compared with all other groups when detected with 8051 ($p < 0.0005$) and compared with the inactive RA and spondyloarthropathy group when detected on the blood vessels with Mab 805 ($p < 0.0005$).

Table 6. Mean Semi-quantitative Scores and Digital Image Analysis Results (IOD and MOD) for the Expression of OPG using Mabs 805 and 8051 in Synovial Tissue from RA, Spondyloarthropathy, and OA Patients and Normal Subjects.

	Mab 805				**Mab 8051**		
	SQA *	**% OPG +ve BV ***	**MOD ***	**IOD***	**SQA ***	**MOD ***	**IOD ***
Active RA	0.5 (0.9)	4.9 (8.4)	0.0099 (0.0071)	720 (336)	0.2 (0.4)	0.17 (0.08)	61.1 (92.8)
Inactive RA	3.3 (0.9)#	71.3 (14.7)#	0.0442 (0.0318)§	2104 (724)#	2.7 (1.1)#	0.25 (0.1)	6094.8 (4741.2)#
SpA	3.3 (0.6)#	79.3 (4.8)#	0.5 (0.091)#	5782.1 (2086.6)#	2.9 (1)#	0.34 (0.13) §	5980 (6399.5)#
OA	3.6 (0.5)#	70.7 (8)#	0.46 (0.04)#	892.5 (405.8)	4.1 (0.7)#	0.29 (0.06) §	7648.4 (2651.5) #
Normals	3.3 (0.7)#	72.7 (11.5)#	0.61 (0.18)#	556.9 (226.2)	3.6 (1)#	0.26 (0.08)	5276 (4716.4)#

RA- Rheumatoid Arthritis, SpA- Spondyloarthropathy, OA- Osteoarthritis

IOD - Image Optical Density
MOD- Mean Optical Density
SQA- Semi-Quantitative Assessment
BV- Blood Vessels

* The data represent: mean (SD)
§ $p < 0.005$ compared to active RA group
# $p < 0.0005$ compared to active RA group

The percentage of blood vessels expressing OPG was significantly reduced in tissues from the active RA patients compared with samples from all other groups ($p < 0.0005$). There was no significant difference in OPG protein, as detected by either Mab 805 or Mab 8051, between inactive RA, SpA, OA and normal groups. Overall, these findings suggest that OPG is reduced on the synovial lining and the endothelial cells of blood vessels in the active stage of RA.

Dual immunohistochemical labelling with an antibody to Factor VIII (a cell lineage marker for endothelial cells) demonstrated that the Mab 805 detected OPG on endothelial cells (Figures 8E and F). The amount of OPG on the blood vessels was clearly reduced in tissue from active RA patients (Figure 8E) compared with the tissue from an OA sample (Figure 8F). Dual immunohistochemical labelling on in active RA (Figures 9 E and G) and OA tissue (Figure 9F and H) with lineage specific Mabs for macrophages (CD68), fibroblast-like synoviocytes (Mab 67) shows that the Mab 8051 detects OPG on the intimal macrophages (Figures 9E and 9F) rather than the FLS (Figures 9G and H).

3.3.6 RANKL and OPG Protein in the Synovium During Treatment for Active RA

Biopsies from patients with active RA were collected at different stages of their treatment and disease activity in order to investigate changes in RANKL and OPG protein. RANKL appeared to be reduced in the synovium of the patients as they were followed through treatment. At initial consultation during treatment, high levels of RANKL were present in the synovium of some patients for up to 14 months (Figures 10A, C and E). In active RA patients that achieved remission, RANKL levels were markedly reduced in synovial tissues (Figure 10G).

While RANKL protein was expressed at high levels in the synovium from active RA patients at the initiation of treatment (Figure 10), the expression of OPG protein was reduced or absent (Figure 10B). Levels of OPG protein were increased in the synovial tissues from RA patients with treatment and were highest when the affected joints were in remission (Figures 10F and H). Future analysis will be carried out on OPG and RANKL staining in these patients using digital image analysis and SQA. OPG and RANKL levels will be compared levels in serial samples taken at different stages of disease activity to give more meaningful results. In order to determine whether the rise or fall in RANKL or OPG expression correlates with disease activity, the results from this study will be correlated to signs of bone erosion on x-rays of the joint from which the biopsies were taken.

3.3.7 Detection of OPG on Cultured Human Endothelial (HUVE) Cells

HUVE cells are an accepted model system for studying endothelial cells. However, it must be noted that to what extent they reflect endothelial cells in RA tissues is not known. To verify the production of OPG protein in the endothelial cell population, immunohistochemical staining was used to detect OPG protein on cultured HUVE cells. Immunohistochemical staining was carried out using the two Mabs to OPG to verify the form of OPG produced by these cells. OPG was detected strongly on endothelial cells with the Mab 805 (Figure11A) but barely detected or absent with Mab 8051 (Figure 11C), consistent with the expression in the human tissues. In addition, RANKL Mab 626 did not detect RANKL on either the treated or untreated HUVE cells with similar results to the negative control (Figure 11D).

It is likely that TNF and other inflammatory cytokines in RA tissue have an effect on OPG and RANKL expression. Therefore, the effect of recombinant hTNF-α, on OPG release by HUVE cells was also investigated.

Surprisingly, the amount of OPG protein detected on the surface of HUVE cells (by Mab 805) was reduced following 24 hours treatment with TNF-α (Figure 11B). Whereas, OPG released into the supernatant, as detected in ELISA assays, was increased (personal communication with Christopher Holding). Recent *in vitro* studies have shown TNF-α to be a stimulator of OPG mRNA and protein levels in human osteoblastic cells (Brandstrom et al., 1998a, Hofbauer et al., 1998). It is therefore possible that TNF increases the production of OPG but that OPG is then released by the endothelial cells. This is under further investigation by our group.

3.4 DISCUSSION

Osteoclasts are responsible for the resorption of bone during normal bone metabolism as well as the resorption of bone seen in pathologies such as RA. Resorption of bone occurs at the cartilage-pannus junction in RA. This tissue contains many chronic inflammatory cells including macrophages, lymphocytes and plasma cells. These cells may contribute to osteolysis by releasing a variety of cytokines that stimulate osteoclast formation and bone resorption. Cytokines in the soft tissue and fluid of the RA joint include IL-1α and β, IL-6, IL-11, IL-17, and TNF-α (Deleuran et al., 1992, Chu et al., 1992, Kotake et al., 1996, Kotake et al., 1999). In addition, inflammatory chemokines produced in the inflamed joint may also contribute to osteoclast formation by upregulating cytokine production as well as recruiting osteoclast precursors (Zheng et al., 1998, Nanki et al., 2001).

Previous studies have shown that TRAP-positive multinuclear cells isolated from rheumatoid can resorb bone *ex vivo* (Fujikawa et al., 1996c). This indicates that pre-osteoclasts, as well as mature osteoclasts, may be present in the soft tissues of rheumatoid joints. In this study TRAP was also used as a marker of osteoclasts. TRAP mRNA was expressed by the total cell population isolated from the majority of the RA samples. Consistent with this, large numbers of TRAP positive cells were among the adherent cells cultured from the synovial lining layer of active RA. In addition, immunohistochemical staining in RA synovial tissue identified high numbers of TRAP positive cells *in situ*, which is consistent with previous reports (Suzuki et al., 2001). These studies corroborate an earlier study in which TRAP mRNA was detected in the multinucleated and mononuclear cells of invading inflammatory tissues using *in situ* hybridisation (Gravallese et al., 2000). These results support the contention that osteoclast precursors and mature osteoclasts are present in the pannus and synovium of RA joints.

It was interesting to note that in this study TRAP mRNA expression did not always correlate with the formation of resorption pits by cells isolated from the same samples. This is consistent with the results in Chapter 2 and suggests that such cells may not be fully differentiated osteoclasts or may be inactive osteoclasts. This is not surprising since results from previous studies have suggested that TRAP may not be a definitive marker for mature osteoclasts (Hattersley and Chambers, 1989a). It has been suggested that TRAP positive cells, that are away from the bone and therefore not located in Howship's lacunae, should be designated "osteoclast-like cells" or "osteoclast precursors" (Chun et al., 1999). Therefore, the expression of additional markers was investigated to further support evidence of pre-osteoclasts and mature osteoclasts in the soft tissue.

The interaction between RANKL and its receptor, RANK has been identified in many studies as pivotal in osteoclast formation (Yasuda et al., 1998b, Nakagawa et al., 1998, Hsu et al., 1999). RANK, is present on both pre-osteoclasts and osteoclasts and mediates osteoclast differentiation and activation stimulated by RANKL (Nakagawa et al., 1998, Burgess et al., 1999, Hsu et al., 1999, Myers et al., 1999). The expression of RANK mRNA in the total cell population isolated from RA synovial tissue further supports the notion that precursors or mature osteoclasts may be present in the soft tissue of the RA joint adjacent to osteolytic zones. *In situ* hybridisation identified mononuclear cells within inflammatory infiltrates as positive for RANK expression. Although dual labelling was not carried out with the RA tissues, results from the *in vitro* studies in Chapter 2 suggest that many of the cells expressing RANK mRNA may be of the monocyte/macrophage lineage. This is consistent with the concept that osteoclasts differentiate from cells of the monocytes/macrophage lineage under appropriate conditions *in vivo* (Fujikawa et al., 1996a, Quinn et al., 1998c, Fujikawa et al., 1996b).

Although the expression of TRAP or RANK may suggest the presence of pre-osteoclasts as well as osteoclasts in RA synovium, CTR expression may be a more definitive indicator of mature osteoclasts (Hattersley and Chambers, 1989a). It may, therefore, be important to note that CTR mRNA was expressed in all of the pannus samples but expressed in only one of the synovial membrane samples, which is consistent with the erosive effect pannus has on bone *in vivo*. In addition, CTR was consistently expressed in rheumatoid tissues from which mature osteoclasts readily formed. This is in accordance with the notion that CTR expression is associated with the later stages of osteoclast differentiation. It is noteworthy that the rapid rise and reduction in serum calcium that has been reported following administration of RANKL or OPG respectively suggests these may also be mature osteoclasts that have not been activated (Akatsu et al., 1998b, Lacey et al., 1998). Dual labelling with antibodies to CTR and TRAP or staining in sequential sections would be required to confirm the observation that CTR expression is more specific to mature osteoclasts in this pathology but at the time of this study antibodies to human CTR were not widely available.

It is likely that cytokines in the soft tissue and fluid such as TNF-α, IL-1α, IL-6 and PGE_2 can effect osteoclast formation indirectly by regulating newly identified mediators of osteoclast formation, RANKL, RANK and OPG (Horwood et al., 1998, Nakashima et al., 2000, Hofbauer et al., 1999, Wani et al., 1999, Collin-Osdoby et al., 2001). The interaction between RANKL and RANK may be inhibited by OPG, which acts as a soluble decoy receptor by binding to RANKL and thereby inhibiting osteoclast differentiation and activation (Lacey et

al., 1998, Simonet et al., 1997). It is significant that RANKL and OPG mRNA expression were observed in the cells isolated from RA pannus and synovium. These results are supported by recent reports showing the expression of these mediators in tissues from human rheumatoid joints (Gravallese et al., 2000, Takayanagi et al., 2000) and in animal models (Kong et al., 1999a, Romas et al., 2000, Mori et al., 2002). This suggests that RANKL and OPG may be key factors involved in regulation of osteoclast formation in RA tissue adjacent to sites of osteolysis.

