Regulation of osteoclast activity: differential adhesion of osteoclasts to the bone surface

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

Elizabeth Ann O'Brien

December 2000

Abstract

Excessive bone loss and risk of fracture occurs in several common diseases including osteoporosis, rheumatoid arthritis, hypercalcaemia of malignancy and hyperparathyroidism. The purpose of this project was to shed light on the mechanism whereby bone loss occurs.

The non-steroidal anti-inflammatory drug, indomethacin inhibits bone resorption *in vivo* and in the mouse parietal bone *in vitro* by blocking prostaglandin production. This drug had the effect of reducing the number of tartrate-resistant acid phosphatase-positive osteoclasts (TRAP+OC) on the surface of the bone and increasing those on the adjacent endocranial membrane. This observation was made by peeling back the membrane from the bone so that the respective numbers could be counted. The stimulators of bone resorption, prostaglandin E_2 (PGE_2) and 1,25-dihydroxyvitamin D_3 ($1,25D_3$) and parathyroid hormone (PTH) reversed this effect, producing large numbers of TRAP+OC on bone at the expense of those on the membrane.

When freshly isolated bones were stained for the β3 subunit of the integrin αvβ3, β3-positive osteoclasts (β3+OC) were only seen on the bone surface. When bones were incubated with indomethacin very few β3+OC were seen on the membrane and none on the bone. The stimulators of bone resorption caused the re-appearance of β3+OC on the bone surface. This process could be blocked with the disintegrin, echistatin and by function-blocking antibody to β3. The conclusion was drawn that αvβ3 integrin was necessary for osteoclasts to translocate to the bone surface from the membrane.

PGE_2, PTH and $1,25D_3$ are known to act through osteoblast-like cells to activate osteoclasts. One candidate for the intermediary produced by osteoblasts that stimulates osteoclasts is osteoprotegerin ligand (OPGL). OPGL has been shown to stimulate osteoclast differentiation and activation. Soluble recombinant human OPGL brought about the translocation of osteoclasts from the endocranial membrane to the parietal bone surface. PGE_2, $1,25D_3$ and PTH all increased the expression of mRNA for OPGL in parietal bones compared with indomethacin-treated controls, after 6 h exposure.

Recombinant human OPG (rhOPG) was found to cause osteoclasts to detach from the bone surface and attach to the membrane. rhOPG binds strongly to OPGL and inhibited the stimulatory effects of PGE_2 and $1,25D_3$ on osteoclast adhesion to the bone. OPGL and a function blocking antibody to OPG both inhibited the effect of indomethacin, leaving active osteoclasts on the bone. Messenger RNA for OPG was not down-regulated by stimulators of bone resorption in mouse calvaria, contrary to reports with some bone cell lines. However, secretion of OPG was inhibited. OPG activity was detected by an enzyme-linked immunoassay in the culture medium from indomethacin-treated bones. PTH, PGE_2, $1,25D_3$ and dexamethasone all inhibited the production of OPG activity.

In conclusion, PTH, PGE_2 and $1,25D_3$, act to induce OPGL production which brings about a change in adhesion of osteoclasts from the membrane to the bone surface mediated by the integrin αvβ3. OPG is produced by bone lining cells in the absence of specific stimulators of bone resorption, for instance when prostaglandin production is inhibited by indomethacin. Thus, OPG inhibits bone resorption by blocking OPGL and causes osteoclasts to detach from the bone surface.

Table of Content

Materials and Methods

5. Effect of osteoprotegerin on osteoclasts

Abbreviations

AMV	Avian myeloblastosis virus
ATP	Adenosine triphosphate
β3+OC	β3 positive osteoclasts
BDU	Bromodeoxyuridine
bg	Beige gene
BMPs	Bone morphogenetic proteins
BMU	Basic multicellular unit
cAMP	Cyclic 3'-5'-adenosine monophosphate
Cbfa	Core binding factor gene
cDNA	Complementary DNA
CFU-M	Colony forming unit macrophage
CFU-GM	Colony forming unit granulocyte/ macrophage
CFU-GEMM	Colony forming unit granulocyte/erythrocyte/ macrophage/megakaryocyte
CO_2	Carbon dioxide
COX-2	Cyclo-oxygenase-2
$1,25D_3$	1,25 dihydroxyvitamin D_3
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleoside triphosphates
EDTA	Ethylenediaminetetra-acetic acid
GM-CSF	Granulocyte macrophage colony stimulating factor
HCl	Hydrochloric acid
HSC	Haematopoietic stem cell factor
h	Hour
hu	Human
IGF	Insulin-like growth factor
Ihh	Indian hedgehog gene
IL-1	Interleukin-1
IMS	Industrial methylated spirit

Ind	Indomethacin
m-CSF	Macrophage colony stimulating factor
MEM	Minimum essential medium
MES	2-(N-morpholino) ethane sulfonic acid
$MgCl_2$	Magnesium chloride
mi	Microphthalmic gene
min	Minutes
MMPs	Matrix metalloproteinases
mu	Mouse
NaCl	Sodium chloride
NSAIDS	Non-steroidal anti-inflammatory drugs
OCIF	Osteoclastogenesis inhibitory factor
ODF	Osteoclast differentiation factor
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein
PVDF	Polyvinyl difluoride
RANKL	Receptor activator of NF-κB ligand
RGD	Arginine/glycine/aspartic acid
Rh	Recombinant human
RNA	Ribonucleic acid
s	Soluble
SCF	Stem cell factor
Taq	Thermus aquaticus
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinases
TMB	3,3'5,5' Tetramethylbenzidine substrate
TNF-α	Tumor necrosis factor alpha
TR1	TNF receptor-like molecule 1

TRANCE	Tumor necrosis factor receptor activation induced cytokine
TRAP	Tartrate-resistant acid phosphatase
TRAP+OC	TRAP positive osteoclasts
TRAF	TNFR-associated factor
VDR	Vitamin D receptor

List of Figures

Acknowledgements

I would like to thank Dr Michael Davie for giving me the opportunity to undertake this study at the Charles Salt Research Centre and for financial support to attend meetings.

I would also like to thank my supervisor at the Charles Salt Research Centre Dr Michael Marshall for his guidance and support throughout, his infinite patience and his friendship during my time at Oswestry.

My thanks also to my supervisor at Chester College Dr John Williams for advice and helpful discussions with particular regard to my thesis.

I would like to thank all members of staff at the Charles Salt Centre for useful discussions and advice and moral support during my studies.

Finally I would particularly like to thank my husband John O'Brien for his unfailing support and sense of humour.

Introduction

1. Disorders of bone

The purpose of investigating the regulation of bone resorption is to shed light on the mechanisms of bone disorders and to indicate possible therapeutic targets for their treatment. There are a variety of disorders which may affect bone and they may be congenital or acquired, primary or secondary. Those which involve an increase in the number or an increase in the activity of the osteoclast include osteoporosis, Paget's disease, primary hyperparathyroidism and osteolytic metastases.

1.1 Osteoporosis

Osteoporosis has been defined as "a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture" (World Health Organization, 1994). In this country osteoporosis affects one in three women and one in twelve men and costs the UK National Health Service approximately £1.5 billion per year (National Osteoporosis Society 2000). A major risk factor for women is the decline in oestrogen levels following the menopause and currently the most effective way to decrease the risk of fracture is hormone replacement therapy. The cause of the bone loss in osteoporosis is an imbalance between the amount of bone removed by the osteoclast and the amount of bone replaced by the osteoblast during remodelling.

1.2 Paget's disease

Paget's disease is a highly localized disorder of bone remodelling. It is thought to begin with an increase in bone resorption by osteoclasts which leads to an increase in the amount of new bone laid down by osteoblasts. This results in a disorganized structure of immature and mature bone at the affected site. The result is that the bone enlarges in size and is more prone to deformity and fracture (Rodan

& Martin, 2000). The cause is unknown and it is more commonly diagnosed in older people as an incidental finding during other investigations.

1.3 Primary hyperparathyroidism

Primary hyperparathyroidism is a common cause of hypercalcaemia and is a relatively frequent endocrine disorder with an incidence of 1 in 500 to 1 in 1000 in the United States (Silverberg *et al*, 1995). It results from an excessive secretion of parathyroid hormone (PTH) from one or more parathyroid glands most commonly caused by a benign tumour. The PTH causes an increase in both the differentiation and activation of the osteoclast leading to increased bone resorption and a subsequent rise in serum calcium. The cause is unknown and it may be managed surgically or medically.

1.4 Osteolytic metastases

Osteolytic metastases are a common complication arising from a malignant tumour. In some cases the tumour will secrete a PTH related protein (PTHrP) which indirectly activates osteoclasts to resorb bone to make room for the growing tumour. Alternatively the tumour itself may directly activate the osteoclast to make space in the bone for the tunour. Both cases can result in humoral hypercalcaemia of malignancy, a syndrome characterized by an increase in serum calcium and often occurs during the final stages of the disease (Stewart, 1996).

2. Bone : function and structure

Bone is a dynamic tissue, which is continually being replaced as it becomes aged or damaged, by a process known as remodelling. The skeleton performs several important functions in the body:

- Provides protection for vital organs such as the lungs and brain.
- Allows for attachment of muscle for movement, support and locomotion.
- Houses the bone marrow, which in the adult is the major site of haematopoiesis.

- Acts as a reservoir for minerals such as calcium, magnesium, and phosphate, which is important in maintaining mineral homeostasis.

Two types of bone may be found in the skeleton, dense or cortical bone, which covers the surface shell of bone, and spongy cancellous or trabecular bone, which is enclosed by the cortical bone (Fig 1.1). The general features of both types of bone are similar. Both may consist of woven (primary) or lamellar (secondary) bone. The embryonic skeleton consists of woven bone, which is gradually resorbed and replaced with mature bone. Woven bone is rarely seen in the human skeleton after the age of five years though it may reappear in certain circumstances. These include soft tissue injury, treatment which stimulates the formation of bone, metabolic and neoplastic disease or inflammation. Collagen fibrils of woven bone are randomly arranged with irregular mineralization whereas in lamellar bone the collagen lies in parallel sheets with regular mineralization (Buckwalter *et al*, 1995). Both compact and trabecular bone consists of a mineralized matrix. In trabecular bone the matrix is organized in the form of thin spicules whereas in cortical bone it is organized in Haversian systems or osteons. These osteons consist of a canal containing a blood vessel surrounded by a ring of lamellae. As well as describing bones according to their architecture, they may also be described according to their method of formation during development. The majority of bones develop *via* a mechanism of cartilage replacement called endochondral ossification. These may be distinguished from bones which form directly in a process called intramembranous ossification.

The periosteum is the membrane that covers the external surfaces of bone and is of biological importance. It constitutes an important part of the blood supply to the bone and periosteal cells can resorb and form bone in response to local and systemic stimuli. The periosteum consists of two layers, the outer layer being more dense and fibrous and the inner layer less dense and more vascular and cellular. The inner layer consists of cells that are capable of becoming osteoblasts and so this may be referred to as the osteogenic layer. These cells are also able to form cartilage and they help to form the fracture callus during fracture healing. As the bone grows they secrete organic matrix which enlarges the diameter of the bone. The outer layer has fewer cells and more collagen. The periosteum undergoes changes as it ages. In

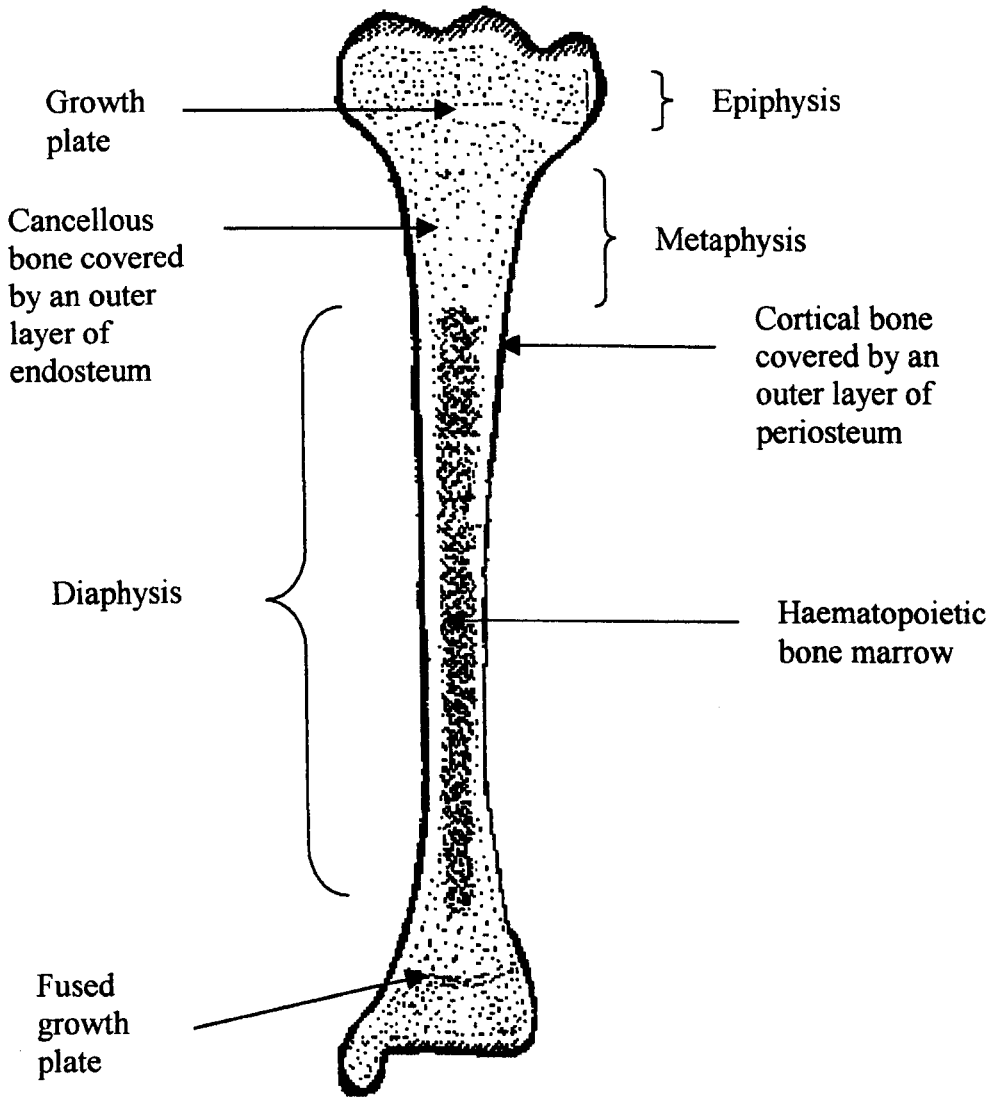


Figure 1.1 Diagrammatic view of a longitudinal section through a long bone

children the inner layer readily forms new bone which can be seen following injury or disease to the diaphysis when a new diaphysis may be formed. In later life the periosteum thins and loses to some extent its capacity to form new bone. However, the periosteum continues to form new bone throughout life.

3. Bone matrix

Bone matrix is extremely durable such that it can retain much of its strength for many years after death. Matrix usually makes up more than 90% of the volume of bone with the remaining being cells, cell processes and blood vessels canals and canaliculae. Matrix consists of an organic and inorganic component and is a composite material. The inorganic component makes up approximately 65% of the wet weight of bone, the organic component 25% of the wet weight and water makes up approximately 10%. The organic content of the bone is mainly collagen which gives the bone its form and allows it to resist tension whereas the inorganic content allows it to resist compression. A demineralized bone becomes extremely pliable and resistant to fracture whereas a bone which has had its organic content removed becomes extremely brittle and easily fractured.

Collagen makes up approximately 90% of the organic matrix being predominantly type I along with small amounts of types V and XII. The collagen fibres are organized in sheets or around an osteon and all lie in the same direction in a single lamellae. However, in adjacent lamellae they lie in different directions. In mineralized tissues, the long edge of the apatite crystals lie parallel to the axis of the collagen fibres which allows the crystals to add to the strength of the bone. Type I collagen has a unique amino acid sequence and inherited abnormalities of collagen type I occur due to amino acid substitutions. These patients suffer from fragile bones due to either a decreased amount of normal type I collagen or an abnormality of the structure of type I collagen. Bone also contains several non-collagenous proteins involved in the mineralization of bone. These proteins include osteocalcin, osteonectin, bone sialoprotein, bone phosphoproteins and small proteoglycans. Water makes up a further 5 to 10% of the weight of bone tissue and is needed for both nutrition and function of cells.

The mineral content of bone performs two essential functions in that it serves as a reservoir of ions and it gives bone most of its strength. Roughly 90% of body calcium, 85% of phosphorous and 40 to 60% or sodium and magnesium are associated with bone mineral crystals. This pool is the major source of ions for exchange with the extracellular fluid and helps to maintain the concentrations of these ions within the narrow ranges needed for vital functions in the body such as nerve conduction and biochemical reactions. Bone mineral or apatite crystals are relatively small in size which allows the incorporation and adsorption of ions such as carbonate and acid phosphate groups as well as enabling the mineral to dissolve in the acid environment created by the osteoclast during bone resorption.

4. The cells of bone

The cells that form bone (osteoblasts) are derived from mesenchymal stem cells found in the bone marrow (Friedenstein, 1973). These stem cells are capable of differentiating into osteoblasts and may also form cells which give rise to cartilage, adipose tissue and muscle. Undifferentiated mesenchymal cells which have the potential to become osteoblasts may also be found in the bone canals, endosteum, and the periosteum (Buckwalter *et al*, 1995). Early work by Friedenstein *et al* (1967) showed that cells of marrow origin found in peritoneal exudates formed bone in diffusion chambers under the influence of bladder mucosa. However, the cells were not self-sustaining. Further work by Friedenstein *et al* (1968) showed that in a similar experiment bone was not formed from cells from different connective tissues. Further, bone marrow placed in diffusion chambers was capable of forming bone without the presence of bladder mucosa or any other inducing agent and that this bone was self-sustaining (Friedenstein *et al* 1966). The conclusion from these and other experiments was the existence of "a determined osteogenic precursor cell" which is able to form bone without any inducer substances, and an "inducible osteogenic precursor cell". Mesenchymal cells remain undifferentiated until they are stimulated to proliferate and differentiate into osteoblasts. Following a fracture growth factors such as bone morphogenetic proteins (BMPs) are released which stimulate these mesenchymal cells to differentiate into fibroblasts and osteoblasts which heal the fracture.

4.1 Osteoblasts

The osteoblast is the cell type that forms bone and is required, along with the chondrocyte of the growth plate, for longitudinal bone growth (Warshawsky 1982; cited in Karsenty, 1999). Osteoblasts line the surface of bone and pack tightly together against adjacent osteoblasts. Osteoblasts secrete type I collagen and bone matrix proteins towards the mineralizing front of the tissue (Buckwalter *et al*, 1995). Osteoblastic differentiation and function is regulated by the interaction of the cell with matrix proteins. Cytoplasmic processes of the osteoblast pass through the osteoid matrix in order to contact osteocytes that are found within the mineralized matrix. So far only two genes have been found which control osteoblastic differentiation directly or indirectly and these are *Cbfa 1* (Speck & Stacy, 1995) and *Indian hedgehog* (*Ihh*) (Bitgood & McMahon, 1995). *Cbfa 1* is the earliest and most specific marker of osteoblastic differentiation found thus far (Karsenty, 1999). The genetic deletion of *Cbfa 1* in mice leads to animals in which the skeleton is comprised entirely of cartilage with no sign of calcified bone (Komori *et al*, 1997). Mutations of the *Cbfa 1* gene in humans have been found in patients affected with cleidocranial dysplasia (Lee *et al*, 1997).

Active osteoblasts may follow one of three fates. They may remain on the surface of bone where they cease activity and become flattened bone lining cells or they may be surrounded by matrix and become osteocytes or they may undergo apoptosis (Buckwalter *et al*, 1995).

Mineralization of the bone collagen fibrils takes place in an organized fashion. Mineral first appears in specific regions of the collagen fibrils separated by unmineralized regions. As mineralization progresses, mineral appears in an increasing number of regions of the collagen matrix and ultimately mineral fills all the available space within the collagen fibrils. Once it has begun mineralization proceeds fairly rapidly with approximately 60% of the total mineral formed within hours. As mineralization proceeds the water and non-collagenous protein concentrations decrease but the collagen concentration remains unchanged. Increased mineralization and organization of the matrix, maturation of the bone crystals and the replacement of woven bone with lamellar bone leads to increasing stiffness of the bone (Buckwalter *et al*, 1995). Mineralization is impaired in vitamin

D deficient rickets and osteomalacia. Both conditions illustrate the importance of mineralization for normal skeletal function. In such cases the osteoblasts secrete osteoid but the osteoid does not mineralize. As the amount of unmineralized matrix increases as a proportion of the mineralized matrix, the bone progressively weakens and may deform or fracture.

4.2 Osteocytes

Osteocytes make up more than 90% of the bone cells in the mature human skeleton (Cooper *et al*, 1966). As mineralized matrix envelops the osteoblast, morphological changes occur and the osteoblast progresses towards its terminally differentiated stage, the osteocyte. Osteocytes have long branching cytoplasmic processes that extend through canaliculae in the matrix and come into contact with cytoplasmic processes from other cells. It has been suggested that the interconnecting network between the osteocytes may allow the osteocytes to detect mechanical loading in the bone (Turner *et al*, 1994). Osteocytes may also be able to control the movement of ions between the mineralized matrix and the extracellular fluid spaces of the bone (Buckwalter *et al*, 1995).

4.3 Bone lining cells

Bone lining cells are flattened cells which lie directly against the bone matrix. They have extensions which penetrate the bone matrix and contact other extensions of the osteocytes (Buckwalter *et al*, 1995). They may be referred to as resting osteoblasts or surface osteocytes. Lining cells and osteocytes have fewer organelles and less cytoplasm than active osteoblasts (Buckwalter *et al*, 1995). Bone lining cells may function like osteocytes as a means of detecting mechanical stress and thereafter stimulating new bone formation or resorption.

4.4. Osteoclasts

In the mid nineteenth century Robin (cited in Hancox, 1972) was the first to describe multinucleate giant cells in bone and to distinguish them from

megakaryocytes. However, it was Kolliker (1873; cited in Gothlin and Ericsson, 1976) who called them osteoclasts and proposed that they may resorb bone. It is now known that osteoclasts carry out bone resorption and it seems to be their only function (Vaananen, 1996). Work by Bromley & Woolley (1984) showed the presence of multinucleate tartrate-resistant acid phosphatase (TRAP) positive cells adjacent to the calcified cartilage matrix in the large joints of patients with rheumatoid arthritis. These cells were termed "chondroclasts" and were thought to contribute largely to subchondral bone loss. During evolution osteoclasts first appear in bony fish but not in sharks (Glowacki *et al*, 1986). The function of these osteoclasts in bony fish appears to be fracture repair and mineral homeostasis.

The osteoclast shows characteristic specializations that distinguishes it from other cells including monocytes and macrophages (Chambers, 1985). With transmitted electron microscopy osteoclasts can be seen on bone surfaces associated with a resorption pits or Howship's lacunae (Miller, 1981). The osteoclast shows a prominent ruffled or brush border made up of microvilli juxtaposed to the lacunae and a so-called clear zone that runs around the perimeter of the osteoclast. The clear zone is devoid of organelles such as the mitochondria, lysosomes and the Golgi complexes that typify osteoclasts. The clear zone is also called the sealing zone because it is thought to act to limit the diffusion of the acid and proteolytic enzymes secreted by the ruffled border (Baron *et al*, 1985). Osteoclasts are capable of great motility (Hancox & Boothroyd, 1961) which can be rapidly inhibited by calcitonin (Chambers, 1982). Using scanning electron microscopy, Jones & Boyde (1977) demonstrated that not all osteoclasts were associated with resorption lacunae and some of these were not in contact with bone. In this case osteoclasts were not flat and ovoid in shape as when on bone but elongated and extensively branched. These latter osteoclasts were thought to be inactive osteoclasts. Resting osteoclasts were also described by Lasfargues & Saffar (1983) in a study of the effects of indomethacin on remodelling of alveolar bone in hamster.

The nuclear morphology of the osteoclast is attributed to fusion of mononuclear cells rather than to endomitosis. There are no reports of mitosis in osteoclasts (Loutit & Nisbet, 1982) and tritiated thymidine (Young, 1962) and bromodeoxyuridine (Marshall & Davie, 1991) labelling studies have described instances of the presence of single labelled nuclei amongst other unlabelled nuclei

within an osteoclast, suggesting fusion of mononuclear precursors occurs and not mitosis. Evidence that osteoclasts derive from circulating monocytes was obtained by injecting tritiated thymidine labelled monocytes isolated from blood into mice in which osteoclast formation had been stimulated by 1-α-hydroxycholecalciferol (Tinkler *et al*, 1981). Labelled osteoclasts were seen in autoradiographs of sections of femur. The formation of osteoclasts by fusion of monocytes from circulating blood was directly observed in primary culture (Zambonin Zallone *et al*, 1984).

5. Bone remodelling

The replacement of bone in the adult skeleton is carried out by a process known as remodelling. Old and damaged bone is removed by the osteoclast and new bone is deposited by the osteoblast in the same place (Fig 1.2). The concept of the BMU or basic multicellular unit has been put forward to try to explain the integration in time and space of the remodelling process (Parfitt, 1994). The BMU is a temporary structure consisting of several different types of cells. In the healthy adult skeleton, whether normal or osteoporotic, all osteoclasts and osteoblasts belong to a BMU and isolated cells are not considered to exist (Parfitt, 2000). New osteoblasts only appear at sites which have recently undergone resorption and the link between resorption and new bone formation is generally referred to as coupling. The reasons for remodelling may be, as already stated, to remove damaged bone but it is also a means by which demands for calcium can be met in order to maintain calcium homeostasis. Most important perhaps is the adaptation to changes in stress so that bone is put on where needed and taken off where it is not needed. In addition bone remodelling is required for growth. Cortical bone remodelling is more likely to be due to the need for damage repair whereas cancellous remodelling is more concerned with calcium homeostasis. In cortical bone the process is known as osteonal remodelling and the BMU is an elongated cylindrical structure which burrows through the bone and is approximately 2 mm long and 0.2 mm wide. In humans it moves at a rate of about 20 – 40 μm per day. The front end of the BMU is the cutting cone which consists of about nine osteoclasts each with about nine nuclei which resorb the bone in front of them (Jaworski *et al*, 1981). Following on from the cutting cone is the transitional zone which is lined with

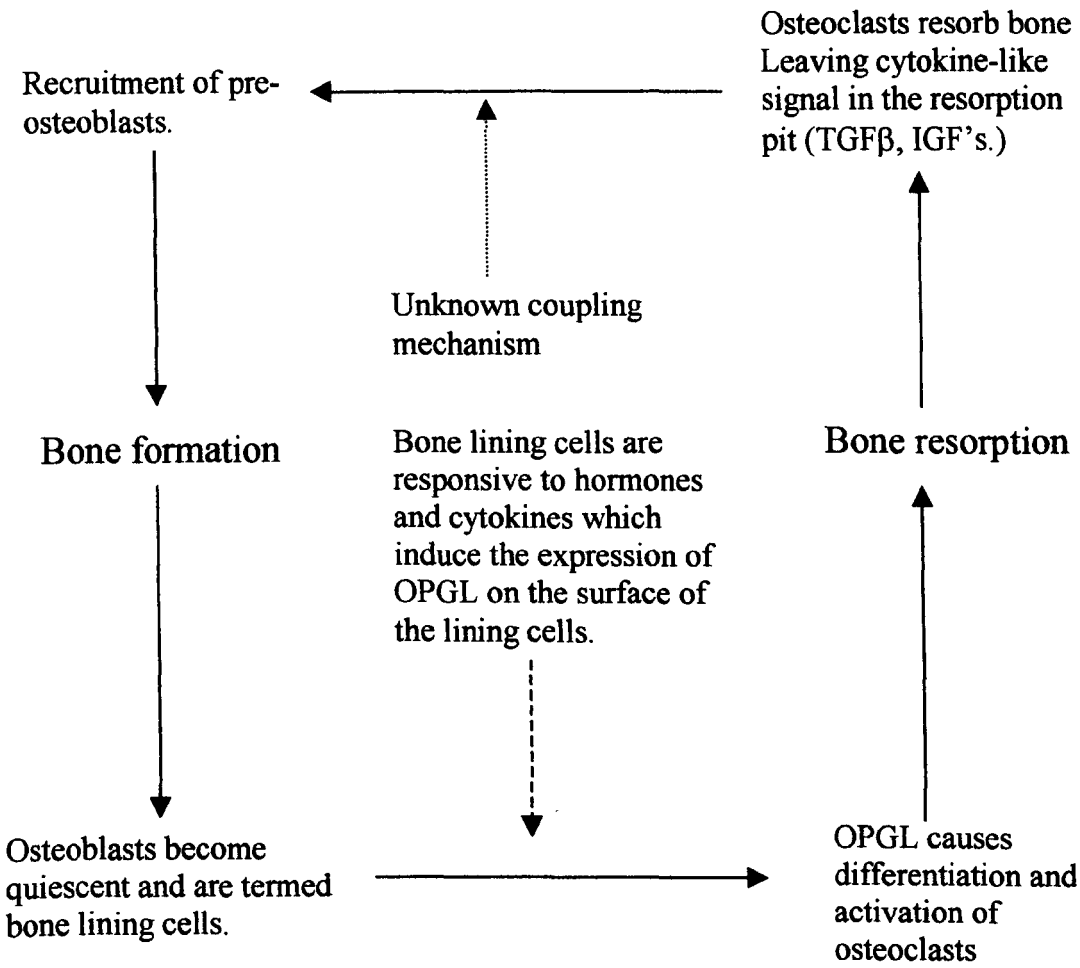


Figure 1.2 Mechanism for coupling bone resorption to bone deposition. Active osteoblasts lay down new bone and then become inactive and form lining cells. These cells are capable of responding to stimulators of bone resorption and express the cytokine OPGL which causes differentiation and activation of osteoclasts to resorb bone. Signal released by resorption including TGF-β and IGF's may recruit pre-osteoblasts to lay down new bone and fill the resorption pit.

spindle-shaped cells which lay down cement onto the cavity wall. Behind the transitional zone is the closing cone, which contains about 2,000 osteoblasts that form bone within the cavity. In the centre of the cavity is a blood capillary loop from which new osteoclast mononuclear cells are recruited. An osteoclast at the cutting cone may remain the same for as long as the BMU exists but it would change its nuclei many times over by the fusion of new pre-osteoclasts (Parfitt, 1994). The turnover of nuclei is approximately 8% measured by tritiated thymidine labelling (Jaworski *et al*, 1981). The nuclei undergo apoptosis (Boyce *et al* 1993). Thus the osteoclasts at the cutting cone would be made up of cells of different ages. In contrast the osteoblasts at the closing cone will all be of the same age. A group of new osteoblasts is recruited at the speed required to ensure that the closing cone remains the same distance from the cutting cone. These osteoblasts come from the spindle-shaped precursor cells that line the BMU behind the cutting cone. They undergo cell division before becoming osteoblasts (Jaworski *et al*, 1980). Once the osteoblast had finished secreting bone matrix it may either become a lining cell within the canal, it may become embedded in the new bone as an osteocyte or it may undergo apoptosis.

In cancellous bone remodelling is known as hemi-osteonal remodelling and the process is very similar to what occurs in cortical bone. The osteoclasts move across the surface of the bone rather than burrowing through the bone and the cavity created is filled by osteoblasts following behind. Again the osteoclast nuclei are renewed throughout the cycle and successive teams of osteoblasts arrive at the junction of the transitional zone and the osteoid seam to fill in the cavity with new bone, although this has not been as well studied as in cortical bone. The osteoblasts are derived from preosteoblasts that line the transitional zone. The osteoblasts may undergo one of the same three fates as those involved in osteonal remodelling. It is still not fully understood how there is an increased availability of osteoblasts at the site of previous bone resorption. It may be due to the release of growth factors embedded in the matrix as resorption takes place, such as insulin-like growth factor II (IGF-II) (Mohan *et al*, 1988) or transforming growth factor-beta (TGF-β) (Bonewald & Mundy, 1990). Other theories include a response to mechanical strain on the bone or changes in local cytokine concentration which lead to an increase in formation of pre-osteoclasts thus leading to an increase in osteoblast production. A

more recent theory put forward by Parfitt (2000) suggests that the blood vessel found in every BMU may act as a source of osteoblasts as well as a source of signaling factors for osteoblast recruitment. It may be that this "parallel pathway" acts in concert with other events previously described.

6. Origin of the osteoclast.

The earliest suggestion that osteoclasts were derived from blood leucocytes was seen in the model system of regenerating newt limb. When animals were injected with tritiated thymidine one day prior to amputation of a limb, labelled osteoclasts were seen in the limb stumps between ten and twenty days later (Fischman & Hay, 1962). Prior to this, the only other labelled cells seen in the limb tissue were extra vascular blood leucocytes. These authors concluded that osteoclasts in this model were derived by fusion of mononucleated leucocytes, probably monocytes. A similar conclusion was obtained in rats that had been surgically joined so that they shared circulating cells (parabiosis) (Gothlin & Ericsson, 1972). One of the parabiotic rats was lethally irradiated to ablate haematopoiesis and then both animals given a fracture of the femur. The cross-circulation was arrested by a clamp and tritiated thymidine injected into the shielded rat. The clamp was removed twenty minutes later. Labelled monocytes and osteoclasts were seen in both fracture sites but labelled fibroblasts, chondroblasts and osteoblasts were seen only in the fracture site of the shielded rat. The conclusion was drawn that osteoclasts were derived from the circulating precursors from the shielded rat whereas the connective tissue cells were locally derived.

The study of congenital defects in mice has led to a better understanding of the origin of the osteoclast. There are at least four naturally occurring recessive mutations in mice of which two, *mi* and *op*, have been particularly informative. The microphthalmic (*mi/mi*) mouse, amongst other symptoms, shows impaired bone resorption leading to the obliteration of the marrow cavities with primitive bone (osteopetrosis). The first report of therapeutic resolution of this defect came from Walker (1972). He joined in parabiosis *mi/mi* mice with their unaffected siblings (*mi/+*) which led to resorption of the marrow cavities in about two weeks. That this was brought about by the transfer of cells rather than a humoral effect was shown by

injecting bone marrow from a normal littermate into lethally irradiated *mi/mi* mice (Walker, 1975) which also resolved the osteopetrosis. The irradiation was necessary because the strain of mice was not sufficiently inbred, leading to rejection of the grafted cells (Loutit & Nisbet, 1982). However, this work did not prove that the osteoclast population in the *mi/mi* was being replaced by a donated normal population. For this a cytoplasmic marker of donor cells was used which could be seen in the osteoclast population. The beige mouse (*bg*) shows giant lysosomes in cells of the myeloid series and when bone marrow from this mouse was injected into lethally irradiated osteopetrotic mice then the osteopetrosis was cured and giant lysosomes were seen in the resulting osteoclast population (Ash *et al*, 1981). The *mi* gene has been shown to code for a basic helix-loop-helix-zip type of transcription factor (Steingrimsson *et al*, 1994) but the exact mechanism for the failure of bone resorption in the *mi* mouse remains to be elucidated.

The *op* mouse shows a similar osteopetrosis to the *mi* but this is not resolved by a graft of normal compatible bone marrow (Marks *et al*, 1984). The *op* mouse showed drastically reduced numbers of mature macrophages and unlike the *mi* mouse, the defect could not be transferred to lethally irradiated normal mice by the injection of spleen cells (Wiktor-Jedrzejczak *et al*, 1982). Cultured osteoblasts and fibroblasts from the *op* mouse did not secrete macrophage colony stimulating factor (mCSF), unlike normal mice. When this growth factor was administered to the *op* mouse resorbing osteoclasts were induced and the marrow cavity was restored (Felix *et al*, 1990). The *op* gene was shown to code for mCSF and the mutant had a single base pair insertion which prevented the formation of functional protein (Yoshida *et al*, 1990). The *mi/mi* mice had normal numbers of osteoclasts but were unable to resorb bone whereas the *op/op* mice had low numbers of osteoclasts due to a lack of mCSF leading to reduced recruitment and differentiation of osteoclasts. Thus, there were two requirements for functioning osteoclasts, the intrinsic machinery of the osteoclast and the appropriate mechanisms for their recruitment and differentiation.

7. Differentiation of the osteoclast

Osteoclasts are derived from circulating monocytes which in turn are derived from the haematopoietic stem cell (Fig 1.3) as described in the previous section.