M-CSF has also been identified as an essential co-factor for the induction of osteoclast differentiation by RANKL, and is a limiting factor in human osteoclastogenesis in culture (Lacey et al., 1998). M-CSF has also been shown to induce RANK mRNA expression in monocytes in culture (Arai et al., 1999). Therefore, although M-CSF mRNA was observed to be abundant in cells digested from RA tissues, M-CSF was included in all the cell cultures, consistent with other studies (Fujikawa et al., 1996b, Itonaga et al., 2000b, Takayanagi et al., 2000). This enabled the investigation of the activities of RANKL independent of a requirement for M-CSF.

RANKL is essential for osteoclast formation in bone and is normally provided by osteoblast-like cells (Yasuda et al., 1998b, Lacey et al., 1998). Previous reports have found that osteoblast-like cells (Fujikawa et al., 1996b, Fujikawa et al., 1996a, Itonaga et al., 2000b) or RANKL (Itonaga et al., 2000b) are required for RA synovial macrophage and blood-derived monocytes to differentiate into bone resorbing osteoclasts. However, it is important to note that in this study, functional osteoclasts, capable of resorbing bone, were generated in culture even in the absence of osteoblast-like cells and without the addition of RANKL. In addition, the levels of RANKL mRNA were high in the tissues from which osteoclasts readily formed. The reason for the difference in these findings may be due to the fact that this study used cells isolated from tissue adjacent to bone, corresponding to the invading pannus, whereas the previous studies (Fujikawa et al., 1996b, Itonaga et al., 2000b) used monocytes isolated from the synovial tissue of RA patients, that was not closely associated with bone.

As discussed below, the expression of RANKL mRNA in these rheumatoid samples in the present study supports earlier reports that RANKL may be produced by other cell types, such as synovial fibroblasts (Gravallese et al., 2000, Takayanagi et al., 2000) or lymphocytes present in the rheumatoid tissues (Horwood et al., 1999, Gravallese et al., 2000, Kotake et al., 2001). This suggests that pre-osteoclasts in the RA tissue may not need to be in contact with the bone to become fully mature osteoclasts. In addition, the levels of other cytokines present

in the tissues from different parts of the joint might alter the sensitivity to given levels of RANKL (Fuller et al., 2002).

The need for RANKL to induce osteoclast formation in this disease is underlined by the fact that OPG inhibition of RANKL can completely prevent cartilage and bone loss in an arthritic animal model (Kong et al., 1999a). Furthermore, the differentiation of cells isolated from RA tissue into functional osteoclasts has been shown to be RANKL dependent as demonstrated by its dose-dependent inhibition by OPG (Itonaga et al., 2000b). These studies suggest a major role for RANKL in the resorption of bone, which is balanced by the production of its inhibitor, OPG. The present study provides evidence for the significance of RANKL mRNA expression in determining osteoclast formation in the tissue adjacent to bone loss in human RA joints. Central to this was the finding that there was a strong correlation between the ratio of RANKL:OPG mRNA, in the total cell population isolated from these tissues, and osteoclast formation *ex vivo*, by the adherent cell population isolated from the same samples. Therefore, these findings support the concept that the relative levels of RANKL and its inhibitor, OPG, are key factors in determining the level of osteoclastic bone resorption (Horwood et al., 1998, Nagai and Sato, 1999, Hofbauer et al., 2000, Kotake et al., 2001).

In addition to osteoclasts markers, RANKL and OPG, TRAIL mRNA expression was also investigated in cells isolated from synovial and pannus RA tissue. TRAIL is a TNF-α-related molecule that induces apoptosis (Wiley et al., 1995, Pitti et al., 1996, Walczak and Krammer, 2000). However, TRAIL has also been shown to bind OPG, and is able to suppress the inhibitory action of OPG in osteoclast formation (Emery et al., 1998). It is important to note that TRAIL mRNA was expressed consistently by the cells isolated from RA tissues adjacent to sites of bone erosion. TRAIL expression in the RA joint may represent an additional pro-osteoclastic influence. TRAIL may have other important activities in RA besides interacting with OPG. This is demonstrated in a recent report showing that TRAIL may suppress lymphocyte proliferation and have anti-inflammatory activity in an animal model of arthritis (Song et al., 2000). The findings presented here support the need for further studies in humans to determine whether TRAIL produced in the RA joint is involved in regulating both bone loss and inflammation in this pathology.

The results of RNA analysis were strongly supported by immunohistochemical studies used to investigate RANKL and OPG protein in the RA tissue adjacent to osteolytic zones. Since it is likely that a number of cell types present in the soft tissue and bone matrix are capable of

producing these factors, dual labelling was also carried out to identify the cells associated with OPG and RANKL production.

The immunohistochemical findings provided additional evidence that RANKL is involved in the pathogenesis of RA. RANKL protein was detected in aggregates of mononuclear cells and fibroblast-like cells in the regions below the synovial lining of the synovial membrane. RANKL protein was not detected on blood vessels, nor was it present in the lining of the synovial membrane. Although the levels varied, RANKL was most abundant in the synovial tissue from RA and spondyloarthropathy patients with active synovitis. It is also important to note that immunohistochemical staining demonstrated that RANKL protein is absent from or present in very low levels in the synovial tissue lining from inactive RA, OA and normal patients. In contrast to these results, Gravellese *et al* found that tissue from normal or OA patients did not express any RANKL mRNA (Gravallese et al., 2000). However, results from the present study were in agreement with observations of Kotake *et al* (Kotake et al., 2001), which showed that RANKL was expressed by some of the cells in the synovial tissue from OA patients.

Previous immunohistochemical studies have shown that cell aggregates in RA tissue vary in size and contain different proportions of T and B lymphocytes, as well as macrophages (Yanni et al., 1992). RANKL mRNA expression has been shown in a subset of $CD3^+/CD4^+$ T cells within the lymphoid follicle-like cell aggregates (Horwood et al., 1999, Kotake et al., 2001). Previous studies have also shown that activated T cells derived from RA joints express RANKL mRNA (Gravallese et al., 2000) and human activated T cells support differentiation of adherent murine spleen cells (Horwood et al., 1999) and human monocytes in co-culture (Kotake et al., 2001). These findings make it likely that infiltrating T lymphocytes support osteoclast differentiation from monocyte/macrophages present in the inflamed RA joint.

Dual labelling in this study verified that a subset of CD3-positive T lymphocytes was associated with RANKL protein in human RA joints. In addition, this study was the first to analyse the production of RANKL protein by activated, memory T cells (CD45Ro positive cells) in human RA. CD45Ro positive cells were present in synovial samples from each of the patient groups but were more abundant in the lymphoid aggregates in the synovium from the active RA and spondyloarthropathy patients. This is in consistent with the findings of Sakkas *et al* (Sakkas et al., 1998) who showed that there were higher numbers of CD45Ro positive cells in the synovial tissues of RA patients than in OA patients. A recent study

showed that the high number of CD45Ro and CD68 cells in active rheumatoid arthritis patients was reduced in patients who were assessed as in clinical remission (Smith et al., 2001). In the present study, sequential staining showed that RANKL coincided with many CD45Ro positive cells in the active RA joint. These findings suggest that RANKL can be produced by activated, memory T lymphocytes in human RA.

The role of B cells in the inflammatory process and bone lysis in RA remains unclear. Based on results in an animal model of inflammatory arthritis it has been suggested that B lymphoid lineage cells may be able to support osteoclastogenesis through the production of RANKL (Manabe et al., 2001). However, in this study detection of RANKL protein in the synovium from active RA patients was not found on CD22 positive B cells. It is also interesting to note that recent report suggests that B cells may be able to become osteoclasts (Grcevic et al., 2001) but this was not noted here. Therefore the results obtained here with patient synovial tissue do not suggest a role for B cells in the production of RANKL or support the possibility that B cells may develop into osteoclasts in RA in humans.

The current consensus is that monocytes and macrophages are not a major source of RANKL (Hakeda et al., 1998). However, in this study many cells expressing the macrophage marker CD68 expressed RANKL protein. Without *in situ* hybridisation it was not possible to verify whether these cells were the source of RANKL or merely binding to RANKL that was produced by other cells. However, evidence that these cells can produce RANKL was presented in Chapter 2, showing that human adherent peripheral blood mononuclear cells (PBMC) in culture can express RANKL mRNA when stimulated by prosthetic wear debris. Furthermore, Horwood *et al* (1999) noted macrophage-like cells expressing RANKL mRNA in RA tissue (Horwood et al., 1999).

Previous *in vitro* studies have shown that synovial fibroblasts from RA patients, stimulated with 1,25 dihydroxy vitamin D_3 are able to support osteoclast formation via RANKL expression (Takayanagi et al., 2000). Kotake *et* al noted occasional fibroblast-like cells expressing RANKL protein (Kotake et al., 2001). However, in the study described here cells expressing the fibroblastic cell marker, CD55, did not express RANKL protein. While it is possible that CD55 is not expressed on all fibroblastic cells, this study supports the contention that activated T lymphocytes, and not fibroblasts, are the major cell type that supports osteoclast formation by expressing RANKL in the synovial tissue of patients with active RA. The possibility that fibroblast produce RANKL in tissues adjacent to localised bone loss was discussed in more detail in Chapter 2.

OPG protein production was also investigated in RA and other joint tissues using immunohistochemical staining. Abundant OPG protein was seen in the synovial tissue lining of normal joints. The pattern of staining for OPG was similar to that observed and discussed in detail in Chapter 2. There were different staining patterns for OPG when detected within the joint using the two antibodies, one of which detected OPG predominantly found in macrophages in the synovial lining and weakly in endothelial cells (as detected with Mab 8051), while the other detected OPG exclusively associated with blood vessels (as detected with Mab 805). As discussed in detail in Chapter 2 it is possible that the different binding patterns may be due to different molecular forms of OPG being recognised by the two monoclonal antibodies.

This study demonstrated that OPG is expressed in blood vessels in the synovial tissue of normal subjects and in both inflammatory and degenerative arthritides, but appeared reduced in tissues from the active RA patients. OPG was also detected (both vascular and synovial lining) in the synovial membrane of RA patients with no active synovitis at the time of synovial biopsy. This is in accordance with the decreased OPG levels seen in RA synovial fluid compared with OA as measured in ELISA assays (Kotake et al., 2001).

Dual labelling verified the types of cells associated with the forms of OPG detected with the two different antibodies. Consistent with the results shown in Chapter 2, dual labelling with the monoclonal antibodies demonstrated that OPG detected with Mab 805 was associated with Factor VIII positive endothelial cells, while OPG detected with Mab 8051 was predominantly associated with CD68 positive cells in the synovial lining. As discussed in detail in the previous chapter, it is possible that the different binding patterns may be due to different molecular forms of OPG being recognised by the two monoclonal antibodies.

The RT PCR and *ex vivo* studies support the contention that the levels of RANKL and OPG mRNA are likely to be important in determining the osteoclastic potential of cells residing in and recruited to the soft tissues of RA joints. To investigate this further, the levels of RANKL and OPG protein detected in human active RA synovial tissue, using immunohistochemical staining, were compared with those seen in other arthritides and normal tissues. Analysis of staining was carried out using semi-quantitative assessment (SQA) and quantitative (digital image analysis) measurement techniques.

The observation that RANKL staining is higher in active RA was supported by statistical assessment of the IOD analyses of RANKL. This study found staining to be significantly higher in tissue from active RA patients than in tissue from inactive RA, OA and normal patients. Surprisingly, statistical analysis of the SQA did not show that RANKL was significantly higher in active RA or spondyloarthropathy tissues compared with the control tissues. However, it is important to note that SQA grades were designated according to the proportion of positive cells in the tissues, and not the absolute amount of RANKL protein or number of positive cells in each tissue. Therefore it is likely that the amount of RANKL detected in the active RA patients is underestimated using SQA values when compared to the IOD values. This is supported by previous immunostaining analysis studies where an IOD measurement by digital image analysis was shown to be more sensitive to change than SQA (Kraan et al., 2000).

In addition to these findings, comparison of the IOD values for RANKL between the active RA and spondyloarthropathy groups did not show a statistically significant difference. However, it must be noted that the values were consistently lower in the spondyloarthropathy group than the active RA group.

Semi-quantitative and quantitative analysis of OPG detected with both Mab 805 and 8051 supported observations that there was a notable reduction in OPG in tissue from the RA patients with active synovitis at the time of synovial biopsy. Statistical analysis of SQA found OPG detected with both antibodies to be significantly reduced in active RA tissues compared with samples from all other groups. However, it must be noted that the SQA reflects the proportion of positive staining vessels and not the amount of staining per vessel. Analysis of MOD more accurately reflects the amount of staining per vessel and statistical assessment of this parameter supported observations that the OPG produced per vessel was significantly reduced in the disease state. The IOD values of OPG when detected with 8051 were also significantly reduced in the active RA tissues compared with tissues from all other groups. This was also the case when IOD values of OPG detected on the blood vessels with Mab 805 were compared with tissues in the inactive RA and spondyloarthropathy group. Overall, the findings support the contention that RANKL is present in the inflamed tissues adjacent to sites of bone loss, while OPG is reduced. The levels of these mediators may therefore have an important role in stimulating osteoclast formation and activity in RA joints where bone loss occurs.

A unique aspect of this work was the ability to study synovial specimens from patients at different stages of disease during treatment. The results showed a reduction in RANKL protein and a concurrent increase in OPG in several of the patients being followed through treatment. This suggests that successful treatment of RA may correspond with an increase in OPG and a decrease in RANKL protein. This may have major implications for the progression of bone erosion in this disease. Future work will involve comparison of the levels of RANKL and OPG protein with radiological outcome measures in hand and feet X-rays to determine any correlation**.**

Consistent with these studies showing the production of OPG in human vasculature, OPG knockout mice have been shown to develop arterial calcification (Bucay et al., 1998, Min et al., 2000), in addition to severe osteoporosis. This suggests that vascular endothelial expression of OPG may have a role in the homeostasis of bone and blood vessels (Min et al., 2000). This may mean that OPG may have a role in regulating levels of extracellular matrix calcification in blood vessels (Schinke et al., 1999) and raises the possibility that alterations in OPG expression on endothelial cells in RA patients may play a role in the excess cardiovascular morbidity and mortality in RA (Raynauld et al., 1993).

OPG expression by vascular endothelial cells has been described recently and shown to be upregulated by inflammatory cytokines IL-1β and TNF-α (Collin-Osdoby et al., 2001). If this is also the case *in vivo* then we might expect that OPG protein expression would increase during active RA where elevated levels of these cytokines are reported. However, raised levels of OPG were not observed in the present study, indicating that other mechanisms regulate the levels of OPG protein in RA tissue. It may also be possible that following chronic exposure to inflammatory cytokines the OPG protein may have all been secreted into the serum or formed complexes with RANKL or TRAIL. Low levels of OPG in synovial fluid from RA patients, compared with OA, trauma and gout patients has been reported in a recent paper (Kotake et al., 2001), which supports the results found in synovial tissue in this study.

TNF is an important inflammatory cytokine that is produced in abundant amounts in inflamed tissue such as adjacent to prosthetic joint implants and in RA joints (Maini et al., 1993, Xu et al., 1996, Jiranek et al., 1993, Appel et al., 1990, Chiba et al., 1994, Perry et al., 1997). Current treatments for RA involve using antagonists to TNF (Maini et al., 1993, Moreland et al., 1997). TNF-α has been shown to upregulate RANKL expression (Nakashima et al., 2000) in culture. In addition, TNF-α may induce osteoclastogenesis by direct stimulation of

macrophages exposed to low levels of RANKL normally too low to stimulate osteoclast formation alone (Lam et al., 2000). It has been suggested that TNF-α may have an additional major role in regulating osteoclast formation, through the TNF receptor type I (Abu-Amer et al., 2000, Zhang et al., 2000). TNF may also activate osteoclasts directly, independent of RANKL (Fuller et al., 2002). Collin-Osdoby *et al* (1998) also detected an increase in RANKL protein and mRNA in endothelial cells in response to TNF. However, in this study RANKL protein was not detected on endothelial cells with immunohistochemical staining.

In this study the effects of TNF on the levels of OPG protein associated with endothelial cells was investigated and OPG was found to reduce following the addition of TNF *in vitro*. However, the results of ELISAs carried out on supernatants from these cultures showed an increase in OPG protein release into the media. Recent *in vitro* studies have also shown that TNF stimulates OPG mRNA and protein expression in both human osteoblastic cells (Brandstrom et al., 1998a, Hofbauer et al., 1998) and OPG in endothelial cells (Collin-Osdoby et al., 2001). It should be noted that expression of OPG mRNA in TNF stimulated endothelial cells was found to be rapid and transient (Collin-Osdoby et al., 2001). This may explain why in this study the levels of OPG detected in the supernatants were higher following TNF treatment but were lower when detected with immunohistochemistry. The OPG may have been rapidly released from the endothelial cells and may then be dispersed into the serum. OPG production in HUVE cells needs to be further studied to understand its relationship to OPG produced by blood vessels *in vivo*.

This study supports the contention that RANKL and OPG play an important role in osteoclast formation in the tissue adjacent to sites of pathological bone loss in RA joints. Immunohistochemical staining and RT PCR showed expression of these mediators and osteoclast markers in RA tissue. Significantly, the ratio of RANKL mRNA expression to its inhibitor, OPG, in the RA tissues correlated with the formation of functional osteoclasts. Using dual labelling, OPG protein was detected on endothelial cells and type A synovial-lining cells, while RANKL protein detected on a subset of T cells and some macrophages. The reduction of OPG protein in active RA synovial tissue and the significantly higher levels of RANKL protein in the same tissue further demonstrated that these molecules are likely to be key regulators of bone loss in active RA. This is important as it may correspond to the disease state and erosion. The relative levels of both RANKL and OPG are likely to be important in determining the levels of bone loss in health and disease.

It is possible that bone lysis, and the cytokines that cause bone lysis, also contribute to the progression of inflammation in RA. Using the rodent model of adjuvant arthritis Kong *et al* (Kong et al., 1999a) demonstrated that inhibition of RANKL by OPG treatment *in vivo* reduced joint destruction in a model of adjuvant arthritis in rats (Kong et al., 1999a). However, although treatment with OPG reduced both cartilage and bone destruction it did not reduce inflammation (Kong et al., 1999a). This has yet to be demonstrated for human RA. However, in the light of our results here, the separate consideration of treatment for inflammation and for bone loss may result in better treatments for these two components of human RA.

Bone loss is a major cause of the loss of function of the human RA joint and inhibiting this bone loss would be of great assistance to patients with rheumatoid arthritis. Alone, or in combination with anti-inflammatory therapies, these treatments to inhibit osteoclast formation and activity may help maintain joint function (Joosten et al., 1999, Lubberts et al., 2000). The results presented here further support the exciting possibility that OPG, or a structural mimetic of OPG, might be useful in maintaining joint function for sufferers of rheumatoid arthritis.

4 PERIODONTAL DISEASE

4.1 INTRODUCTION

Periodontitis is one of the most prevalent human diseases associated with the destruction of alveolar bone and is a major cause of tooth loss in humans. Gingivitis is a non-specific inflammatory reaction of the gingival tissues that localises to the more superficial gingival tissues. Both diseases are characterised by an inflammatory reaction in the gingival tissues and can occur concurrently, however, only periodontitis is associated with alveolar bone destruction, whereas gingivitis is localised to the soft tissue (Jeffcoat, 1994, Schwartz et al., 2000).

The progression from gingivitis to periodontitis has been suggested to be associated with the movement of an inflammatory infiltrate towards alveolar bone (Graves et al., 1998). The inflammation and tissue destruction seen in periodontitis is associated with granulomatous tissue containing inflammatory cells, including T and B lymphocytes, plasma cells and cells of the monocyte/macrophage lineage. These cells are thought to produce a variety of inflammatory mediators and high levels of several inflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, prostaglandin (PG)E_2 and tumour necrosis factor (TNF)-α have been found in the tissue and gingival crevicular fluid of patients suffering advanced periodontitis (Page and Schroeder, 1976, Moskow and Polson, 1991, Iwasaki et al., 1998, Graves et al., 1998, Schenkein, 1999). Similar findings have been reported in animal models of periodontitis (Assuma et al., 1998, Masada et al., 1990, Stashenko et al., 1991, Ishihara et al., 1997, Rasmussen et al., 2000, Bickel et al., 2001).

There is a correlation between the severity of inflammation in the periodontal tissue and the percentage of alveolar bone loss in corresponding areas (Kardachi et al., 1979). However, little is known about the process of alveolar bone loss that occurs in periodontitis. Gingival crevicular fluid is reported to stimulate bone resorption *in vitro* (Lerner et al., 1998). This indicates that the cytokines that promote osteoclast formation are present in this disease (Chu et al., 1991, Chu et al., 1992, Deleuran et al., 1992, Suda et al., 1995, Kotake et al., 1996, Kobayashi et al., 2000a, Schwartz et al., 2000). Inflammatory chemokines may also contribute to osteoclast formation by attracting monocyte and macrophages, which are osteoclast precursors, to the area (Fujikawa et al., 1996b, Quinn et al., 1998c).

Inflammatory cytokines, such as those present in crevicular fluids of patients with periodontitis, are reported to stimulate production of receptor activator of nuclear factor κB ligand (RANKL), a mediator of osteoclastogenesis (Hofbauer et al., 1999, Nakashima et al.,

2000). The importance of RANKL and its receptor, RANK, in regulating osteoclast formation (Lacey et al., 1998, Yasuda et al., 1998b, Nakagawa et al., 1998, Hsu et al., 1999, Burgess et al., 1999, Kong et al., 1999b, Gravallese et al., 2001) and the immune system (Wong et al., 1997a) has been discussed in previous chapters of this thesis. However, the role of RANKL and RANK has not been reported in human periodontitis.

Osteoprotegerin (OPG), the naturally occurring inhibitor of osteoclast differentiation (Lacey et al., 1998, Simonet et al., 1997, Yasuda et al., 1998a) is reported to be produced by human periodontal ligament cells, gingival fibroblasts and epithelial cells (Sakata et al., 1999). Like RANKL, OPG is modulated by inflammatory cytokines present in periodontitis (Brandstrom et al., 1998a, Hofbauer et al., 1999). Since OPG and RANKL are believed to be key factors regulating bone metabolism, it is likely that they are key factors regulating alveolar bone destruction in periodontitis.

TRAIL (TNF-related apoptosis-inducing ligand) is a member of the TNF family that is also able to bind to OPG (Pitti et al., 1996, Emery et al., 1998, Walczak and Krammer, 2000) and may regulate osteolysis by preventing OPG from interacting with RANKL. The presence of TRAIL in soft tissue adjacent to areas of osteolysis may modulate the availability of OPG and therefore affect the ratio of RANKL and OPG in the periodontal tissue.

There have been few reports investigating key mediators of bone resorption in periodontitis in humans. This chapter investigates the hypothesis that the bone destruction seen in periodontitis is associated with elevated levels of RANKL and/or lower levels of OPG protein in the tissues adjacent to active bone resorption. The expression of mRNA for key mediators of osteoclast formation and osteoclastic markers was determined in periodontitis tissues from sites adjacent to alveolar bone loss and compared to that seen in non-periodontitis (normal/mildly inflamed gingival) tissues obtained from patients without bone loss. RANKL and OPG protein was also detected with immunohistochemical staining in the control and diseased tissue. The levels of RANKL and OPG protein in periodontitis tissues and non-periodontitis tissues were assessed semi-quantitatively and compared. Dual labelling was carried out to determine the cell lineages associated with OPG and RANKL protein production *in situ*.