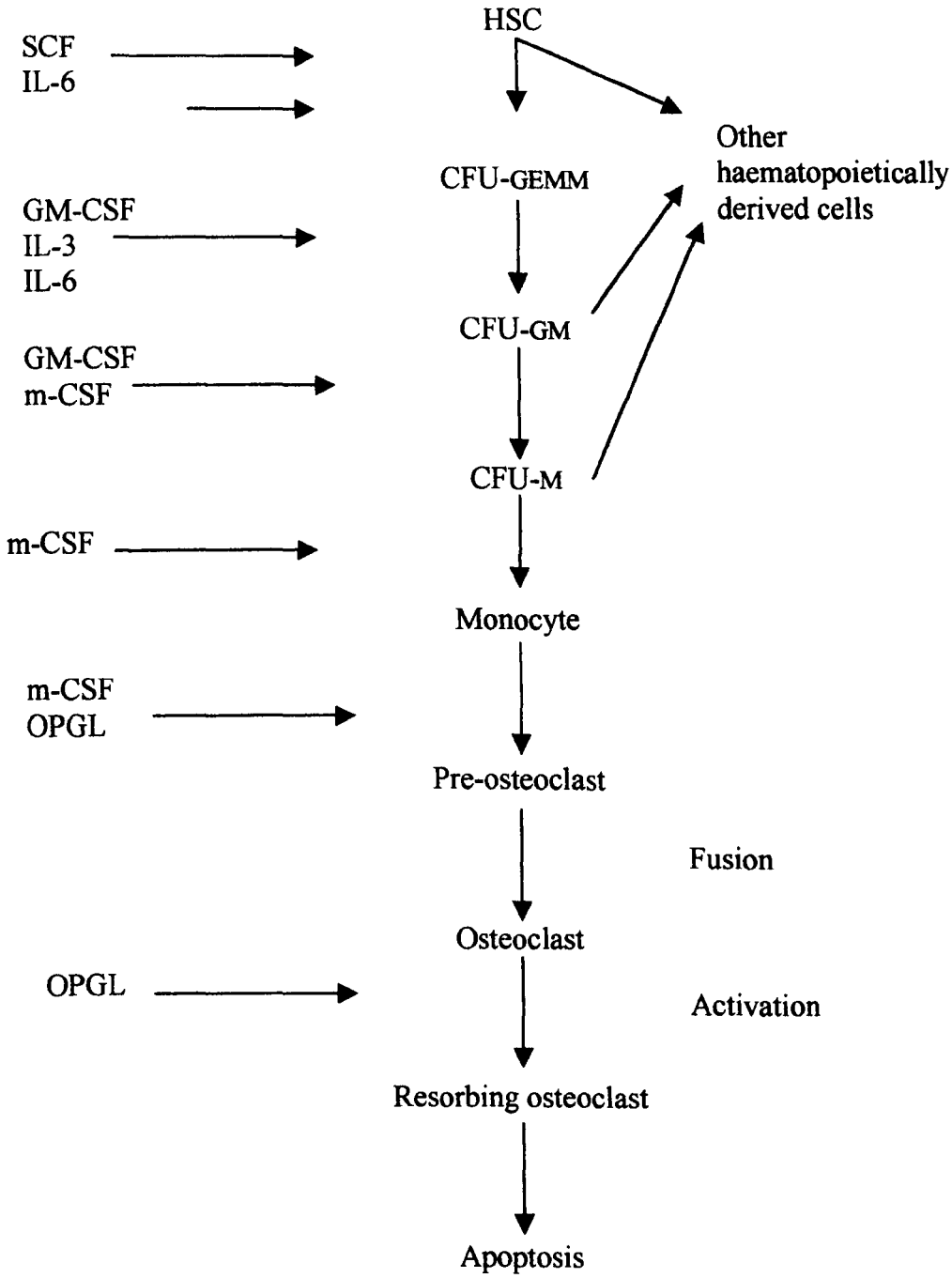


Figure 1.3 Osteoclast differentiation. Pluripotent stem cell in bone marrow give rise to granulocyte/erythrocyte/macrophage/megakaryocyte colony forming units (CFU-GEMM) which in turn lead to the formation of granulocyte/macrophage colony forming units (CFU-GM). These latter cells give rise to macrophage colony forming units (CFU-M) which form monocytes that circulate in the blood. Final differentiation and activation of the osteoclast takes place under the influence of m-CSF and OPGL.

The intermediate proliferative cell types between the haematopoietic stem cell and the monocyte become progressively restricted in their potential to differentiate. They include the colony forming units for the granulocyte, erythrocyte, monocyte and megakaryocyte. Their differentiation and proliferation is determined by specific growth factors and cytokine combinations (Hoffbrand & Pettit, 1995). The factors controlling the process of commitment and differentiation of the osteoclast have only recently started to be elucidated. One of the earliest demonstrations that osteoclasts could be formed *in vitro* (Marshall *et al*, 1986) demonstrated a requirement for osteoblastic stromal cells. This need for monocytes to come into contact with osteoblastic cells before osteoclast differentiation could take place was confirmed in cell culture studies by Takahashi *et al* (1988). Stimulators of bone resorption such as 1,25D3, PTH and PGE2 were found to act on the osteoblastic cells, not directly on the osteoclast precursors (Suda *et al*, 1995). Thus, a mediator appeared to be produced by osteoblastic cells in response to stimulators of bone resorption that when in contact with monocytes brought about osteoclast differentiation. Macrophage colony stimulating factor (mCSF), although necessary for osteoclast differentiation as demonstrated in the *op/op* mouse (Takahashi *et al*, 1991), was insufficient in this regard. The long sought after growth factor was discovered by two independent groups of workers (Lacey *et al*, 1998, Yasuda *et al*, 1998a) and termed osteoprotegerin ligand (OPGL) and osteoclast differentiation factor (ODF) respectively. The structure and properties of this cytokine will be discussed in detail in a later section (10.14). A soluble form of this membrane bound protein was found to stimulate bone resorption by isolated osteoclasts and, in the presence of mCSF, could induce the differentiation of circulating- (Quinn *et al*, 1998) or bone marrow derived- (Wani *et al*, 1999) monocytes into multinucleate osteoclasts which could resorb bone. Some of the phenotypic changes that the monocyte undergoes when differentiating into an osteoclast have recently been described (Takeshita *et al*, 2000). A cell population that was mCSF dependent was isolated from mouse bone marrow which in the presence of OPGL produced osteoclasts with high efficiency, in particular, all the cells were TRAP positive. This mCSF-dependent population of cells had many of the features of the monocyte/macrophage, they phagocytosed particles and they were positive for macrophage antigens, Mac-1 and 2, F4/80 and Fc receptor. In the presence of

OPGL and high levels of mCSF these surface antigens declined and proteins characteristic of the osteoclast were expressed, including calcitonin receptor, carbonic anhydrase II, cathepsin K, matrix metalloproteinase 9, TRAP and the integrin $\alpha v\beta 3$. The multinucleate cells that formed were capable of resorbing bone.

Several transcription factors and protein kinases have been found to be important in osteoclast differentiation. Tondravi *et al*, (1997) reported that the transcription factor *PU.1* was critical for osteoclast differentiation. They showed *in vitro* that *PU.1* expression increased as marrow macrophages became phenotypically osteoclastic. They also found that *PU.1* deficient mice are osteopetrotic and that marrow transplanted from normal mice restored normal osteoclastic and macrophage differentiation. *c-fos* is a proto-oncogene which is normally associated with osteosarcomas. Grigoriadis *et al* (1994) showed that mice lacking *c-fos* develop osteopetrosis but have normal macrophage differentiation. Udagawa *et al* (1996) reported that when co-cultures of mice bone marrow with primary osteoblastic cells were treated with *c-fos* antisense oligomers during the first four days of culture, osteoclast formation was inhibited. However, if the antisense oligomers were added during the second four day culture period osteoclast formation was unaffected. Together these findings suggest that *c-fos* is critical in the differentiation of osteoclast precursors at the point where the osteoclast lineage diverges from the macrophage.

Nf-κB is another transcription factor important in the differentiation of osteoclasts. It is in fact a family of transcription factors and mice deficient in two of the subunits, p50 and p52 develop osteopetrosis. Xing *et al* (1997) showed that the animals lack normal osteoclastic development. Nf-κB is also important in the expression of several cytokines involved in osteoclast differentiation including IL-1, TNFα, IL-6 and GM-CSF.

c-src is another proto-oncogene and plays an important role in the ability of osteoclasts to resorb bone. In mice lacking the *c-src* gene osteoclast formation is normal but they develop osteopetrosis as they are unable to resorb bone (Soriano *et al*, 1991). The osteoclasts do not form ruffled borders (Boyce *et al*, 1992). The osteopetrosis can be reversed by transplantation of bone marrow from normal mice expressing *c-src* (Lowe *et al*, 1993).

8. Mechanism of bone resorption.

The development of an *in vitro* model to study bone resorption using isolated osteoclasts and mineralized bone or dentine slices as a matrix allowed detailed studies of bone resorption to take place (Boyde *et al*, 1984; Chambers *et al*, 1984). The sequence of events which takes place during bone resorption is called the "resorption cycle." It covers the events which occur from when the osteoclast migrates to the site of resorption, attaches to the bone, becomes polarized and forms new membranes, dissolves the apatite and degrades the organic matrix, removes the products of resorption and finally either undergoes apoptosis or reverts to a non-resorbing state Fig 1.4). Following migration of the osteoclast to the site for resorption a membrane domain called the sealing zone forms underneath the osteoclast. The exact details of the interaction between the osteoclast plasma membrane and the bone matrix are unknown. However it has been shown that integrins play an important role at this early stage of the resorption cycle. There are at least four different integrins expressed on the osteoclast: $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 1$, and $\alpha 2\beta 1$ (Nesbitt *et al*, 1993). One of these integrins $\alpha v\beta 3$ has received much attention as it has been found that antibodies against $\alpha v\beta 3$ as well as peptides which contain the arginine/glycine/aspartic acid (RGD) amino acid motif such as echistatin and kistrin, are potent inhibitors of bone resorption both *in vivo* and *in vitro* (Horton *et al*, 1991; King *et al* 1994; Fisher *et al*, 1993). The $\alpha v\beta 3$ integrin is highly expressed in mature osteoclasts but the exact part that the integrin plays in the resorption cycle is unknown. Some workers have found that the integrin mediates the attachment of the sealing zone to the bone surface (Nakamura *et al*, 1996). However, others have failed to show the presence of the integrin at the sealing zone although it was present in the focal adhesions of migrating osteoclasts (Lakkakorpi *et al*, 1991). Other workers have also failed to show the presence of the integrin at the sealing zone using labelled echistatin (Masarachia *et al*, 1998). The precise molecules involved in the attachment of the sealing zone to the bone matrix have yet to be fully described.

Following attachment of the osteoclast to the bone matrix *via* the sealing zone another membrane domain, the ruffled border is formed. It is formed by the fusion of intracellular acidic vesicles together with the part of the plasma membrane

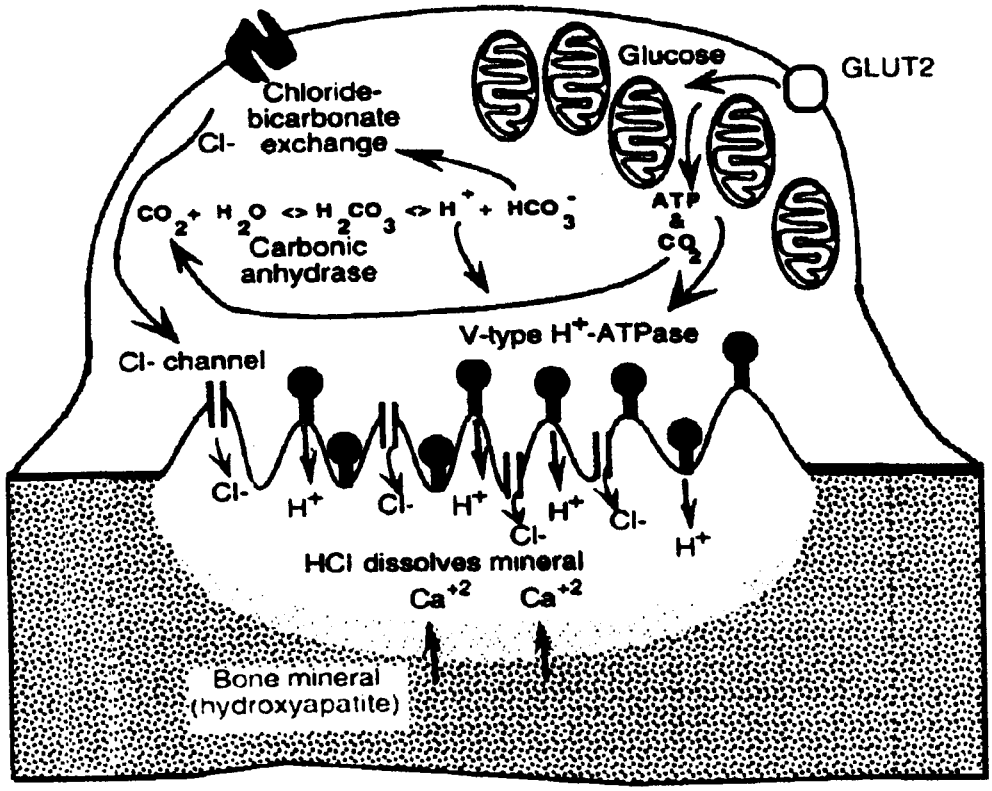


Figure 1.4 Diagrammatic representation of a resorbing osteoclast. The energy required to drive acid secretion by the osteoclast is derived from the oxidative phosphorylation of glucose, taken up by a GLUT2 transporter, in mitochondria. The source of protons for acid secretion is carbonic acid derived from carbon dioxide and water catalysed by carbonic anhydrase. In order to maintain intracellular pH within physiological limits, a passive chloride/bicarbonate exchange occurs at the basolateral membrane.

facing the bone. The membrane then forms long projections which enter the bone matrix. At this stage of the resorption cycle the osteoclast is highly polarized. The osteoclast can then begin to resorb bone. This involves the dissolution of the crystalline apatite as well as proteolytic cleavage of the organic matrix which is rich in collagen. The first process which must occur is the dissolution of the apatite crystals and this occurs through secretion of hydrochloric acid through the ruffled border into the lacuna below. The lacuna is the space between the ruffled border and the bone matrix and is kept separate from the extracellular fluid by, it was thought, the tight attachment of the sealing zone in order to prevent leakage of acid (Vaananen & Horton, 1995). However, a recent paper has challenged this view of the tight sealing zone and has suggested that it is not as tight as predicted (Stenbeck & Horton, 2000). They propose that the protons secreted through the ruffled border to dissolve the mineral phase become tethered to inorganic content of the bone matrix by ion exchange. In this situation there would be no need to prevent the leakage of acid with a tight sealing zone. In addition any products of degradation could diffuse out of the sealing zone. This system would allow bone resorption and cell movement to take place at the same time. The acidification of the resorption lacunae is achieved with a vacuolar proton pump which utilizes ATP and is found both at the ruffled border and in intracellular vacuoles (Vaananen *et al*, 2000). If the action of the proton pump is disrupted with bafilomycin A1, bone resorption is inhibited both *in vivo* (Sundquist & Marks, 1994) and *in vitro* (Sundquist *et al*, 1990). The protons for the pump are produced by cytoplasmic carbonic anhydrase II which is synthesized in large amounts in the osteoclast (Gay & Mueller, 1974). Excess bicarbonate which accumulates in the cell is removed by a chloride/bicarbonate exchanger which is found in the basolateral membrane located at the opposite end of the osteoclast to the ruffled border (Hall & Chambers, 1989). Chloride channels in the ruffled border allow chloride ions to flow from the cell into the resorption lacunae in order to maintain electrical neutrality.

Following dissolution of the mineral phase, the organic bone matrix is degraded by the action of several proteolytic enzymes. Cysteine proteinases play an important role in bone resorption demonstrated by the fact that E-64, a fungal inhibitor of cysteine proteinases, acts as an inhibitor of bone resorption both *in vitro* and *in vivo* (Vaes, 1988). Other workers also found that cystatin C, a mammalian

inhibitor of cysteine proteinases, also inhibited bone resorption (Lerner & Grubb, 1992). Lerner (2000) concludes that these and other studies show that these inhibitors work by inhibiting the activity of cysteine proteinases released into the resorption lacunae and that demineralization is unaffected. However, when bone mineral dissolution takes place without proteolytic breakdown of bone matrix, it is limited and so bone mineral release is eventually inhibited (Lerner, 2000). Several cathepsins which belong to the cysteine proteinase family have been found in osteoclasts and it is not known which are important to bone resorption. One of these *cathepsin K* has recently been cloned and is selectively expressed in osteoclasts (Inaoka *et al*, 1995). It has a pH optimum of 6-6.5 but does have activity over a broader range and is active at neutral pH (Clark & Murphy, 1999). Several authors have shown that cathepsin K plays a crucial role in bone resorption. For example Votta *et al* (1997) have shown that specific inhibitors of cathepsin K inhibit bone resorption both *in vivo* and *in vitro*. In addition Inui *et al* (1997) showed that treatment of isolated rabbit osteoclasts with antisense specific for cathepsin K resulted in decreased bone resorption.

Other enzymes which play a part in bone resorption are the matrix metalloproteinases or MMPs. These enzymes are maximally active at physiological pH. Stimulators of bone resorption increase levels of collagenase (MMP-1) (Vaes, 1988) and the response is seen mainly in the osteoblast. This suggests that collagenase is involved in the early stage of bone resorption, when the osteoid layer is removed by the osteoblasts to allow attachment of the osteoclast (Lerner *et al*, 2000). Several MMPs are thought to be present in osteoclasts and a tissue inhibitor of MMPs, TIMP-1, has been found in osteoclasts (Bord *et al*, 1999). Everts *et al* (1992) reported that in mouse calvaria treated with inhibitors of MMP's there was an accumulation of demineralized non-degraded bone matrix in the lacunae. These results suggest that MMPs are involved bone resorption. However, other workers have shown that inhibitors of MMPs do not inhibit resorption of dentine or bone slices by isolated osteoclasts (Delaisse *et al*, 1987). A recent study by Everts *et al* (1999) suggested that MMPs are important in bone resorption in calvaria but not in long bones, which may account for the differing results obtained by other people. In addition, there is no osteoid on bone or dentine slices, as there is on bone.

The manner in which the degradation products of osteoclastic bone resorption are removed is not well established. It may be that the osteoclast detaches from the bone surface before reattaching in order to allow degraded products to be removed. On the other hand following degradation of the bone matrix by cathepsins and MMPs, the resulting products may be removed by a process known as transcytosis. Vesicles pass from the ruffled border to the basolateral membrane where they are freed into the extracellular space (Nesbitt & Horton, 1997). Large amounts of bone matrix are removed by this method as the size of the resorption lacunae is often larger than the cell itself. It is not known to what extent the degradation of bone matrix takes place inside the vesicles or how much takes place in the extracellular compartment. It has been suggested recently that the enzyme TRAP is found in the transcytotic vesicles and can generate reactive oxygen species which are able to degrade collagen (Halleen *et al*, 1999).

9. Integrins and bone resorption

Integrins are a family of heterodimeric adhesion molecules which are involved in cell-matrix and cell-cell interactions and they regulate a variety of cell functions including leucocyte homing and activation, clot retraction, tumour metastases as well as bone resorption. They are composed of two subunits, α and β, and each combination has its own binding specificity and signalling properties. Each subunit has a large extracellular domain, a membrane spanning domain and a short cytoplasmic domain. It is thought that the short cytoplasmic domains of the α and β integrin subunits do not have enzyme activity but are able to interact with cytoplasmic proteins including cytoskeletal and signaling molecules (Yamada & Miyamoto, 1995). Members of the family are made of combinations of the 16 different α and 8 different β subunits which have so far been identified (Duong & Rodan, 1999). Osteoclasts express high levels of the integrin $\alpha v\beta 3$ as well as lower levels of other integrins $\alpha 2\beta 1$, a collagen/laminin receptor and $\alpha v\beta 1$, a fibronectin receptor (Duong & Rodan, 1999). When osteoclasts adhere to the bone surface there is an interaction between the cell and the extracellular matrix proteins within the bone matrix such as vitronectin, osteopontin and bone sialoprotein. These proteins have in common an RGD amino acid motif to which the $\alpha v\beta 3$ and other integrins

can bind. The importance of the αvβ3 integrin in bone resorption has been illustrated by the fact that blocking antibodies to αvβ3 block bone resorption *in vitro* (Horton *et al*, 1991). Blocking of bone resorption *in vivo* has also been shown using the disintegrin echistatin which blocked a PTH-induced increase in serum calcium (Fisher *et al*, 1993). Echistatin is a snake venom peptide which contains the amino acid motif RGD and is able to bind to integrins and block their action. The work of Fisher *et al* (1993) was later confirmed using another disintegrin, kistrin (King *et al*, 1994). Although the αvβ3 has been found to be critical in bone resorption, its exact mechanism of action in the osteoclast is not fully known. Nakamura *et al* (1998) showed that αvβ3 was important in the migration of osteoclast precursors prior to their fusion during osteoclast differentiation in culture. Some studies have found the αvβ3 in the clear zone of resorbing osteoclasts suggesting that the integrin may mediate the attachment of the sealing zone (Zambonin Zallone *et al*, 1989). However, other workers have been unable to locate the integrin in the sealing zone (Lakkakorpi *et al*, 1991). Instead the integrin was found to accumulate at the basolateral membrane, in intracellular vesicles and at the ruffled border of the resorbing osteoclasts. It is now thought that the formation of a seal between the osteoclast and the bone surface is not the most important function of the αvβ3 integrin (Tietelbaum, 2000). It is more likely that the transmission of signals from the bone matrix leading to the rearrangement of the cytoskeleton and formation of the ruffled border is of most importance (McHugh *et al*, 2000). In addition, the structural features of the integrin do not suggest that it forms the tight seal between the osteoclast and the bone surface (Horton, 1997). In agreement with these findings Masarachia *et al* (1998) found that the number of osteoclasts on the bone surface was not decreased in mice treated with echistatin suggesting that the blocking of bone resorption did not occur due to osteoclasts detaching from the bone surface. The αvβ3 integrin has been shown to play a role in the regulation of osteoclast activity in mouse parietal bones (Holt & Marshall, 1998). When bone resorption was inhibited *in vitro* with the non-steroidal anti-inflammatory drug indomethacin, osteoclasts were shown to detach from the bone surface and attach to the adjacent periosteum. When stimulators of bone resorption (PGE2, PTH and 1,25D3) were then added, osteoclasts re-attached to the bone surface and resorption commenced. Immunostaining of the β3 integrin subunit showed an increase from

the low level seen while osteoclasts were on the periosteum. Also this re-attachment was inhibited by echistatin and antibodies to the β3 subunit leaving β3 positive osteoclasts on the periosteum. Thus, it appears that this integrin can be modulated under the control of stimulators and inhibitors of bone resorption so that osteoclasts can attach to and detach from the bone surface (Holt & Marshall, 1998).

Several changes take place in the osteoclast once it has recognized and adhered to an RGD containing peptide or protein on the bone surface. These include alterations in intracellular calcium, protein tyrosine phosphorylation and cytoskeletal reorganization (Duong & Rodan, 1999). Duong *et al*, (2000) have proposed the following sequence of events based on current knowledge. Osteoclasts make contact with mineralized extracellular matrix which induces the αvβ3 integrins to cluster together. This in turn leads to phosphorylation of the adhesion kinase PYK2 by the proto-oncogene *c-src* (Duong *et al*, 1998). This signalling leads to actin reorganization and the association of several proteins with the cytoskeleton. These proteins include vinculin, paxillin (Duong *et al*, 1998) and Rho (Zhang *et al*, 1995) plus other kinases and phosphatases. This activity leads to the formation of the sealing zone which contains many of these proteins but not αvβ3 (Horton, 1997) or *c-src* (Tanaka *et al*, 1996). At the same time the ruffled border is formed followed by the pumping out of protons in order to dissolve the inorganic matrix and the release of enzymes to digest the organic matrix. The mechanism by which bone resorption is brought to an end has not yet been fully elucidated. In conclusion, the αvβ3 integrin is a natural target for the development of therapies which may help to block the excess bone resorption of osteoporosis.

10. Regulation of bone resorption

Adaptation of the skeleton to mechanical stresses placed upon it depends on bone remodelling. This is the controlled removal of bone by resorption followed by deposition of new bone by osteoblasts. If bone resorption exceeds bone deposition then osteoporosis will result and if bone deposition exceeds bone resorption then osteopetrosis will result. There are a variety of local growth factors and cytokines as well as systemic peptide and steroid hormones which regulate both the differentiation of precursor cells and the activity of mature cells in both the

osteoclastic and osteoblastic lineage. The majority of these factors act indirectly on the osteoclast *via* the osteoblast. A recently discovered cytokine, osteoprotegerin ligand (OPGL -see later section), has been found to be a potential link between the osteoblast and the osteoclast

10.1 Systemic hormones – PTH, 1,25D_3 and oestrogen

Parathyroid hormone (PTH) is synthesized in the parathyroid gland as a 115-amino acid single chain polypeptide and is called preproparathyroid hormone. The pre sequence is cleaved by a signal peptidase on the endoplasmic reticulum and the pro sequence is removed in the Golgi body. The resultant parathyroid hormone is 84- amino acids. It is important in the maintenance of normal calcium homeostasis and achieves this by stimulating osteoclastic bone resorption and increasing renal reabsorption of calcium. The first 34 amino acids are necessary and sufficient for the stimulation of resorption. The stimulation of bone resorption by PTH was first demonstrated by Barnicot, 1948. Later studies showed a similar effect of PTH in an organ culture system (Raisz & Nieman, 1967; Reynolds & Dingle, 1969). The primary target for PTH seems to be the osteoblast (Rodan & Martin, 1981). Isolated osteoclasts do not resorb bone when incubated with PTH and only did so when osteoblasts or osteoblastic cell lines were added to the cultures (McSheehy & Chambers, 1986). An *in vivo* study carried out by Uy *et al* (1995) showed that PTH had no effect on early osteoclast precursors but did increase the number of mononuclear osteoclast progenitors as well as mature osteoclasts. It is thought that PTH may activate *via* the cyclic 3'-5'-adenosine monophosphate (cAMP) pathway and increase secretion of IL-6 by osteoblasts (Greenfield *et al*, 1996). However, it has also been reported that PTH is a mitogen for highly purified human osteoclast precursors (Kurihara *et al*, 1991). PTH-rP is a major mediator of humoral hypercalcaemia of malignancy and is produced by several different types of cells. It has 70% homology for the first thirteen amino acids of PTH and it binds to the PTH receptor and activates cAMP (Reddy & Roodman, 1998). Although PTHrP was discovered to cause hypercalcaemia of malignancy it is also expressed by a large number of normal foetal and adult tissues.

Osteoblasts are known to express PTH receptors (Pilam *et al*, 1982) and recently Fuller *et al* (1998) showed that tumour necrosis factor receptor activation induced cytokine (TRANCE). TRANCE, a member of the TNF family and identical to OPGL, is expressed on the surface of osteoblastic cells and can account for the stimulation of bone resorption brought about by PTH. Lee & Lorenzo (1999) have shown that expression of mRNA for TRANCE was increased by the addition of PTH to murine bone marrow cultures. It was previously thought that osteoclasts did not possess PTH receptors but it has been reported that murine or avian osteoclasts express PTH receptors (Agarwala & Gay, 1992 ; Teti *et al*, 1991). It has also been shown that radio-labelled PTH binds to osteoclasts *in vitro* (Teti *et al*, 1991). Others workers have demonstrated the presence of mRNA for the PTH/PTHrP receptor in isolated rat and mouse osteoclasts (Tong *et al*, 1995).

10.2 1α,25 dihydroxyvitamin D_3 ($1,25D_3$)

The active metabolite of vitamin D, 1α25dihydroxyvitamin D_3 ($1,25D_3$), has many actions but its main role is related to bone metabolism and mineral homeostasis. The actions of $1,25D_3$ are mediated by a nuclear vitamin D receptor (VDR) which is a member of the family of steroid/thyroid hormone receptors. It had not been shown conclusively whether $1,25D_3$ acts directly on mature osteoclasts or indirectly through osteoblasts. However, mature osteoclasts have been shown to express vitamin D receptors (Meena *et al*, 1997). It is known to promote fusion of mononuclear osteoclast precursors (Kurihara *et al*, 1990a). The formation of osteoclast-like cells in co-cultures of bone marrow or spleen together with osteoblastic stromal cells is dependent on the presence of $1,25D_3$ (Carmeliet *et al*, 1999). The critical factor in this process is the presence of the stromal cells of the osteoblastic lineage. McSheehy & Chambers (1987) have shown that when calvarial osteoblasts were stimulated with $1,25D_3$ a soluble factor was released which was able to increase osteoclastic bone resorption *in vitro*. Further support for this mechanism was seen in co-culture experiments with VDR-deficient mice. When VDR-deficient osteoblastic cells were cultured with normal spleen cells in the presence of $1,25D_3$ no osteoclasts were formed. However, when normal osteoblastic cells were cultured with VDR-negative spleen cells normal osteoclasts were seen

(Takeda *et al*, 1997). The treatment of osteoclasts in culture with $1,25D_3$ is only required during the last two days of a six day culture period in order to produce significant numbers of osteoclasts (Takahashi *et al*, 1994). This suggests that $1,25D_3$ acts at the later stages of osteoclast differentiation. It is interesting to note that $1,25D_3$ is not needed *in vivo* for osteoclast formation as VDR-deficient mice have normal numbers of osteoclasts. Yasuda *et al* (1998a) have shown that $1,25D_3$ along with PTH and PGE_2 can increase expression of mRNA for OPGL in mouse primary osteoblasts which in turn stimulates osteoclastic activity. Presumably these other stimulators of bone resorption can compensate for the lack of VDR in VDR-deficient mice.

10.3 Oestrogen

The sex steroids have major effects on bone and calcium metabolism (Khosla *et al*, 1999). Many studies have been carried out into the effects of sex steroids, especially oestrogen, on osteoblasts and osteoclasts. It is not clear what the relative contributions of androgens and oestrogens to bone metabolism are but this section will concentrate on the effects of oestrogen. The low levels of oestrogen which occur in women following the menopause are a major cause of osteoporosis and so the mechanism of action of oestrogen in preventing bone loss has been the subject of much investigation. Oestrogen replacement therapy had been shown to be effective in the treatment and prevention of osteoporosis (Riggs & Melton, 1992). Oestrogen receptors have been identified on osteoblasts (Eriksen *et al*, 1988) and this led to the suggestion that oestrogen had direct effects on bone rather than working indirectly through a mediator such as another hormone. Oestrogen receptors have also been identified on osteoclasts (Oursler *et al*, 1994). As well as potentially having a direct effect on the osteoclast, oestrogen has been shown to increase the production of TGF-β by both osteoblasts (Oursler *et al*, 1991) and osteoclasts (Robinson *et al*, 1996), which may then inhibit osteoclastic bone resorption. In addition, it has been shown that oestrogen induces apoptosis in osteoclasts and this may be mediated by TGF-β (Hughes *et al*, 1996).

As well as the possible direct effects of oestrogen on osteoclasts, oestrogen may also regulate the production of cytokines, either by osteoblasts or bone marrow

cells, which in turn have an effect on osteoclasts (Manolagas & Jilka, 1995). It was reported by Pacifici *et al* (1991) that the production of IL-1 by peripheral blood monocytes was increased in women who had undergone ovariectomy and that this could be corrected by the administration of oestrogen. Another study showed that an antagonist to IL-1 receptor, which blocks the action of IL-1 when given to ovariectomized rats, could inhibit bone resorption (Kimble *et al*, 1994). In another study investigating the effects of TNF-α, when a soluble type 1 receptor which inhibits the action of TNF-α was given to ovariectomized rats, bone loss was inhibited (Kitazawa *et al*, 1994). All this data suggests that both IL-1 and TNF-α play a part in the bone loss which follows oestrogen deficiency.

Another cytokine which has been investigated as a candidate for mediating the effects of oestrogen on bone resorption is macrophage colony stimulating factor (m-CSF) which stimulates the proliferation and differentiation of osteoclast precursors (Tanaka *et al*, 1993). Stromal cells taken from mice following ovariectomy produce larger amounts of m-CSF than normal controls (Romas & Martin, 1997). In addition *in vivo* treatment with either oestrogen or a combination of antagonist to IL-1 receptor and TNF-α binding protein stopped the increase in m-CSF by the stromal cells *in vitro* from the ovariectomized mice (Kimble *et al*, 1996). The above results suggest that the increase in production of both IL-1 and TNF-α following ovariectomy led to an increase in m-CSF which in turn led to an increase in the number of osteoclast precursors.

Other investigators have found that IL-6 may also play an important role in mediating bone loss following oestrogen deficiency. It has been reported that oestrogen can suppress the IL-6 production induced by IL-1 or TNF-α in mouse bone marrow and osteoblastic cells (Girasole *et al*, 1992). Later Jilka *et al*, (1992) showed that a neutralizing antibody to IL-6 could block the increase in osteoclastogenesis seen in ovariectomized rats. Another study showed that oestrogen inhibited the production of mRNA for IL-6 as well as the protein induced by IL-1 and TNF-α in human osteoblastic cells (Kassem *et al*, 1996). It has also been shown that IL-6 gene knockout mice do not suffer from ovariectomy induced bone loss (Poli *et al*, 1994).

Thus it seems that several cytokines may mediate the effects of loss of oestrogen on bone and that they may act in concert rather than one cytokine

accounting for all the effects of oestrogen deficiency on bone. It has been shown that neutralizing antibodies to IL-6 may only partly inhibit bone resorption *in vivo* and in addition the bone resorption was inhibited by indomethacin which may indicate the involvement of prostaglandins in bone loss following ovariectomy (Miyaura *et al*, 1995). More recent work has shown that oestrogen can stimulate mRNA for osteoprotegerin, a member of the TNF receptor family, as well as protein production in human osteoblastic cells (Hofbauer *et al*, 1999a). Osteoprotegerin blocks the action of osteoprotegerin ligand which in turn induces the differentiation and activation of osteoclasts (see later section). The effects of oestrogen on bone formation are less clear.

10.4 Local factors - Prostaglandins

Prostaglandins act as local regulators of bone metabolism and may influence local bone cell activity (Buckwalter *et al*, 1995). Prostaglandins are a product of cyclo-oxygenases (COX-1&2) action on arachidonic acid. Prostaglandin E_2 was first found to stimulate bone resorption in foetal rat long bones thirty years ago (Klein *et al*, 1970) and further work has shown that prostaglandins play an important role in the regulation of bone metabolism. Prostaglandins are primarily made by osteoblastic cells but can also be produced by the bone marrow, vascular and connective tissue, and cells involved in the inflammatory process and this may also influence skeletal tissues. Results of early work showed that PGE_2 was the most abundant eicosanoid in bone (Raisz *et al*, 1979) and that prostaglandins of the E group are the most potent stimulators of bone resorption (Raisz *et al*, 1989). *In vivo* studies in animals and humans have shown that prostaglandins may be responsible for hypercalcaemia of malignancy (Minkin *et al*, 1981), bone loss in periodontal disease (Harris *et al*, 1973) and rheumatoid arthritis (Robinson *et al*, 1975).

Studies of the effects of prostaglandins on isolated osteoclasts showed an initial inhibitory effect (Fuller & Chambers, 1989), but this was found to be transitory and its importance is unknown. This rapid inhibition is in contrast to the slower stimulation of bone resorption which requires the differentiation of preosteoclasts (Collins & Chambers, 1992). In marrow cultures the addition of prostaglandin can increase the number of osteoclast-like cells (Akatsu *et al*, 1989).

Non-steroidal anti-inflammatory drugs (NSAIDS) such as indomethacin,which suppress prostaglandin production, can inhibit the activation of osteoclast formation by stimuli which suggests a role for endogenous prostaglandin production (Akatsu *et al*, 1989).

When endogenous prostaglandin synthesis is inhibited by indomethacin in mouse calvaria *in vitro*, bone resorption is inhibited (Lerner, 1987) and there is a decrease in the number of osteoclasts on the bone surface (Marshall *et al*, 1995; Garrett & Mundy, 1989). This was shown to be due to a detachment of osteoclasts from the bone and adhesion to the adjacent endocranial membrane (Marshall *et al*, 1996). This change in adhesion was reversed if PGE_2 was added to the culture medium (Marshall *et al*, 1995). Adhesion of the osteoclasts to the bone surface was accompanied by the appearance of the integrin subunit β3 on the osteoclast (Holt & Marshall, 1998).