RANK, TRAP and CTR mRNA expression was investigated using RT PCR to identify the presence of osteoclastic cells in periodontitis and non-periodontitis tissues. In addition, the TRAP enzyme and RANK mRNA was detected in periodontitis tissue sections using

immunostaining and *in situ* hybridisation techniques. This study identifies osteoclast precursors in inflamed periodontal tissues and contributes to understanding the underlying mechanisms of osteoclast differentiation in human alveolar bone loss, a common bone pathology, which may lead to tooth loss.

4.2 METHODS

4.2.1 Tissue Processing for RT PCR, Immunohistochemistry and *In situ* Hybridisation

The non-periodontitis tissue samples consisted of gingival tissue resected from patients undergoing crown-lengthening or canine exposures and not affected by periodontitis. Microscopic analysis showed that the non-periodontitis tissue samples occasionally contained areas of mild inflammation consistent with low-grade chronic gingivitis (Figure 1A and D). Tissue samples, taken from patients with severe chronic localized periodontitis, consisted of granulation tissue (Figure 1B and E) that was removed from bony defects (as shown in Figure 1C) during periodontal flap surgery carried out for the treatment of the lesions. The tissues used in this study would usually have been discarded after surgery. This study was approved by the University of Adelaide Ethics Committee, in accordance with the guidelines of the National Health and Medical Research Council of Australia.

Tissue samples were either fixed in 10% buffered formalin for paraffin embedding or snap-frozen in Tissue-Tek OCT (Miles Diagnostics, Elkhart, IN, U.S.A) within 1 hour of surgery. Where possible, a portion of the tissue was processed to extract total RNA for the RT PCR studies.

Paraffin embedded blocks were stored at room temperature and frozen blocks stored at -80^{O}C until used. The first section cut (5μm) from each of the frozen and paraffin embedded blocks was stained with haematoxylin and eosin for routine histological assessment. Sections with good morphology were included in the study. Five-micrometer sections were mounted on APTS (Sigma, St Louis, MO) coated glass slides. The sections cut from frozen blocks were fixed for 4 minutes in cold acetone and dried at room temperature. The glass slides were boxed and stored at -20^{o}C until processed for immunohistochemical staining or *in situ* hybridisation.

4.2.2 RNA Extraction

Normal and diseased tissue samples were placed in RNAse free 1.5ml tubes and immersed in 500μl of Trizol (Gibco BRL, Life Technologies) reagent. The tissue was then homogenised in the reagent to lyse the cell population so that the total RNA could be extracted. The suspension was transferred to a fresh RNAse free 1.5ml tube. Total RNA was prepared according to the manufacturer's instructions. This is described in detail in the methods section of Chapter 2. Demographic details of the patients included in the RT PCR studies and their current medications at time of surgery are shown in Table 1.

Table 1. Demographic and Clinical Details of Patients and Tissue Samples Included in the RT PCR Experiments.

Periodontitis*	**Age (years)**	**Sex (M/F)**	**Erosions (yes/no)**	**Current medication**
1	48	M	Yes	none
2	38	M	Yes	none
3	48	M	Yes	none
4	50	F	Yes	none
5	53	F	Yes	HRT[1]
6	61	F	Yes	none
7	50	M	Yes	none
8	50	M	Yes	none
Non-periodontitis**	**Age (years)**	**Sex (M/F)**	**Erosions (yes/no)**	**Current medication**
9	14	F	No	none
10	41	M	No	none
11	45	F	No	none

* Markedly inflamed periodontal tissue adjacent to bone loss
** Normal/mildly inflamed gingival tissue
[1] Hormone Replacement Therapy

4.2.3 Preparation of RNA for the Reverse Transcription Polymerase Chain Reaction (RT PCR)

cDNA was synthesized using an AMV RT cDNA kit (Promega, Madison, WI, USA). cDNA was then amplified by PCR in a thermal cycler (Eppendorf, Hamburg, Germany), to generate products corresponding to mRNA encoding the gene products listed in Table 2 in the methods section of Chapter 3. Twenty-two cycles of PCR were performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 29-36 cycles for the other primer pairs**.** Primer sequences and predicted PCR product sizes are also listed in Table 2 in the methods section of Chapter 3.

Amplification products were resolved by electrophoresis on a 2% w/v DNA grade agarose gel and post-stained with SYBR Gold for 20 minutes (Molecular Probes, Eugene, OR, USA). The PCR products were scanned using a Molecular Imager Fx fluorescent scanner and the density of the bands was quantified using Quantity-One software (Bio Rad RAD, Hercules,

California, USA). Amplified products were expressed as a ratio of the respective GAPDH PCR product.

Preliminary RT PCR experiments were performed on several RNA samples extracted from the periodontitis tissues to ensure that the number of PCR cycles was within the exponential phase of the amplification curve. Methods for RT PCR are described in detail in the methods section of Chapter 2.

4.2.4 *In situ* Hybridisation

In situ hybridisation with digoxigenin (DIG)-labelled riboprobes was used to investigate the expression of RANK mRNA in periodontitis tissues from 6 patients. The method used to generate DIG-labelled riboprobes is described in detail in the methods section of Chapter 2**.** The method of *in situ* hybridisation detection was based on a previously published method (Smith et al., 1997) and is described in the methods section of Chapter 2.

4.2.5 Immunohistochemistry

Patients.

Clinical and demographic data for the patients, from which periodontitis and non-periodontitis tissue was used for immunohistochemical staining, are included in Table 2. For semi-quantitative assessment, tissues from 9 patients undergoing periodontal surgery were investigated along with tissues from 5 non-periodontitis patients**.** This study was approved by the University of Adelaide Ethics Committee, in accordance with the guidelines of the National Health and Medical Research Council of Australia.

Antibodies

Serial sections were stained with mouse monoclonal antibodies (Mab) from commercial sources with known antigen specificities and are listed in Table 4 of Chapter 3. To eliminate variability in immunohistochemical staining, all sections to be stained with a particular antibody were processed concurrently and incubated with the antibody for the same incubation period. Immunohistochemical staining was carried out using appropriate controls to study the antibodies. Inactive and active RA synovial tissues, with a previously defined staining pattern, were stained for the positive control. The negative controls consisted of omission of the primary antibody and substituting with an irrelevant Mab similar immunoglobulin isotype and subclass for the specific antibodies.

Table 2. Demographic and Clinical Details of Patients and Tissue Samples Included in the Immunohistochemical Studies

Periodontitis*	**Age (years)**	**Sex (M/F)**	**Erosions (yes/no)**	**Current medication**
1	53	F	Yes	None
2	48	M	Yes	Zantac, Retinol,
3	17	F	Yes	None
4	44	F	Yes	Zenecal,
5	32	M	Yes	None
6	64	F	Yes	Prednisolone, HRT[1]
7	65	F	Yes	Arthroid glucosamine phosphate, Ca^{++} mineral for OSP[2], NSAIDs[3]
8	42	M	Yes	None
9	42	M	Yes	None
Non-periodontitis**	**Age (years)**	**Sex (M/F)**	**Erosions (yes/no)**	**Current medication**
10	64	F	No	Prednisolone, HRT[1]
11	60	M	No	None
12		F	No	Avapro
13	65	F	No	Arthroid glucosamine phosphate, Ca^{++} mineral for OSP[2], NSAIDs[3]
14	59	M	No	None

* Markedly inflamed periodontal tissue adjacent to bone loss
** Normal/mildly inflamed gingival tissue
[1] Hormone Replacement Therapy
[2] Osteoporosis
[3] Non-steroidal anti-inflammatory drugs

4.2.6 Double Enhancement Aminoethylcarbazole (DE AEC) Immunohistochemistry

Immunohistochemical staining with a double enhancement method was carried out on sequential sections with an anti-RANKL antibody (Mab 626) as well as two OPG antibodies (Mab 805 and Mab 8051). In addition, several sections from each group were stained with anti-CD45Ro to detect activated, memory T cells.

Following a primary step of incubation with the Mabs to RANKL, OPG and CD45Ro, bound antibody was detected according to a three-step immunoperoxidase method (Kraan et al., 2000, Parker and Smith, 1999, Tak et al., 1995). Horseradish peroxidase (HRP) activity was detected using hydrogen peroxide as the substrate and aminoethylcarbazole (AEC) as the dye.

Slides were counterstained briefly with haematoxylin solution and mounted in Gurr Aquamount (BDH, Poole, UK). The method of immunohistochemical staining is described in detail in the methods section of Chapter 2.

4.2.7 Dual Immunohistochemical Labelling: DE AEC and Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP)

Dual immunohistochemical labelling was performed, as previously described (Parker and Smith, 1999, Wikaningrum et al., 1998). In brief, tissue was incubated first with the primary antibody (Mab RANKL 626, OPG 805 or OPG 8051) and subsequent steps in a standard three-step immunoperoxidase method were performed, developing the final colour product using AEC. Cell markers were detected with monoclonal antibodies using an alkaline phosphatase anti-alkaline phosphatase staining (APAAP) method, developed with Fast Blue.

APAAP

Between the first and second immunohistochemical reactions the tissue was washed and blocked with Tris-glycine to prevent cross-reaction between the first primary and the second linking antibody.

The second primary antibody: anti-CD68, anti-CD55 (dual labelled with RANKL Mab 626 and OPG Mab 8051), anti-Factor VIII (dual labelled with OPG Mab 805), anti-CD3 or anti-CD22 (dual labelled with RANKL Mab 626), was incubated with the sections overnight at 4^{o}C. A standard immunoalkaline phosphatase method was used to detect antibodies to the cell lineage markers (Tak et al., 1995, Parker and Smith, 1999), developing the colour reaction with Fast Blue. No counterstain was used and the sections were mounted in an aqueous mounting medium. The dual labelling method is described in detail in the methods section of Chapter 2.

4.2.8 TRAP Staining in Periodontitis Tissues

To identify osteoclastic cells in tissue sections, TRAP was detected in serial frozen sections from the same periodontitis and non-periodontitis patients included in the RANKL and OPG immunostaining studies. A commercial acid phosphatase leukocyte kit (Sigma-Aldrich, St Louis MO) was used and sections were counterstained with Methyl Green. Sections were then air-dried and mounted in DPX medium.

4.2.9 Quantitation of Immunohistochemical Staining

After immunohistochemical staining, sections dual labelled with Mab OPG 805 and anti-Factor VIII and sections stained with Mab RANKL 626 were scored in random order by two independent observers by semi-quantitative assessment (SQA) on a 5 point scale, as described previously (Tak et al., 1995, Kraan et al., 2000). The number of blood vessels dual labelled with the antibody to OPG and Factor VIII were counted and expressed as a percentage of all Factor VIII positive blood vessels. Digital image analysis of RANKL and OPG could not be carried out in the periodontitis tissues due to the small size of the sections.

4.2.10 Statistical Analysis.

A Mann-Whitney test for non-parametric data was used to analyse the mean ranks of the semi-quantitative scores for RANKL and OPG. The number of blood vessels detected with Mab 805, the number of Factor VIII positive blood vessels and the percentage of OPG positive vessels were normally distributed and were analysed with non-parametric and parametric tests. The number of OPG (Mab 805) blood positive vessels, Factor VIII positive blood vessels and the percentage of OPG positive blood vessels were analysed with a Mann Whitney non-parametric test as well as 2 tailed T-tests for independent samples. A p value of < 0.05 was considered significant.

4.3 RESULTS

4.3.1 mRNA Expression of Regulators of Osteoclast Formation and Osteoclast Markers in Cells Extracted from Periodontitis Tissue

PCR products were generated by RT PCR from total RNA extracted from cells isolated from periodontitis and non-periodontitis tissues sampled from 11 patients (listed in Table 1). Figure 2 shows that OPG mRNA was expressed by the total population of cells isolated from each of the periodontitis and non-periodontitis tissues. RANKL mRNA was expressed at varying levels in all of the periodontitis tissues and weakly in two of the three non-periodontitis tissues. TRAIL mRNA was strongly expressed in all of the samples tested.

RANK mRNA was expressed in 6 of the 8 periodontitis samples. Surprisingly, RANK mRNA was also expressed by the three non-periodontitis tissues. CTR was expressed in three of the diseased samples and none of the non-periodontitis tissues. This reflects the more localised expression of CTR by more differentiated pre-osteoclasts. Unfortunately antibodies to human CTR are not readily available so the production of CTR on the osteoclasts-like cells could not be verified.

4.3.2 Detection of TRAP and RANK mRNA in Osteoclast-Like Cells in Periodontitis Tissue Sections

Experiments were performed to detect RANK mRNA and TRAP activity in periodontitis and non-periodontitis tissues to identify pre-osteoclasts and osteoclasts *in situ*. Large numbers of cells expressing RANK mRNA were detected in the mononuclear cell aggregates in the periodontitis tissues using an antisense riboprobe (Figure 3A). The sense riboprobe was used as a negative control for specificity and did not stain any of the sections (Figure 3B).

Serial frozen tissue sections of the periodontitis and non-periodontitis samples were stained for the presence of TRAP. Mononuclear and some multinuclear TRAP positive cells were detected within the cell infiltrates in the periodontitis tissues (Figure 3C) while TRAP was absent in the non-periodontitis tissues (Figure 3D).

4.3.3 Detection of RANKL Protein in Periodontitis

Strong staining for RANKL protein was seen in leukocytes present in large mononuclear cell infiltrates in the granulation tissue from periodontitis lesions (Figures 4A, C and G). RANKL expression was low or absent in the non-periodontitis tissues (Figures 4B, D and H).

Activated, memory T cells (CD45Ro positive) were often abundant in some of the mononuclear cell aggregates within the periodontitis tissue (Figure 4E). Few CD45Ro positive cells were present in the non-periodontitis tissues (Figure 4F) and these did not produce RANKL (Figure 4D). Aggregates of RANKL positive cells (Figure 4C) corresponded with areas containing cells both positive and negative for CD45Ro (Figure 4E) in sequential tissue sections. It must also be noted that although many CD45Ro positive cells corresponded with cells positive RANKL, in many cases aggregates of CD45Ro positive cells corresponded with cells negative for RANKL staining in sequentially stained sections (Figures 4C and E).

Dual immunohistochemical labelling with the RANKL antibody was carried out in combination with cell lineage specific Mabs for pan T cells (CD3), B cells (CD22), macrophages (CD68) and fibroblasts of the synovial lining (FLS) (Mab 67). Many T lymphocytes (CD3 positive cells) scattered throughout the periodontitis tissues but the majority were seen in the large mononuclear cell aggregates. Approximately 30% of the cells expressing CD3 also expressed RANKL but many cells not expressing CD3 also expressed RANKL (Figure 4G). Fewer cells expressing CD3 were seen in the tissues obtained from non-periodontitis patients (Figure 4H).

Small numbers of B lymphocytes (CD22 positive cells) were seen in the mononuclear cell aggregates in the periodontitis and non-periodontitis tissues and these cells did not express RANKL (Figures 5A and B respectively). Dual staining experiments with antibodies directed against RANKL and CD68 showed that macrophages were present in the mononuclear cell aggregates in the periodontitis tissues but these were less abundant than the CD3 positive cells. Approximately half of these CD68 positive cells present also expressed RANKL (Figure 5C). Few cells expressing CD68 were seen in the non-periodontitis tissues (Figure 5D).

Although RANKL was present on some cells with a fibroblastic phenotype, these cells did not express the FLS marker, CD55 (Figures 5E and F) when dual labelled. The antibody to CD55 also detects blood vessels, which did not express RANKL in the non-periodontitis (Figure 5F) and periodontitis tissues.

4.3.4 Detection of OPG in Periodontitis Tissue

Staining for OPG using Mab 805 demonstrated that OPG was associated with cells lining blood vessels in both types of tissues. However, OPG staining in the blood vessels was much weaker in periodontitis tissues (Figure 6A) than in non-periodontitis tissues (Figure 6B). Antibodies directed against Factor VIII were used to verify that the cells expressing OPG in the blood vessels were endothelial cells. Only weak expression of OPG protein was detected on Factor VIII expressing cells in the periodontitis tissues (Figure 6C). However, OPG was strongly expressed by Factor VIII positive cells in blood vessels throughout the non-periodontitis tissues (Figure 6D).

OPG protein was not often detected using Mab 8051 in the periodontitis tissues (Figure 7A, C and E). However, in non-periodontitis tissues OPG protein was often associated with cells in the epithelial lining (Figure 7B). Blood vessels stained weakly when OPG was detected with a higher concentration of Mab 8051 in the non-periodontitis tissues. In some areas of the non-periodontitis tissue, OPG co-localised with CD68 positive cells of the monocyte/macrophage lineage (Figures 7D) but did not co-localise with CD55 positive cells (Figure 7F).

4.3.5 Statistical Analysis

Semi-quantitative scoring for RANKL reflected the proportion of positive cells in the periodontitis tissue. Results of the analysis of RANKL and OPG staining are in Table 3.

Table 3. Mean Semi-quantitative Scores for the Expression of RANKL and OPG Protein in Tissue from Periodontitis and Non-Periodontitis Patients.

	RANKL SQA*	**OPG 805 SQA***	**Number of OPG +ve BV***	**Number of F8 +ve BV***	**%OPG/F8***
P¤ n=9	3.33 (1.12)#	1.89 (1.05)§	33.11 (28.31)#	63.22 (19.29)#	49.59 (29.37)#
NP¤¤ n=5	1.80 (0.45)	4.00 (0.00)	80.20 (20.60)	87.80 (15.06)	90.79 (12.25)

P-Markedly inflamed periodontal tissue adjacent to bone loss
NP-Normal/mildly inflamed gingival tissue

SQA -Semi-Quantitative Assessment
BV- Blood Vessels
F8- Factor VIII

* The data represent: mean (SD)
# p< 0.05 compared to non-periodontitis tissues
§ p< 0.005 compared to non-periodontitis tissues

RANKL expression was found to be significantly higher in the periodontitis tissues than in non-periodontitis tissues (p =0.0227). Conversely, SQA scores for the proportion of Factor VIII positive blood vessels with any OPG protein were significantly greater in the non-periodontitis tissues compared with the periodontitis tissues (p=0.0048). The percentage of blood vessels (detected with Factor VIII antibodies) expressing OPG was also significantly more in the non-periodontitis tissues with both the Mann-Whitney (p=0.015) and the 2-tailed T-test (p=0.012).

4.4 DISCUSSION

The mechanism of osteoclast formation and bone resorption in human periodontitis is poorly understood. The infiltration of chronic inflammatory cells into the gingival tissues is likely to be a key step in the inflammatory process leading to alveolar bone lysis. Studies have shown that blood-derived monocyte can become functional osteoclasts when cultured under the right conditions, suggesting that when recruited to the tissue these cells have the potential to directly cause osteolysis by differentiating into osteoclasts (Fujikawa et al., 1996b, Quinn et al., 1998c). More mature cells of the monocyte/macrophage lineage isolated from the soft tissues of arthritic joints (Fujikawa et al., 1996b, Itonaga et al., 2000b, Suzuki et al., 2001) and peri-prosthetic tissue (Sabokbar et al., 1997, Itonaga et al., 2000a), are also capable of becoming bone resorbing osteoclasts. Based on these reports and the findings presented in the previous chapters of this thesis, it may also be possible that monocytes recruited to, and macrophages residing in, inflamed periodontal tissue become mature osteoclasts.

The finding that cells expressing TRAP were present in tissues from patients with periodontitis is consistent with a previous report (Rody et al., 2001). Rody *et al* observed TRAP positive cells in the periodontal ligaments of rats in response to application of force and it was suggested that these cells originated from fusion of pre-osteoclasts recruited from the bone marrow (Rody et al., 2001). In the study reported here, TRAP mRNA was expressed in cells isolated from both periodontitis and non-periodontitis tissues from human patients. However, when TRAP activity was detected in frozen sections only a select number of the periodontitis tissues, and none of the non-periodontitis tissues, contained cells positive for TRAP. Although TRAP mRNA may be expressed by pre-osteoclasts in periodontitis tissues these cells may require further stimulation to actively produce the TRAP enzyme. Due to the small size of the periodontitis samples, the tissue could only be used for either RT PCR or immunostaining experiments. TRAP mRNA expression could, therefore, not be correlated with TRAP staining *in situ* or the *ex vivo* studies investigating formation from cells as in other studies in this thesis.

RANK, the receptor for RANKL, is present on pre-osteoclasts and osteoclasts and mediates osteoclast differentiation and activation stimulated by RANKL (Hsu et al., 1999, Nakagawa et al., 1998, Burgess et al., 1999, Myers et al., 1999). The expression of RANK mRNA in the total cell population isolated from the majority of the periodontitis tissues was confirmed with *in situ* hybridisation. *In situ* hybridisation identified mononuclear cells present within inflammatory infiltrates as expressing RANK mRNA. Although dual labelling was not carried out, the morphology of the cells and results from *in vitro* studies in Chapter 2 suggest

these cells may be of the monocyte/macrophage lineage. These results suggest that there may be cells of the osteoclast lineage in the periodontitis tissues adjacent to alveolar bone.

As discussed in the previous chapters, CTR expression may be a more definitive marker of mature osteoclasts (Hattersley and Chambers, 1989a). In the present study, CTR mRNA was expressed by only three of the eight periodontitis tissues and was absent in all the non-periodontitis tissues. This supports the contention that the CTR is only present on more mature osteoclasts and is consistent with the results presented in Chapters 2 and 3. Overall, results from RT PCR, *in situ* hybridisation and immunohistochemical staining suggest that there are likely to be osteoclast precursors present within the periodontitis tissues and in several cases there may even be cells that have already differentiated into mature osteoclasts. However, cell culture studies similar to those carried out with the peri-prosthetic and RA tissues would need to be carried out to confirm the presence of pre-osteoclasts and mature osteoclasts in periodontitis tissues. Cell culture results would need to be compared to the RT PCR results to assess the significance of TRAP, RANK and CTR expression and osteoclasts formation in these tissues.

Recent animal experiments using a chimeric model of periodontitis support the view that RANKL and OPG are key factors regulating bone destruction in periodontitis (Teng et al., 2000). Using NOD/SCID mice seeded with human CD4+ T cells, it was demonstrated that bacteria associated with periodontal disease stimulated RANKL production by the CD4+ T cells and this correlated with an increase in osteoclast numbers in periodontal tissues of the recipient mice (Teng et al., 2000). The addition of OPG, the natural inhibitor of RANKL, was reported to inhibit alveolar bone destruction and reduce the numbers of osteoclasts adjacent to alveolar bone in this murine model of periodontitis (Teng et al., 2000). This is in accordance with the lack of tooth eruption that occurs in RANKL -/- mice where there is a lack of the functional osteoclasts required to resorb for eruption to occur (Kong et al., 1999b). More recently, a study in mice has also shown that OPG expression is reduced in the dental follicle (loose connective tissue sac surrounding unerrupted teeth) to enable osteoclast formation and activation during tooth eruption (Wise et al., 2002). In the present study it is important to note that RANKL mRNA and OPG mRNA were expressed by all of the tissue samples from human patients with periodontitis. The production of RANKL and OPG in the tissue adjacent to human alveolar bone may therefore be an important feature in tooth loss in both health and disease.