Most factors which stimulate bone resorption also stimulate prostaglandin production and appear to act through the induction of COX-2, an enzyme involved in the synthesis of prostaglandins (Lorenzo & Raisz, 1999). One exception is TGF-β which inhibits osteoclasts directly but stimulates prostaglandin production (Bonewald & Mundy, 1990).

In bone organ culture prostaglandins have been found to have both inhibitory and stimulatory effects on collagen synthesis (Blumenkrantz & Sondergaard, 1972; Raisz and Koolemans-Beynon, 1974). At low concentrations or in the presence of glucocorticoids, PGE_2 increased collagen synthesis but at high concentration or in the presence of IGF-1, collagen synthesis was decreased (Chyun *et al*, 1984; Raisz and Fall, 1990). The major effect of administered prostaglandins in humans and animals seems to be the stimulation of bone formation (Akamine *et al*, 1992; Jorgensen *et al*, 1988).

The response of bone cells to mechanical forces may be prostaglandin dependent. Both stretching of bone cells (Harell *et al*, 1977) and fluid shear stress (Reich & Fangos, 1991) result in an increase in prostaglandin production. The increase in bone formation in response to impact loading has been shown to be prostaglandin dependent in avian and rodent models (El Haj *et al*, 1990; Pead and Lanyon, 1989). Immobilization rapidly leads to bone loss and may be prostaglandin dependent. Indomethacin inhibited tenotomy-induced bone loss in rats (Thompson

& Rodan, 1988) and NSAIDS can reduce urinary calcium excretion in immobilized patients (Filipponi *et al*, 1988).

10.5 Leukotrienes

Arachidonic acid may also be metabolized into leukotrienes. There has been much less investigation into the effects of leukotrienes on bone compared to prostaglandins but there is evidence to show that leukotriene B4 (LB4) can stimulate bone resorption and that other leukotrienes may have similar but less potent effects (Garcia *et al*, 1996). Other workers have examined the role of leukotrienes in bone resorption by inhibiting their synthesis with BWA4C, an inhibitor of 5-lipoxygenase. Rats treated with this compound showed few TRAP+ preosteoclasts compared with a the control group (Franchi-Miller & Saffar, 1995). They also found reduced numbers of mature osteoclasts which may illustrate the importance of leukotrienes in the recruitment and differentiation of osteoclast precursors.

10.6 Cytokines

Numerous cytokines have been found to be involved in osteoclastogenesis and osteoclast activity. Cytokines are commonly produced following leucocyte cell activation and they act on target cells through interaction with specific receptors on the surface of the target cells. Cytokines and their receptors can occur in soluble, membrane bound, or intra-cytoplasmic forms or they may be associated with the extracellular matrix (Muller-Newen *et al*, 1996). Soluble receptors may be generated either by proteolytic cleavage of the membrane bound form or by alternative splicing of the gene which codes for the membrane bound form. These receptors can modulate the activities of cytokines either by binding to their ligands (cytokines) and so blocking binding of the cytokine to a membrane bound receptor, or by enhancing ligand binding to the membrane bound receptor and stimulating the activity of the cytokine. These receptor proteins are critical to the activity of cytokines. Two of the most important cytokines to act upon the osteoclast are OPGL and m-CSF.

10.7 Macrophage colony stimulating factor (m-CSF)

Macrophage colony stimulating factor, sometimes referred to as CSF-1, is produced by osteoblastic/stromal cells and is vital, in combination with OPGL, for the enhancement by stromal cells of osteoclast development in co-cultures. The important role of m-CSF in the production of osteoclasts had been illustrated in work done on the *op/op* (osteopetrotic) mouse. These mice lack functionally active m-CSF due to a genetic mutation (Yoshida *et al*, 1990) which results in the osteopetrotic phenotype. *In vivo* administration of m-CSF restores the normal phenotype. *In vitro* work has also shown the vital role of m-CSF in osteoclastogenesis. When osteoblastic cells from normal mice were co-cultured with spleen cells from the osteopetrotic *op/op* mouse, normal osteoclast formation occurred. However, when normal spleen cells were co-cultured with osteoblastic cells from *op/op* mice no osteoclasts were formed. The treatment of co-cultures of *op/op* osteoblastic cells and normal spleen cells with m-CSF was essential for osteoclast formation (Tanaka *et al*, 1993). It was also found that absence of m-CSF during either the first four days of culture (proliferative phase) or the last two days (differentiation phase) prevented osteoclast formation. These results show that the defect in the *op/op* mouse is due to a fault in m-CSF production by the osteoblastic cells not the spleen cells (Takahashi *et al*, 1991).

It has been reported that the treatment of embryonic stem cells with an antibody to the m-CSF receptor, *c-fms*, resulted in a large decrease in the number of osteoclasts produced. The antibody did not affect the formation of any other haematopoietic cell types from the embryonic stem cells (Yamane *et al*, 1997). Others have reported that m-CSF itself can down regulate the expression of *c-fms* on osteoclast progenitors in mouse bone marrow cultures (Fan *et al*, 1997).

Mbalaviele *et al* (1995) showed that when human cord monocytes were cultured in the presence of rh m-CSF and stripped metatarsals, they became mature osteoclasts. In similar work macrophages were isolated from the synovium and monocytes were isolated from the peripheral blood of rhematoid arthritis patients. When cultured with rat osteoblast-like cells, m-CSF and $1,25D_3$, they differentiated into mature osteoclasts (Fujikawa *et al*, 1996). Sarma & Flanagan, (1996) have examined the effects of m-CSF on the formation of human osteoclasts *in vitro*.

They found that the basal levels of m-CSF in the marrow cultures were insufficient for large numbers of osteoclasts to form. The addition of m-CSF was needed to induce osteoclast formation and bone resorption above that was induced by $1,25D_3$.

10.8 Tumour necrosis factor (TNF)

Tumour necrosis factor (TNF) is the most potent stimulator of bone resorption yet identified (Thomson *et al*, 1987). TNF alpha and beta both stimulate osteoclast formation in mouse and human marrow cultures (Pfeilschifter *et al*, 1989). It seems to stimulate both the proliferation and differentiation of osteoclast precursors in addition to activating mature osteoclasts to resorb bone (Thomson *et al*, 1987). TNF binds to two cell surface receptors, TNF receptor 1 (p55) and TNF receptor 2 (p75) and both are capable of transmitting responses (Fiers, 1993). TNF may mediate the effects of oestrogen on bone. Rickard *et al* (1992) found that oestrogen could modulate TNF production in human osteoblastic cultures. However, others did not find any effects of oestrogen on TNF protein production in human osteoblast-like cell cultures (Chaudhary *et al*, 1992). TNF production in peripheral monocytes from women who had recently undergone ovariectomy was increased compared to those levels pre-ovariectomy (Pacifici *et al*, 1991). Kimble *et al* (1995) found that mice treated with soluble recombinant TNF receptor, an inhibitor of TNF, did not suffer from bone loss following ovariectomy. Similar results were obtained with mice which over-expressed a soluble form of TNF receptor 1 in that they did not lose bone following ovariectomy (Ammann *et al*, 1997).

10.9 Interleukin 1 (IL-1)

Interleukin-1 is produced by a variety of cells including monocytes, macrophages, and stromal cells (Reddy & Roodman, 1998). It may also be produced by osteoblasts (Keeting *et al*, 1991) and osteoclasts (O'Keefe *et al*, 1997). IL-1 is an extremely potent stimulator of bone resorption *in vivo* (Lorenzo *et al*, 1987) and can also stimulate bone resorption *in vivo* (Sabatini *et al*, 1988). IL-1 can also cause an increase in prostaglandin production in bone (Sato *et al*, 1986).

However, indomethacin does not block the effects of IL-1 *in vivo* which suggests that the effects of IL-1 are not entirely mediated by prostaglandins (Boyce *et al*, 1989). IL-1 may be involved in the development of post menopausal osteoporosis as one group had found that *in vivo* treatment with oestrogen reduced the amount of IL-1 released from cultured peripheral blood monocytes (Pacifici *et al*, 1989). Others have shown that the activity of IL-1α was increased in the marrow of ovariectomized mice compared to control mice or ovariectomized mice treated with oestrogen (Miyaura *et al*, 1995). This did not involve a change in the levels of IL-1α detected in the serum.

A natural inhibitor of IL-1 is known as IL-1 receptor antagonist (IL-1ra). It binds to but does not activate the IL-1 receptor (Arend *et al*, 1990). IL-1ra can block IL-1 stimulation of bone resorption and increased synthesis PGE_2 in organ cultures (Seckinger *et al*, 1990).

Studies have been done on the effects of oestrogen on IL-1 in humans. One group has shown that the levels of IL-1 were increased in serum four weeks after ovariectomy (Fiore *et al*, 1994). The administration of IL-1ra to ovariectomized rats inhibited bone loss with the greatest effect seen four weeks following surgery (Kimble *et al*, 1994). Additional evidence of the role of IL-1 in bone loss following oestrogen deficiency came from studies on mice which lack the IL-1 receptor type 1. They do not lose bone following ovariectomy unlike normal mice (Lorenzo *et al*, 1998). The finding that oestrogen treatment upregulates the production of IL-1 type 2 receptor (Sunyer *et al*, 1997) has been put forward as a theory as to how oestrogen might influence the IL-1 system. If IL-1 receptor type 2, which acts as a decoy receptor for IL-1, is increased by oestrogen without any effects on IL-1 production, it may decrease the response of bone to IL-1and so reduce the bone resorption caused by IL-1 (Lorenzo & Raisz, 1999).

10.10 Interleukin 6 (IL-6)

Interleukin 6 is produced by many cells in the bone environment including marrow stromal cells, monocytes, macrophages, osteoclasts, and osteoblasts (Roodman, 1999). The receptor for IL-6 is composed of two parts, a binding protein which can be either soluble or membrane bound, and gp-130, a protein which is used

by many cytokines as a receptor. The soluble receptor binds IL-6 and together they can activate the target cell *via* the gp-130 receptor. The role of IL-6 in osteoclast activity has not been fully elucidated. In mice it does not appear to cause osteoclast differentiation or bone resorption in calvarial organ cultures although levels were increased in response to PGE_2 and PTH (Holt *et al*, 1994). However, it does cause osteoclast formation *in vitro* in murine marrow cultures if soluble IL-6 receptor is added to the cultures (Tamura *et al*, 1993). IL-6 receptors have been found on human osteoclasts (Ohsaki *et al*, 1992) and IL-6 can induce osteoclast-like cell formation in human marrow cultures without added IL-6 receptors (Kurihara *et al*, 1990b).

The role of IL-6 in post menopausal bone loss had been investigated. The administration of a monoclonal antibody to IL-6 into ovariectomized mice for four weeks blocked both the bone loss and the increase in osteoclast formation (Jilka *et al*, 1992). They also found that the levels of IL-6 in bone marrow cultures were increased in ovariectomized mice compared to intact mice. However, other workers were unable to repeat either of these findings (Kitazawa *et al*, 1994; Kimble *et al*, 1997).

10.11 Interleukin 11 (IL-11)

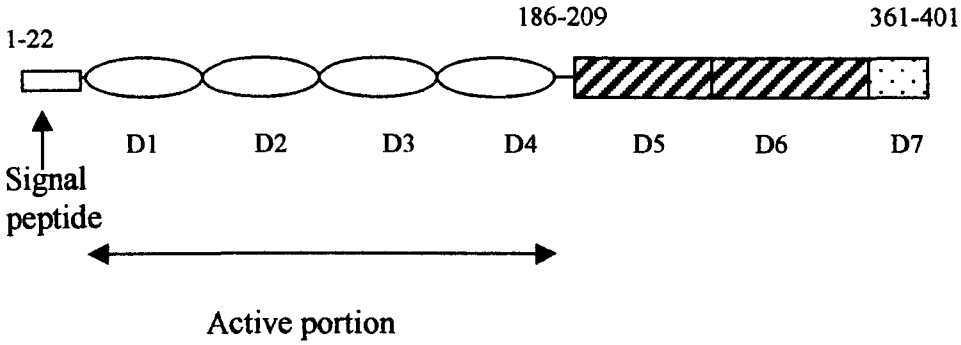
Interleukin-11 can be produced by stromal cells of the bone marrow (Paul *et al*, 1990). Girasole *et al* (1994) reported that IL-11 could induce the formation of osteoclasts in co-cultures of murine bone marrow and calvarial cells. Resorption lacunae were visible on calcified matrices suggesting that these were active osteoclasts. The study also showed that an antibody against IL-11 could suppress osteoclast development induced by $1,25D_3$, PTH, IL-1 or TNF. The effects of IL-11 appeared to be mediated through the induction of prostaglandin synthesis as indomethacin blocked the effects of IL-11 on osteoclast development. Others have also shown that osteoclast differentiation can be stimulated in porcine marrow cultures by recombinant human IL-11 (rhIL-11) and PGE_2 (Galvin *et al*, 1996).

10.12 Direct effectors of bone resorption

It was known for many years that many if not all the local cytokines and systemic hormones described above acted indirectly on the osteoblast or stromal cell to produce a factor which acts directly on the osteoclast or its precursor to bring about differentiation and activation. Recently, this factor named OPGL, TRANCE, ODF, or RANKL by different authors, has been discovered and has been the subject of intensive investigation (Fig. 1.6). In addition a second factor has been found OPG or OCIF (Fig. 1.5) which blocks the effects of the stimulating factor and has itself become a focus for attention as a possible therapy for the treatment and prevention of osteoporosis. Both factors have been independently discovered by several different groups and so several names are in general use for what are the same molecules.

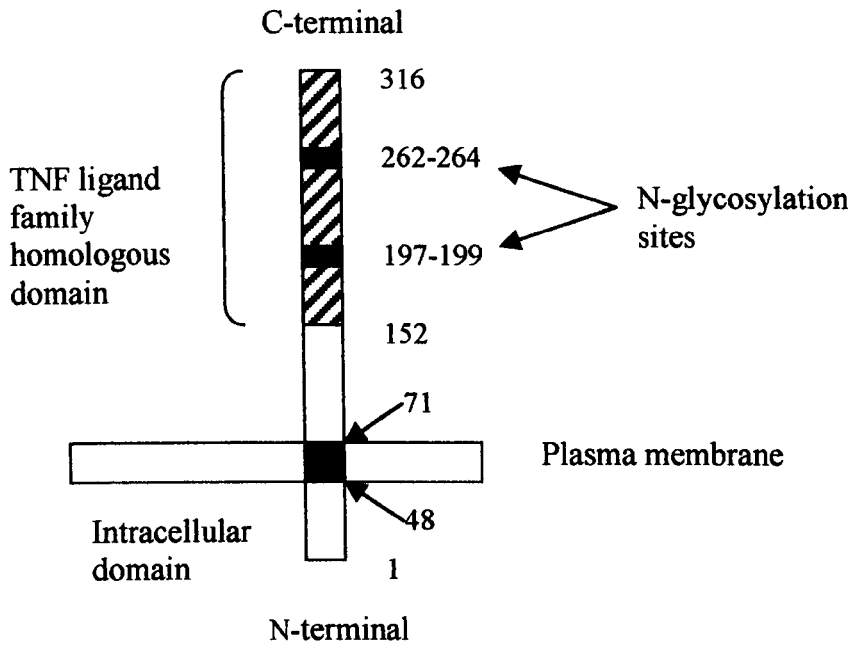
10.13 Osteoprotegerin (OPG) and osteoclastogenesis inhibitory factor (OCIF)

OPG (Fig 1.5) was independently identified by two different groups. Simonet *et al* (1997) identified OPG as a possible member of the TNF receptor family whilst sequencing foetal rat intestine cDNA. It was found to encode a 401 amino acid polypeptide which had several features of a secreted glycoprotein. They were unable to find any matches in the database but it showed sequence homology with members of the TNF receptor family. However, it did not contain a membrane-spanning sequence suggesting that it was a secreted protein. In further work, mouse and human cDNA for OPG was also found to encode 401 amino acid proteins. The mouse and human proteins are 85% and 94% identical to the predicted rat protein so the OPG gene has been highly conserved. It was found to be synthesized as a 55 kDa monomer which then becomes linked *via* a disulfide bridge to another monomer to form a homodimer of approximately 110 kDa. Another group independently identified a factor in the conditioned media of human embryonic lung fibroblasts, IMR-90, which could inhibit osteoclast-like cell development (Tsuda *et al*, 1997). It was termed osteoclastogenesis inhibitory factor (OCIF). It was found to be a heparin-binding basic glycoprotein and existed as a monomer and a homodimer.



Nomenclature	Alternative nomenclature
OPG Osteoprotegerin	OCIF Osteoclastogenesis inhibitory factor
	TRI TNF receptor-like molecule 1

Figure 1.5 Diagrammatic representation of the structure of mouse OPG. It is composed of 401 amino acids. Four cysteine rich domains exist between residues 22 and 186 (D1-D4). Two death domain homologous regions exist between residues 209-361 (D5-D6). A cysteine residue at 400 is essential for dimer formation. D7 is a heparin binding domain.



Nomenclature	Alternative nomenclature
OPGL Osteoprotegerin ligand	RANKL Receptor activator of NF-κB ligand
	ODF Osteoclast differentiation factor
	TRANCE TNF-related activation induced cytokine

Figure 1.6 Diagrammatic representation of the structure of mouse OPGL. It is composed of 316 amino acids. The predicted transmembranous region is between residues 48 and 71. The TNF homologous region is between residues 152 and 316. Two possible N-glycosylation sites exist at residues 197-198-199 and residues 262-263-264.

Kwon *et al* (1998) identified a factor from a search of an expressed sequence tag database which encoded a 401 amino acid secreted glycoprotein with a molecular weight of 62 kDa. It was called TNF receptor-like molecule 1 (TR1). Tan *et al* (1997) also identified TR1 following a search of a cDNA database. This protein is identical to OPG and will be referred to as OPG throughout.

Following translation of the gene, the 21 amino acid signal peptide which indicates that the protein is to be secreted, is cleaved leaving the OPG protein with 380 amino acids (Simonet *et al*,1997). The mature human protein has been found to consist of seven separate domains. The N-terminal portion contains four cysteine-rich domains which are then followed by two death domain homologous regions. The final domain contains the heparin binding site and amino acid 400 is always a cysteine which is essential for dimer formation (Suda *et al*,1999). The formation of a dimer is not critical for the activity of OPG as the substitution of the cysteine residue with serine did not affect the inhibitory action of OPG (Suda *et al*,1999).

OPG is expressed in many different tissues and cell lines and is not restricted to bone (Simonet *et al*,1997). In human foetal tissue mRNA for OPG is most highly expressed in the lung, kidney, liver, and brain (Yasuda *et al*,1998b). In adult human high levels of mRNA for OPG have been found in the heart, lung, kidneys and bone (Simonet *et al*,1997). Other tissues which express mRNA for OPG are the placenta, liver, thyroid, spinal cord, and the brain (Yasuda *et al*,1998b). In mice the highest levels of mRNA for OPG were detected in the heart, lung, kidney, liver, brain, placenta, stomach, intestine, skin, and calvaria (Simonet *et al*,1997). Various human and mouse primary cells and cell lines express mRNA for OPG such as mouse stromal cell line ST2 (Yasuda *et al*,1998b), mouse osteoblastic MC3T3 (Yasuda *et al*, 1998b), human osteoblastic TF274 (Tan *et al*, 1997) and the human marrow stromal preosteoblastic cell line hMS (Hofbauer *et al*, 1998). The osteosarcoma cell line MG-63 produces the largest amounts of OPG among the osteoblastic cell lines (Vidal *et al*, 1998b).

Two model systems have been used to investigate the role of OPG in the skeleton, the OPG knockout mouse and the transgenic mouse overexpressing OPG. Bucay *et al* (1998) and Mizuno *et al* (1998) have both generated OPG knockout mice. They found increased postnatal mortality which was associated with an increased incidence of vertebral and endochondral bone fractures. The mice were

normal at birth suggesting that OPG deficiency did not affect embryogenesis or organogenesis but they did show decreased bone mineral density with thin cortical bone at the femur and the pelvis. They also suffered from multiple fractures especially of the long bones. Older mice suffered from compression fractures of the vertebrae leading to severe deformities (Bucay *et al*, 1998). At two months of age the trabecular bone mineral density of the OPG deficient mice was decreased by 45% and the cortical bone density by 19% compared to normal littermates (Bucay *et al*, 1998). It was also found that the OPG deficient mice had arterial calcification at two weeks of age which became much more pronounced by two months of age in up to 60% of the mice (Bucay *et al*, 1998). The overexpression of OPG in transgenic mice results in early osteopetrosis at various sites including the long bones, vertebrae, and the pelvis (Simonet *et al*, 1997). They showed no other abnormality other than splenomegaly which was due to extramedullary haematopoiesis. There was an increase in mineralized trabecular bone including the spaces normally occupied by active marrow.

Many studies have been carried out investigating the effects of OPG *in vitro*. Simonet *et al* (1997) showed that an OPG-Fc fusion protein could inhibit the development of TRAP+OC and TRAP activity in a spleen cell and bone marrow co-culture system. The OPG fusion protein at a concentration of 10 to 100 ng cm^{-3} could completely prevent the formation of osteoclasts whereas 50% could be inhibited by a concentration of 1 ng cm^{-3} (Simonet *et al*, 1997). Another group using OPG purified from medium used to culture foetal lung fibroblasts, found that osteoclastogenesis could be inhibited at concentrations of 1 to 40 ng cm^{-3} (Tsuda *et al*, 1997). Over an eleven day culture period the sensitive period for the action of OPG *in vitro* was shown to be between days five and eleven as addition of OPG between days zero and three or on day seven or eight had no effect on osteoclastogenesis (Akatsu *et al*, 1998). Yasuda *et al* (1998b) reported that osteoclast development was inhibited when OPG was added for days three and four of a six day culture. A recent study has also shown that OPG could decrease osteoclast survival by inducing apoptosis (Akatsu *et al*, 1998). OPG has also been shown to inhibit osteoclastogenesis induced by $1,25D_3$, PGE_2, PTH, IL-11, and IL-1 (Kwon *et al*, 1998).

In addition to inhibiting osteoclastogenesis, OPG was also found to inhibit the activity of mature osteoclasts. Kwon *et al* (1998) found that OPG inhibited the ability of osteoclasts to form pits and Tsukii *et al* (1998) found that OPG inhibited calcium release from mouse foetal long bones in culture when stimulated by $1,25D_3$, PGE_2, PTH, and IL-1α. Further evidence that OPG inhibits both osteoclastogenesis and the activity of mature osteoclasts came from Fuller *et al* (1998) who showed that OPG inhibited both bone resorption and the stimulation of osteoclasts by osteoprotegerin ligand (OPGL). Hakeda *et al* (1998) reported that the effects of OPG on isolated rabbit osteoclasts were independent of the OPGL mechanism in that OPG could directly affect the formation of the F-actin ring, a cytoskeletal structure necessary for bone resorption, and so inhibit the activity of mature osteoclasts.

Work has also been carried out to investigate the effects of administered OPG *in vivo*. Four week old mice were treated with daily injections of recombinant mouse OPG for seven days which resulted in an increase in trabecular bone mass at the proximal tibial metaphysis (Simonet *et al*, 1997). Similar results were obtained by Yasuda *et al* (1998b) using rats. Simonet *et al* (1997) also showed that OPG could prevent the bone loss associated with oestrogen deficiency. When OPG was administered to mice for two weeks following ovariectomy there was an increase in bone volume and a decrease in the number of osteoclasts compared to untreated ovariectomized controls.

As previously described, various cytokines and hormones have the effect of either stimulating or inhibiting osteoclastogenesis. Since the discovery of OPG many studies have been carried out to see whether OPG acts a mediator for any of these cytokines or hormones. It has been shown in various human osteoblastic cell systems that OPG mRNA and protein can be stimulated. Hofbauer *et al* (1998) showed an increase of 90% and 50% in OPG mRNA expression following treatment with $1,25D_3$ in a foetal osteoblastic cell line (hFOB) and normal trabecular osteoblastic cells, respectively. However, they found no effect in marrow stromal preosteoblastic cell line. They also found that BMP-2, IL-1β, and TNF-α increased OPG mRNA levels in hFOB by 4, 6 and 4 fold, respectively. Treatment with $1,25D_3$, BMP-2, IL-1β, and TNF-α increased OPG protein production in hFOB cells by 60, 390, 300, and 80%, respectively. Vidal *et al* (1998b) have shown that

IL-1α caused an increase in OPG mRNA in MG-63 cells. Brandstrom *et al* (1998a) showed that TNF-β could also increase levels of OPG mRNA in MG-63 cells. Studies using primary mouse osteoblasts have shown that mRNA levels for OPG can be down-regulated with $1,25D_3$, IL-1α, and PTH (Murakami *et al*, 1998). Other studies using mouse osteoblastic/stromal cells showed a decrease in OPG mRNA in marrow stromal cell lines ST-2 and tsJ2 by $1,25D_3$ (Yasuda *et al*, 1998b; Horwood *et al*, 1998) but not in primary osteoblasts (Horwood *et al*, 1998). Some of these differences may be due to species differences or may be a result of differences in the cell systems with regard to their stage of differentiation. The inhibitor of osteoclastic bone resorption TGF-β (Pfeilshifter *et al*, 1988) is also able to stimulate the production of mRNA for OPG in bone marrow stromal cell lines as well as primary osteoblasts and to inhibit osteoclastogenesis (Murakami *et al*, 1998; Takai *et al*, 1998). Both groups of workers also showed that an antibody to OPG could prevent the inhibitory effects of TGF-β on osteoclastogenesis suggesting that OPG could directly mediate the effects of TGF-β. It has been thought by some that OPG may play a role in bone loss which occurs following the menopause and decreased levels of oestrogen. A recent study has shown that 17β-oestradiol can stimulate an increase in the concentrations of both mRNA and OPG protein in human foetal osteoblastic cell line in a dose and time dependent manner (Hofbauer *et al*, 1999a). Treatment with an oestrogen antagonist could block these effects.

The effects of glucocorticoids on levels of OPG have been investigated as long term use of these agents can lead to osteoporosis (Canalis, 1996). The exact mechanism by which glucocorticoids induce osteoporosis has not been fully elucidated and as OPG has been shown to inhibit bone resorption, recent work has been carried out to investigate whether OPG has a role in bone loss following prolonged use of glucocorticoids. Vidal *et al* (1998a) showed that mRNA for OPG could be down-regulated by glucocorticoids in primary human osteoblast-like cells. Other workers using mouse cell systems showed that the glucocorticoid dexamethasone decreased OPG mRNA levels in the stromal cell line ST-2 whereas in primary osteoblasts they saw an initial decrease in OPG levels followed by an increase (Murakami *et al*, 1998). Thus, the newly discovered protein, OPG, has the unexpected property of inhibiting osteoclast differentiation and is itself negatively regulated by bone resorptive agents.

10.14 Osteoprotegerin ligand (OPGL)

Following the discovery of OPG the search was on for its ligand. Using an OPG-Fc fusion protein as a probe Lacey *et al* (1998) found a binding site for the protein on the surface of a murine myelomonocytic cell line 32D. They then were able to clone a 316 amino acid gene product in mammalian cells termed OPGL (Fig. 1.6). This was closely followed by cloning of human OPGL from a lymph node cDNA library. At about the same time Yasuda *et al* (1998a) cloned osteoclast differentiation factor (ODF) from a library of the mouse marrow stromal cell line ST-2 after treatment with $1,25D_3$ and dexamethasone. Both groups concluded that OPGL and ODF were identical to two other members of the TNF family which had been independently cloned, namely TNF related activation-induced cytokine (TRANCE) and receptor activator of nuclear factor NF-κB ligand (RANKL) (Wong *et al*, 1997; Anderson *et al*, 1997).

The human and mouse OPGL are 317 and 346 amino acids respectively with the human OPGL having an 87% homology with the mouse OPGL indicating conservation throughout evolution (Lacey *et al*, 1998). Analysis of OPGL showed a transmembranous region (Lacey *et al*, 1998). Human OPGL has a cytoplasmic domain, a transmembrane domain and an extracellular region consisting of two domains, a stalk region and the active ligand site (Lacey *et al*, 1998). OPGL may be found in the cell-bound form and also in a soluble form (Lacey *et al*, 1998). The soluble form is derived from the cell-bound form by post-translational processing at amino acid position 139 (murine OPGL) and 140 or 145 (human OPGL) (Lacey *et al*, 1998). It is not known whether the cleavage occurs by the action of an enzyme and what the distribution of this enzyme might be.

Expression of OPGL is abundant in the skeleton and in the lymphoid tissues (Lacey *et al*, 1998). Other sites of mRNA for OPGL include heart, placenta, skeletal muscle, stomach, thyroid, and peripheral blood leucocytes (Anderson *et al*, 1997). In rats mRNA for OPGL is highly expressed in lymph nodes, spleen, thymus, and the cerebellum (Lacey *et al*, 1998). In addition message has also been detected in murine marrow cell lines ST-2 and MC3T3-E1, the rat osteosarcoma cell line ROS (Lacey *et al*, 1998), primary murine calvarial osteoblasts (Yasuda *et al*, 1998a) as well as several murine lymphoid cell lines.

To help study the role of OPGL in the skeleton, OPGL knockout mice have been generated. They appear normal at birth but there is an increase in bone mineral density throughout life and the long bones lack a normal marrow space leading to extramedullary haematopoiesis in the spleen (Kong *et al*, 1999). They lack mature osteoclasts and have shorter broad and flat bones (Kong *et al*, 1999). These mice also have abnormalities of the immune system including a defect of T and B cell maturation (Kong *et al*, 1999). Administration of OPGL can reverse the bone abnormalities and causes the differentiation of osteoclasts indicating that the precursor cells are present but in the absence of OPGL they are unable to differentiate (Kong *et al*, 1999).

Many studies have looked at the *in vitro* effects of OPGL the major ones being the promotion of differentiation, fusion, activation and survival of osteoclasts (Lacey *et al*, 1998; Jimi *et al*, 1999). It has been shown that the addition of OPGL and m-CSF to bone marrow monocytes *in vitro* without any additional hormones, cytokines or stromal cells being present, is able to induce the differentiation of mature osteoclasts (Lacey *et al*, 1998). OPGL is also able to increase TRAP activity and the number of TRAP+OC (Lacey *et al*, 1998). During differentiation of osteoclasts, OPGL increases the levels of mRNA for some proteins which are used to characterize cells of the osteoclastic lineage namely TRAP, β3 integrin, cathepsin K, *c-src*, and the calcitonin receptor (Lacey *et al*, 1998). OPGL has more recently been shown to be able to stimulate the differentiation of osteoclasts from human monocytes derived from peripheral blood and these osteoclasts were capable of bone resorption (Quinn *et al*, 1998). As well as promoting the differentiation of osteoclasts, OPGL has been shown to be capable of activating mature osteoclasts to resorb bone. Lacey *et al*, (1998) showed that OPGL was able to activate mature rat osteoclasts to form resorption lacunae on bovine bone slices. OPGL was also able to increase calcium release in an organ culture system indicating bone resorption was occurring and when OPG was added to the system the calcium release stimulated by the OPGL was abolished (Tsukii *et al*, 1998).

The *in vivo* effects of administered OPGL have been studied and in mice are manifested as severe hypercalcaemia within one day indicating the activation of mature osteoclasts (Lacey *et al*, 1998). Radiographs of these mice showed a marked bone loss which was associated with the presence of larger than normal osteoclasts

with an increased number of nuclei which covered a larger than normal area of bone. There was no increase in the total number of osteoclasts (Lacey *et al*, 1998).

In general hormones and cytokines which stimulate bone resorption have been found to increase the production of OPGL. The up-regulation of mRNA for OPGL has been shown to occur in the presence of dexamethasone in the mouse bone marrow derived stromal cell line ST2 (Yasuda *et al*, 1998a). $1,25D_3$ also produced an increase in mRNA for OPGL in ST2 cells and mouse primary osteoblasts (Yasuda *et al*, 1998b; Horwood *et al*, 1998). In addition, IL-11and PTH brought about an increase in mRNA for OPGL in mouse primary osteoblasts (Horwood *et al*, 1998; Yasuda *et al*, 1998b). PGE_2 also increased mRNA for OPGL in mouse primary osteoblasts (Yasuda *et al*, 1998b). More recently the pro-inflammatory cytokines IL-1β and TNF-α have been shown to increase the production of mRNA for OPGL in normal and immortalized marrow stromal cell lines and in the osteosarcoma cell line MG-63 (Hofbauer *et al*, 1999b). Another study adds to the evidence that some hormones and cytokines indirectly regulate bone resorption by the production of OPGL. When bone resorption was stimulated by $1,25D_3$, PTH, PGE_2, and IL-1α, the addition of OPG or neutralizing antibodies to OPGL, blocked the bone resorption (Tsukii *et al*, 1998). However, the effects of IL-α were not as marked as those of the other effectors. Takai *et al* (1998) found that TGF-β1 suppressed the expression of mRNA for OPGL whilst at the same time increasing levels of mRNA for OPG in mouse cell lines. Formation of osteoclasts was inhibited by TGF-β1 and this could be reversed by the addition of neutralizing antibodies to OPG. Thus, OPG production is stimulated by a variety of bone resorbing factors and it acts directly on the osteoclast precursor to bring about differentiation and activation of the osteoclast.

10.15 Receptor activator of NFκB (RANK)

RANK is the receptor for RANKL or OPGL and is found on the osteoclast (Fig 1.7). It was first characterized in dendritic cells by Anderson *et al*, (1997). It is a transmembranous protein consisting of 616 amino acids which form a signal peptide, an extracellular domain, a transmembrane domain and a cytoplasmic domain (Anderson *et al*, 1997). A signal peptide is a short amino acid sequence that

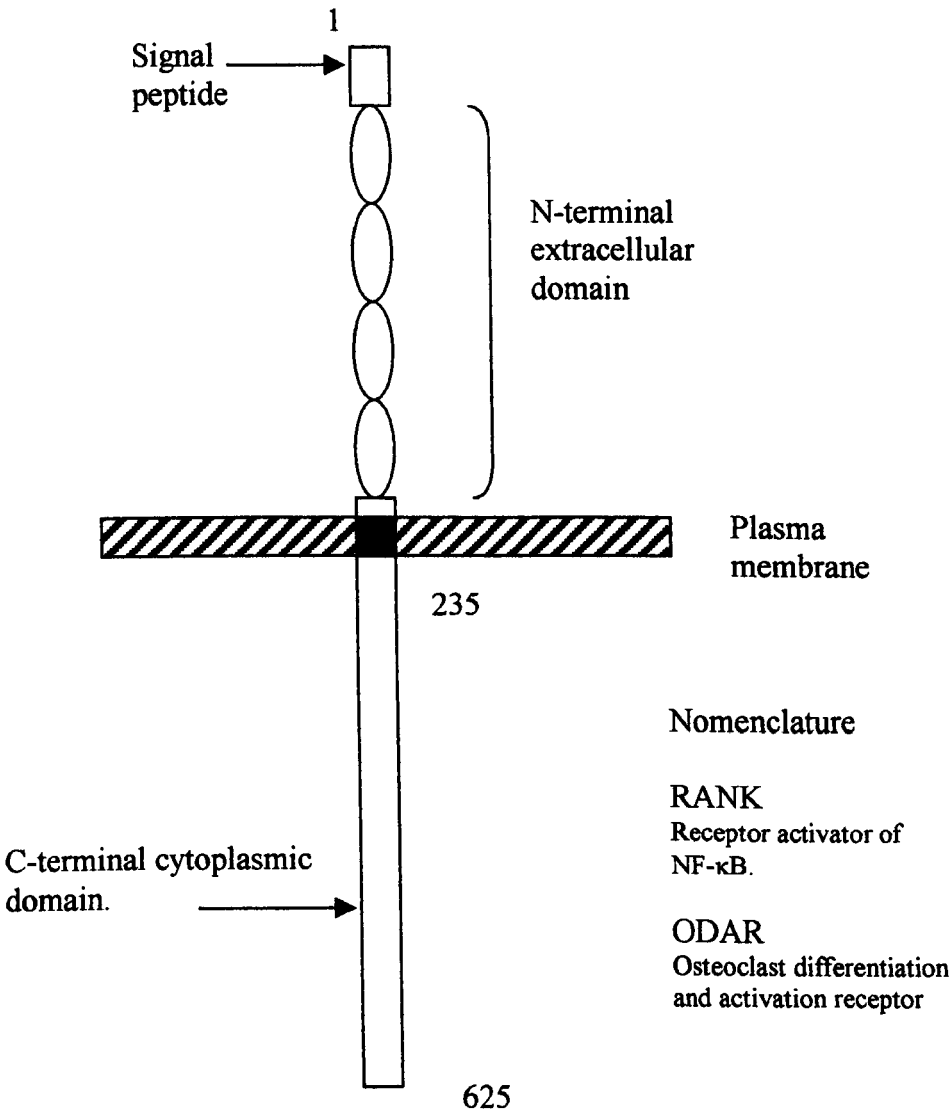


Figure 1.7 Diagrammatic representation of the structure of mouse RANK. It is a tumour necrosis receptor family member and is composed of 625 amino acid residues. Like OPG it has four extracellular cysteine rich domains. Human RANK is 70% homologous to mouse RANK. Of the C-terminal domain, 93 amino acids which bind TRAF 2, TRAF 5 and TRAF 6 are essential for NF-κB activation.

directs a protein to a specific site within the cell. The human RANK amino acid sequence is 70% homologous to the mouse form (Anderson *et al*, 1997). The receptor was more recently identified and characterized in normal mouse osteoclast progenitors by Hsu *et al*, (1999) and called osteoclast differentiation and activation factor (ODAR).

RANK mRNA is expressed in many tissues including skeletal muscle, liver, small intestine, colon, thymus and adrenal gland (Anderson *et al*, 1997). It is also expressed in lymphocytic cell lines (B lymphoblastoid cell line) and non-lymphocytic cell lines (K-562 chronic myeloid leukaemia cell line, A-172 glioblastoma, HFF human foreskin fibroblasts, and WI-26 (SV40-transformed lung fibroblasts) as well as in osteoclasts (Anderson *et al*, 1997; Hsu *et al*, 1999). Although the message for RANK is found in many tissues the expression of protein is found only in osteoclasts, dendritic cells, B and T cell lines, and fibroblasts (Anderson *et al*, 1997). It has been established by at least two groups that OPGL binds to RANK and that it does so with a high affinity (Nakagawa *et al*, 1998; Hsu *et al*, 1999). Both groups also showed that OPGL binding to RANK is both necessary and sufficient for osteoclastogenesis. Strong evidence that RANK is the only receptor for OPGL was provided by Nakagawa *et al* (1998) who used a genetically engineered soluble form of RANK, a polyclonal antibody to RANK, and a fragment of the antibody (Fab) to investigate whether any of them would induce osteoclast formation in mouse spleen cells. They showed that the antibody to RANK was able to induce osteoclast formation whereas both the soluble form of RANK and the antibody fragment blocked the binding of OPGL to RANK and so suppressed osteoclast formation normally induced by OPGL. The study also suggested that the clustering of RANK is needed for activation of osteoclasts as the Fab fragment was unable to cross-link and cluster antigens and did not result in osteoclastogenesis.

When OPGL binds to RANK intracellular signals are passed *via* the receptor which lead to the activation of the osteoclast (Anderson *et al*, 1997). RANK interacts with members of a family of proteins called TNFR-associated factor (TRAF) in order to activate the nuclear transcription factor NF-κB and the protein kinase c-Jun N-terminal kinase (JNK) (Wong *et al*, 1998). NF-κB is a member of a family of transcription factors that regulate the expression of a series of target genes

involved in development and immunity (Abu-Amer & Tondravi, 1997). The TRAF family consists of six members and they are known to regulate cell survival, proliferation, and apoptosis *via* activation of NF-κB and other transcription factors (Arch *et al*, 1998). The intracellular domain of RANK is thought to interact with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 (Wong *et al*, 1998). A recent study has shown that when OPGL binds to RANK which itself is bound to TRAF6, NF-κB becomes dissociated from I κB to which it is bound in the cytoplasm (Wong *et al*, 1999). NF-κB then translocates to the nucleus. Together with other transcription factors NF-κB promotes osteoclast differentiation by activating specific genes Abu-Amer & Tondravi, 1997). A recent study showed that TRAF6 deficient mice had osteopetrosis and abnormal tooth eruption (Lomaga *et al*, 1999). The osteoclasts did not form ruffled borders suggesting an important role for TRAF6 in osteoclast activation. In conclusion, RANK is the receptor of OPGL on the osteoclast precursor and brings about differentiation *via* activation of NF-κB.

11. Aims of the study

The purpose of the study was to elucidate the mechanisms controlling bone resorption with a view to identifying potential targets for therapeutic intervention in diseases where excessive bone loss occurs.

The aim was to understand the mechanism whereby osteoclasts differentiate from monocytes and then become activated to resorb bone. The model chosen to investigate these processes was the mouse calvarial culture system. Previous work with this system (Marshall *et al* 1995, 1996) had shown that when spontaneous production of prostaglandins was inhibited by indomethacin osteoclasts detached from the surface of the bone and attached to the adjacent periosteum. This process could be reversed by the addition of prostaglandin E2 which caused osteoclasts to re-attach to bone and start to resorb. PTH and 1,25D3 could also reverse the inhibition by indomethacin and stimulate bone resorption. It was known that these stimulators did not act directly on the osteoclast or its precursor but on osteoblastic cells.

Objectives:

- to identify the intermediary that passes on the signal from the osteoblast to the osteoclast or its precursor that causes adhesion to and resorption of bone by the osteoblast
- to identify the mechanism that causes osteoclasts to detach from the bone surface
- to identify the adhesion molecules whereby osteoclasts attach to the surface of bone
- to identify the adhesion molecules whereby the osteoclast attaches to the adjacent periosteum

Materials and Methods

2.1 Materials

Antibiotics

Benzylpenicillin. Glaxo, Greenford, UK.

Streptomycin. Evans Medical Ltd., Greenford, UK.

Antibodies

Biotin conjugated hamster anti-mouse CD61 (Integrin β3 chain) monoclonal antibody product number 01862D. Cambridge Bioscience, Cambridge, UK

Biotin conjugated hamster IgG monoclonal immunoglobulin isotype standard anti-trinitrophenol product number 11122C. Cambridge Bioscience.

Polyclonal IgG anti-human osteoprotegerin antibody product number AF805. R&D Systems, Abingdon, Oxon, UK.

Biotinylated IgG anti-human osteoprotegerin antibody product number BAF805. R&D Systems, Abingdon, Oxon, UK.

Apoptosis detection

ApoAlert™ Annexin V apoptosis detection kit. Clontech, Palo Alto, California.

Culture media

BGJ (Fitton-Jackson modified) medium, foetal bovine serum, HEPES buffered 199 medium. Imperial Laboratories, Andover, UK.

MEM alpha medium with ribonucleotides and deoxyribonucleotides. GIBCOBRL Life Technologies, Paisley, Scotland.

Culture vessels

96 well plates. Sterile 6-well plates 9 cm^2. Sterile culture flasks 25 cm^2 Appleton Woods, Birmingham, UK.

Sterile 24 well plates 2 cm^2. ICN Biomedicals, High Wycombe, UK.

Cytokines

Recombinant human sRANKL, Insight Biotechnology, Middlesex, UK. Recombinant human osteoprotegerin rhOPG:Fc, Alexis Corporation, Nottingham, UK)

Echistatin

Sigma, Poole, UK.

Electrophoresis

NuPage 4-12% Bis-Tris gels, sample buffer, 2-(N-morpholino) ethane sulphonic acid (MES) running buffer, transfer buffer, Polyvinylidene difluoride (PVDF) membrane, Novex, San Diego, California.

ELISA plates

96-well plates Appleton Woods, Birmingham, UK.

Heparin

CP Pharmaceuticals, Wrexham, UK.

Hormones

Vitamin D($1\alpha,25(OH)_2D_3$) Leo Pharmaceutical Products, Denmark. Prostaglandin E_2, Parathyroid hormone, Sigma, Dorset, UK.

Industrial Methylated Spirits

De Puy Healthcare, Leeds, UK.

Leitz Dialux 20 microscope

Leitz Wetzlar, Germany.

Mounting media

Apathy's mountant, Aquamount mountant, BDH Laboratory Supplies, Poole England.

Phosphate buffered saline

Pharmacy department, Orthopaedic Hospital, Oswestry, UK

Polaroid 667 black and white film.

Appleton Woods, Birmingham, UK.

RNA analysis

SV Total RNA Isolation kit, reverse transcription system, *Taq* polymerase, dNTP's, $MgCl_2$, 10 x Thermo buffer. Promega, Southampton, UK. Restriction enzymes: Eco R1, Pst 1, Pvu II and Stu 1 all from Promega, Southampton, U.K.

Steroids

Dexamethasone, Sigma.

Trypsin/EDTA

0.5 g porcine trypsin, 0.2 g EDTA 4Na made up to 1 dm^3 in Hepes buffered saline solution.

Skimmed milk powder

J Sainsbury Ltd. UK.

SYBR Green II nucleic acid stain.

Molecular Probes, Leiden, Netherlands.

TMB

3,3'5,5' Tetramethylbenzidine substrate, Pierce Chemical Company, Ilinois, USA.

All other chemicals were of analytical grade and were purchased from either Sigma, Poole, UK., BDH Poole, UK or Fisher Scientific, Loughborough, UK.

2.2 Methods

2.2.1 Animals

The mice used in this study were inbred BALB/c. They were housed as set down by the Home Office, Animals (Scientific Procedures) Act 1986. The ambient temperature was maintained at 18-21°C and the air was changed twenty times an hour. There was a daily cycle of 12 h light and 12 h dark. Food and water were freely available.

2.2.2 Removal of mouse parietal bones.

All dissection was carried out in a laminar flow cabinet using equipment which had been boiled for 5 min to ensure sterility. Seven-day old mice were taken and killed by decapitation. Skin on the mouse head was sterilized by spraying with 70% industrial methylated spirit (IMS) which was allowed to dry prior to immobilizing the head with a knob pin through the nasal region. The skin was lifted at the back of the head using fine forceps and cut centrally to the front of the head. The two skin flaps were pulled to the side to expose the top of the skull and secured with knob pins. (Fig.2.1). The skull was bathed in 200 µl of 10 U cm^{-3} heparin, 5 mM diaminoethanetetra-acetic acid diammonium salt (EDTA) in phosphate buffered saline (PBS, 137 mM sodium chloride, 10 mM potassium phosphate pH 7.4) in order to prevent platelet aggregation which may interfere with the organ culture. The sagittal suture was cut from the back of the head to the front using small dissecting scissors followed by further cuts along the lamboid and coronal sutures (Fig 2.1). The bone was lifted away from the underlying soft tissue using watch makers forceps and final cuts were made along the masto-parietal, squamous and spheno-parietal sutures to free the parietal bone from the remainder of the skull. Parietal bones were dissected out in pairs and washed in 0.5 cm^3 HEPES buffered 199 medium containing 10% foetal bovine serum in 2 cm^2 wells in sterile 24-well plates. Where whole calvaria were taken, cuts were made to include the interparietal bone and part of the frontal bone as shown in Fig 2.1.

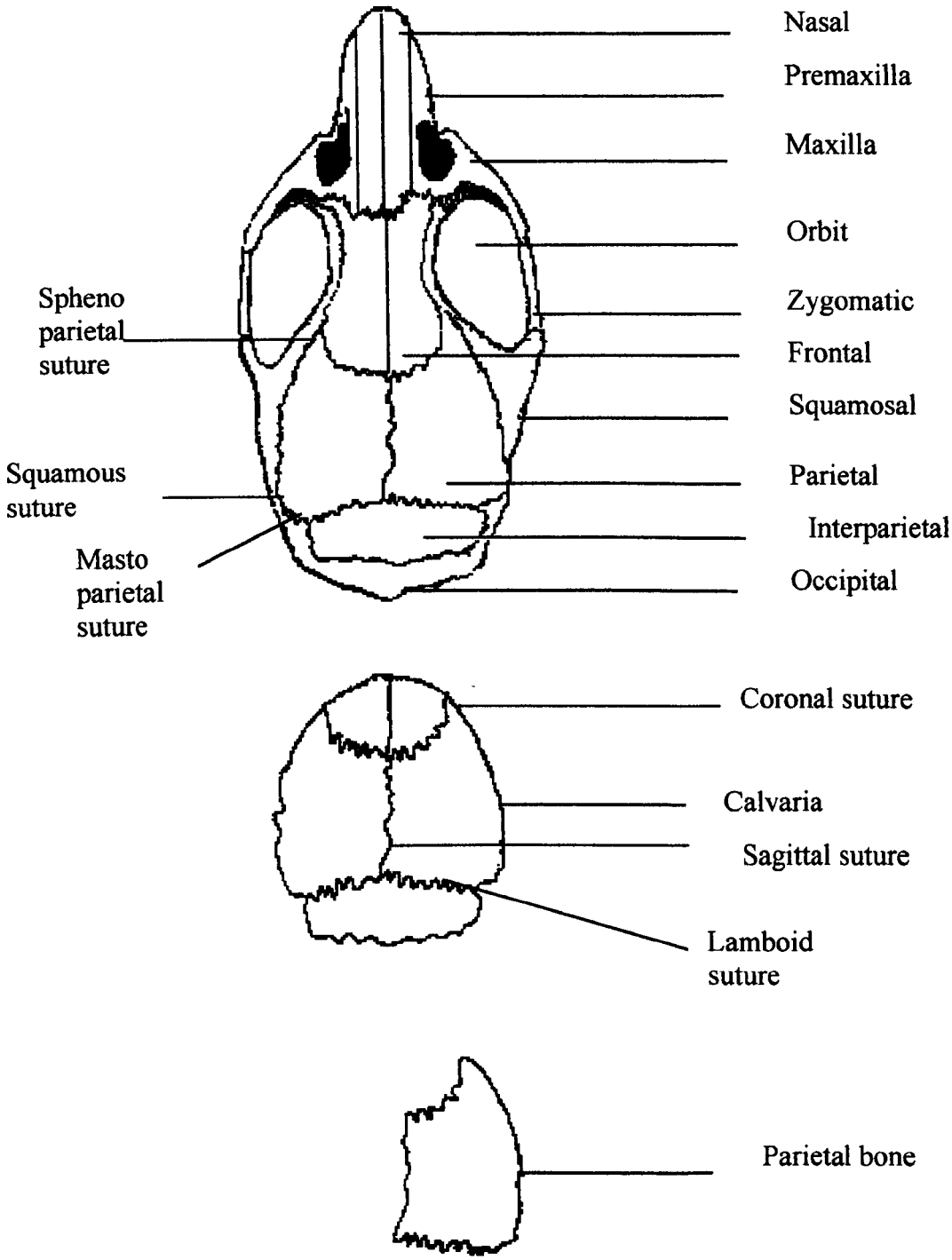


Figure 2.1 Dorsal aspect of the mouse skull

2.2.3 Culture conditions for whole calvaria and parietal bones

Prostaglandins have been found to stimulate bone resorption in organ culture. Lerner and Fredholm (1985) found maximal calcium release in mouse calvaria in culture, in the presence of PGE_2 at a concentration of 1 μM. Concentrations at or above 10 nM and maximally at 1 μM, caused a significant increase in the number of TRAP+OC on bone compared to control bones (Holt *et al* 1994). Indomethacin, an inhibitor of cyclo-oxygenase, suppressed the production of prostaglandins. Lerner and Fredholm (1985) showed that at a concentration of 1 μM, indomethacin inhibited prostaglandin synthesis to undetectable levels in mouse calvarial cultures. Parietal bones or whole calvaria were removed and placed in culture wells as previously described (Section 2.2.2). The 199 medium was removed from each well and replaced with 0.5 cm^3 of BGJ (Fitton-Jackson modified) medium containing 10% decomplemented foetal bovine serum, 100 U cm^{-3} benxylpenicillin and 100 μg cm^{-3} streptomycin. In some experiments, as indicated in the results, bones were incubated in the presence of 1 μM indomethacin for 24 h and then put into fresh media containing factors to be tested. Cultures were incubated in a humidified atmosphere of 5% carbon dioxide (CO_2) in air at 37°C. Test factors used in individual experiments are explained at the beginning of each method. In order to minimize the variation in osteoclast number, where possible each experiment used mice only from one litter. Where this was not possible, bones from different litters were included in each treatment so as to gain a representative sample.

2.2.4 Determination of Tartrate-resistant acid phosphatase-positive osteoclasts (TRAP+OC)

Following the culture period in indomethacin, one bone was incubated in indomethacin and the other half in prostaglandin E_2 (PGE_2) for 24 h. Bones were fixed for 5 min in 95% IMS, 5% glacial acetic acid in a 96-well plate. Unless otherwise stated, the ectocranial membranes were removed and discarded and the endocranial membranes were peeled back to the sagittal suture. It was important to

ensure that the endocranial surface of a bone could be identified and mounted towards the microscope objective. To achieve this a small triangle of bone was cut away using a scalpel from the top left hand corner opposite the sagittal suture with the endocranial side uppermost, leaving a diagonal cut edge. Following fixation, the bones were washed in two changes of phosphate buffered saline (PBS 137 mM sodium chloride 10 mM potassium phosphate pH 7.4) and stained for TRAP activity. The TRAP reagent consisted of 1 mM Naphthol AS-BI phosphoric acid, 100 mM acetic acid, 26.8 mM L(+) tartaric acid and 0.15 mg cm^{-3} Fast Garnet GBC salt pH 5.2. Bones were incubated in this reagent for 30 min at 37°C and were then washed in PBS. Bones were then decalcified in 1 M hydrochloric acid (HCl) with 12.5% glutaraldehyde for 5 min followed by washing in three changes of PBS. Bones were orientated using the dissecting microscope and fine forceps with the endocranial surface uppermost and the membrane flattened out away from the bone. They were mounted using Aquamount mountant. Each parietal bone was scanned completely in a rasta fashion using a Leitz Dialux 20 transmitted light microscope with a total magnification of x 200. TRAP+OC were counted as the number of cells which were stained red.

2.2.5 Immunocytochemical identification of β3 positive osteoclasts (β3+OC)

Following an initial culture period and further incubation in either indomethacin or PGE_2 as previously described, (Section 2.2.4) bones were fixed in 95% IMS, 5% glacial acetic acid for 5 min in a 96-well plate. The ectocranial membrane was stripped and the endocranial membrane peeled back. The bones were washed in PBS and decalcified in 10% EDTA pH 6.6 with mixing for 30 min. The EDTA was then removed and replaced with fresh EDTA for another 30 min of gentle mixing. Bones were then washed in three changes of skimmed milk blocking buffer, each 15 min, in order to block non-specific binding of the antibody. The skimmed milk blocking buffer consisted of 154 mM NaCl, 10 mM Tris HCl. pH 7.6, 0.2 mg cm^{-3} thiomersal and 2.5% skimmed milk. Individual bones were then placed in 25 µl of the primary antibody diluted 1 in 100 in blocking buffer to give a concentration of 5 µg cm^{-3} and then incubated in a humidified atmosphere at 37°C

for 1 h. The primary antibody was a biotin-conjugated hamster anti mouse CD61 (Integrin β3 chain) monoclonal antibody. A biotin-conjugated hamster IgG monoclonal immunoglobulin isotype standard, anti-trinitrophenol product number 11122C was used as a negative control. This antibody was an isotype control IgG2κ. Bones were washed for 15 min in three changes of blocking buffer. After washing, each bone was placed in 25 μl of ExtrAvidin peroxidase conjugate (Sigma) diluted 1 in 100 in blocking buffer to give 20 μg cm^{-3} and incubated at 37°C for 1 h. Following further washing in three changes of blocking buffer as above, the peroxidase activity was detected by incubating in a solution of 0.5 mg cm^{-3} 3,4,3,4 tetra-aminophenyl hydrochloride, 1 mM nickel chloride, 1 mM cobaltous chloride and 0.012% hydrogen peroxide in 25 mM citric acid 16 mM di-sodium hydrogen orthophosphate pH 6.4 for 20 min at 37°C. Finally, bones were briefly washed in three changes of PBS and mounted, endocranial surface uppermost with the membrane flattened out, in Apathy's mountant. β3+OC stained black and were counted as described previously for TRAP+OC.

2.2.6 Effect of Echistatin on osteoclast adhesion.

In order to further investigate the role of β3 in the translocation process, the disintegrin echistatin was utilized in the calvarial culture system. Echistatin is a snake venom disintegrin which contains the amino acid motif arginine/glycine/aspartic acid (RGD). A disintegrin is a molecule which interferes with integrin ligand interactions and echistatin blocks the binding of the αvβ3 and other integrins to the RGD sequence which is found in several plasma and matrix proteins. The disintegrin echistatin is not specific for αvβ3 but it does bind with a high affinity. The integrin αv β3 appears to be important in the translocation of osteoclasts from the endocranial membrane to the bone surface seen the presence of PGE_2. Following the initial culture period in indomethacin, half the bones were incubated in 1 μM PGE_2 alone to act as a control, and the other half in PGE_2 plus 10 nM echistatin. Cultures were incubated at 37°C, 5% CO_2 for a further 24 h. In another experiment following the initial culture period, bones were incubated for a further day in the presence of 1 μM indomethacin alone or with sOPGL

(100 ng cm^{-3}) with or without 10 nM echistatin. Bones were then stained for the presence of the integrin subunit β3 as previously described (Section 2.2.5).

2.2.7 Effect of osteoprotegerin ligand.

Freshly isolated parietal bones were cultured in the presence of 1 μM indomethacin and varying concentrations of the soluble extracellular domain of osteoprotegerin ligand (sOPGL, recombinant human sRANKL, Insight Biotechnology, Middlesex, UK). Also, after a one day incubation with indomethacin, PGE_2 (1 μM) or sOPGL (100 ng cm^{-3}), in the presence of 1 μM indomethacin, was added for one day. In a similar experiment, bones were incubated first with indomethacin, then the endocranial membranes of the bones were peeled back to the sagittal suture prior to incubation with sOPGL for 6 h. Control bones were left intact.

2.2.8 Effect of osteoprotegerin

OPG, the decoy receptor for OPGL has been found to block bone resorption both *in vivo* (Simonet *er al*, 1997) and *in vitro* (Fuller *er al*, 1998). Bones were cultured in the presence of 1 μM indomethacin for one day followed by incubation for a further day in the presence of 1 μM PGE_2 or 10 nM $1,25(OH_2)D_3$ or 4 nM PTH each with or without 100 ng ml^{-1} osteoprotegerin (OPG, recombinant human rhOPG:Alexis Corporation, Nottingham,UK)

2.2.9 Extraction of total RNA

Following one day in indomethacin, half the bones were placed in media containing indomethacin 1 μM and the other half in PGE_2 (1 μM) or $1,25D_3$ (10 nM) Both sets were incubated at 37°C, 5% CO_2 for 4 h. Total RNA was extracted using the SV Total RNA isolation kit from Promega following the manufacturer's instructions. With the use of a dissecting microscope, the ectocranial membranes were removed and discarded, the endocranial membranes being left intact. Four

bones from each treatment were placed in a sterile microcentrifuge and 175 µl of RNA lysis buffer was added to solubilize the RNA. This was left for 5 min at room temperature. Following the addition of 350 µl of dilution buffer and thorough mixing, the samples were incubated at 70°C for 3 min. To remove the bones and cellular debris, the tubes were centrifuged at 8,000 *g* for 10 min. Part of the supernatant (350 µl) was removed and placed into a clean microcentrifuge tube. The RNA was precipitated by the addition of 200 µl of 95% ethanol with gentle mixing and the lysate transferred to a spin basket. The spin basket consisted of a sterile plastic tube into which a second plastic tube was inserted. The second tube contained a porous membrane to which the RNA became bound. The spin basket was centrifuged at 8,000 *g* for 1 min to allow the RNA to bind to the membrane incorporated in the spin basket. The membrane was then washed with 600 µl of wash solution. A 15 min incubation at room temperature with DNase mixture, 50 µl per basket, removed any contaminating DNA. Following removal of the DNase with a stop solution, the membrane was washed with 600 µl of wash solution and then a further 250 µl of wash solution. The RNA was eluted with two volumes of 50 µl of nuclease- free water giving a total volume of 100 µl. Great care was taken at each step to avoid contamination with RNases by working in a class 2 flow cabinet and the use of gloves at all times. All plastic tubes and pipette tips were guaranteed RNase free by the manufacturers. The RNA solution was stored at -20°C

2.2.10 Assay of RNA with SYBR Green II stain.

SYBR Green II was diluted 1 in 100 in nuclease free water and then 1 in 100 in 10 mM Tris HCl, 1 mM EDTA pH 8.0. Test RNA was added (10 µl) and the fluorescence measured in a Locarte fluorimeter with a zinc lamp. It was fitted with cut-off filters BG12 and PY to give the excitation wavelength of SYBR Green II (468 nm). An interference filter with a 4 nm band pass of 525 nm was used to detect emitted light. A standard curve was constructed using a total RNA preparation from bone marrow. The concentration of RNA in this preparation was determined by measuring the OD at 260 and 280 nm. in a spectrophotometer. RNA

has an OD_{260} of 1 at a concentration of 40 $\mu g\ cm^{-3}$ and high purity RNA will give a 260/280 ratio of 2. RNA concentrations in test solutions were calculated by reference to this standard.

2.2.11 Reverse transcription.

The reverse transcription of RNA into cDNA was performed using the Promega reverse transcription kit, as per the manufacturer's instructions, the only difference being the substitution of random hexa deoxyribonucleotides (p(dN)6) for the supplied oligo (dT) primers. This allows for superior priming along the length of the RNA strand. The kit uses avian myeloblastosis virus (AMV) reverse transcriptase. The total volume of the reaction mixture was 40 µl. This consisted of 2.5 mM $MgCl_2$, 0.5 mM final concentration of each dNTP, 12.5 $\mu g\ cm^{-3}$ p(dN)6 primers, 0.5 U μm^{-3} rRNasin (ribonuclease inhibitor), 13.8 U reverse transcriptase and 200 ng RNA in times five reverse transcription buffer. Equal volumes of test RNA were added to each reaction mixture so that 400 ng of RNA were included in each reaction. The mixture was left at room temperature for 10 min to allow the primers to anneal to the RNA and was further incubated at 42°C for 1 h to allow transcription to proceed to completion. The cDNA was stored at -20°C.

2.2.12 Polymerase chain reaction

The process of PCR amplification of a target sequence of DNA entails the denaturation of the double stranded DNA at 94°C, the annealing of DNA primers at a lower temperature (Ta) usually about 60°C, followed by elongation of the primers by the enzyme *Taq* polymerase at 72°C, the optimum temperature for the enzyme reaction. The final volume per tube was 20 µl containing 2 mM $MgCl_2$, 0.2 mM each dNTP's, Thermo buffer, 1µM final concentration each primer and 2 µl cDNA. The whole mixture was covered with 10 µl mineral oil to minimise evaporation. The PCR reaction was carried out with a Techne Progene thermocycler. The machine was heated to 94°C for 1 min in order to heat the lid to prevent condensation on the inside of the reaction tubes. The tubes were placed in the

cycler and heated to 94°C for 3 min at the end of which 0.5 U of *Taq* were added through the oil. The programe continued for a total of 25 cycles which consisted of heating to 94°C for 1 min, cooling to the annealing temperature appropriate to the primers used for one min and heating to 72°C for 1 min to allow extension of the DNA strand. Negative controls included all but cDNA.

Primers used are shown below, they were dissolved in sterile 10 mM Tris HCl, 1 mM EDTA pH 8.0

Primer	Sequence 5' - 3'	Conc. μM	Annealing Temp (Ta)	Product Length
5'muβActin	TGTGATGGTGGGAATGGGTCAG	1.0	60°C	514bp
3'muβActin	TTTGATGTCACGCACGATTTCC			
5' muOPG	GGAACCCCAGAGCGAAACACAGT	1.0	58°C	515bp
3' muOPG	TTCCCAGGCAGGCTCTCCATCAAG			
5' muOPGL	CCATCGGGTTCCCATAAAGTCAC	1.0	57°C	407bp
3' muOPGL	AAAGCCCCAAAGTACGTCGCATCT			
5' huOPG	GTGCGCCCCTTGCCCTGACC	1.0	58°C	556bp
3' huOPG	TGAGCTGTGTTGCCGTTTTATCCT			

RNA sequences for β Actin and β3 were obtained from the National Centre for Biotechnology Information site (NCBI @www.ncbi.nlm.nih.gov/) using ENTREZ software. The PCR primers for mouse β3 were designed with PrimerSelect DNASTAR Inc. Primers for mouse β Actin were designed by Stratagene UK Ltd. Other primers were synthesized by Genosys, Biotechnologies Inc. Cambridge, UK. The housekeeping gene β Actin was used to demonstrate, after electrophoresis of the β Actin PCR products, that equal amounts of RNA were included in the reverse transcription reaction. Equal band intensities should be obtained if equal amounts of cDNA were present in the PCR reaction. This assumes that the amount of PCR product was proportional to the amount of starting cDNA. This was established by determining the range of cDNA concentrations which gave a directly proportional amount of product. If a band intensity exceeded the maximum limit of linearity, this PCR reaction was repeated with less cDNA present.

2.2.13 Electrophoresis of PCR products

A 2% agarose gel was made using 89 mM Tris Borate, 2.5 mM EDTA pH 8.3. The agarose was dissolved in the buffer by placing a conical flask containing the agarose and buffer into a glass beaker containing water. The water was brought to the boil on a heated metal plate and allowed to continue boiling until the agarose had dissolved. After cooling, the agarose was poured into a gel tray 10 x 7.5 cm with a comb and allowed to set for 30 min. PCR product (10 µl) was mixed with 2 µl of loading buffer and 10 µl of that solution was loaded on to the gel. The loading buffer consisted of 89 mM Tris HCl, 2.5 mM EDTA and 89 mM Boric acid pH 8.3 with 0.1% bromophenol blue and 50% glycerol. In addition, 2 µl of DNA ladder was mixed with 1.5 µl of loading buffer and 3 µl of that solution loaded onto the gel. The gel was placed in the electrophoresis tank with 200 cm^3 of running buffer which was the same as that used to make the agarose gel. The gel was run at 8 V/cm for 1 h. The gel was post-stained for 30 min in ethidium bromide 10 ng cm^{-3} in running buffer. The gel was destained in running buffer for 30 min and visualized under UV light and photographed with a Polaroid DS-34 camera with 667 black and white film. The band intensities were measured by image analysis using the Sight System (Foster Finlay Associates Ltd) Freelance Version 5.2. with an Hitachi CCTV analogue camera. This involved thresholding the digitized image and using this as a mask, the area and the mean Grey level were determined. The band intensity was the product of these two measurements.

2.2.14 Restriction enzyme digest

Restriction enzyme digests were carried out in order to verify that the PCR product was likely to be that which was expected. Restriction enzymes were identified using PrimerSelect DNASTAR Inc. Enzymes were chosen which cut the PCR product at a unique site and approximately 100bp from either end of the product so that the two cut products were easily identifiable on the electrophoresis gel. The appropriate enzyme (1 µl) was placed in a tube together with 1 µl of buffer, 5 µl of cDNA and 3 µl of nuclease free water to make the total volume up to

10 µl. The buffer used with Pvu II consisted of 6 mM Tris, 6 mM Mg Cl_2, 50 mM Na Cl and 1 nM DTT pH 7.5. The buffer used with enzyme Pst I consisted of 90 mM Tris, 10 mM $MgCl_2$, 50 mM NaCl pH 7.5..Controls consisted of one tube with no enzyme and one tube with an enzyme which did not cut the product. All three tubes were then incubated at 37°C for 1 h. At the end of the incubation, 2 µl of electrophoresis buffer was added to each tube and the products underwent electrophoresis as described above.

The enzymes used and where they cut are shown below.

Enzyme and concentration	PCR product verified	Recognition site	Product lengths
Pvu II (Proteus vulgaris) 8-12 u/µl	OPGL 407 bp	CAG/CTG GTC/GAC	190 bp plus 217 bp
Pst I (Recombinant E. coli strain) 10 u/µl	OPG 515 bp	CTGA/G G/ACGTC	100 bp plus 415 bp

2.2.15 Assay of osteoprotegerin

Freshly isolated parietal bones were put into culture media containing either 1 µM indomethacin or 1 µM PGE_2 and after 3, 6 and 9 h of culture a sample of the medium was removed, frozen, and replaced with fresh medium containing the relevant effector of bone resorption. After 24 h in culture, the media was removed and frozen. In a further experiment calvaria were cultured in 1 cm^3 of media containing either 1 µM indomethacin or 1 µM PGE_2 or 4 nM PTH or 10 nM $1,25D_3$ or 100 nM dexamethasone for 20 h. The media was then frozen until required. A 96-well ELISA plate was twice washed in distilled water followed by thorough shaking to remove any remaining water. The plates were incubated overnight at 4°C

following the addition of 100 µl of 200 ng cm^{-3} goat anti-human OPG antibody (R&D Systems, Abingdon, UK) in 0.1 M sodium bicarbonate buffer pH 8.6 to each well (O'Brien *et al*, 2001). Following removal of the antibody, each well was blocked with 200 µl of 0.25% skimmed milk in 0.1 M Tris HCl pH 7.6 (skimmed milk) and incubated at 4°C for one hour. After two brief washes in skimmed milk, 100 µl of standards, unknowns and controls were added to the plate. The standards consisted of dilutions of recombinant mouse OPG in BGJ culture media to give 0,1,2 and 4 ng cm^{-3} of OPG. Controls consisted of goat IgG (20 ng per well) coating the well, no secondary antibody and no avidin peroxidase. All samples were assayed in triplicate. The plate was incubated at 4°C for 1h. After three washes in skimmed milk, 100 µl of a biotinylated goat anti-human OPG antibody (R&D Systems, Abingdon, UK) in skimmed milk was added to the wells at a concentration of 200 ng cm^{-3} and incubated at 4°C for 1 h. Following three washes in skimmed milk, 100 µl of 2 ug cm^{-3} Extravidin Peroxidase was added to the wells and incubated at 4°C for 1 h. After four washes in skimmed milk, 100 µl of 3,3'5,5' Tetramethylbenzidine (TMB) substrate was added and incubated at room temperature for 60 min. The reaction was stopped with 100 µl of 10% sulphuric acid and the optical densities were determined using a microtitre plate reader (Dynatech Laboratories) at 450 nm.

2.2.16 Staining for apoptotic cells with annexin V

Freshly isolated parietal bones were cultured for 24 hours in the presence of 1 µM indomethacin. Following culture the membranes were treated as previously described and the bones were incubated for 30 min at 37°C in annexin V reagent (Clontech) as per the manufacturer's instructions. All apoptotic cells were counted on both the bone and the endocranial membrane using a fluorescent microscope. After counting, the bones were stained for TRAP as previously described and TRAP+OC on the bone and membrane were also counted.

2.2.17 Cell culture.

The cell lines used in this project were MG63, human osteosarcoma cells and MC3T3-E1. The latter were spontaneously immortalized cells derived from neonatal mouse calvarial bones. Cells were cultured in α MEM with 10% decomplemented foetal bovine serum (FBS), 100 U cm^{-3} benxylpenicillin, 100 μg cm^{-3} streptomycin and 1% L-Glutamine in 25 cm^3 flasks. The foetal bovine serum was decomplemented by heating the serum to 56°C for 1 h. Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. Culture media was changed every four days until the cells were confluent. When cells became confluent different effectors were added to the media depending on the experiment being carried out. These included 1 μM indomethacin, 1 μM PGE_2, 4 nM PTH, 10 nM $1,25D_3$ or 100 nM dexamethasone. Culture times varied and will be described at the beginning of each result. In some experiments the culture media was removed from the cells and frozen for future assay of osteoprotegerin levels as previously described (Section 2.2.14). In other experiments the total RNA was extracted in order to look for levels of messenger RNA using RT-PCR. In order to extract the RNA the cells were washed with 2 cm^3 of sterile PBS followed by the addition of 2 cm^3 of Trypsin/EDTA. The cells were then left at 37°C for approximately 5 min until all the cells became detached from the bottom of the flask. The cells were then transferred to sterile centrifuge tubes and centrifuged at 4,000 *g* for 5 min after which the supernatent was removed and replaced with 175 μl of RNA lysis buffer. The samples were thoroughly mixed to resuspend the cells and following the addition of 350 μl of RNA dilution buffer, heated to 70°C for exactly 3 min. The cells were then processed as previously described (Section 2.2.9).