M-CSF is an important mediator required for osteoclast formation (Quinn et al., 1997a, Quinn et al., 1998a, Tsurukai et al., 2000), therefore the expression of M-CSF mRNA was also investigated. Consistent with results in peri-prosthetic and RA tissues (as discussed in Chapters 2 and 3), M-CSF was demonstrated in each of the periodontitis tissues. It is important that M-CSF has also been shown to induce RANK expression in monocytes (Arai et al., 1999) and prolong the survival of mature osteoclasts as well as stimulating their osteoclastic activity *in vitro* (Fuller et al., 1993). The expression of M-CSF in periodontitis tissue may therefore promote osteoclast formation and alveolar bone resorption.

TRAIL has not been investigated in either animal or human periodontitis tissues. As discussed in previous chapters, TRAIL is a TNF-like molecule that induces apoptosis (Wiley et al., 1995, Pitti et al., 1996, Walczak and Krammer, 2000). TRAIL is also able to bind OPG and suppress the inhibitory action of OPG in osteoclast formation (Emery et al., 1998) and may therefore play a role in bone regulation. This study demonstrated high levels of TRAIL mRNA expression in both periodontitis and non-periodontitis tissues. Along with elevated RANKL expression, an increase in TRAIL expression in periodontal tissues may induce the resorption of alveolar bone. The results from RT PCR suggest that the formation of osteoclasts in the periodontitis tissue may be influenced by expression of RANKL, OPG, M-CSF and TRAIL produced as part of the inflammatory response.

The expression of RANKL mRNA by periodontitis tissues suggests that there may be various cell types in the inflamed tissues adjacent to alveolar bone producing RANKL. In a previous study, human periodontal ligament cells were reported to express RANKL mRNA *in vitro* (Kanzaki et al., 2002). In the present study, immunohistochemical staining confirmed the RT PCR results and showed high levels of RANKL protein in the samples from periodontitis patients. It is important to note that RANKL protein was associated with aggregates of mononuclear leukocytes and fibroblast-like cells throughout the tissue but was not present on the blood vessels in these tissues.

Dual labelling and sequential staining was used to identify the cell types producing RANKL in the periodontal tissues. The cells associated with expression of RANKL in periodontitis tissues were similar to that seen in the active RA patients described in Chapter 3. RANKL protein was associated with a large proportion of pan T cells expressing CD3 in the periodontitis tissues. RANKL staining also corresponded with areas of CD45Ro positive, activated memory T lymphocytes in sequential sections of the periodontitis tissues. The observation that not all CD45Ro cells produce RANKL suggests that a subset of the CD45Ro

cells may not be able to produce RANKL or may be in a quiescent state. These results support previous studies carried out on tissues from patients with rheumatoid arthritis, which have shown that activated T cells in the rheumatoid joint express RANKL mRNA (Gravallese et al., 2000). Taken together, these findings indicate that T cells may have an important role in regulating bone lysis in a variety of pathologies. This is consistent with the fact that activated human PBMC-derived T cells can support differentiation of adherent murine spleen cells (Horwood et al., 1999) and human monocytes in co-culture (Kotake et al., 2001). This may mean that T lymphocytes regulate osteoclast formation in periodontitis in a manner similar to that seen in rheumatoid arthritis.

There are few reports that monocytes and macrophages produce RANKL. It is interesting to note that results in this study were similar to those presented in Chapter 2 in that periodontitis tissues contained cells expressing CD68, a marker for macrophages. About 50% of these cells also expressed RANKL. However, these macrophages may not necessarily be the source of RANKL since these macrophages may have soluble RANKL bound to RANK on their surface that is derived from other cells (Horwood et al., 1999). Findings from the *in vitro* studies, discussed in detail in Chapter 2, showed that human monocytes could be stimulated to express RANKL mRNA.

The role B cells have in the inflammatory process and bone lysis in periodontal disease is unclear. As discussed in previous chapters, B lymphoid cells may be able to support osteoclastogenesis through production of RANKL (Manabe et al., 2001). However, the results obtained with human periodontitis tissue show that B cells do not produce RANKL and indicate that B cells do not have a role in stimulating osteoclast formation in this disease. It is also interesting to note that recent report suggests that B cells may be able to become osteoclasts (Grcevic et al., 2001) but like the results shown in peri-prosthetic and RA tissues, this was not noted here.

This study is unique in that the levels of RANKL protein could be assessed in the periodontitis and non-periodontitis tissues by semi-quantitative analysis to determine any difference between diseased and non-periodontitis tissues. RANKL protein levels were markedly higher in the tissues from patients with periodontitis than the tissues from patients without periodontitis. This was confirmed by SQA, in which detection of RANKL was absent (graded as 0) or very low (graded as 1) in the non-periodontitis tissues. It is important to note that statistical analyses of the SQA verified that RANKL staining was significantly

higher in the periodontitis tissues compared to tissue from non-periodontitis patients. This suggests the role RANKL may have in alveolar bone loss adjacent to sites of inflammation.

Immunohistochemical staining was also used to investigate production of OPG protein in periodontitis and non-periodontitis tissues. Few studies have been carried out to determine the cell types involved in the production of OPG in human periodontitis. OPG production has been demonstrated in human periodontal ligament cells, gingival fibroblasts and epithelial cells (Sakata et al., 1999). In the present study, slightly different staining patterns were observed with the two OPG monoclonal antibodies. Mab 805 only detected OPG in blood vessels, while Mab 8051 could detect OPG in the epithelial lining cells as well as blood vessels. This is consistent with the studies with RA and peri-prosthetic tissues and may be because the two monoclonal antibodies to OPG recognise different forms of the OPG molecule (this was discussed in detail in Chapter 2 and further in the concluding chapter).

Dual labelling was again used to verify the types of cells associated with OPG production in periodontitis and non-periodontitis tissues. Factor VIII staining co-localised with staining for OPG detected by Mab 805, indicating that the dimeric form of OPG was associated with vascular endothelial cells, like that shown in the joint tissues (as discussed in Chapters 2 and 3). OPG as detected by Mab 8051 was seen to co-localise with CD68 positive monocyte macrophages along the periphery of the tissue. OPG, most likely monomeric, was also detected with Mab 8051on the surface of the epithelium but dual labelling with an epithelial cell marker was not carried out.

In contrast to RANKL, OPG protein was present in non-periodontitis tissue with a notable reduction seen in the tissues from the periodontitis patients. Statistical analyses of the SQA of OPG detected with Mab 805 confirmed the observation that OPG levels were significantly reduced in diseased compared to control samples. In addition, the percentage of OPG-positive blood vessels was significantly lower in the periodontitis tissues. However, the role that the OPG produced by blood vessels plays in bone metabolism is not yet known and this is discussed further in the concluding chapter.

It is interesting to note that OPG mRNA has also been reported as expressed by odontoblasts, ameloblasts and pulp cells in mice (Rani and MacDougall, 2000). In addition, it has been reported that RANKL mRNA is expressed by odontoclasts located on dentine surfaces being resorbed (Oshiro et al., 2001). While the present studies did not include cells present in the mineralised tissues, the observation that the reduction of OPG protein is associated with cells

in the granulomatous tissues adjacent to bony defects, is consistent with the concept that RANKL and OPG produced in the soft tissue regulation resorption of bone in periodontitis.
The results shown here demonstrated the expression of RANKL and OPG mRNA and protein in human periodontal diseased and non-periodontitis tissues. In addition to this, a subset of T cells and macrophages were identified as cells associated with RANKL production while endothelial cells and possibly epithelial cells produced OPG. This study is unique as for the first time levels of RANKL and OPG in the granulomatous tissue in chronic periodontitis were compared with gingival tissue from patients without periodontitis. A significant difference in the expression of key mediators of osteoclast differentiation was noted, similar to that seen in the previous chapters in this thesis. These results suggest similar mechanisms of bone loss in the three diseases investigated in this thesis.

The findings of this study, therefore, strongly support the original hypothesis that there is an elevation of RANKL and a reduction of OPG in granulation tissue adjacent to alveolar bone loss. This identifies the OPG/RANKL/RANK pathway of osteoclast differentiation as a possible target for regulating the bone destruction in human periodontitis. In addition, the inhibition of RANKL by the addition of OPG inhibited alveolar bone destruction in a murine model (Teng et al., 2000), further supports a therapeutic role in advanced human periodontal disease. Future investigations should compare the expression of these factors in the tissues from patients with severe chronic gingivitis but no evidence of bone loss to periodontitis tissues. Findings from this study may help us understand why some patients with gingivitis develop periodontitis but many others do not.

5 CONCLUSION

Bone loss is a serious complication of a number of common human diseases. Excessive bone resorption by osteoclasts is likely to be a significant factor causing this osteolysis in disease. This study has investigated the cellular and molecular mechanisms regulating osteoclast activity around orthopaedic implants, in the joint erosions of rheumatoid arthritis (RA), and in periodontal disease. Numerous cytokines, physical influences and other factors have been implicated in osteolysis in these conditions but at the commencement of this work the underlying cellular mechanisms of localised pathological bone loss were not yet understood. The work described in this thesis sheds considerable light on these questions, and the major findings are summerised below. It should be noted that while the studies described here focus on the formation and activity of bone resorbing cells in the tissues adjacent to osteolysis, the effects of factors in these tissues on bone formation also needs to be considered.

An important part of these studies was the investigation of whether cells of the osteoclast lineage were present in tissues adjacent to sites of pathological bone loss. Consistent with the idea that pre-osteoclasts and osteoclasts are present in the soft tissues adjacent to localised osteolysis (Hummel et al., 1998, Gravallese et al., 1998, Gravallese et al., 2000), RANK mRNA and TRAP were found to be consistently expressed in these tissues, in each of the pathologies examined. TRAP staining, and *in situ* hybridisation, to detect RANK mRNA, identified both mononuclear and multinuclear cells as cells of the osteoclast lineage in these tissues. Cells of the osteoclast lineage express these molecules abundantly, but are not the only TRAP and RANK positive cells (Efstratiadis and Moss, 1985, Bianco et al., 1987, Hattersley and Chambers, 1989a, Nakagawa et al., 1998). A more definitive marker of this cell type is the calcitonin receptor (CTR).

CTR mRNA was expressed in all three pathologies, but less often than RANK and TRAP mRNA. This finding supports the contention that CTR is a more definitive marker of mature osteoclasts than either of these markers (Efstratiadis and Moss, 1985, Bianco et al., 1987, Hattersley and Chambers, 1989a). However, it must be noted that the expression and regulation of the CTR in human osteoclast-lineage cells, especially *in situ*, is less well understood than in the mouse counterparts. Ideally, osteoclast-like cells would be identified by labelling with antibodies to CTR, however, antibodies to human CTR were not readily available during the course of this study.

The immunohistology findings suggested, but did not prove, the presence of osteoclast-lineage cells in the tissues adjacent to osteolysis. In support of the idea that osteoclast

differentiation occurred in these tissues, cells isolated from tissues adjacent to peri-prosthetic osteolysis and in active RA joints were capable of becoming osteoclasts when cultured with human osteoblast-like cells, as has now been reportet by several other groups (Fujikawa et al., 1996c, Fujikawa et al., 1996b, Sabokbar et al., 1997, Neale et al., 1999a, Itonaga et al., 2000a, Itonaga et al., 2000b, Suzuki et al., 2001). In addition, the rapid appearance of resorption pits in the experiments using cells isolated from the RA and peri-prosthetic tissues suggests that mature functional osteoclasts may already have been present in several of these tissues. In fact, and in contrast to the earlier reports (Fujikawa et al., 1996b, Neale et al., 1999b), mature osteoclasts formed from cells isolated from several of these tissues adjacent to aggressive osteolysis, even in the absence of added osteoblast-like stromal cells. Correlating with the functional activity of the osteoclast-like cells present in the rheumatoid tissues was the consistent expression of CTR mRNA.

Prior to beginning this work, the receptor activator of NFκB ligand (RANKL), its receptor RANK, and inhibitor Osteoprotegerin (OPG) were newly discovered as key factors regulating osteoclast formation in normal bone metabolism. However, work at that time was primarily in rodents and described *physiological* events. During the period of this work there have been major advances in understanding the role of these molecules in skeletal *pathology*, to which the findings of this thesis have made important contributions. On the premise that RANKL, RANK and OPG would be important in pathologies associated with localised bone loss, experiments were designed to investigate the expression of these osteoclastic mediators in cells isolated from peri-prosthetic, RA and periodontitis tissues. A major finding of the studies described here was that RANKL and OPG, as well as macrophage-colony stimulating factor (M-CSF), an apparently essential co-factor of RANKL, were consistently expressed, at the mRNA level, in tissues adjacent to osteolytic zones in all of the disease states.

Since TRAIL binds to and inhibits the actions of OPG (Emery et al., 1998), the expression of TRAIL mRNA was also investigated in tissues adjacent to each of the bone loss pathologies. It is interesting that TRAIL mRNA was abundantly expressed in each case. This is the first report of TRAIL in human tissues adjacent to localised bone loss. The role of TRAIL in tissues adjacent to sites of bone loss could be to induce apoptosis, the major known activity of this molecule (Wiley et al., 1995, Pitti et al., 1996, Walczak and Krammer, 2000) since cells in inflammatory tissues are likely to be both proliferating and undergoing apoptosis. Further studies are required to determine whether TRAIL might be involved in inflammation *per se* or whether it may represent an additional pro-osteoclastogenic influence by reducing the local concentration of OPG.

The studies described here are unique, in that expression of RANKL and RANK were compared with osteoclastogenesis in cells isolated from RA and peri-prosthetic tissues. Thus, the highest levels of RANKL and RANK mRNA were expressed in cells isolated from the pannus of active RA, or tissues adjacent to aggressive bone loss in patients with silastic implants, from which osteoclasts more readily formed. This is consistent with the notion that RANKL has an essential role in the localised bone loss observed in these inflammatory diseases.

During the course of this study it was suggested that the differentiation of pre-osteoclasts into mature, active osteoclasts, is determined primarily by the relative levels of RANKL and OPG (Hofbauer et al., 1999). The results described here, show for the first time, a significant correlation between osteoclast formation *ex vivo* and the ratio of RANKL to OPG mRNA levels in cells isolated from the RA tissues. Consistent with this, the ratio of RANKL to OPG mRNA was also higher in cells isolated from peri-prosthetic tissues, from which osteoclasts readily formed. It is interesting that cells from several of these samples formed active osteoclasts without additional osteblast-like/stromal cells. These results provide strong evidence that a high ratio of RANKL to OPG in tissue adjacent to bone may stimulate macrophages to become mature osteoclasts without the need for them to be in contact with osteoblast cells.

Examination of peri-prosthetic tissues suggested that the types of wear particles present in the tissues might influence pro-osteoclastic cytokines, particularly RANKL. These histology findings were extended by *in vitro* experiments, in which circulating monocytes were exposed to particles of prosthetic materials. These results show for the first time upregulation of RANKL, M-CSF, RANK, and OPG mRNA expression by blood-derived monocytes in response to wear particles. In addition, there was a notable variation between the expression of the osteoclast mediators in response to particles of CoCr, TiAlV and 316L SS *in vitro*. These findings are consistent with reports that the expression of pro-inflammatory and pro-osteoclastic cytokines (Haynes et al., 1998, Haynes et al., 1993, Rogers et al., 1997) vary depending on the type and material of the prosthetic wear particles present in the tissue adjacent to orthopaedic implants. The findings are important because they indicate that prosthesis manufacture should take into account the biological activity of prosthetic particulate debris likely to be produced from orthopaedic implants, in addition to the bio-compatibility of the intact materials.

As antibodies to RANKL and OPG became commercially available during the course of these studies, immunohistochemical staining was used to confirm the production of RANKL and OPG protein in peri-prosthetic, RA and periodontitis tissues. RANKL and OPG protein was also investigated in various control tissues. In physiological bone turnover, RANKL and OPG appear to be primarily produced by osteoblastic cells. However, the results presented here suggest that a number of cell types may be important in the ectopic production of RANKL in the tissues adjacent to bone and affect localised bone loss. RANKL staining was predominantly detected within inflammatory cell infiltrates in the tissues adjacent to pathological bone loss. Significantly, the expression of RANKL in the diseased tissues appeared greater than in the relevant controls. This was strongly supported by semi-quantitative assessment (SQA) and digital image analysis of RANKL, which found a significant increase in RANKL staining in the tissues adjacent to bone loss.

RANKL was often associated with CD68 positive monocyte/macrophages. This was seen in all the pathologies studied but was most apparent in the peri-prosthetic tissues. In active RA and periodontitis tissues the predominant cell type associated with RANKL production was a subset of T cells, a large proportion of which expressed CD45Ro. This is significant, as while previous studies have identified activated T cells as capable of producing RANKL (Horwood et al., 1999, Kotake et al., 2001), monocyte/macrophage cells are not generally accepted as associated with RANKL production. Since monocyte/macrophages can express RANK and may also become osteoclasts, it is possible that by producing RANKL these cells may "auto-stimulate" themselves to become osteoclasts.

While T cells and macrophages were the main cell types identified in this thesis as associated with the production of RANKL, the production of RANKL by fibroblasts is also possible. Although cells with the appearance of fibroblasts were associated with RANKL staining, they did not express CD55. However, it is likely the antibody to CD55 did not identify all fibroblasts in the tissues as it specifically identifies fibroblast-like synoviocytes. Another important finding was the absence of RANKL expression on the endothelial cells in the human tissues examined in the present studies. This was in contrast to a previous report of isolated microvascular endothelial cells (Collin-Osdoby et al., 2001) but is consistent with studies from this laboratory that have shown that HUVE cells do not express RANKL. Taken together, the results of this study suggest that RANKL-producing cells may either be recruited to sites of inflammation, for example lymphocytes, or the local inflammatory environment may cause up-regulation of RANKL in cells such as fibroblasts.

Two different antibodies to OPG were employed in these studies to investigate the production of OPG protein and identify the cells associated with its production. As discussed in Chapter 2, these antibodies were found to detect different forms of OPG; Mab 8051 detected dimeric and monomeric OPG, while Mab 805 detected only dimeric OPG, which was not bound to TRAIL or RANKL. It might be significant that these antibodies differentially identified OPG on particular cell types within the tissues investigated. Mab 805 detected OPG only in blood vessels (on Factor VIII positive endothelial cells), whereas Mab 8051 predominantly detected OPG in the synovium (on CD68 positive type A synoviocytes) in RA and peri-prosthetic tissues, as well as the epithelium in several periodontal tissues. Whether OPG produced by endothelial cells or the type A synoviocytes is able to block RANKL and inhibit the formation of osteoclasts in these tissues is yet to be determined. However, it is significant that the expression OPG was greatly reduced in the blood vessels and synovium of the diseased tissues compared with the controls. In addition, it is important to note that the reduction in OPG expression in active RA tissues resolved with disease resolution. Although the role of OPG production in blood vessels is not yet understood, the difference in OPG expression between diseased and control tissues suggests that the production of OPG in the diseased tissues might be important.

It is possible that the OPG produced by endothelial cells in the blood vessels of the tissues investigated has a role in regulating the vasculature in health and disease. This is supported by previous studies, which have shown that OPG null mice exhibit extensive inflammation and calcification of the aorta and renal arteries (Min et al., 2000). OPG has also been reported to be a survival factor for endothelial cells and may be involved in angiogenesis and tissue ingrowth (Malyankar et al., 2000). Angiogenesis is an important component of inflammation as it enables cytokines, inflammatory cells and possibly pre-osteoclasts to infiltrate the tissues. This is important, as vascular endothelial cells are involved in bone development and influence osteoclast recruitment, formation and activity. If endothelial cells are a major source of OPG in tissue adjacent to bone, the reduction in OPG detected on blood vessels in peri-prosthetic, RA and periodontal tissues may explain why there is enhanced bone resorption in the bone adjacent to these tissues.

Preliminary results, not shown here, indicate that TRAIL protein is also associated with various cell types in these same diseased and control tissues. It was particularly interesting to note co-localisation of OPG and TRAIL staining in blood vessels. The levels of TRAIL in these vessels might influence bone loss as TRAIL can bind to and inhibit OPG from inhibiting osteoclast activity (Emery et al., 1998). Alternatively, OPG may be preventing TRAIL from

stimulating apoptosis in the tissues (Emery et al., 1998). Future work will investigate the levels of TRAIL produced in tissues adjacent to bone loss in the diseases studied here.

The simultaneous up-regulation of RANKL and down-regulation of OPG in the peri-prosthetic, RA and periodontitis tissues further supports a likely role for the balance of RANKL and OPG in determining osteoclastic activity in these bone loss pathologies. Further studies to determine the levels of RANKL and OPG expressed in tissues sampled from patients with active RA during treatment for RA are currently being investigated. Levels of OPG and RANKL are being compared with X-rays of affected joints used to assess bone erosions at different times during treatment. It is hoped the results from this analysis will provide more insight into the significance of RANKL and OPG in patients with active RA and those that obtain remission.

As this laboratory and others have previously shown (Chu et al., 1991, Geivelis et al., 1993, Yamazaki et al., 1994, al-Saffar and Revell, 1994, Arend and Dayer, 1995, Xu et al., 1996) (Kotake et al., 1996, Xu et al., 1998, Neale et al., 1999a, Merkel et al., 1999) TNF and other cytokines associated with osteolysis are present in high levels in RA, peri-prosthetic and periodontitis tissues. TNF-α can regulate both OPG and RANKL expression (Brandstrom et al., 1998a, Horwood et al., 1998, Hofbauer et al., 1999, Nakashima et al., 2000), which may influence pathological bone loss adjacent to these tissues. In addition it has been suggested TNF may stimulate osteoclast formation directly (Kobayashi et al., 2000a) or work in synergy with RANKL by making cells more sensitive to low levels of RANKL (Lam et al., 2000). In this study, the effect of TNF-α on OPG production by endothelial cells was investigated *in vitro*. The finding that TNF-α reduces OPG detected on endothelial cells but increases OPG released into the supernatant may be relevant to inflammatory joint diseases. It is possible that although TNF might initially upregulate OPG production, chronic exposure to inflammatory cytokines *in situ* might exhaust the production of OPG by endothelial cells. TNF-antagonists and antibodies are now an accepted treatment regime for active RA. Not only does inhibition of TNF activity reduce the inflammatory response (Assuma et al., 1998) but blocking TNF-α may also enhance the differentiation and proliferation of bone cells in disease (Haynes et al., 1997). It is therefore important to understand how TNF may affect OPG in the tissues adjacent to bone loss and whether the monomeric and dimeric forms of OPG are regulated differently by TNF.