Other cells used in this project were calvarial cells removed from whole mouse calvaria. Eight whole calvaria were dissected out as previously described (Section 2.2.2) and placed in 5 cm^3 of 199 media, 10% FBS, 100 U cm^{-3} benxylpenicillin and 100 μg cm^{-3} streptomycin. Following completion of dissection the media was removed and replaced with 5 cm^3 of 199 media without FBS. This was in order to remove the FBS and reduce any excess protein which may interfere with the action of the collagenase. Following two further washes the media was

replaced with 4 cm^3 of 199 media and 1 cm^3 of collagenase 1 mg cm^{-3} in sterile PBS. The calvaria were then incubated at 37°C for 1 h. Following incubation the media was removed from the bones and placed in a sterile centrifuge tube and using a pasteur pipette the cells were released by repeatedly drawing up and expelling the media into the tube. The cells were then centrifuged at 2,000 *g* for 5 min and the supernatant removed. The cells were then resuspended in 5 cm^3 of 199 media with FBS and were again centrifuged in order to wash away the collagenase. The supernatent was removed and replaced with 8 cm^3 of BGJ (Fitton-Jackson modified) media. The cells were mixed carefully and 1 cm^3 of the cell suspension placed in each well of a 12-well plate. The cells were then placed in a humidified atmosphere of 5% CO_2 in air at 37°C. The following day the plate was swirled in order to remove any non-adherent cells and the media removed and replaced with fresh media. The cells were returned to the incubator and grown to confluency. Various effectors were then incubated with the cells and will be described at the beginning of each experiment.

Mouse bone marrow was used to try to suppress mRNA for OPG with PTH. One five to eight week old mouse was killed with diethyl ether inhalation and the femora and tibia aseptically removed. Bones were washed in 199 medium containing 10% FBS, 100 U cm^{-3} benxylpenicillin and 100 μg cm^{-3} streptomycin. The marrow cavities were then flushed into a sterile centrifuge tube by slowly injecting 199 medium at one end of the bone using a sterile 15-gauge needle. Any cell clumps were disaggregated into a single cell suspension using a fine tipped plastic transfer pipette. The cells were centrifuged at 150 *g* for 5 min. The supernatent was decanted and the cells resuspended in 4 cm^3 α MEM with 10% FBS, 100 U cm^{-3} benxylpenicillin, 100 μg cm^{-3} streptomycin and 1% L-Glutamine. A cell count was determined using a Neubauer counting chamber at a 1/10 dilution in white cell counting fluid (2% acetic acid, 50 μg cm^{-3} gentian violet). Six million cells were added to each of four wells of a 9 cm^2 well sterile plate. Wells were topped up to give a total volume of 3 cm^3 with α MEM medium. The plate was then incubated for 24 h at 37 °C in 5% CO_2 in air. The plate was then shaken to remove any non-adherent cells and the medium replaced with α MEM containing 4 nM PTH. The medium was replaced with α MEM containing 4 nM PTH every three

days up to twelve days. At the end of this period the RNA was extracted from the cells as previously described.

2.2.18 Electrophoresis

Electrophoresis and Western blotting were carried out in order to illustrate the presence of osteoprotegerin protein in the media of cultured cells following incubation with various effectors and inhibitors of bone resorption described at the beginning of each experiment. Each sample was mixed with sample buffer in a ratio of 20 μl of sample with 5 μl of sample buffer. The sample buffer consisted of 4 g glycerol, 0.682 g Tris base, 0.666 g Tris HCL, 0.8 g sodium dodecyl sulphate (SDS), 0.006 g EDTA, 0.75 cm^3 of a 1% solution of Serva Blue G250, 0.25 cm^3 of phenol red made up to 10 cm^3 with distilled water. The buffer was adjusted to pH 8.5. After thorough mixing the final sample solution was heated to 70°C for 10 min. followed by brief vortex prior to loading 20 μl onto the gel. With each run a 7 μl sample of molecular weight standard was loaded in two separate positions so that following blotting the membrane could be cut in half with both halves containing a standard. The standard used was a multi-coloured standard with loading buffer of Tris HCL, formamide, SDS and phenol red. The gel used was a NuPAGE 4-12% Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel and was placed in the XCell II™ Mini-Cell (E19001) according to the manufacturer's instructions (Novex, San Diego, California). The running buffer used was NuPAGE MES (2-(N-morpholino) ethane sulfonic acid) times twenty stock made up to 1 dm^3. The inner chamber of the electrophoresis tank was filled with running buffer prior to loading the samples. The outer chamber of the tank was then filled with running buffer and electrophoresis was carried out at 200 V, 110-125 mA for 35 min. When the run was complete the power was disconnected and the gel removed from the mini-cell.

2.2.19 Western blotting.

The gel was processed according to the Western Transfer Protocol from Novex. The running buffer used consisted of 850 cm^3 distilled water, 50 cm^3

NOVEX NuPAGE transfer buffer undiluted at times twenty concentration and 100 cm^3 methanol. The blotting membrane used was PVDF from Novex. The blotting was carried out at 30 V for 1 h. After blotting the membrane was removed from the blotting apparatus and cut in half with one half used as a test membrane and the other half as a control membrane for staining purposes.

2.2.20 Immunochemical staining for osteoprotegerin.

Following Western blotting of the gel, the two halves of the membrane were placed in 5 cm^3 of 0.25% skimmed milk blocking buffer as previously described (Section 2.2.14). These were incubated with mixing for 1 h at room temperature. After blocking the skimmed milk buffer was removed and replaced with 5 cm^3 of skimmed milk containing 0.1 $\mu g\ cm^{-3}$ biotinylated goat anti-human osteoprotegerin with one half of the membrane or 0.1 $\mu g\ cm^{-3}$ goat IgG with the other half to act as a control. These were incubated with mixing for 1 h at room temperature followed with washing in three changes of skimmed milk for 5 min each. Both membranes were then placed in 5 cm^3 skimmed milk containing 2 $\mu g\ cm^{-3}$ ExtrAvidin Peroxidase and incubated with mixing for 1 h at room temperature. Both membranes were then washed as previously described and the peroxidase activity was detected by incubating in a solution of 0.5 $mg\ cm^{-3}$ 3,4,3,4 tetra-aminophenyl hydrochloride, 1 mM nickel chloride, 1 mM cobaltous chloride and 0.012% hydrogen peroxide in 25 mM citric acid 16 mM di-sodium hydrogen orthophosphate pH 6.4 for 20 min at room temperature. The membranes were then washed in two changes of PBS to remove the excess stain and allowed to dry at room temperature overnight.

2.2.21 Statistics

All data is presented as the mean ± standard error of the mean (s.e.m) of at least four replicates. The statistical significance of results was determined by Student's two-tailed t-test. Probability (p) values of less than 0.05 were considered significant.

3. Results: Effects of PGE_2 and indomethacin on osteoclasts.

3.1 Effect of indomethacin and PGE_2 on TRAP+OC on freshly isolated parietal bones.

When mouse parietal bones were incubated with 1 μM indomethacin for one day there was a significant decrease in the number of TRAP+OC seen on the bone surface ($p<0.05$) compared to the media control (Fig 3.1). In contrast there was a significant increase in the number of TRAP+OC seen on the endocranial membrane ($p<0.005$) compared to the control (Fig 3.1). When parietal bones were incubated with 1 μM PGE_2 there was a significant increase in the number of TRAP+OC seen on the bone surface ($p<0.001$) compared to the control (Fig 3.1). At the same time there was a decrease in the number of TRAP+OC seen on the endocranial membrane but this was not statistically significant.

There was no significant difference between the total numbers of osteoclasts seen with any of the treatments. Indomethacin seems to cause osteoclasts to detach from the bone surface and to adhere to the endocranial membrane where they are non-resorbing and further experiments were carried out to determine whether these osteoclasts could return to the bone surface and commence bone resorption.

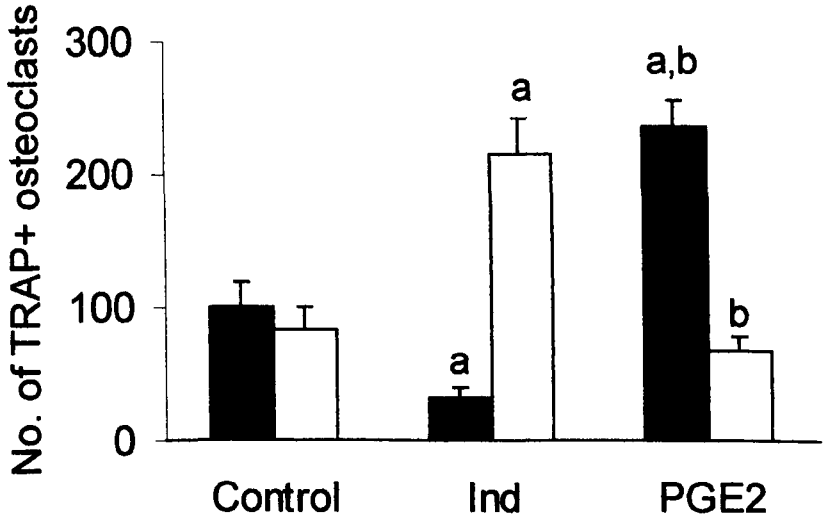


Figure 3.1: The effect of 1 μM indomethacin and 1 μM PGE_2 on the numbers of TRAP+OC seen on the bone surface (■) and the endocranial membrane (□). Freshly isolated mouse parietal bones were incubated for 24 h in either a BGJ (Fitton-Jackson modified) medium control, medium containing 1 μM indomethacin (Ind) or 1 μM PGE_2. The numbers of histochemically stained TRAP+OC were determined as described in the materials and methods and are shown as the mean and the SEM. For each culture condition n = 6.

a, $p<0.05$ compared to the control

b, $p<0.001$ compared to indomethacin

This experiment was performed on three separate occasions and these are representative results.

3.2 Effect of indomethacin and PGE_2 on TRAP+OC previously incubated with indomethacin.

When parietal bones were incubated in the presence of 1 μM indomethacin for one day followed by a further day in media containing either 1 μM indomethacin (control) or 1 μM PGE_2, there was a significant recovery in the number of TRAP+OC on the bone surface with PGE_2 ($p<0.001$) compared to the indomethacin control (Fig 3.2). At the same time there was a significant decrease in the number of TRAP+OC on the endocranial membrane with PGE_2 ($p<0.001$) compared to the control (Fig 3.2). There was a significant difference between the total number of osteoclasts with indomethacin compared to PGE_2. The variability in the numbers of osteoclasts counted is a result of biological variation and/or variation in counting efficiency. The osteoclasts were counted over the whole surface of the bone and endocranial membrane including the edges of the bone where the membrane may have become detached during the dissection procedure. This may account for the failure of some osteoclasts to translocate from the membrane to the bone in the presence of PGE_2. In addition, only those osteoclasts which are TRAP+ were counted. Unstained (TRAP negative) osteoclasts were sometimes seen on the endocranial membrane but were not counted.

Following incubation with PGE_2, the TRAP+OC on the bone surface were stained red throughout the cell although the apparently leading edge of the cell is more intensely stained. The leading edge of the cell was probably the direction in which the cell was moving indicated by the resorption site left behind at the rear of the cell. The osteoclasts appear to be in resorption lacunae and TRAP stain may be seen more intensely at the periphery of the lacunae (Fig 3.3a). In contrast, following incubation with indomethacin, osteoclasts on the endocranial membrane appear stellate in shape presumably due to cytoplasmic retraction. They are stained evenly throughout the cell with no leading edge and as they are not on bone, they are not associated with resorption lacunae (Fig 3.3b).

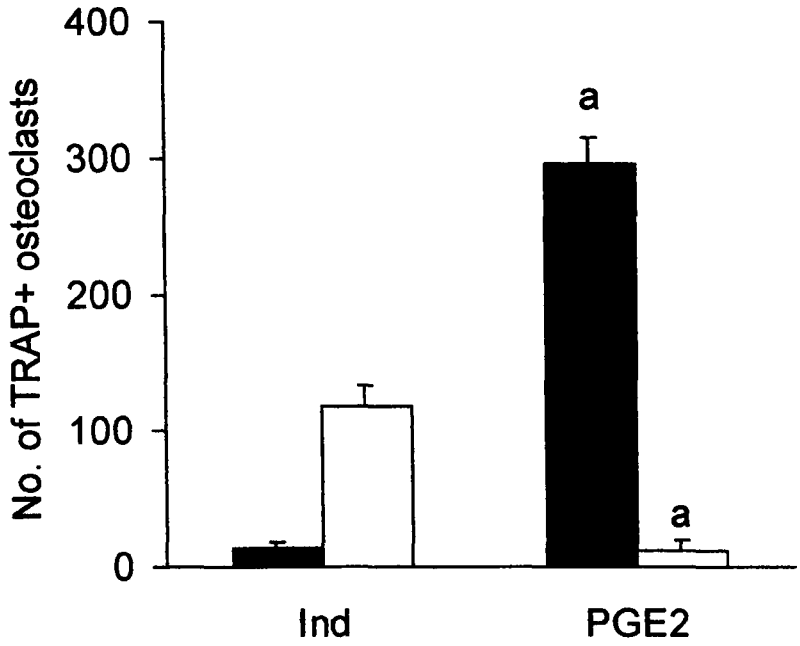
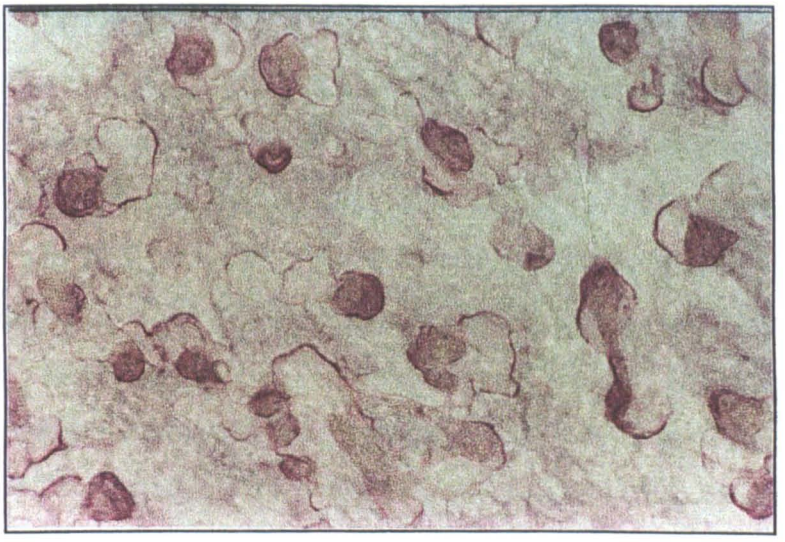


Figure 3.2: The effect of 1 μM indomethacin and 1 μM PGE_2 on the numbers of TRAP+OC on the bone surface (■) and the endocranial membrane (□). Bones were cultured for 24 h in medium containing 1 μM indomethacin and then a further 24 h in 1 μM indomethacin (Ind, control) or 1 μM PGE_2. The numbers of TRAP+OC are shown as the mean and the SEM. For each culture condition n = 5. a, $p<0.001$ compared to indomethacin

a



b

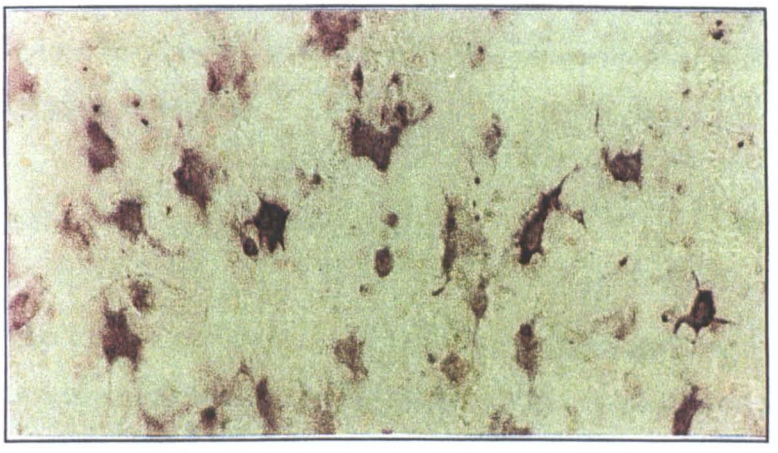


Figure 3.3: The effect of indomethacin and PGE_2 on TRAP+OC on the endocranial surface of bone and the endocranial membrane. a) Bones were incubated in medium containing 1 μM indomethacin for 24 h and a further 24 h in 1 μM PGE_2 and then stained for TRAP following removal of the endocranial membrane. Osteoclasts appear rounded in shape, stained red and are often associated with resorption lacunae. b) Bones were incubated in medium containing 1 μM indomethacin for 24 h and the endocranial membrane peeled back to the sagittal suture before staining for TRAP. The osteoclasts stain red and are stellate in shape. Total magnification x 283.

3.3 Effect of PTH and $1,25D_3$ on TRAP+OC

In addition to PGE_2, other stimulators of bone resorption include PTH and $1,25D_3$. Parietal bones were incubated in the presence of 1 μM indomethacin for one day followed by a further day in either indomethacin (control) or indomethacin together with 1 μM PGE_2, 10 nM $1,25D_3$, or 4 nM PTH. There was a significant increase in the number of TRAP+OC seen on the bone surface ($p<0.005$) with all three effectors compared to the indomethacin control (Fig 3.4). At the same time there was a significant decrease in the number of TRAP+OC in the endocranial membrane ($p<0.05$) compared with indomethacin (Fig 3.4). There was no significant difference between the total numbers of osteoclasts (bone + endocranial membrane) seen with indomethacin compared to PTH, however there was a significant increase ($p<0.05$) with PGE_2 and $1,25D_3$ (Fig 3.4). There was no significant difference between the total numbers of osteoclasts seen with any of the three effectors of bone resorption

3.4. Effect of indomethacin and PGE_2 on β3+OC

In a similar experiment, following incubation in either indomethacin or PGE_2 after an initial one day in indomethacin, the parietal bones were stained for the presence of the integrin subunit β3 (Fig 3.5). Osteoclasts were the only cells staining intensely for β3 in line with the observations of other workers (Holt and Marshall, 1998). There were significantly more β3+ osteoclasts on the bone ($p<0.001$) compared with the indomethacin control (Fig 3.5). There were very few β3+ osteoclasts seen on the endocranial membrane. The osteoclasts stained black and appeared to be resorbing as they were associated with resorption lacunae (Fig 3.6a).

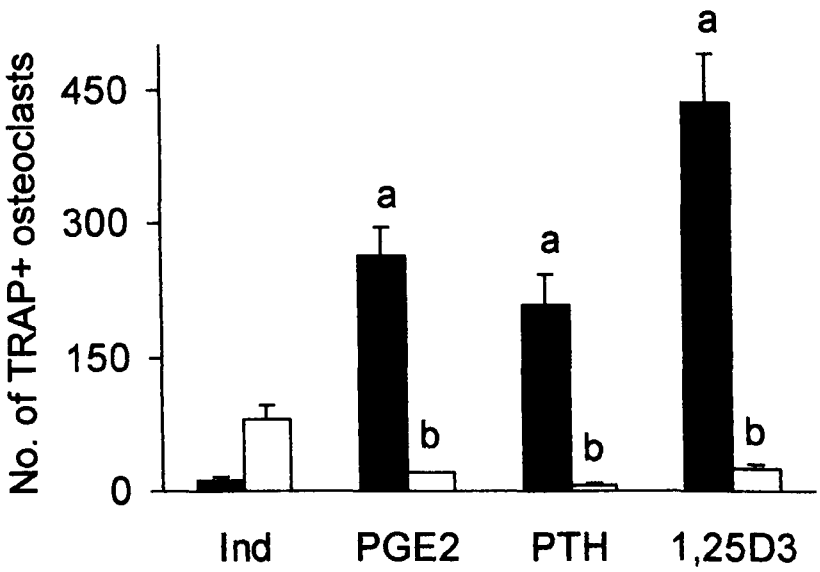


Figure 3.4: The effect of 4 nM PTH and 10 nM $1,25D_3$ on the numbers of TRAP+OC on the bone surface (■) and the endocranial membrane (□). Bones were cultured for 24 h in medium containing 1 μM indomethacin (Ind) and then a further 24 h in 1 μM indomethacin (control) or indomethacin together with 1 μM PGE_2, 4 nM PTH, or 10 nM $1,25D_3$. The numbers of TRAP+OC are shown as the mean and SEM. For each culture condition n = 5 or 6.

a, $p<0.005$ compared to indomethacin

b, $p<0.05$ compared to indomethacin

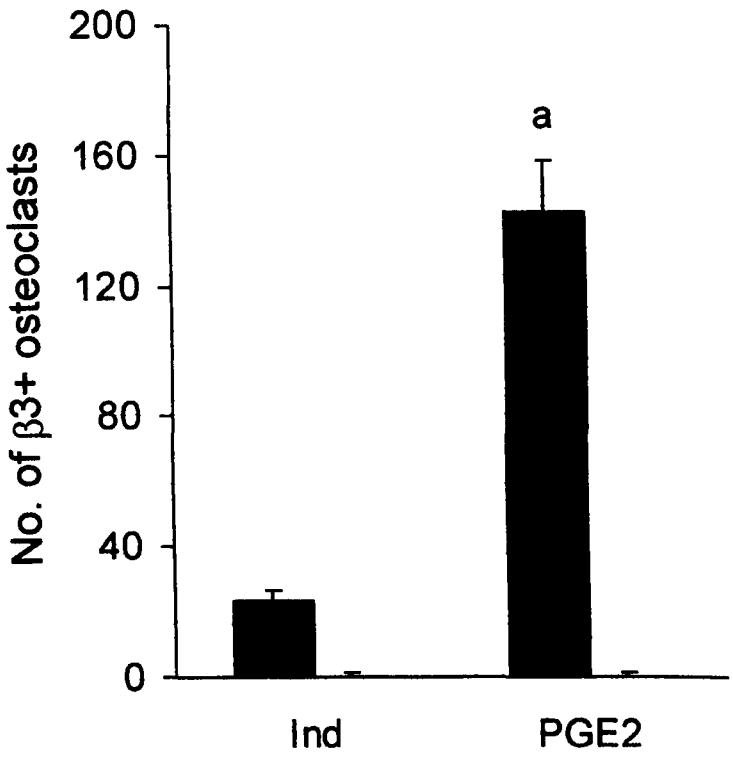
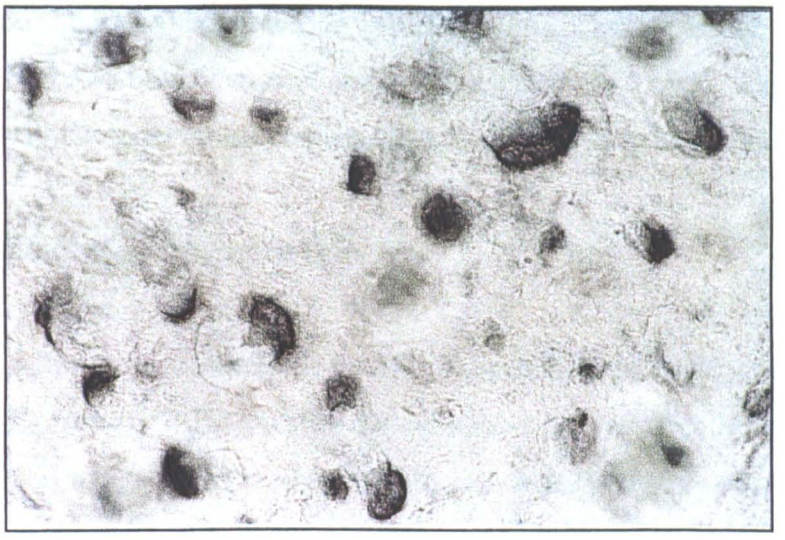


Figure 3.5: The effect of 1 μM indomethacin and 1 μM PGE_2 on β3+OC on the endocranial surface of bone (■) and the endocranial membrane. Bones were cultured for 24 h in medium containing 1 μM indomethacin and then for a further 24 h in 1 μM indomethacin (Ind, control) or 1 μM PGE_2. The numbers of β3+OC are shown as the mean number of osteoclasts and the SEM. For each culture condition n = 5.

a, $p<0.001$ compared to indomethacin

a



b

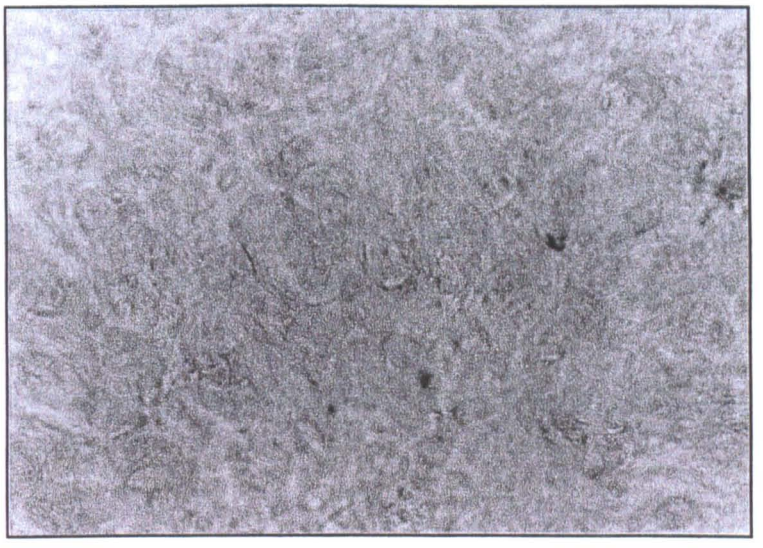


Figure 3.6: The effect of PGE_2 on β3+OC on the endocranial surface of bone. Bones were incubated in medium containing 1 μM indomethacin for 24 h and a further 24 h in 1 μM PGE_2. They were then immunostained using either monoclonal anti-β3 antibody or a control antibody. a) Osteoclasts stain a pale diffuse black throughout the nuclei and most of the cytoplasm but with a much darker leading edge away from the resorption lacunae. They are multinucleate and are often seen in resorption lacunae. b) Control antibody showing no β3 staining. Total magnification x 283.

A control bone is shown which was stained in a similar fashion but using an isotype control shows no evidence of stained osteoclasts (Fig 3.6b). The control antibody was a monoclonal biotin-conjugated hamster antibody to trinitrophenol.

3.5. Effect of echistatin on β3+OC

Bones were incubated in indomethacin for one day followed by a further day in PGE_2 alone or PGE_2 plus 10 nM echistatin. Echistatin blocks the translocation of β3+OC osteoclasts to the bone surface which normally happens in the presence of PGE_2 and significantly fewer osteoclasts were seen on the bone ($p<0.05$) in the presence of echistatin (Fig 3.7). Conversely, significantly more β3+OC osteoclasts ($p<0.05$) were seen on the membrane $p<0.05$ (Fig 3.7). The osteoclasts on the surface of the bone were of irregular shape and stained black throughout the cell but with more intense staining of the membrane (Fig 3.8a). The osteoclasts on the membrane also stained black throughout the cell again with more intense staining of the membrane. They showed greater irregularity of shape (Fig 3.8b).

3.6. The endocranial membrane is the source of osteoclasts on mouse parietal bones.

The previous experiments suggest that the endocranial membrane is the source of the osteoclasts seen on the bone surface and that in the presence of PGE_2 osteoclasts translocate from the membrane to the bone surface. Earlier work has shown that when the endocranial membrane is not in contact with the bone surface, the increase in the number of osteoclasts seen on the bone following incubation with PGE_2 does not occur. In order to obtain further evidence that this was the case, bones were incubated with indomethacin for one day after which the endocranial membranes of some bones were peeled back to the sagittal suture. Other bones were left intact. All bones were incubated for a further one day in the presence of PGE_2 and were stained for the presence of TRAP+OC.

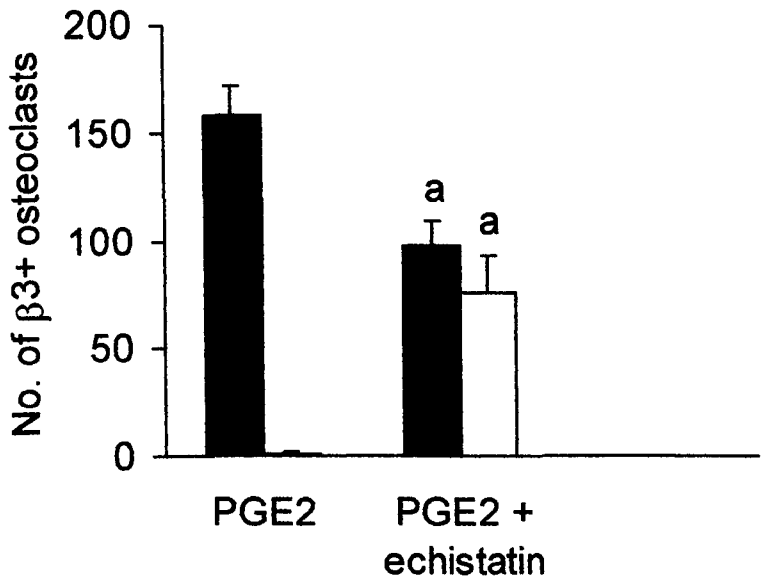
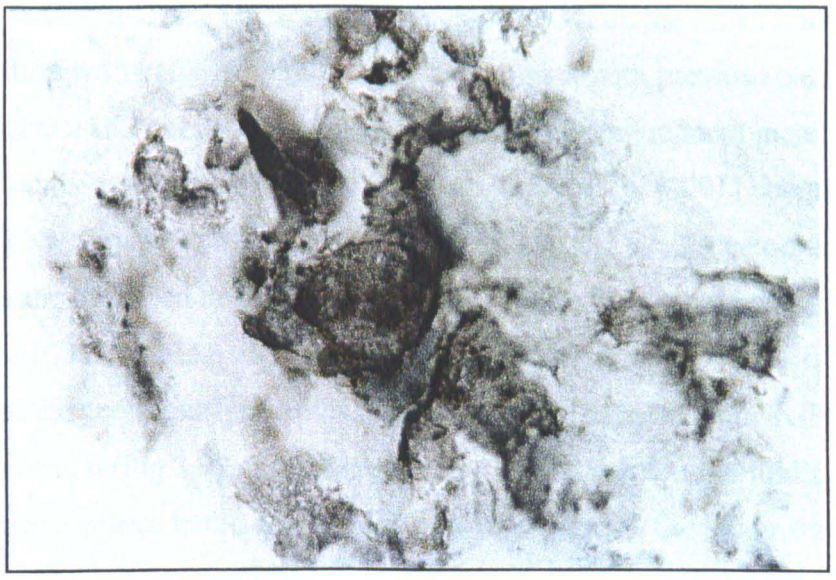


Figure 3.7: The effect of 10 nM echistatin on β3+OC on the bone surface (■) and endocranial membrane (□). Bones were cultured for 24 h in medium containing 1 μM indomethacin and then a further 24 h in 1 μM PGE_2 (control) or 1 μM PGE_2 together with 10 nM echistatin. The numbers of β3+OC are shown as the mean number of osteoclasts and the SEM. For each culture condition n = 5.
a, $p<0.05$ compared to PGE_2

a



b

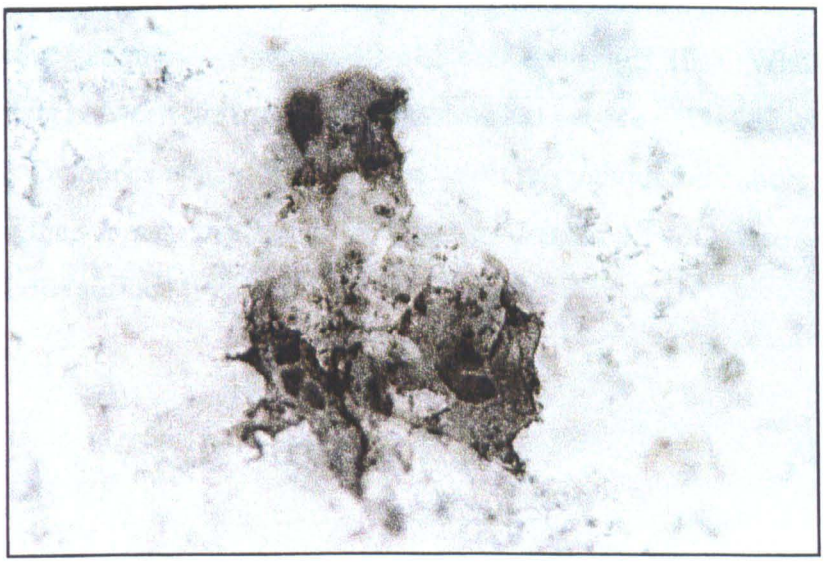


Figure 3.8. The effect of echistatin on β3+OC on the endocranial surface of bone and the endocranial membrane. Bones were cultured in medium containing 1 μM indomethacin for 20 h and a further 20 h in 1 μM PGE_2 plus 10 nM echistatin. They were then immunostained for β3. a) β3+OC on the endocranial surface of bone. They show black staining of the cytoplasm with more intense staining of the membrane. They are irregular in appearance. b) β3+OC on the endocranial membrane. They also show intense black staining more especially at the cell membrane and greater irregularity in appearance. Total magnification x 884.

There was an increase in the number of TRAP+OC on the intact bone following incubation with PGE_2 (Fig 3.9). This is consistent with previous results. However, this increase did not occur on bones which had the endocranial membranes peeled back and there were significantly fewer on the bone ($p<0.001$) compared to the intact bone (Fig 3.9). Significantly more TRAP+OC were seen on the endocranial membrane of peeled bones ($p<0.05$) cultured with PGE_2 compared to indomethacin (Fig 3.9). There was however a significant increase in the number of TRAP+OC seen on the peeled surface of the bones ($p<0.001$) cultured with PGE_2 compared to indomethacin (Fig 3.9). This increase presumably reflects the finding that not all osteoclasts adhere to the endocranial membrane during the indomethacin culture period. These osteoclasts which remain on the bone during the culture period with indomethacin appear to be capable of being re-activated by PGE_2. β3+OC were seen on the surface of bones which had half the membrane peeled back where the membrane had remained in contact with the bone (Fig 3.10a). Where there had been no contact between the membrane and the bone very few β3+OC were seen (Fig 3.10a). On bones which had remained intact throughout the culture period i.e. had not had their membranes removed, large numbers of β3+OC were seen covering the entire bone surface (Fig 3.10b).

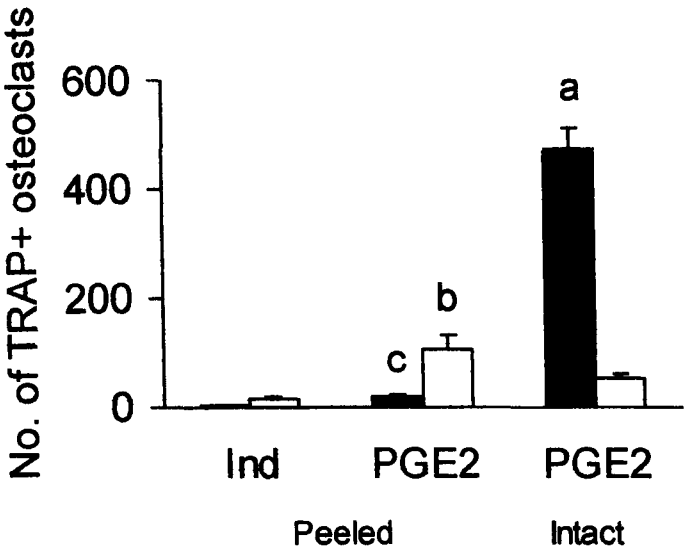


Figure 3.9: The effect of peeling the endocranial membrane on the number of TRAP+OC on the bone surface (■) and the endocranial membrane (□). Bones were cultured for 20 h in medium containing 1 μM indomethacin (Ind) following which some had their endocranial membranes peeled back to the sagittal suture, others were left intact. Peeled bones were then incubated for a further 20 h in either 1 μM indomethacin or 1 μM PGE_2 whilst intact bones were incubated in 1 μM PGE_2 alone. The numbers of TRAP+OC are shown as the mean and SEM. For all conditions n = 5.

a, $p<0.001$ compared to the peeled bone cultured with PGE_2

b, $p<0.05$ compared to indomethacin

c, $p<0.001$ compared to indomethacin

a

b

Figure 3.10: The effect of peeling back the endocranial membrane on β3+OC on the endocranial surface of bone and the endocranial membrane. Bones were cultured in medium containing 1 μM indomethacin for 20 h after which some of the endocranial membranes were peeled back to halfway across the bone keeping the membrane in contact with half the bone. Both intact and peeled bones were then incubated for a further 20 h in medium containing 1 μM PGE_2. They were then stained for β3+OC. a) A peeled bone showing β3+OC on half of the bone where the membrane remained in contact with the bone. The other half of the bone which did not have contact with the endocranial membrane shows very few β3+OC. b) A bone which remained intact throughout the culture period showing large numbers of β3+OC over the entire surface of the bone. Total magnification x 28.

4. Effect of osteoprotegerin ligand on osteoclasts.

4.1 Effect of sOPGL on TRAP+OC on freshly isolated parietal bones.

Previous results have shown a large decrease in the number of TRAP+OC counted on the bone surface when mouse parietal bones are incubated in the presence of indomethacin for one day (Fig 3.1). Concurrently, there was an increase in the number of TRAP+OC seen on the adjacent endocranial membrane. When parietal bones were incubated together with the soluble form of recombinant human osteoprotegerin ligand, (sOPGL) in the presence of indomethacin, there was an inhibition of this translocation of osteoclasts from the bone surface. There were significantly more TRAP+OC on the bone ($p<0.05$) after a one day culture with all concentrations from 10 ng cm^{-3} up to 100 ng cm^{-3} sOPGL compared to the indomethacin control (Fig 4.1). There appeared to be a dose-response effect up to 50 ng cm^{-3}. No significant trend was seen for the number of TRAP+OC seen on the endocranial membrane. The morphology of the TRAP stained osteoclasts in the presence of sOPGL is very similar to that seen with PGE_2, the osteoclasts stained red throughout the cell, appeared rounded and were seen in resorption lacunae (Fig 4.2)

4.2. Effect of sOPGL on the number of TRAP+OC on the surface of bone following a one day incubation in indomethacin.

Previous results showed that following a one day incubation with indomethacin and a further one day incubation in PGE_2 the numbers of TRAP+OC on the bone surface are significantly increased (Fig 3.5). A similar experiment was carried out to investigate whether sOPGL would have the same effect on osteoclasts adhered to the endocranial membrane after a one day incubation with indomethacin. Following a one day incubation with 1 μM indomethacin and a further one day

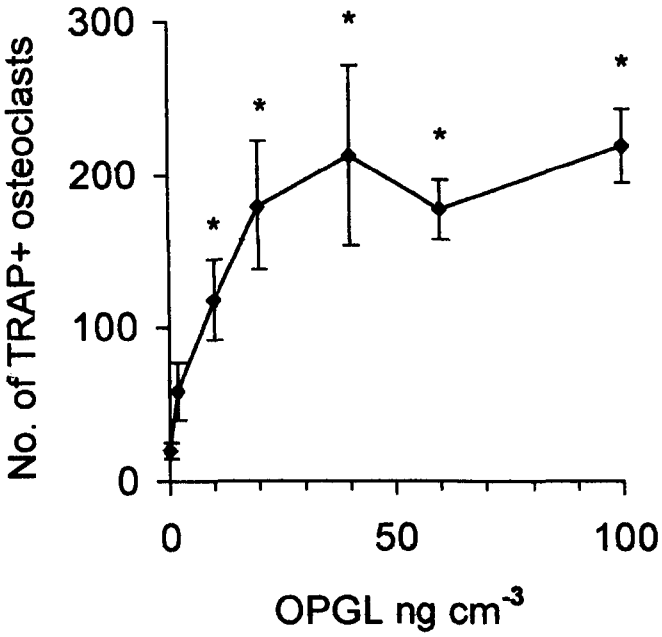


Figure 4.1: The effect of sOPGL on the number of TRAP+OC on the bone surface Freshly isolated parietal bones were cultured for 24 h in medium containing1 μM indomethacin together with varying concentrations of sOPGL. Values are shown as the mean and SEM of the number of TRAP+OC on the bone surface. For each culture condition n = 4 to 8.

*, $p<0.05$ compared to the zero sOPGL control

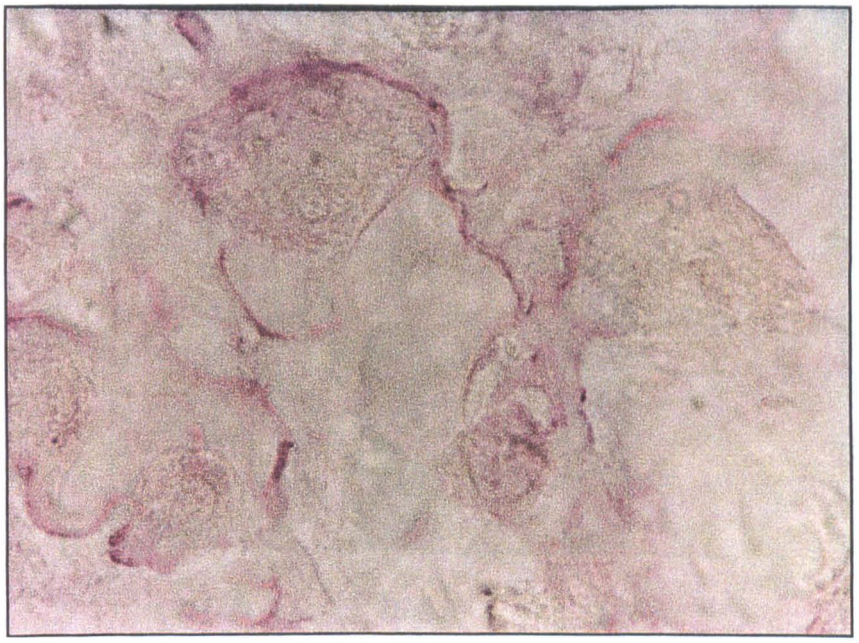


Figure 4.2: The effect of sOPGL on TRAP+OC on the endocranial surface of bone. Freshly isolated parietal bones were incubated in medium containing varying concentrations of sOPGL together with 1 μM indomethacin for 24 h. The bones were stained for TRAP following removal of the endocranial membrane. Osteoclasts are stained red, appear rounded in shape and can be seen associated with resorption lacunae. Total magnification x 884.

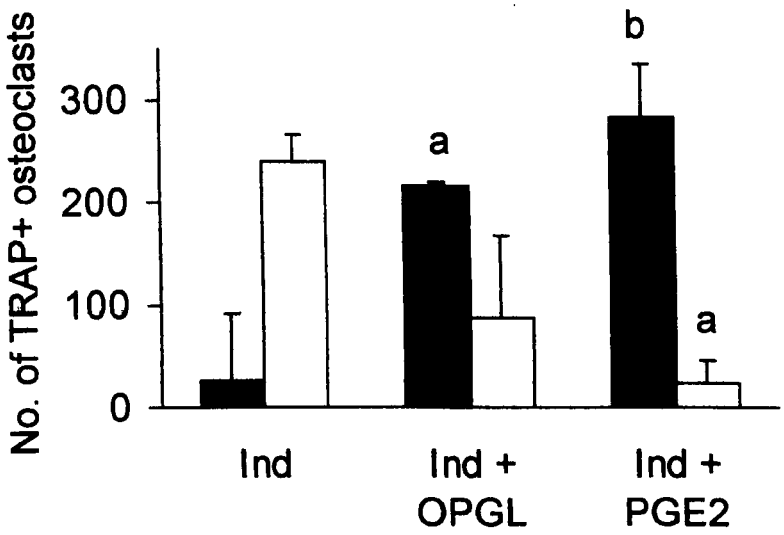


Figure 4.3: The effect of sOPGL on the number of TRAP+OC in the presence of indomethacin seen on the bone surface (■) and the endocranial membrane (□). Bones were cultured for 24 h in medium containing 1 μM indomethacin (Ind) followed by a further 24 h in the presence of either indomethacin alone, indomethacin plus 100 ng cm^{-3} sOPGL or indomethacin plus 1 μM PGE_2. The numbers of TRAP+OC are shown as the mean and the SEM. For each culture condition n = 5 to 6.

a, $p<0.05$ compared to indomethacin alone

b, $p<0.005$ compared to indomethacin alone

incubation with indomethacin together with 1 μM PGE_2 there was a recovery of TRAP+OC on the bone surface ($p<0.005$) confirming previous results (Fig 4.3). There was a significant decrease in the number of TRAP+OC on the endocranial membrane in the presence of PGE_2 ($p<0.05$) compared with indomethacin alone. sOPGL in the presence of indomethacin also stimulated this translocation of osteoclasts from the endocranial membrane to the bone surface and there was a significant increase in TRAP+OC on the bone ($p<0.05$) compared with indomethacin alone (Fig 4.3). However there was no significant decrease in the number of TRAP+OC seen on the endocranial membrane compared to indomethacin alone.

4.3 The endocranial membrane is the source of osteoclasts when mouse parietal bones are incubated with sOPGL.

The previous experiments have shown that sOPGL causes an increase in the number of osteoclasts on the bone surface. sOPGL is known to cause differentiation of osteoclasts so in order to provide evidence that the increase in the number of TRAP+OC was due to translocation from the endocranial membrane to the bone, following incubation for one day in indomethacin, the membranes of some bones were peeled back to the sagittal suture. Bones were then incubated for a further 6 h in the presence of sOPGL. The osteoclasts were then stained for the presence of the integrin β3 subunit. As expected from previous results there were large numbers of β3+OC in intact bone and few on the endocranial membrane. Significantly more β3+OC were seen on the intact bone compared to the peeled bone $p<0.001$ (Fig 4.4). Conversely there were significantly fewer β3+OC on the endocranial membranes of the intact bones compared to the peeled bones $p<0.001$ (Fig 4.4). Those β3+OC which were seen on the endocranial membrane of the intact bones after 6 h with sOPGL, disappeared by 24 h in culture and may be increasing their expression of β3 prior to adhering to the bone surface. The osteoclasts on the endocranial membrane of the peeled bones were rounded with the cytoplasm retracting as if detaching from the membrane (Fig 4.5).

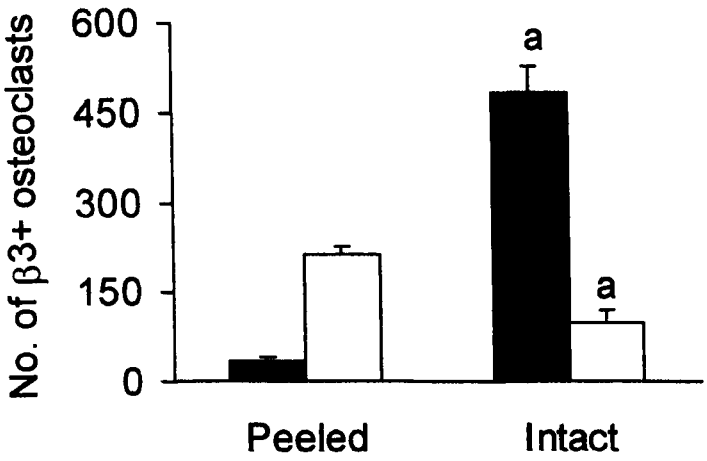


Figure 4.4: The effect of peeling on β3+OC on the bone surface (■) and the endocranial membrane (□) in the presence of sOPGL. Bones were incubated in medium containing 1 μM indomethacin for 24 h after which the endocranial membranes of some of the bones were peeled back to the sagittal suture. All bones were then incubated in the presence of 100 ng cm^{-3} sOPGL for a further 6 h. The numbers of β3+OC are shown as the mean and the SEM. For all culture conditions n = 5 to 6.

a, $p<0.001$ compared to the peeled bones

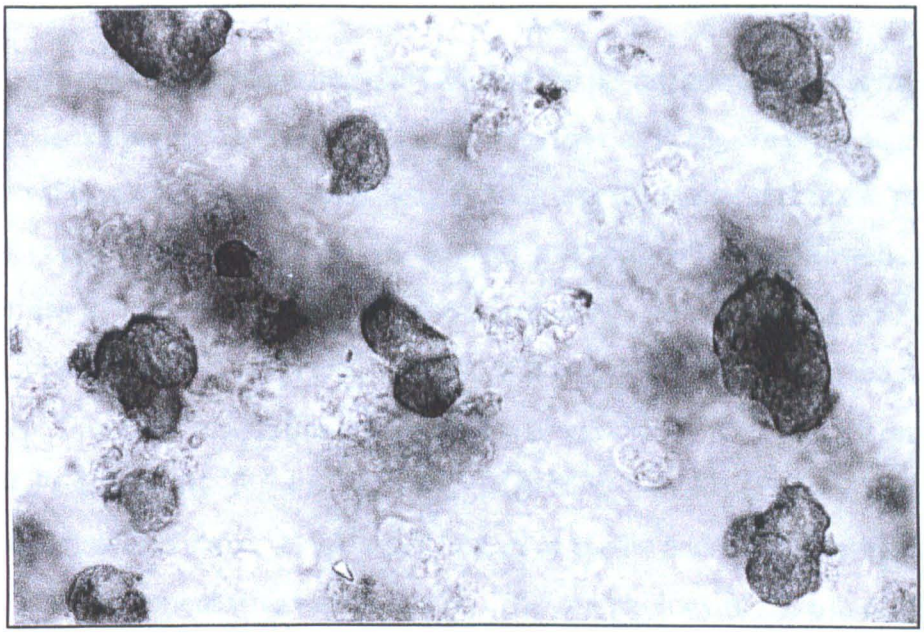


Figure 4.5: The effect of sOPGL on β3+OC on the peeled surface of the endocranial membrane. Bones were cultured in medium containing 1 μM indomethacin for 24 h following which the endocranial membrane was peeled back to the sagittal suture and bones were then cultured in medium containing 100 ng cm^{-3} sOPGL for a further 6 h. They were then immunostained for β3. Osteoclasts appear rounded and loosely attached.

4.4 Effect of sOPGL on β3+OC.

Previous experiments have shown that the β3 integrin subunit is important in the translocation process that occurs in the presence of PGE_2. In order to ascertain whether it is involved in the apparently similar process that occurs in the presence of sOPGL, bones were cultured for one day in 1 μM indomethacin followed by a further day in either indomethacin alone or indomethacin plus 100 ng cm^{-3} sOPGL or indomethacin plus 1 μM PGE_2. Osteoclasts were then immunostained for the β3 subunit. There was a significant increase in the number of β3+OC seen on the bone with sOPGL $p<0.001$ (Fig 4.6) as well as with PGE_2 $p<0.001$ (Fig 4.6) compared with indomethacin alone. No β3+OC were seen on the endocranial membrane which is in agreement with earlier results. The morphology of the osteoclasts after incubation with sOPGL may be seen in Fig 4.7. The osteoclasts appeared to be in resorption lacunae suggesting that bone resorption was taking place.

4.5 Time course of the appearance of β3+OC on the bone surface in response to PGE_2 and OPGL.

In order to investigate the time needed for β3+OC to be appear on the bone surface parietal bones were incubated with 1 μM indomethacin for one day followed by 0 to 4 h, at hourly intervals, in 100 ng cm^{-3} sOPGL. There was a significant increase in the number of β3+OC seen on the bone surface ($p<0.05$) after 3 h (Fig 4.8).

Previous results have shown that PGE_2 significantly increases the number of β3+OC seen on the bone (Fig 4.6) as does sOPGL (Fig 4.6). The time course for the appearance of β3+OC on the bone in response to sOPGL may be seen in Fig 4.8 and an experiment was carried out to compare the amount of time taken for β3+OC to be seen on the bone in response to PGE_2. Parietal bones were incubated with 1 μM

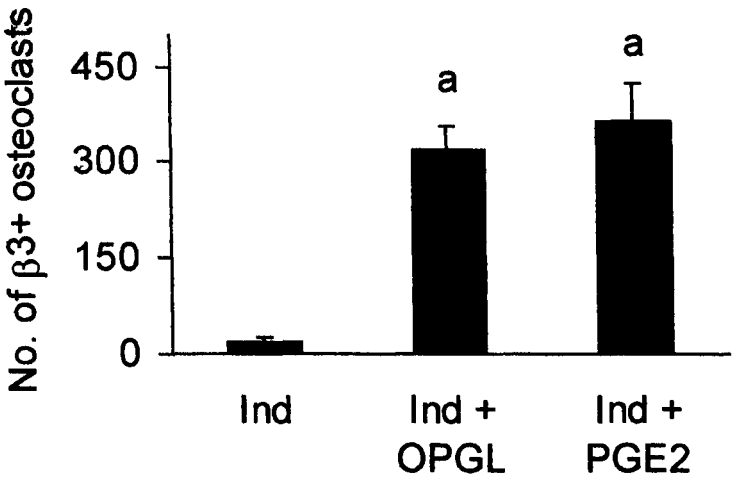


Figure 4.6: The effect of sOPGL on the number of β3+OC seen on the bone surface (■). Bones were cultured for 24 h in medium containing 1 μM indomethacin (Ind) followed by 24 h in either indomethacin alone or indomethacin plus 100 ng cm^{-3} sOPGL or indomethacin plus 1 μM PGE_2. The numbers of β3+OC are shown as the mean and the SEM. For each culture condition n = 4.

a, $p<0.001$ compared to indomethacin alone

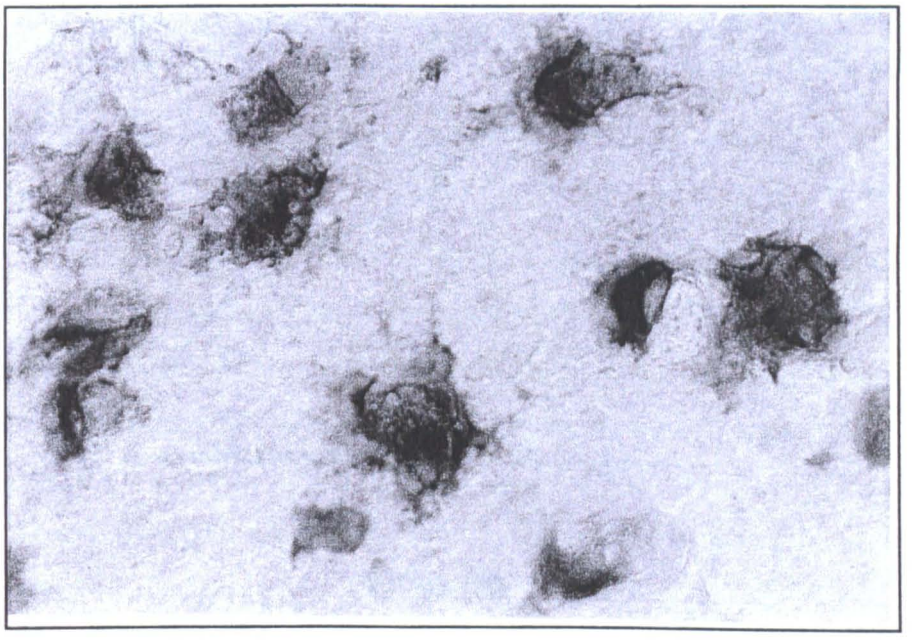


Figure 4.7: The effect of sOPGL on β3+OC on the endocranial surface of bone. Bones were cultured in the presence of 1 μM indomethacin for 24 h followed by 24 h in 100 ng cm^{-3} sOPGL. They were then immunostained for β3. The osteoclasts appear to be in resorption lacunae suggesting that bone resorption is taking place.

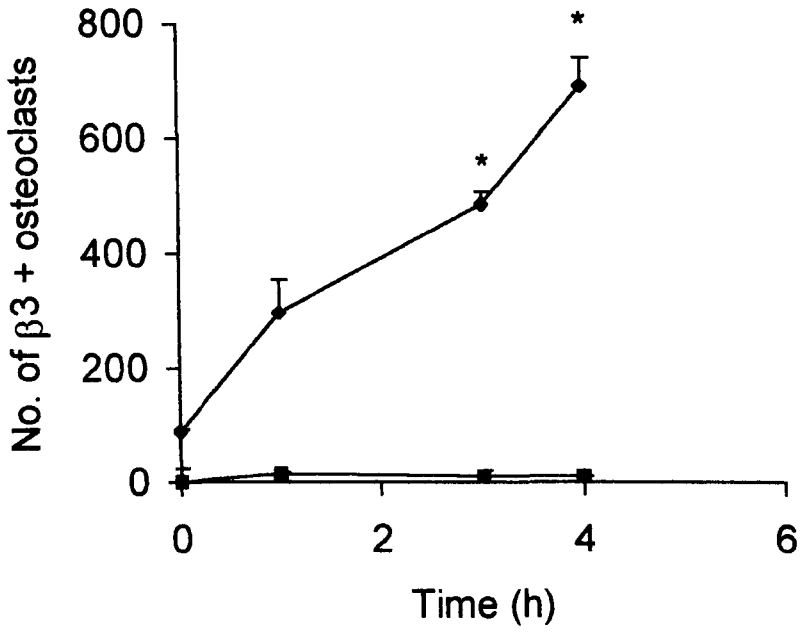


Figure 4.8: Time course for the appearance of β3+OC on the bone surface(◆) and the endocranial membrane (■). Bones were cultured for 24 h in medium containing 1 μM indomethacin followed by the numbers of hours shown in 100 ng cm^{-3} sOPGL. The numbers of β3+OC are shown as the mean and the SEM. For each culture condition n = 3.

*, $p<0.05$ compared to zero time

indomethacin for one day followed by 0 to 24 h at the time intervals shown with 1 μM PGE_2. There was a significant increase in the number of β3+OC seen on the bone surface ($p<0.001$) after 6 h (Fig 4.9). Thus, β3+OC appeared earlier in response to sOPGL than to PGE_2.

4.6 Effect of echistatin on TRAP+OC in the presence of sOPGL.

Previous results have shown that the β3 integrin subunit is important in the translocation of osteoclasts from the membrane to the bone surface with both PGE_2 and sOPGL. In order to assess the role of integrins in the adhesion of osteoclasts to the bone surface under the influence of sOPGL bones were incubated in indomethacin for one day followed by one day in sOPGL together with echistatin. There were significantly more TRAP+OC seen on the bone surface with sOPGL compared to either sOPGL plus echistatin $p<0.001$, or indomethacin alone $p<0.001$ (Fig 4.10). At the same time there were significantly fewer TRAP+OC seen on the endocranial membrane with sOPGL compared with either sOPGL plus echistatin $p<0.001$or indomethacin alone $p<0.001$ (Fig 4.10). In addition there were significantly more TRAP+OC on the bone surface with sOPGL plus echistatin compared with indomethacin alone $p<0.05$ (Fig 4.10). Some osteoclasts are able to translocate to the bone surface in the presence of echistatin but in much reduced numbers. The stained osteoclasts appear very similar to those cultured in the presence of PGE_2 plus echistatin. They were not spread out as osteoclasts do in the absence of echistatin and they were not associated with lacunae (Fig 3.8).

4.7 Assay of mRNA for OPGL.

In order to ensure that the amount of PCR product properly reflected the amount of cDNA in the initial reaction tube a dose-response curve was constructed to show the relationship between the amounts of cDNA added to the PCR reaction and the intensity of the ethidium bromide stained product. It established a linear range for the assay. A sample known to contain OPGL cDNA was diluted 1 in 10 in nuclease- free water and varying amounts were added to the PCR reaction ranging from 0 to 9 μl. The product bands were made visible following staining with

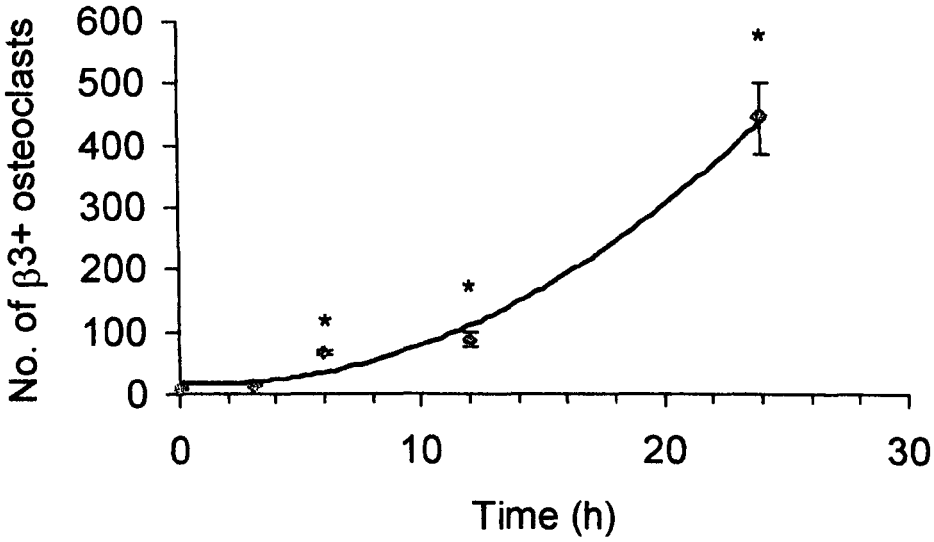


Figure 4.9: Time course for the appearance of β3+OC on the bone surface. Bones were cultured in medium containing 1 μM indomethacin for 24h followed by the number of hours shown in 1 μM PGE_2. The numbers of β3+OC are shown as the mean and the SEM. For each culture condition n = 4.

*, $p<0.05$ compared to zero time

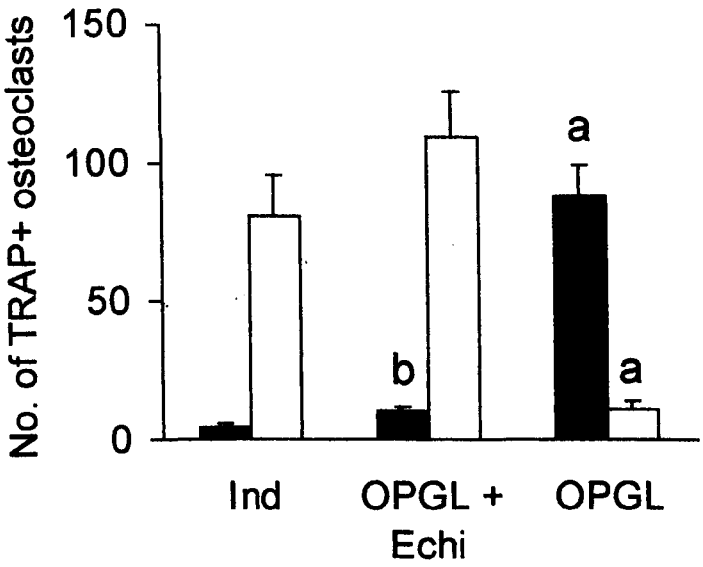


Figure 4.10: Effect of 10 nM echistatin on TRAP+OC on the bone surface (■) and endocranial membrane (□). Bones were cultured for 24 h in the medium containing 1 μM indomethacin (Ind) and then for a further 24 h in either indomethacin alone or 100 ng cm^{-3} sOPGL or sOPGL plus 10 nM echistatin (Echi). The numbers of TRAP+OC are shown as the mean and SEM. For each culture condition n = 6 to 7. a, $p<0.001$ compared with both sOPGL plus echistatin and indomethacin alone b, $p<0.05$ compared with indomethacin alone,

ethidium bromide with UV light. The bands were quantitated using an image analysis system and if a band intensity exceeded the upper limit of linearity the entire PCR comparison was repeated with diluted samples of cDNA. The results show that the upper limit of linearity is 1.5 (Fig 4.11).

4.8 Restriction enzyme analysis of the OPGL product.

The size of the OPGL product was determined by agarose electrophoresis and comparison to a known standard. The size corresponded to that predicted from the sequence obtained from the NCBI internet site described in the materials and methods (Fig 4.12). An NCBI BLAST search revealed that the primers selected were specific to mOPGL. In order to provide further evidence that the product obtained with primers for OPGL is in fact OPGL message, a restriction enzyme digest was carried out on the OPGL product. The product was incubated together with the restriction enzyme PVUII at 37°C for 1 h. One control consisted of one sample with no restriction enzyme present. Another control consisted of an incubation of the OPGL product with an enzyme (StuI) which should not cut authentic OPGL on the basis of the published sequence. The enzyme PVUII cuts after base number 190 of the 407 base pairs of the OPGL product at CAG/CTG on the upper strand and GTC/GAC on the lower strand. The two bands appear on the electrophoresis gel at 190 base pairs and 217 base pairs (Fig 4.13).

4.9 Expression of OPGL mRNA in response to indomethacin and PGE_2.

This experiment was carried out in order to look at the effect of PGE_2 on the expression of mRNA for OPGL. Whole calvaria from mice were cultured with 1 μM indomethacin for one day followed by 4 h in either 1 μM indomethacin or 1 μM PGE_2. RNA was extracted and transcribed into cDNA and amplified with PCR as previously described. Results are shown in Fig. 4.14a and each lane represents RNA extracted from two calvaria. PGE_2 consistently resulted in high levels of product having the correct number of base pairs for the OPGL product.

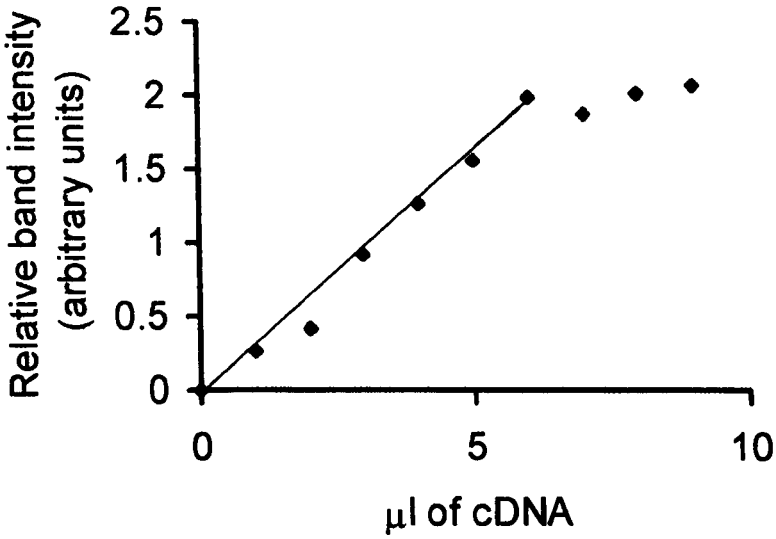


Figure 4.11: Differing volumes of a single cDNA preparation were added to the standard PCR reaction for the OPGL gene product. The products were subjected to electrophoresis, were stained and quantified using the Sight System image analysis method (see Materials and Methods). Band intensities of the OPGL products were expressed relative to that of one of the ladder bands in order to control variations between electrophoresis runs. The line represents the least squares fit. The upper limit for linearity of this dose response curve was taken as 1.5. Any results falling outside this linear range would need to be repeated following dilution.

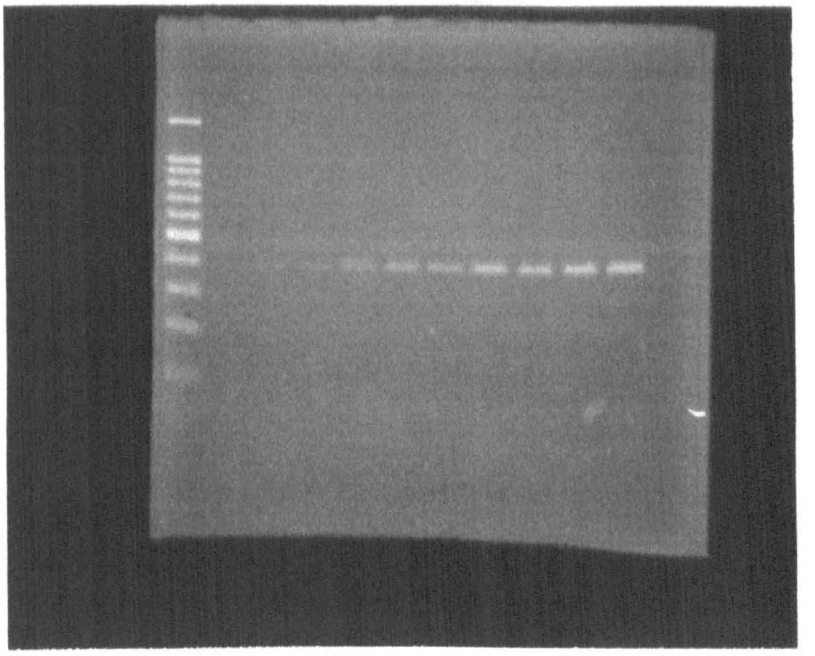


Figure 4.12: The relationship between the volume of cDNA added and product band intensity.

Differing volumes of a single cDNA preparation were added to the standard PCR reaction for the OPGL gene product. The products were subjected to electrophoresis, stained with ethidium bromide and band intensities were quantified using Sight System image analysis. The OPGL product should be 407 bp.

Lane 1 = DNA 100 bp ladder

Lanes 2-11 = Differing volumes of cDNA added to the PCR reaction ranged from 0 to 9 μl.

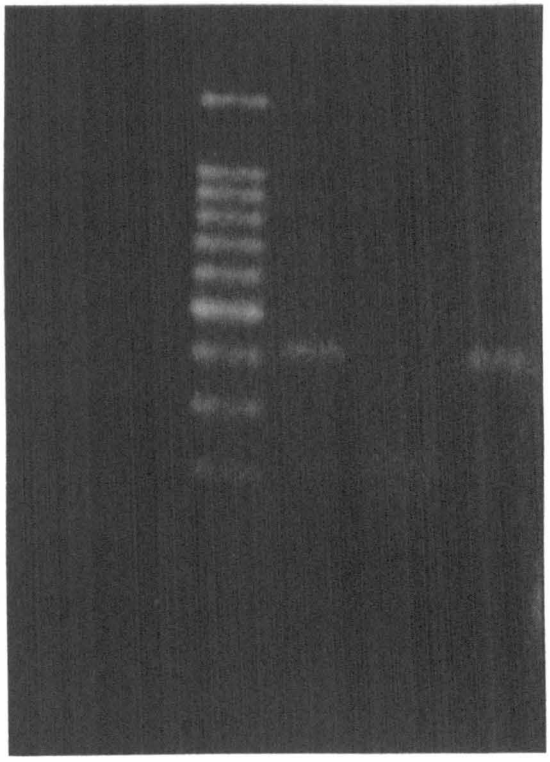


Figure 4.13: Restriction enzyme digest of the OPGL product.

OPGL product was digested with the restriction enzyme PVUII for 1 h. The product was subjected to electrophoresis and stained with ethidium bromide. The enzyme cuts after base number 190 of the 407 base pairs of the OPGL product giving two bands at 190 bp and 217 bp.

Lane 1 = 100 bp ladder.

Lane 2 = no enzyme control

Lane 3 = 217 and 190 bp band products of digestion with PVUII restriction enzyme.

Lane 4 = control incubation with restriction enzyme Stu I which does not cut authentic OPGL gene product.

a

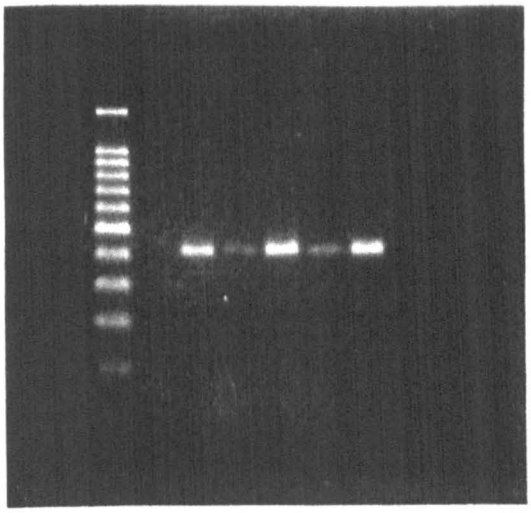


Figure 4.14a: Expression of OPGL mRNA in response to PGE_2 and indomethacin. Total RNA was extracted form whole calvaria cultured in the presence of 1 μM indomethacin for 24 h followed by a further 4 h in the presence of either 1 μM indomethacin or 1μM PGE_2. Following reverse transcription the samples underwent PCR and were subjected to electrophoresis and staining with ethidium bromide. The presence of PGE_2 resulted in consistently higher levels of the OPGL gene product. The OPGL product should be 407 bp.

Lane 1 = 100 bp DNA ladder.

Lanes 2,4,and 6 = RNA from calvaria cultured with indomethacin alone.

Lanes 3,5 and 7 = RNA from calvaria cultured with PGE_2 for 4 h.