A simplified cartoon of osteoclast formation in the tissues adjacent to pathological bone loss, based on our current knowledge and incorporating the findings of this thesis, is summarised in

Figure 1. The activation of the tissue macrophages, after the phagocytosis of prosthetic wear particles or bacteria, initiates the release of chemokines, cytokines and mediators in the soft tissues. Inflammatory cells, including precursors of osteoclasts, are then recruited into the tissues. Among these mediators is M-CSF, which is important for the proliferation of osteoclast progenitors and differentiation into mature osteoclasts (Fujikawa et al., 1996b, Quinn et al., 1997a, Quinn et al., 1998a, Suda et al., 1992, Sarma and Flanagan, 1996, Suda et al., 1999), and has been shown to stimulate RANK expression on osteoclast precursors (Arai et al., 1999). Further to this, TNF-α, IL-1β, IL-1α and other cytokines activate surrounding cells and may stimulate RANKL expression (Nakashima et al., 2000). RANKL is the key stimulator of osteoclast formation and activation. This study showed RANKL expression by T cells, macrophages and fibroblasts in tissues adjacent to pathological bone loss. The interaction between RANKL and RANK results in the formation of mature osteoclasts, leading to pathological bone loss. This interaction can be inhibited by the production of OPG, which acts by binding to RANKL. In this study, OPG was identified on endothelial cells and macrophages in the synovial lining but the role OPG produced by these cells has in osteoclast formation is not known.

The results presented in this thesis show that although the mechanisms of osteolysis around orthopaedic implants, in the joint erosions of RA, and in periodontal disease are similar, different cell types may be involved in OPG and RANKL expression in the tissues adjacent to the pathological bone loss. These studies suggest that there is an elevation of RANKL and a reduction of OPG in tissues adjacent to sites of pathological bone loss. These results identify the RANKL/RANK pathway of osteoclast differentiation as an important pharmaceutical target for regulating the bone destruction in peri-prosthetic loosening, RA and periodontitis.

Therapies based on the inhibition of RANKL by its natural inhibitor OPG (Kim et al., 2001, Itonaga et al., 2000a, Teng et al., 2000), have been tested in *in vivo* and *in vitro* experimental models. OPG inhibits formation of osteoclasts from peri-prosthetic cells(Itonaga et al., 2000a) induced by prosthetic joint fluid (Kim et al., 2001) in culture and treatment of arthritic rats with OPG reduces erosion of the cortical and trabecular bone(Kong et al., 1999a). In a murine model of periodontitis, OPG is reported to inhibit alveolar bone destruction and reduce the numbers of osteoclasts adjacent to alveolar bone (Teng et al., 2000). More recently, a RANK blockade has been shown to be effective in the treatment of titanium particle induced localised bone loss in a murine model (Childs et al., 2002) and suggests a possible treatment to prevent the failure of prosthetic joints in humans. Systemic delivery of

OPG has recently been shown to be effective in reducing the generalised bone resorption seen in postmenopausal women (Becker et al., 2001).

The work in this thesis has shown for the first time that the same mechanisms of osteolysis apply to several diseases in humans. Although peri-prosthetic, RA and periodontitis appear to be quite different diseases, the primary mediators involved in osteolysis, as a complication of these diseases, are common. This study suggests that similar approaches could be used to inhibit osteolysis in localised bone loss. OPG itself is unlikely to be an effective treatment in its natural form for a number of reasons that include the need for it to be delivered by injection and its expense. In the long term it may be more feasible to develop small molecule mimics of OPG or antibodies to RANKL. In the meantime there is scope for more conventional anti-resorptives such as bisphosphonates in the treatment of localised pathological bone loss. Importantly, the studies presented here indicate that treatment with agents that regulate osteoclast differentiation will be effective in pathologies of localised bone loss.

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7 CHEMICALS USED IN THE STUDIES

All chemicals used in the studies were analytical grade

MOPS Solution (10X)

MOPS……..41 gm
sodium acetate…….4.1gm
0.5M EDTA (or 3.72 gm EDTA dissolved)…….20ml
DEPC……1ml
Milli Q water to…….1lt

pH should be 7.9
Autoclave

TAE (50X)

Tris…….242gm
Glacial Acetic acid……57.1ml
0.25M EDTA (pH 8) 200ml or 100ml 0.5M EDTA (18.61gm EDTA)
Milli Q water to…….1lt

Autoclave

RNA Gel (1% agarose gel)

10X MOPS buffer working solution……..4ml
DNA grade agarose…….0.4gm
DEPC H_2O……28.8 ml
- Heat to dissolve ~ 30 seconds
- In fumehood add
Formaldehyde (stock is 37-40%)…….7.2ml
Running Buffer: 1% Mops

2X Loading Buffer

Formaldehyde (37%)……2.6ml
Formamide……7.2ml
MOPS 10X……1.6ml
Bromophenol blue…….800µl
Ethidium Bromide (10mg/ml)……..1.4mg
Water…….1.8ml
Few grains of Brome phenol blue

DNA gel (2% agarose gel)

1X TAE……..200ml
DNA grade agarose ……4 gm

Running Buffer: 1% TAE

6X Loading Buffer

Water……..10ml
40% w/v sucrose…….4gm

0.25% Bromophenol Blue……..25mg

Solutions for *In situ* hybridisation

Tris-glycine

0.1M Tris (BDH, MERK Pty Ltd, Kilsyth, Vic)……..1.2114gm
0.2M Glycine (BDH, MERK Pty Ltd, Kilsyth, Vic,)… 1.5014gm
Milli Q water to ……...100ml
Adjust pH to 7.2 with concentrated HCL
Autoclave and store at 4^{o}C

20X Standard Saline Citrate (SSC) Solution

Sodium Chloride……173gm
Tri Sodium Citrate…….88.2gm
Milli Q water to…….1lt
Adjust pH to 7.0 and autoclave

Proteinase K Buffer

Ethylenediaminetraacetic acid (EDTA)(BDH)……..0.185gm
1X PBS to……100ml
Autoclave and store at room temperature

Prehybridisation Solution: with 2X SSC

Dextran Sulphate (MW 500,000) (Sigma)……. 10%
Polyvinylpyrrolidone (PVP) (Sigma)…… 0.05%
20X SSC……...2X
Formamide, deionized (BDH)……...50%
Triton X-100 (BDH)……0.05%
Store in the freezer
Herring sperm (Boehringer Mannheim) at 500µg/ml is denatured at 90^{o}C for 5-10minutes and chilled on ice before adding to the solution when required. Store the solution in –20^{o}C.

Acid clean coverslips:

Cover coverslips with 0.2N HCL for 20 minutes
Place in 70% alcohol for a few minutes before drying on paper towel

Hybridisation buffer 1:

0.1M Tris……..12.114gm
0.15M Sodium chloride…….8.85gm
Milli Q water to…….1lt
pH to 7.5 with HCL and autoclave

Blocking Serum:

Normal Sheep Serum…….10%
Hyb 1……..90%
NB blocks non-specific binding to proteins

Anti-DIG-Alkaline Phosphatase (Boehringer Mannheim DIG labelling kit):

Antibody at 1/500 dilution with 10% Normal Sheep Serum and Hybridisation Buffer I as diluent

Hybridisation Buffer III:

0.1M Tris……..…..12.114gm
0.1M Sodium chloride……..5.84gm
0.05M Magnesium chloride……..10.16gm
Milli Q water to……....1lt
pH to 9.5 and autoclave

Colour Substrate:

Hyb III……..…5ml
Vial 4 of DIG-kit……..…200µl
(NBT and BCIP, Boehringer Mannheim)
1M Levamisole (Sigma, St Louis, USA)…….1.2mg
N.B Levamisole blocks endogenous alkaline phosphatase activity

Solutions for DE AEC

5X Phosphate Buffered Saline: 1lt
Sodium dihydrogen orthophosphate……. 3.93gm
Di-Sodium Hydrogen orthophosphate……15.2gm
Sodium Chloride (NaCl)…… …….45gm
PH 7.2-7.4
Thimerosal 5mlof 10% Thimerosal/litre of solution
(dissolved in water)

Endogenous Peroxidase Block:

NaN_3 (sodium azide) Labchem, Auburn, Vic Aust…0.1%
H_2O_2……1%
In 1X PBS

Primary antibody:
Diluted in 1 X PBS + 1% BSA

Secondary antibody: - eg goat anti mouse-HRP
Diluted in 1X PBS + 1% BSA + 10% Normal Human Serum as recommended

Tertiary antibody eg swine anti goat-HRP (Biosource International, CA,USA)
Diluted in 1X PBS + 1% BSA + 10% Normal Human Serum as recommended

AEC Development

Tube 1: 740µl Buffer A + 1760µl Buffer B + 2.5ml dH2O
Tube 2: 2.5ml of Tube A + 125µl of AEC
Filter tube 2, then add 10µl of Hydrogen peroxide (H_2O_2) just before use

AEC tablets;- (Sigma)
Diluted in 2.5ml/tablet N,N dimethylformamide

Buffer A: 1.15ml acetic acid in 100ml dH2O

Buffer B: 2.721gm sodium acetate $3H_2$ or 1.64gm sodium acetate water free in 100ml dH2O (make fresh)

Solutions for APAAP

5X Phosphate Buffered Saline:

Sodium dihydrogen orthophosphate……...3.93gm
Di-Sodium Hydrogen orthophosphate……15.2gm
Sodium Chloride……....45gm
Milli Q water to…….….1lt

pH 7.2-7.4
Thimerosal 5ml of 10% Thimerosal/litre of solution
(dissolved in water)

5X Tris-PBS Buffer: 1ltre

Tris……..……..30.275gm
Sodium dihydrogen orthophosphate…….…3.93gm
Di-Sodium Hydrogen phosphate……..15.2gm
Sodium Chloride ……..….….45gm
Milli Q water to……..1lt

pH 7.2-7.4

Endogenous Peroxidase Block:

NaN3 (sodium azide) Labchem, Auburn, Aust ……....0.1%
H_2O_2 ……..……...1% (added before use)
in Tris PBS or T/PBS

Normal Donkey Serum: in T/PBS
Jackson Immuno Research Laboratories Inc, PA

Linking antibody: Donkey anti-mouse IgG (Biotin-SP conjugated Affin Pure anti-mouse) (Jackson)
Use 1/200 dilution in Tris PBS + 1% BSA

APAAP complex (Boehringer Mannheim)**:**
Use 1/50 dilution in Tris PBS + 1% BSA

Fast Blue (or Red) Substrate:
Levamisole (Sigma, St Louis, USA)……..2mg
0.2M Tris/HCL pH 8.2……..9.8ml
Napthol AS-MX (Sigma, St Louis, USA)…….5mg
(added to 200µl DMF (in sm glass beaker))
Fast blue (Sigma, St Louis, USA)…….10mg (just before use)
Filter before using
(Napthol and Fast blue powders are kept at –20^{o}C in the freezer)

APPENDUM

Page 7 line 22, replace “Zannatino A.” with Zannettino A.

Page 13, line 28, replace “levels of these mediators” with “the levels of these mediators”

Page 16, line 28, replace “mineralised tissue” with “mineralised matrix”

Pg 146, line 15 The references should be in the order as follows

These cells are thought to produce a variety of inflammatory mediators and high levels of several inflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, prostaglandin (PG)E_2 and tumour necrosis factor (TNF)-α have been found in the tissue and gingival crevicular fluid of patients suffering advanced periodontitis (Page and Schroeder, 1976; Masada et al, 1990; Stashenko et al 1991; Moskow and Polson, 1991; Ishihara and Nishihara et al 1997; Schenkein 1999; Rasmussen et al, 2000; Bickel et al, 2001). Similar findings have been reported in animal models of periodontitis (Assuma and Oates 1998; Iwasaki and Yoshitaka. 1998; Graves et al, 1998).

Page 168, line 21, replace the word “auto-stimulate” with “autocrine-stimulation”