Lane 8 = no cDNA negative control.

b

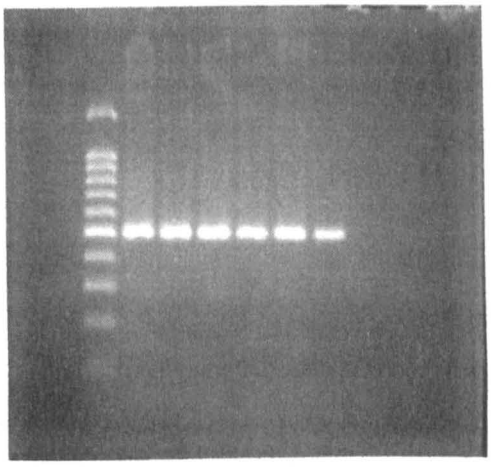


Figure 4.14b: These samples are the same as those shown in figure 4.14a but amplified for the β actin gene product to ensure that the original extraction contained about the same amounts of mRNA for β actin. The β actin product should be 514 bp.

Very little or no OPGL product was detected following incubation with indomethacin. The negative control consisted of a sample of nuclease-free water instead of RNA which had undergone reverse transcription and so should not contain any cDNA. The sample then underwent PCR and electrophoresis and showed that no contamination of the samples with either extraneous RNA or DNA had occurred.

The PCR product for β Actin was similar in all samples and this demonstrated similar amounts of mRNA were included in the reverse transcription step (fig 4.14b).

4.10 Time course of the production of mRNA for OPGL in response to PGE_2.

In order to see how quickly mRNA for OPGL is produced in response to PGE_2 whole calvaria were put into media containing 1 μM indomethacin for 24 h. The following day two calvaria were briefly rinsed in sterile PBS and placed in a microcentrifuge tube and frozen in liquid nitrogen. The other bones had fresh medium containing 1 μM PGE_2 added to them and were incubated for 3,6,12, and 24 h. At the allotted time the bones were removed from the media and were frozen in liquid nitrogen. The following day the bones incubated for 24 h were briefly frozen in liquid nitrogen and then all samples were processed for RNA extraction, reverse transcription, and PCR amplification. The results are shown in Fig. 4.15 with incubation time plotted against band intensity. There appears to be peak levels of RNA for OPGL at between 3 and 6 h. β actin levels were similar in all samples and show that similar levels of mRNA were included in the reverse transcription step (Fig 4.16).

4.11 Induction of mRNA for OPGL with $1,25D_3$ and PTH.

In order to investigate whether other stimulators of bone resorption increase the amount of mRNA for OPGL in mouse calvaria, whole calvaria were put into media containing 1 μM indomethacin for one day followed by a further day in either indomethacin or indomethacin plus 10 nM $1,25D_3$ or indomethacin plus 4 nM PTH.

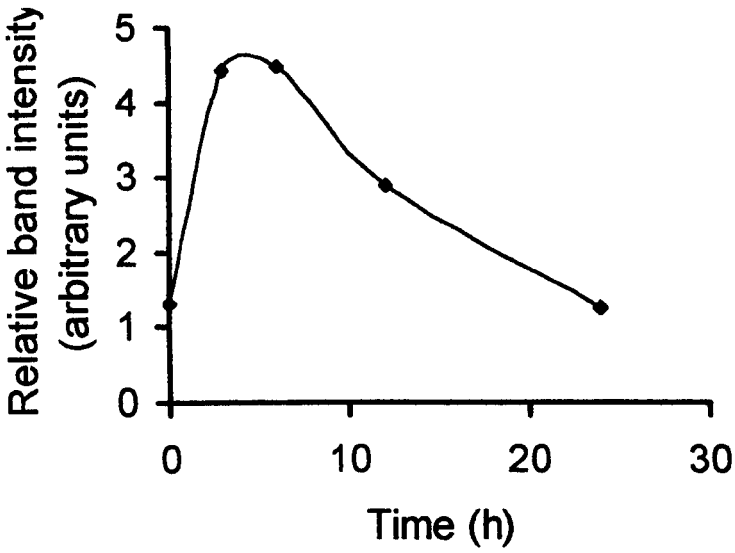


Figure 4.15: Time course for the production of mRNA for OPGL in response to PGE_2

Whole calvaria were incubated in the presence of 1 μM indomethacin for 24 h followed by incubation for varying amounts of time shown in the presence of 1 μM PGE_2. The products were subjected to electrophoresis, then stained and quantified using the Sight System image analysis method. Band intensities of the OPGL products were expressed relative to that of one of the ladder bands in order to control variations between electrophoresis runs.

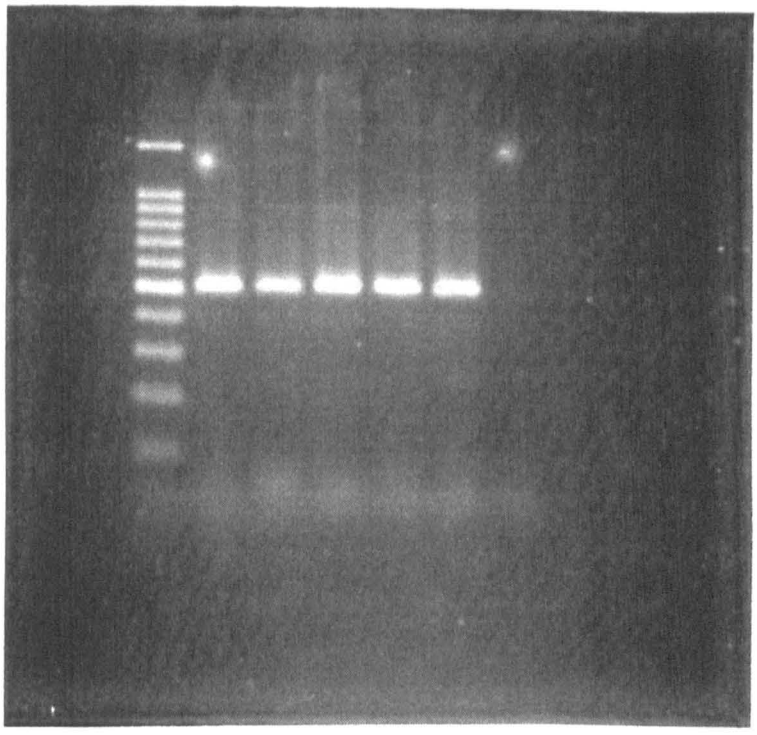


Figure 4.16: β actin levels in total RNA extractions from fig 4.15.

These samples are the same as those shown in fig 4.15 but amplified for the β actin gene product to ensure that the original extractions contained about the same amounts of mRNA for β actin.

Lane 1 = 100 bp DNA ladder.

Lanes 2-6 samples corresponding to time course in figure 4.15.

Lane 7 = no cDNA negative control.

a

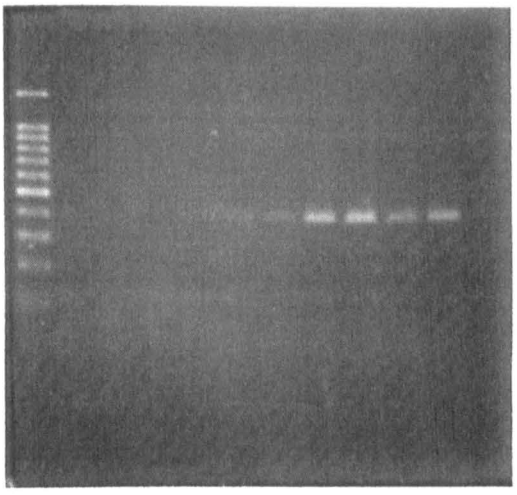


Figure 4.17a: Effect of $1,25D_3$ and PTH on mRNA levels for OPGL in mouse calvaria.

Whole calvaria were cultured for 24 h in 1 μM indomethacin followed by 24 h in 1 μM indomethacin alone or 1 μM indomethacin plus 10 nM $1,25D_3$ or 1 μM indomethacin plus 4 nM PTH. Total RNA was extracted and samples underwent PCR followed by electrophoresis and staining with ethidium bromide.

Lane 1 = 100 bp DNA ladder.

Lanes 6 and 7 = RNA from calvaria cultured with indomethacin.

Lanes 8 and 9 = RNA from calvaria cultured with $1,25D_3$.

Lanes 10 and 11 = RNA from calvaria cultured with PTH.

Lane 12 = no cDNA control.

b

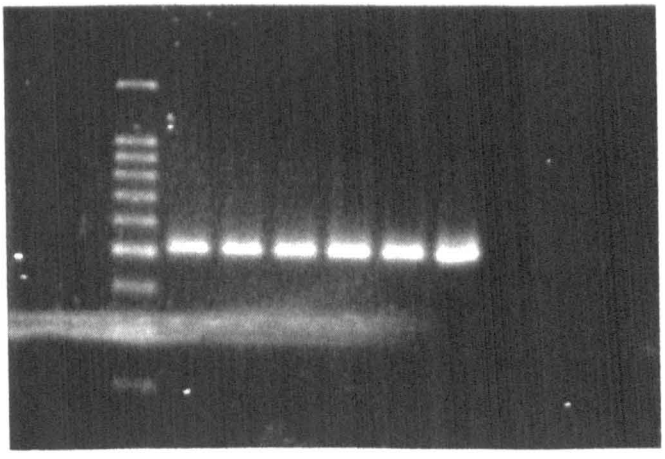


Figure 4.17b: These samples are the same as those shown in fig 4.17a but amplified for the β actin gene product to ensure that the original extractions contained about the same amounts of mRNA.

All samples were then processed for RNA extraction, reverse transcription and PCR amplification. Results are shown in fig 4.17a and each lane represents PCR product from RNA extracted from two calvaria. Both $1,25D_3$ and PTH increased the expression of mRNA for OPGL in mouse calvaria relative to those incubated with indomethacin (Fig 4.17a).

β actin levels were similar in all samples and show that similar levels of mRNA as opposed to total RNA were included in the reverse transcription step (Fig 4.17b).

5. Effect of osteoprotegerin on osteoclasts.

5.1 Effect of rhOPG on TRAP+OC on freshly isolated parietal bones.

When mouse parietal bones were incubated in the presence of recombinant human OPG (rhOPG) for one day there was a significant reduction in the number of TRAP+OC seen on the bone surface ($p<0.05$) for all concentrations compared to the no addition control (Fig 5.1). An increase in the number of TRAP+OC seen on the endocranial membrane ($p<0.001$) was only seen at a concentration of 25 ng cm^{-3} and above compared to the no addition control. However, 10 ng cm^{-3} rhOPG was sufficient to cause osteoclasts to detach from the bone, although larger numbers of osteoclasts were seen on the membrane with higher levels of rhOPG (Fig 5.1). However there was no significant difference between the total number of osteoclasts (bone plus endocranial membrane) with any of the treatments.

5.2 Effect of rhOPG on the number of TRAP+OC on PGE_2 stimulated bones.

Freshly isolated parietal bones were cultured for one day in the presence or absence of 100 ng cm^{-3} rhOPG or in the presence of 1 μM PGE_2 with or without rhOPG. There was a significant decrease in the number of TRAP+OC seen on the bone surface with rhOPG compared to the no addition control $p<0.01$ (Fig 5.2). At the same time there was a significant increase in the number of TRAP+OC seen on the endocranial membrane with rhOPG compared to the control $p<0.005$ (Fig 5.2). Similarly there was a significant decrease in the number of TRAP+OC seen on the bone surface with PGE_2 plus rhOPG compared to the PGE_2 control $p<0.001$ (Fig 5.2). rhOPG caused an indomethacin-like result with TRAP+OC, stellate in appearance, seen mainly on the endocranial membrane (Fig 5.3). The addition of rhOPG with PGE_2 blocked the effect of PGE_2 causing TRAP+OC to accumulate on the endocranial membrane rather than the bone surface (Fig 5.2).

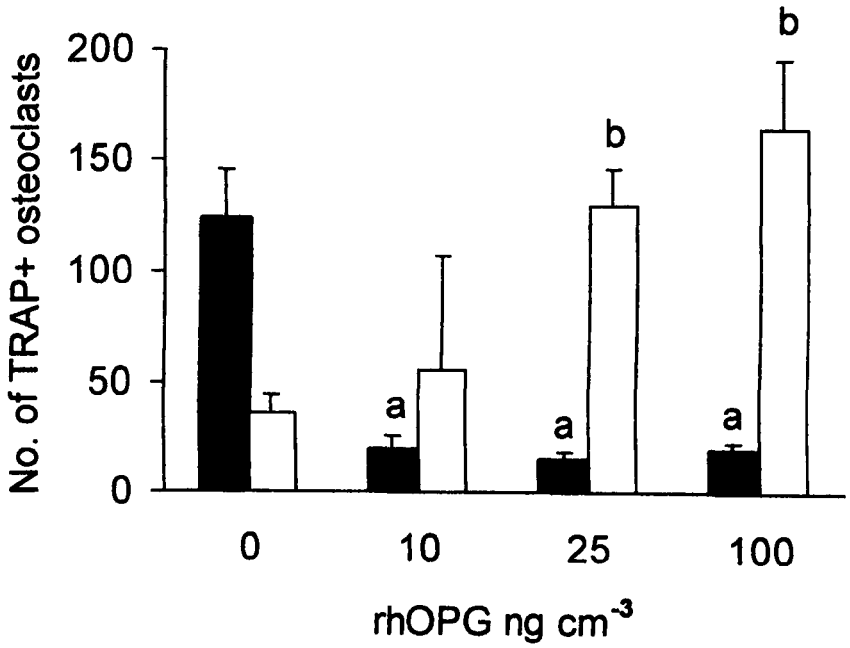


Figure 5.1: Effect of rhOPG on TRAP+OC on the bone surface (■) and the endocranial membrane (□) with freshly isolated parietal bones. Freshly isolated parietal bones were cultured for 24 h in medium containing varying concentrations of rhOPG. Values are shown as the mean and SEM of the number of TRAP+OC. For all culture conditions n = 6 to 10.

a, $p<0.05$ compared to the no addition control

b, $p<0.001$ compared to the no addition control

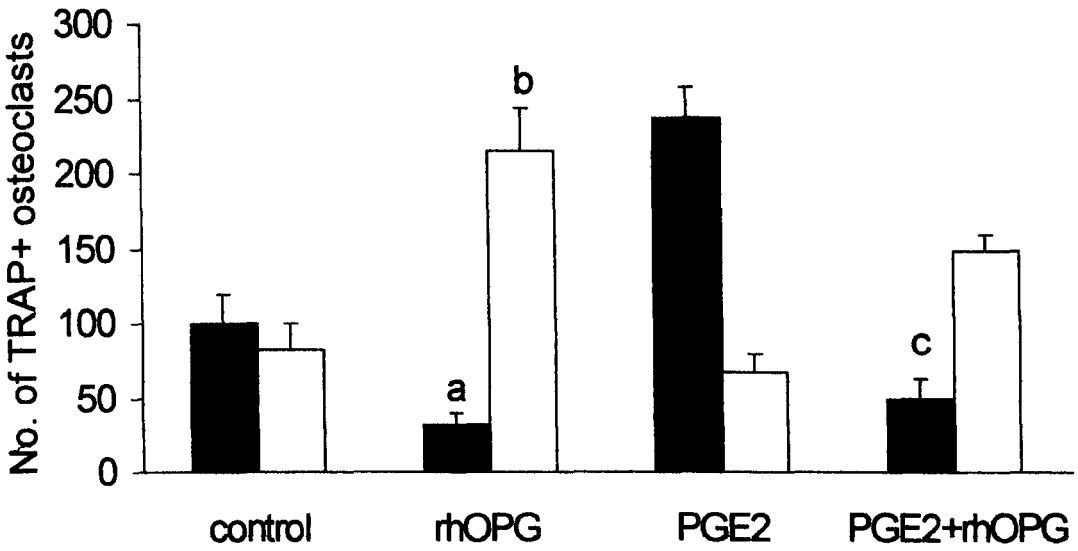


Figure 5.2: Effect of rhOPG on the number of TRAP+OC on the bone surface (■) and the endocranial membrane (□) with PGE_2 stimulated bones. Freshly isolated parietal bones were cultured for 24 h in the presence or absence of 100 ng cm^{-3} rhOPG or in 1 μM PGE_2 with or without rhOPG. Values are shown as the mean and SEM of the number of TRAP+OC. For each culture condition n = 5 or 6.

a, $p<0.01$ compared to the no addition control

b, $p<0.005$ compared to the no addition control

c, $p<0.001$ compared to the PGE_2 control

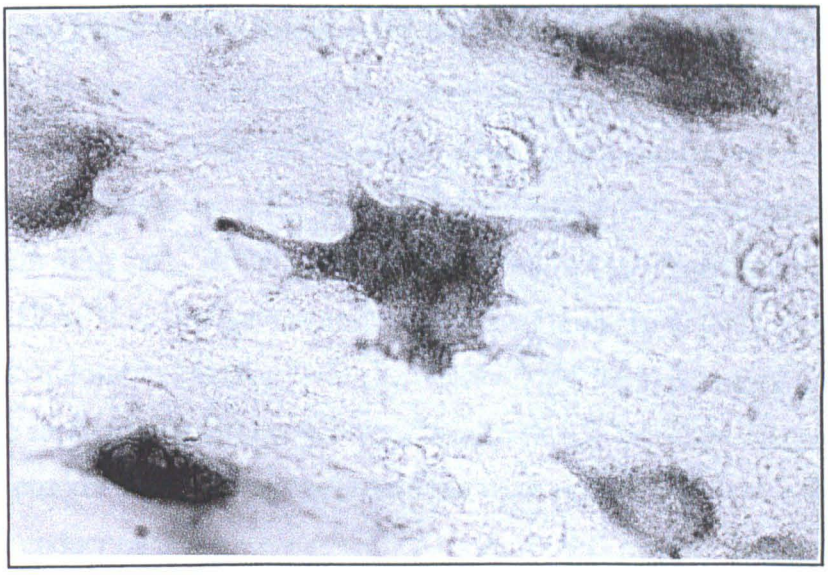


Figure 5.3: The effect of rhOPG on TRAP+OC on the endocranial membrane. Bones were incubated in medium containing 1 μM indomethacin for 24 h followed by 24 h in medium containing 1 μM PGE_2 plus 100 ng cm^{-3} rhOPG. The osteoclasts have taken on a stellate appearance presumably due to a change in adhesive properties causing cytoplasmic retraction. Total magnification x 490.

5.3 Effect of rhOPG on the number of TRAP+OC on $1,25D_3$ stimulated bones.

Parietal bones were cultured in the presence of 1 μM indomethacin for one day followed by one day in either 1 μM indomethacin or 10 nM $1,25D_3$ plus 100 ng cm^{-3} rhOPG or 10 nM $1,25D_3$ alone. There was a significant recovery in the number of osteoclasts seen on the bone ($p<0.001$) with $1,25D_3$ compared to indomethacin (Fig 5.4). At the same time there was a significant decrease in the number of TRAP+OC seen on the endocranial membrane ($p<0.005$) with $1,25D_3$ compared to indomethacin (Fig 5.4). rhOPG inhibited the appearance of TRAP+OC on the bone surface ($p<0.001$) when stimulated with $1,25D_3$ when compared with the $1,25D_3$ control (Fig 5.4). In addition there were significantly more TRAP+OC seen on the endocranial membrane ($p<0.05$) with rhOPG present than with $1,25D_3$ alone (Fig 5.4).

5.4 Effect of rhOPG on the number of TRAP+OC on PTH stimulated bones.

Parietal bones were cultured in the presence of 1 μM indomethacin for one day followed by one day in the presence of indomethacin alone or 4 nM PTH plus 100 ng cm^{-3} rhOPG or 4 nM PTH alone. There was a significant increase in the number of TRAP+OC seen on the bone surface ($p<0.001$) with PTH compared to indomethacin (Fig 5.5). At the same time there was a significant decrease in the number of TRAP+OC seen on the endocranial membrane ($p<0.005$) with PTH compared to indomethacin (Fig 5.5). However, there was no significant difference between the number of TRAP+OC seen on either the bone surface or the endocranial membrane with PTH compared to PTH plus rhOPG. In this case rhOPG does not significantly inhibit the translocation of osteoclasts brought about by PTH from the membrane to the bone unlike PGE_2 and $1,25D_3$.

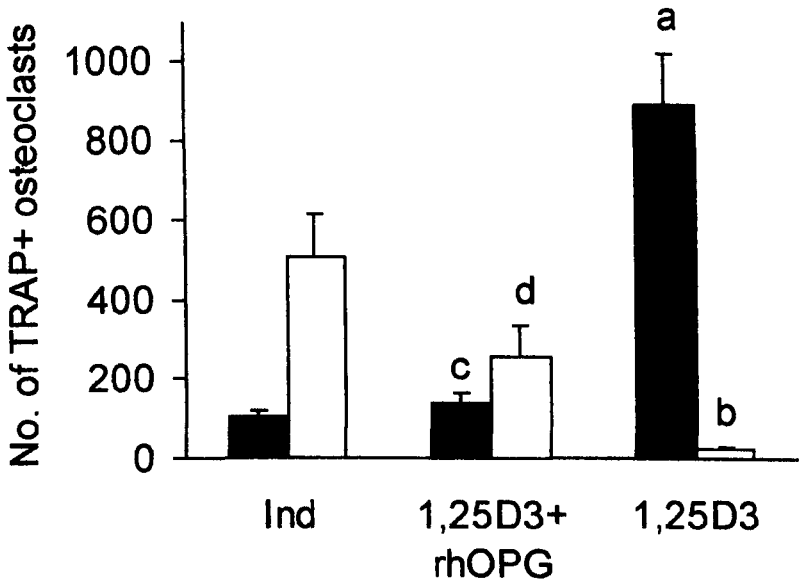


Figure 5.4: Effect of rhOPG on the number of TRAP+OC on the bone surface (■) and the endocranial membrane (□) when stimulated with $1,25D_3$. Parietal bones were incubated in medium containing 1 μM indomethacin (Ind) for 24 h followed by 24 h in either 1 μM indomethacin or 10 nM $1,25D_3$ plus 100 ng cm^{-3} rhOPG or 10 nM $1,25D_3$ alone. The numbers of TRAP+OC are shown as the mean and the SEM. For each culture condition n = 5 or 6.

a, $p<0.001$ compared to the indomethacin control

b, $p<0.005$ compared to the indomethacin control

c, $p<0.001$ compared to the $1,25D_3$ control

d, $p<0.05$ compared to the $1,25D_3$ control

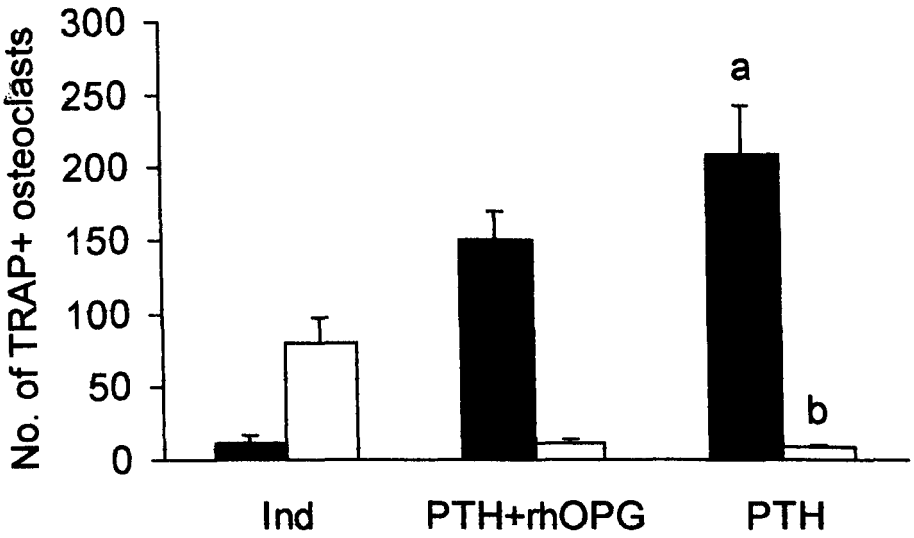


Figure 5.5: Effect of rhOPG on the number of TRAP+OC on the bone surface (■) and the endocranial membrane (□) stimulated with PTH. Parietal bones were cultured in medium containing 1 μM indomethacin (Ind) for 24 h followed by 24 h in the presence of either indomethacin or 4 nM PTH plus 100 ng cm^{-3} rhOPG or PTH alone. The numbers of TRAP+OC are shown as the mean and SEM. For each culture condition n = 6.

a, $p<0.001$ compared to the indomethacin control

b, $p<0.005$ compared to the indomethacin control

5.5 An antibody to OPG blocks the translocation of osteoclasts seen with indomethacin.

Freshly isolated parietal bones were incubated in the presence of 1 μM indomethacin alone or indomethacin together with 10 μg cm^{-3} goat anti-OPG function-blocking IgG antibody, 10 μg cm^{-3} goat IgG, or 1 μM PGE_2. The function-blocking antibody to rhOPG prevented the translocation of osteoclasts from the bone to the endocranial membrane ($p<0.001$) that took place in the presence of indomethacin (Fig 5.6). There was no significant difference between the number of TRAP+OC seen on the bone with indomethacin plus anti-OPG compared with those seen with indomethacin plus PGE_2. (Fig 5.6). Goat IgG at the same concentration had no effect on the number or distribution of TRAP+OC in the presence of indomethacin (Fig 5.6). In addition there were significantly fewer TRAP+OC seen on the endocranial membrane ($p<0.001$) in the presence of anti-OPG plus indomethacin than with indomethacin alone (Fig 5.6) and they did not differ significantly from the number seen with PGE_2.

5.6 Disappearance of β3+OC from bone following treatment with OPG.

Parietal bones were incubated in the presence of PGE_2 for one day and were then placed in fresh medium containing 100 ng cm^{-3} rhOPG for varying times between 0 and 8 h. There was a significant decrease in the number of β3+OC seen on the bone surface ($p<0.05$) at 4 h (Fig 5.7). There were no significant differences between the numbers of osteoclasts on the endocranial membrane at any of the time points. This is in line with previous results when β3+OC were not seen on the endocranial membrane. Staining with annexin V to look for apoptotic cells showed very few on the membrane following 24 h treatment with 100 ng cm^{-3} rhOPG. It appears that the osteoclasts lose their surface β3 expression in the presence of rhOPG.

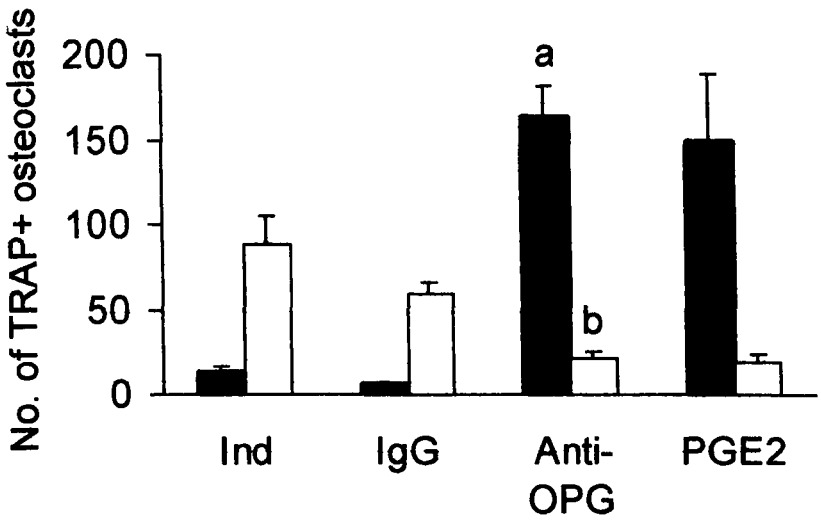


Figure 5.6: Effect of a function-blocking antibody to rhOPG on the number of TRAP+OC on the bone surface (■) and the endocranial membrane (□). Bones were cultured for 24 h in medium containing 1 μM indomethacin (Ind) alone or together with 10 μg cm^{-3} anti-OPG antibody or 1 μM PGE_2. The antibody control consisted of 1 μM indomethacin plus 10 μg cm^{-3} goat IgG. The numbers of TRAP+OC are shown as the mean and SEM. For each culture condition n = 5 or 6.

a, $p<0.001$ compared to the indomethacin control

b, $p<0.01$ compared to the indomethacin control

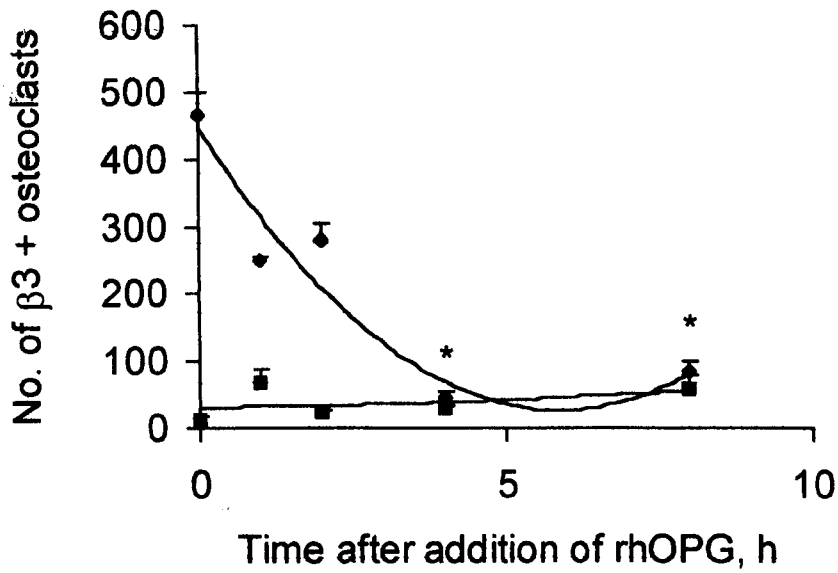


Figure 5.7: Disappearance of β3+OC from the bone surface (◆) and the endocranial membrane (■) following treatment with rhOPG. Parietal bones were incubated for 24 h in medium containing 1 μM PGE_2 followed by incubation for varying times in media containing 100 ng cm^{-3} rhOPG. The numbers of β3+OC are shown as the mean and the SEM. For all culture conditions n = 3.

*, $p<0.05$ compared to zero time

5.7 Assay of mRNA for OPG.

A dose-response curve was constructed as previously described to show the relationship between the amounts of cDNA added to the PCR reaction and the intensity of the ethidium bromide stained product. It established a linear range for the assay. A sample known to contain OPG cDNA was diluted 1 in 10 in nuclease free water and varying amounts were added to the PCR reaction ranging from 0 to 9 µl. The product bands were made visible following staining with ethidium bromide with UV light (Fig 5.8). The bands were quantified using an image analysis system and if a band intensity exceeded the upper limit of linearity the entire PCR comparison was repeated with diluted samples of cDNA. The results show that the upper limit of linearity is 0.8 (Fig 5.9)

5.8 Restriction enzyme analysis of the OPG product.

The size of the OPG product was determined by agarose electrophoresis and comparison to a known standard. The product size corresponded to that predicted from the sequence obtained from NCBI described in the Materials and Methods. An NCBI BLAST search revealed the primers selected were specific to mOPG. A restriction enzyme digest was carried out on the OPG product to provide further evidence that it is in fact OPG. The product was incubated together with the restriction enzyme Pst 1 at 37°C for 1 h. Controls consisted of one sample with no restriction enzyme present and another an incubation of OPG product with the enzyme Eco R1 which should not cut true OPG in the basis of the published sequence. The restriction enzyme Eco R1 appeared to form a complex with the cDNA product as judged by the apparent molecular mass after electrophoresis. It did not however result in a cleavage product. Another restriction enzyme Stu 1 was also used as a control enzyme and this did not result in low molecular mass products (results not shown). The enzyme Pst 1 cuts after base number 100 of the 515 base pairs of the OPG product at CTGCA/G on the upper strand and G/ACGTC on the lower strand. The two bands appear on the electrophoresis gel at 100 base pairs and 415 base pairs (Fig 5.10).

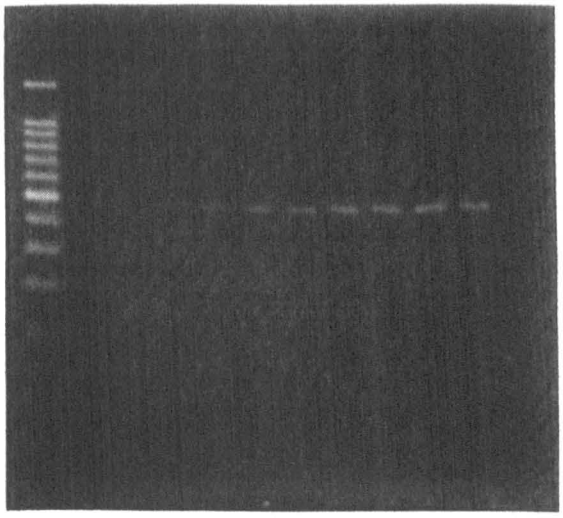


Figure 5.8: The relationship between the volume of cDNA added and product band intensity. Differing volumes of a single cDNA preparation were added to the standard PCR reaction for the OPG gene product. The products were subjected to electrophoresis, stained with ethidium bromide and band intensities were quantified using Sight System image analysis. The OPG product should contain 515 bp.
Lane 1 = DNA 500 bp ladder.
Lanes 2-11 = Differing volumes of cDNA added to the PCR reaction ranged from 0 to 9 µl.

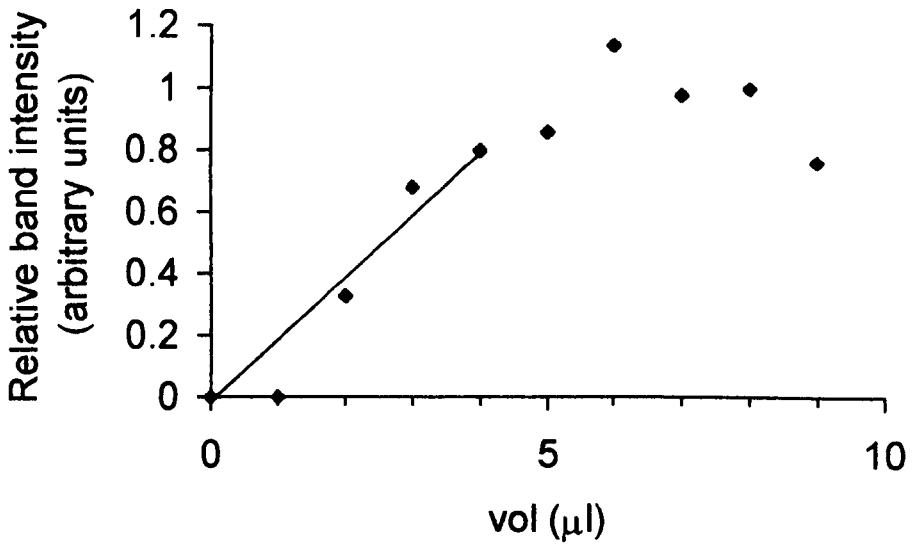


Figure 5.9: Differing volumes of a single cDNA preparation were added to the standard PCR reaction for the OPG gene product. The products were subjected to electrophoresis, were stained and quantified using the Sight System image analysis method (see Materials and Methods). Band intensities of the OPG products were expressed relative to that of one of the ladder bands in order to control variations between electrophoresis runs. The upper limit for linearity of this dose response curve was taken as 0.8. Any results falling outside this linear range would need to be repeated following dilution.

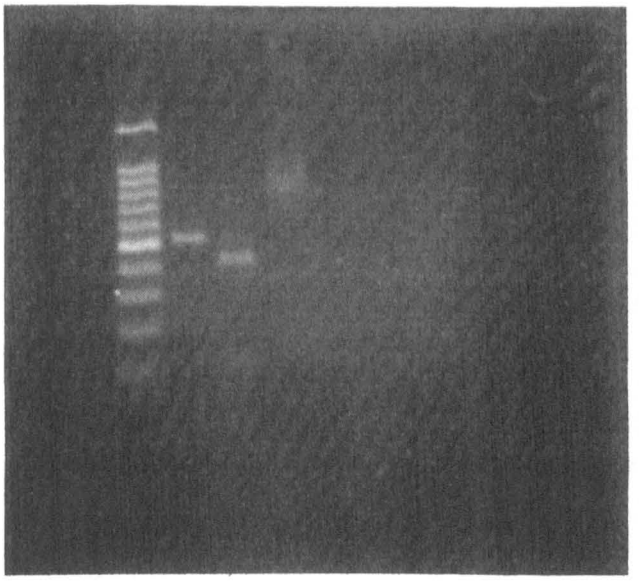


Figure 5.10: Restriction enzyme digest of the OPG product. OPG product was digested with the restriction enzyme Pst 1 for 1 h. The product was subjected to electrophoresis and stained with ethidium bromide. The enzyme cuts after base number 100 of the 515 base pairs of the OPG product giving two bands at 100 bp and 415 bp.

Lane 1 = 100 bp DNA ladder

Lane 2 = no enzyme control

Lane 3 = 415 and 100 bp band products of digestion with Pst 1 restriction enzyme.

Lane 4 = control incubation with restriction enzyme Eco 1 which does not cut authentic OPG gene product.

5.9 Response of mRNA for OPG to stimulators of bone resoption in whole calvaria over 6 h.

Previous results have shown that mRNA for OPGL can be stimulated in whole calvaria when cultured with PGE_2 over a period of 6 h (Fig 4.13). It might be expected that this stimulator of bone resorption would at the same time suppress mRNA for OPG. Whole calvaria were cultured with 1 μM indomethacin for one day followed by 6 h in either 1 μ M indomethacin or 1 μM PGE_2. RNA was extracted and transcribed into cDNA and amplified with PCR as previously described. Results are shown in Fig 5.11 and each lane represents RNA extracted from two calvaria. PGE_2 appeared to have no effect on the levels of mRNA for OPG in these calvaria with similarly high levels seen with both indomethacin and PGE_2. The bands had the correct number of base pairs for the OPG product. The PCR product for β Actin was similar in all samples indicating that similar amounts of mRNA were included in the reverse transcription step (Fig 4.14b).

5.10 Response of mRNA for OPG to stimulators of bone resorption in calvarial cells over 6 h.

It might be expected that stimulators of bone resorption which increase the mRNA for ODF would decrease the mRNA for OPG. Calvarial cells were removed from whole calvaria with collagenase as described in Materials and Methods. When the cells attained confluency the following stimulators of bone resorption were added to the culture media, 1 μM indomethacin (control), 1 μM PGE_2, 4 nM PTH 100 nM dexamethasone. The cells were then incubated for 6 h after which the RNA was extracted and transcribed into cDNA and amplified with PCR as previously described. Results are shown in Fig 5.12a and each lane represents RNA extracted from one 9 cm^2 well. All treatments including indomethacin resulted in similar levels of mRNA for OPG. β actin levels were similar in all samples and show that similar levels of mRNA were included in the reverse transcription step (Fig 5.12b).

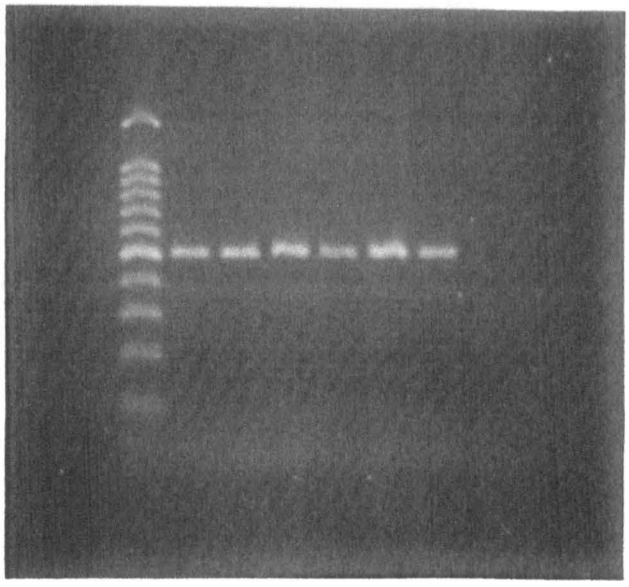


Fig 5.11: Expression of OPG mRNA in response to PGE_2 and indomethacin. Total RNA was extracted from whole calvaria cultured in medium containing 1 μM indomthacin for 24 h followed by a further 6 h in medium containing either 1 μM indomethacin or 1 μM PGE_2. Following reverse transcription the samples underwent PCR and were subjected to electrophoresis and staining with ethidium bromide. The presence of either PGE_2 or indomethacin resulted in similarly high levels of OPG gene product. The OPG gene product should be 515 bp.

Lane 1 = 100 bp DNA ladder.

Lanes 2, 4, and 6 = RNA from calvaria cultured with indomethacin alone

Lanes 3, 5, and 7 = RNA from calvaria cultured with PGE_2 for 6 h.

Lane 8 = no cDNA negative control

β actin levels for these samples can be seen in Fig 4.14b.

a

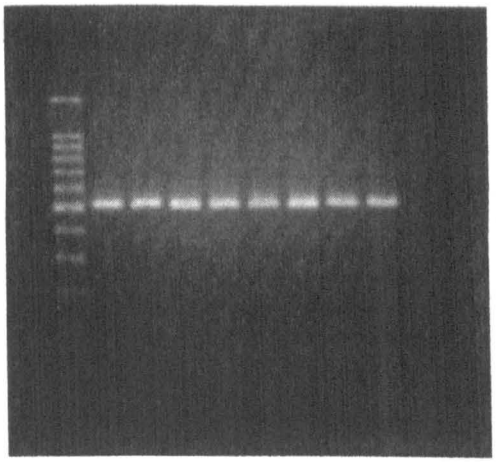


Figure 5.12a: Expression of mRNA for OPG in response to indomethacin, PGE_2, PTH and dexamethasone. Total RNA was extracted from calvarial cells cultured in medium containing 1 μM indomethacin, 1 μM PGE_2 4 nM PTH or 100 nM dexamethasone for 6 h. Following reverse transcription the samples underwent PCR and were subjected to electrophoresis and staining with ethidium bromide. All treatments resulted in similar levels of mRNA for OPG. The OPG product should be 515 bp.

Lane 1 = 100 bp DNA ladder.

Lanes 2 and 3 = RNA from calvarial cells cultured with indomethacin.

Lanes 4 and 5 = RNA from calvarial cells cultured with PGE_2.

Lanes 6 and 7 = RNA from calvarial cells cultured with PTH.

Lanes 8 and 9 = RNA from calvarial cells cultured with dexamethasone.

Lane 10 = no cDNA negative control.

b

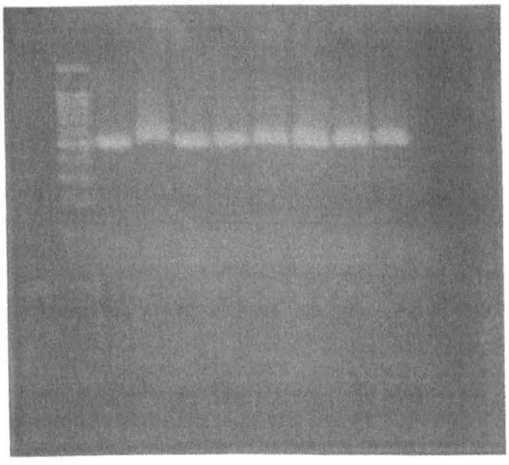


Figure 5.12b: These samples are the same as those shown in fig 5.12a but amplified for the β actin gene product to ensure that the original extractions contained about the same amounts of mRNA. The β actin product should be 514 bp.

5.11 Response of mRNA for OPG to stimulators of bone resorption in calvarial cells over 24 h.

There did not appear to be any suppression of mRNA for OPG during 6 h culture in the presence of various stimulators of bone resorption. It may be that the half-life of mRNA for OPG is longer than 6 h so the experiment was repeated over 24 h. Calvarial cells were removed as previously described and once they had grown to confluency they were incubated with the following stimulators of bone resorption, 1 μM indomethacin (control), 10 nM $1,25D_3$, 4 nM PTH and 100 nM dexamethasone for 24 h. The RNA was extracted and reverse transcribed into cDNA and amplified with PCR as previously described. The results are shown in Fig 5.13a and each lane represents RNA extracted from one 9 cm^2 well. Again all treatments including indomethacin resulted in similar amounts of mRNA for OPG. β actin levels were similar in all samples and show that similar levels of RNA were included in the reverse transcription step (Figure 5.13b).

5.12 Effect of PTH on mRNA for OPG in mouse bone marrow.

Mouse bone marrow was removed from long bones and the stromal cells established and cultured in the presence or absence of 4 nM PTH for 12 days (see Materials and Methods). Following the culture period the total RNA was extracted and underwent reverse transcription and PCR amplification. The results are shown in Figure 5.14a and each lane represents RNA extracted from one 9 cm^2 well. There did not appear to be any suppression of mRNA for OPG in the presence of PTH. β actin levels were similar in all samples which shows that similar amounts of RNA were included in the reverse transcription step (Figure 5.14b).

5.13 Effect of various stimulators of bone resorption on mRNA levels for OPG in MC3T3-E1 cells.

The mouse derived osteoblastic cell line MC3T3-E1 cells were cultured until confluent following which they were incubated in the presence of 1 μM indomethacin, 1 μM PGE_2, 100 nM dexamethasone, or 4 nM PTH for 6 h in order to

a

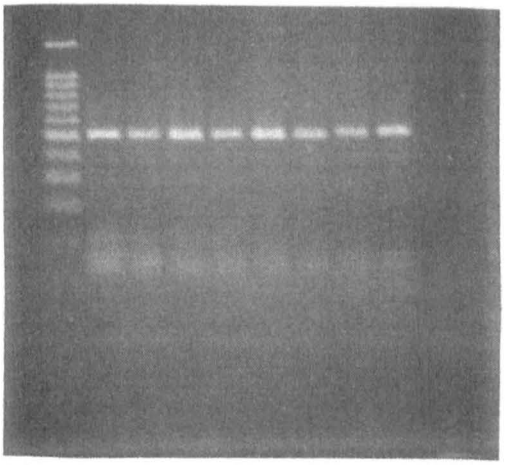


Figure 5.13a: Expression of mRNA for OPG in response to indomethacin, $1,25D_3$, PTH or dexamethasone. Calvarial cells were cultured in medium containing 1 μM indomethacin, 10 nM $1,25D_3$, 4 nM PTH or 100 nM dexamethasone for 24 h. Total RNA was extracted followed by reverse transcription. The samples underwent PCR and were subjected to electrophoresis and staining with ethidium bromide. All treatments resulted in similar levels of RNA for OPG. The OPG product should be 515 bp.

Lane 1 = 100 bp DNA ladder.

Lane 2 and 3 = calvarial cells cultured with indomethacin.

Lane 4 and 5 = calvarial cells cultured with $1,25D_3$.

Lane 6 and 7 = calvarial cells cultured with PTH.

Lane 8 and 9 = calvarial cells culture with dexamethasone.

Lane 10 = no cDNA control.

b

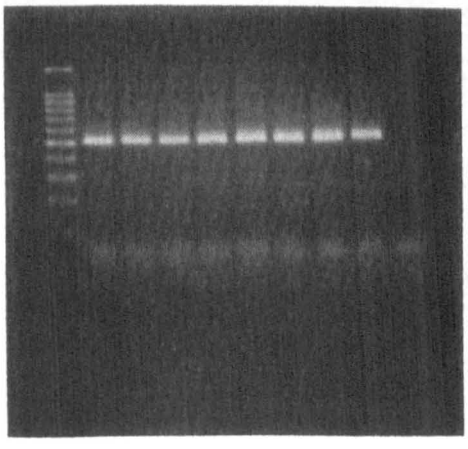


Figure 5.13b: β actin levels in total RNA extractions from figure 5.13. These samples are the same as those shown in fig 5.13 but amplified for the β actin gene product to ensure that the original extractions contained about the same amounts of mRNA. The β actin product should be 514 bp.

a

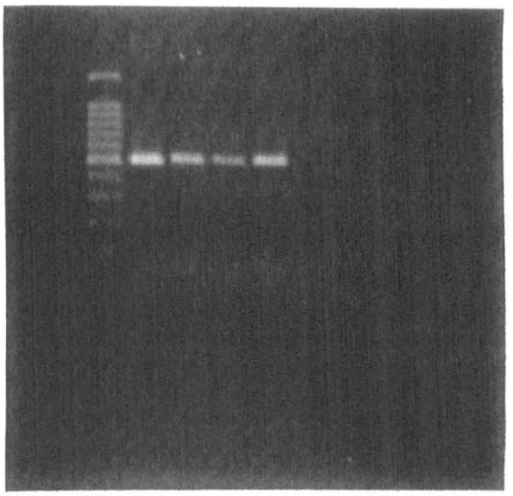


Figure 5.14a: Effect of PTH on levels of mRNA for OPG in mouse bone marrow. Mouse bone marrow was cultured for 12 d in the presence and absence of 4 nM PTH. Total RNA was extracted and following reverse transcription the samples underwent PCR and were subjected to electrophoresis and staining with ethidium bromide. The presence or absence of PTH did not appear to change the expression levels of the OPG gene product. The OPG product should be 515 bp.

Lane 1 = 100 bp DNA ladder.

Lanes 2 and 3 = RNA extracted from bone marrow cultured in the presence of PTH.

Lanes 4 and 5 = RNA extracted from bone marrow cultured in the absence of PTH.

Lane 6 = no cDNA negative control.

b

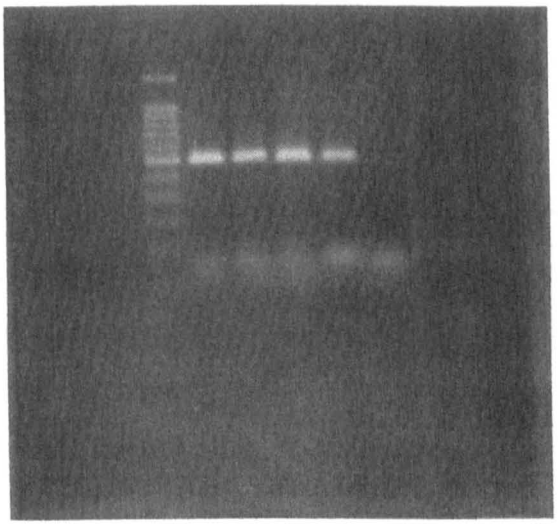


Figure 5.14b: These samples are the same as those shown in figure 5.14a but amplified for the β actin gene product to ensure that the original extraction contained about the same amounts of mRNA. The β actin gene product should be 514 bp.

see whether mRNA levels for OPG could be suppressed. Following the culture period total RNA was extracted which underwent reverse transcription and PCR amplification. The results are shown in Figure 5.15 and each lane represents RNA extracted from one 9 cm^2 well. There did not appear to be any suppression of mRNA levels for OPG with any of the treatments.

5.14 Suppression of mRNA for OPG with dexamethasone.

Previous reports have shown that mRNA for OPG can be suppressed in the human osteosarcoma cell line MG63 cells following 24 h incubation in the presence of dexamethasone (Hofbauer *et al*, 1999). MG63 cells were grown to confluency and incubated in the presence of 1 μM indomethacin or 100 nM dexamethasone for 24 h. Following the culture period the total RNA was extracted prior to reverse transcription and PCR amplification. The size of the huOPG product corresponded to that predicted from the sequence obtained from NCBI and a BLAST search revealed that the primers were specific for huOPG. The results are shown in figure 5.16a. There appeared to be decreased levels of mRNA for OPG in the samples treated with dexamethasone. β actin levels were similar in all the samples which shows that similar amounts of RNA were included in the reverse transcription step (Fig. 5.16b).

5.15 Immunoassay of OPG.

Following on from investigating mRNA levels for OPG, an enzyme-linked immuno sorbent assay (ELISA) was developed to look at the levels of OPG protein in response to stimulators of bone resorption. In order to establish whether the assay would detect OPG, various concentrations of rhOPG-Fc were incubated on a plate which had been coated with an antibody to human OPG (see Materials and Methods). According to the supplier the human antibody showed 50% reactivity with mouse OPG. The ELISA assay consisted of a plate coated with the primary goat anti-human OPG antibody to which the samples and controls were added. Any bound OPG was detected with a biotinylated goat anti-human OPG antibody which in turn was detected with avidin peroxidase. At the end of the assay the plate was

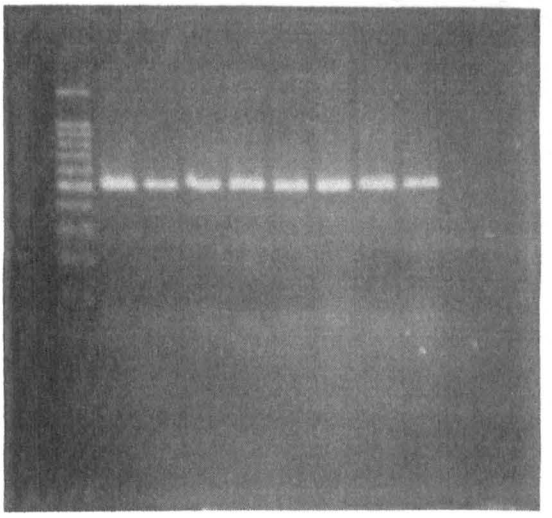


Figure 5.15: Effect of various stimulators of bone resorption on mRNA levels for OPG in MC3T3-E1 cells. Cells were cultured in medium containing 1 μM indomethacin, 1 μM PGE_2, 100 nM dexamethasone, or 4 nM PTH for 6 h. Total RNA was extracted and samples underwent PCR followed by electrophoresis and staining with ethidium bromide. The OPG product should be 515 bp.

Lane 1 = 100 bp ladder.

Lanes 2 and 3 = RNA from cells cultured with indomethacin.

Lanes 4 and 5 = RNA form cells cultured with PGE_2.

Lanes 6 and 7 = RNA from cells cultured with dexamethasone.

Lanes 8 and 9 = RNA from cells cultured with PTH.

Lane 10 = no cDNA negative control.

a

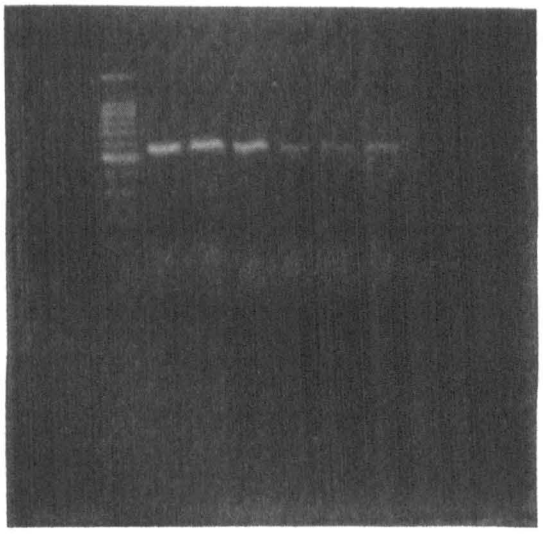


Figure 5.16a: Effect of dexamethasone on mRNA levels for OPG in MG63 cells. MG63 cells were cultured in medium containing1 μM indomethacin or 100 nM dexamethasone for 24 h. Following reverse transcription the samples underwent PCR and were subjected to electrophoresis and staining with ethidium bromide. The presence of dexamethasone resulted in lower levels of the OPG gene product. The OPG product should be 515 bp.

Lane 1 = 100 bp ladder.

Lanes 2-4 = RNA extracted from cells incubated with indomethacin.

Lanes 5-7 = RNA extracted from cells incubated with dexamethasone.

Lane 8 = no cDNA control.

b

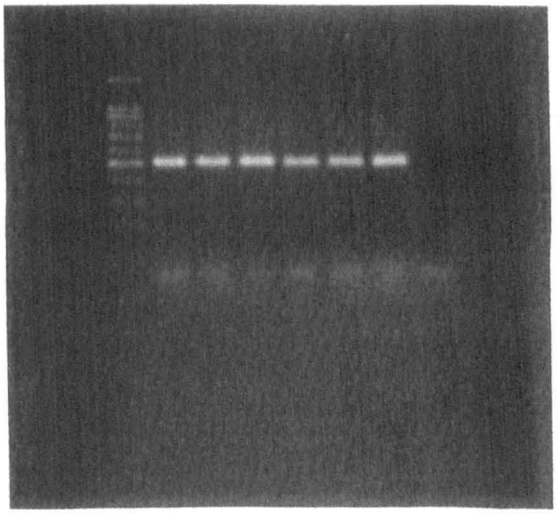


Figure 5.16b: These samples are the same as those shown in figure 5.16b but amplified for the β actin gene product to ensure that the original extraction contained about the same amounts of mRNA. The β actin product should be 514 bp.

read in a plate reader and the optical densities were plotted against the concentration of rhOPG-Fc (Fig 5.17). The assay detected the rhOPG-Fc as the optical density increased linearly with increasing concentrations of rhOPG-Fc. The signal appears to be specific for rhOPG as there is very little signal when a goat IgG is used in place of the anti-human OPG, when no biotinylated anti-human OPG is added instead of the second antibody, or when no avidin peroxidase is added (Fig 5.17).

5.16 Production of OPG over time.

Whole calvaria were cultured in either 1 μM indomethacin or 1 μM PGE_2 for varying amounts of time described in Materials and Methods. The OPG activity of the culture media removed at different times was assayed using the plate method and the results are shown in figure 5.18. OPG activity was greater in media from calvaria incubated for 24 h with indomethacin compared with PGE_2. There appeared to be a short lag of about 2 h followed by a linear increase of OPG activity with time (Fig 5.18). Interestingly, significant amounts of OPG activity were produced even in the presence of PGE_2.

5.17 Suppression of OPG production with different stimulators of bone resorption.

Whole calvaria were incubated in either 1 μM indomethacin, or 1 μM PGE_2, or 4 nM PTH, or 100 nM dexamethasone, or 10 nM $1,25D_3$ for one day. The OPG activity in the media was then assayed and the results can be seen in Table 4.1. PTH, PGE_2 and $1,25D_3$ all resulted in significantly less OPG activity in media compared with indomethacin as shown in Table 4.1 ($p<0.01$. However only dexamethasone resulted in OPG activity that was not significantly different from the culture media control $p<0.05$ (Table 4.1).

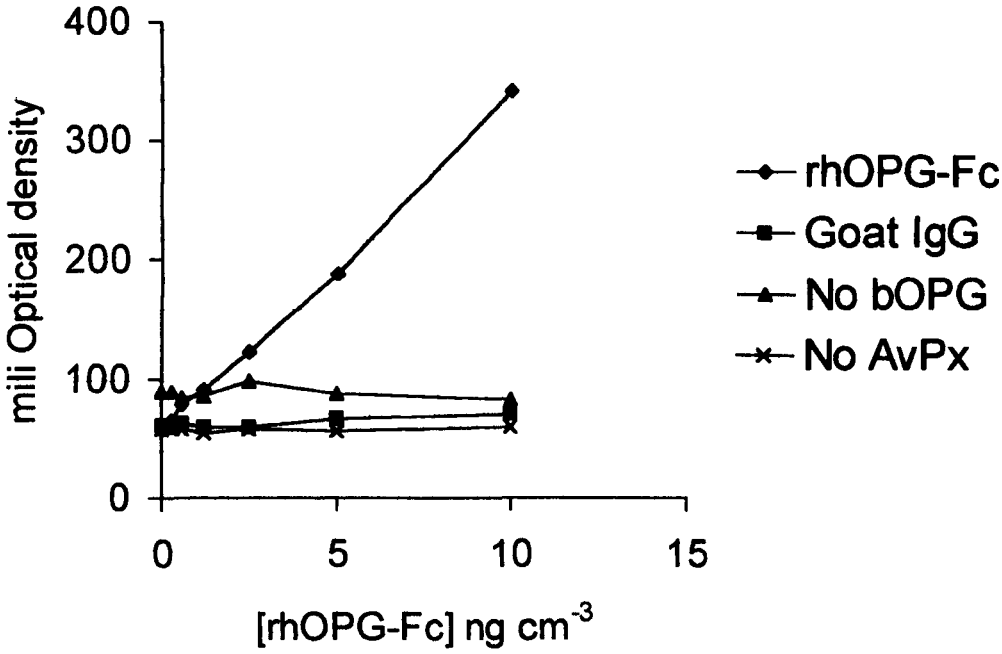


Figure 5.17: Dose response curve for rhOPG-Fc. Various concentrations of rhOPG-Fc were assayed using an enzyme-linked immunoassay. Controls included wells coated with goat IgG in place of goat anti-human OPG (goat IgG), no addition of biotinylated anti OPG (No bOPG) and no addition of avidin peroxidase (No AvPx). All tests were carried out in triplicate.

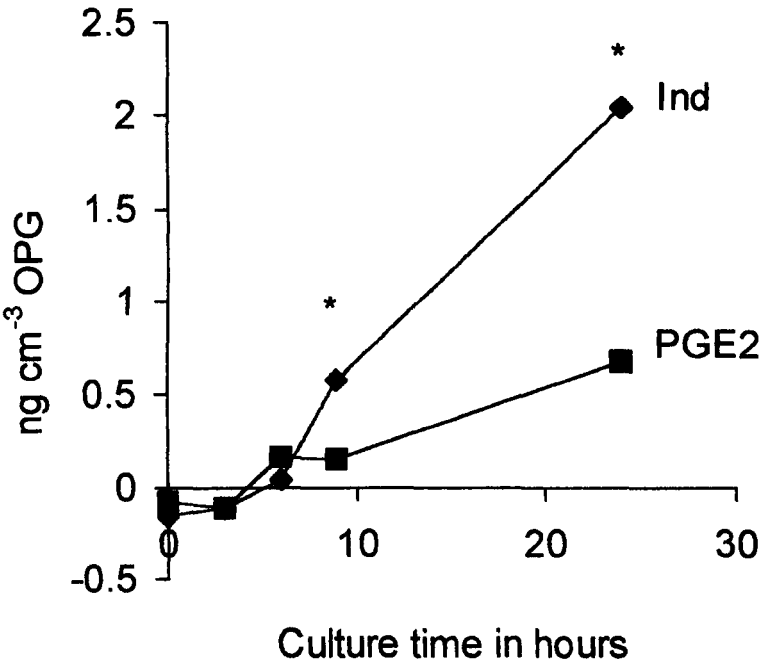


Figure 5.18: The effect of indomethacin and PGE_2 on the production of OPG with time. Whole calvaria were cultured with either 1 μM indomethacin or 1 μM PGE_2 for varying times and the culture medium was removed and assayed for OPG activity. The results shown are the mean and SD of four bones.

*, $p<0.05$ compared to PGE_2

Treatment	Mean ng/ml	SEM	p<
Indomethacin	1.50	0.15	b
PTH	0.43	0.062	a, b
PGE2	0.73	0.007	a, b
1,25D3	0.37	0.066	a, b
Dexamethasone	0.14	0.02	a
Controls	0.09	0.03	a

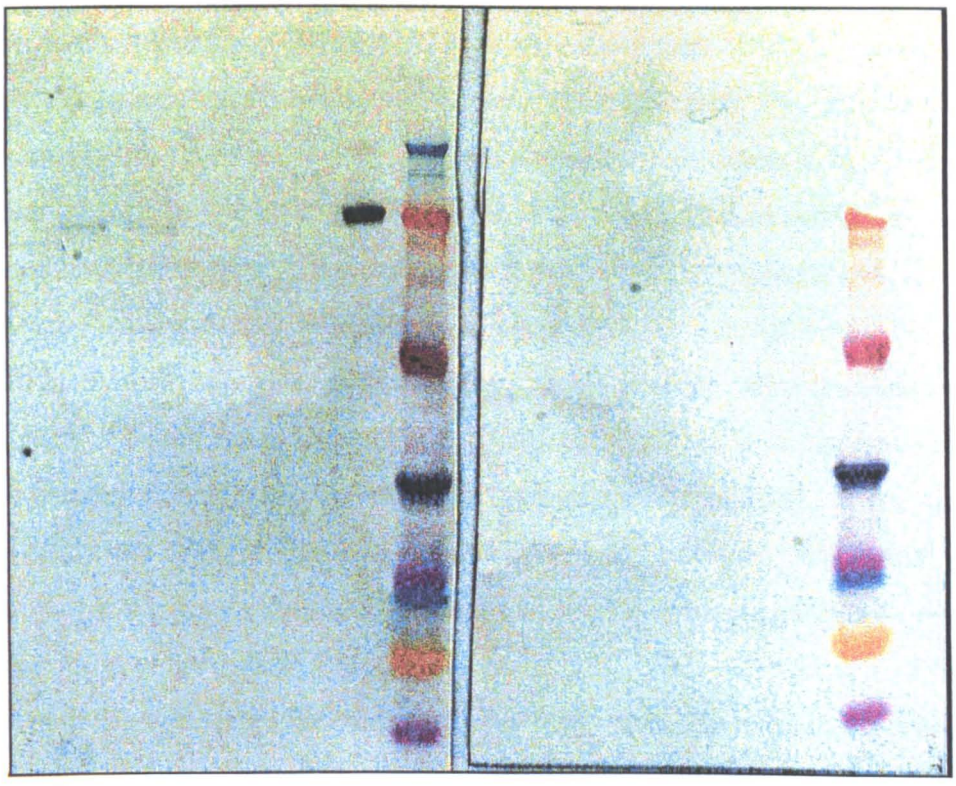
Table 4.1: Effect of modulators of bone resorption on OPG production. Whole calvaria were cultured in medium containing either 1 μM indomethacin, or 4 nM PTH, or 1 μM PGE_2, or 10 nM $1,25D_3$, or 100 nM dexamethasone for 24 h. The media was then assayed for OPG activity. Data represent the mean and SEM. For each culture condition n = 4.

a, significantly different from the indomethacin control $p<0.01$.

b, significantly greater than the control culture medium i.e. in the absence of bones $p<0.05$.

5.18 Western blotting for the detection of OPG.

In order to provide further evidence that OPG is produced in the presence of indomethacin, culture media which had been incubated with MG-63 cells was used to detect OPG using Western blotting. The culture media was incubated with confluent MG-63 cells for 24 h in the presence of either indomethacin or dexamethasone. The cells had been previously used for the extraction of RNA, reverse transcription and PCR, fig, 5.16a. The conditioned media samples were heated to 70°C for 10 min in SDS sample buffer before undergoing SDS electrophoresis and Western blotting followed by immunostaining for OPG. The conditioned media from the cells incubated in the presence of indomethacin showed a band in a similar place to the rhOPG control which migrates with a molecular weight of 122 kDa in SDS-PAGE under non-reducing conditions (Fig 5.19a). Authentic human OPG is reported to have a molecular weight of 120 kDa in the dimeric form. OPG was not detected in the media containing dexamethasone which had been incubated with the cells. The control blot immunostained with goat IgG instead of the biotinylated anti-human OPG did not show any bands (Fig 5.19b).



a b

Figure 5.19a: Effect of indomethacin and dexamethasone on levels of OPG in MG-63 cells detected with Western blotting. MG-63 cells were cultured in medium containing 1μM indomethacin or 100 nM dexamethasone for 24 h. The culture medium was removed and underwent SDS-PAGE and Western blotting followed by immunostaining for OPG.

Lane 1 = culture media from cells incubated in the presence of indomethacin.

Lane 2 = culture media from cells incubated in the presence of indomethacin.

Lane 3 = culture media from cells incubated in the presence of dexamethasone.

Lane 4 = culture media from cells incubated in the presence of dexamethasone

Lane 5 = rhOPG-Fc control.

Lane 6 = molecular mass ladder

Figure 5.19b: Control blot immunostained with goat IgG instead of the biotinylated anti-human OPG antibody. Lanes 7-12 = correspond to lanes 1-6 above.

Discussion

The purpose of this study was to investigate the regulation of bone resorption in order to elucidate the mechanism of bone loss in disease. This may then help to identify therapeutic targets for the treatment of these diseases. Previous work carried out in this department indicated that osteoclasts disappeared from the endocranial surface of mouse parietal bones when prostaglandin synthesis was inhibited with indomethacin and reappeared attached to the adjacent endocranial membrane (Marshall *et al*, 1996, Marshall *et al*, 1995, Holt & Marshall, 1998). It seemed likely that there was an intermediary in this process. Also it was known that stimulators of bone resorption such as PTH did not act directly on the osteoclast but *via* the osteoblast suggesting the existence of another intermediary. The initial aim of this study was to investigate the mechanisms of these effects. While this work was in progress two cytokines (OPGL and OPG) with opposing effects on osteoclast differentiation *in vitro* were discovered (Lacey *et al*, 1998; Simonet *et al*, 1997). The hypothesis was tested that these two cytokines were involved in the mechanism of the translocation of osteoclasts between the bone surface and the endocranial membrane.

In neonatal mouse parietal bones vigorous bone resorption occurs on the endocranial surface at 6 days old to accommodate the rapidly growing brain. The ectocranial surface is, on the other hand, engaged in bone apposition and TRAP+OC are rarely seen on this surface. When bones are excised and placed into culture medium bone resorption occurs spontaneously. This is due to secretion of prostaglandins, which stimulate bone resorption in organ culture (Klein & Raisz, 1970). Indomethacin, a non-steroidal anti-inflammatory drug which blocks prostaglandin synthesis (Vane, 1971), has been shown to inhibit bone resorption both *in vitro* (Lerner, 1987) and *in vivo* (Saffar & Leroux, 1988). In the work presented here indomethacin caused a reduction in the number of TRAP+OC and β3+OC on the bone surface of mouse parietal bones and an increase in the number of TRAP+OC but not β3+OC in the adjacent endocranial membrane (Fig 3.1, 3.5). The osteoclasts appeared stellate in shape presumably due to a change in adhesive properties resulting in cytoplasmic retraction (Fig 3.3b). This phenomena has been previously described by other workers (Marshall *et al* 1996; Holt & Marshall, 1998) who saw a reduction in the number of osteoclasts whether stained for the enzyme

TRAP or the integrin subunit β3 on the bone surface in the presence of indomethacin in murine calvarial cultures.

Earlier studies using a synchronized model of bone remodeling in rat following tooth extraction showed that treatment with indomethacin inhibited osteoclastic bone resorption (Saffar & Leroux, 1988). Histological examination of sections of the rat mandibles showed both actively resorbing osteoclasts, or "on-bone" osteoclasts as well as inactive "off-bone" osteoclasts. The off-bone osteoclasts did not have contact with the bone and were therefore not resorbing. They showed that the ratio of off-bone cells to on-bone cells was as high as 1.87 when animals were treated with indomethacin for the final two days of the four day experiment compared to the controls. Bone resorption was reduced compared to controls as was the mean resorption lacunar surface. These off-bone osteoclasts seen in response to treatment with indomethacin appear to correspond to the osteoclasts seen on the endocranial membrane in this study. This is evidence that this phenomenon is not an artifact of the culture system and occurs *in vivo*.

A study by Garrett and Mundy, (1989) looked at the effects of indomethacin and interleukin-1 on mouse calvarial cultures. They saw fewer osteoclasts in calvaria treated with indomethacin after one day and concluded that this was due to an inhibition of osteoclast formation. However, in the work reported here it appears that the osteoclasts actively leave the bone surface and become attached to the endocranial membrane (Fig 3.1). Important differences exist between these two studies. Garrett and Mundy stained histological sections with haematoxylin and eosin (H&E) which is not specific for osteoclasts and the counted cells may have been either TRAP+OC or TRAP negative OC. In addition the authors did not state whether the osteoclasts were attached to the bone or whether they were detached from the bone. Osteoclasts in histological sections and which have not been stained for TRAP are difficult to identify and may result in a lower estimation of the numbers of osteoclasts present. In the work reported here, osteoclasts were stained for TRAP activity, a specific stain for osteoclasts, on the whole bone rather than sections, allowing easy identification of the osteoclasts. In addition, the endocranial membranes of the bones in the study were physically peeled back to the sagittal suture showing whether the osteoclasts were attached to the bone surface or the endocranial membrane (Fig 3.1). Thus the work of Garrett and Mundy supports the

observation of the depletion of osteoclasts from bone in response to indomethacin treatment.

Several studies have described the *in vivo* effects of indomethacin blocking bone resorption. Adachi *et al* (1991) showed an inhibition of bone resorption with indomethacin in a model of hearing loss in gerbil and Arita *et al* (1989) showed a similar inhibition in maxillary deciduous incisors of neonatal rabbits. Similarly, indomethacin inhibited tenotomy-induced bone resorption in rats (Thompson & Rodan, 1988). Indomethacin was also found to reduce the number of osteoclasts and inhibit bone loss in hamster periodontitis (Lasfargues & Saffar, 1983). Another nonsteroidal anti-inflammatory drug, diclofenac sodium was found to be almost as effective as conjugated oestrogens for decreasing bone loss in post menopausal women (Bell *et al*, 1994).

The work described here showed an increase in the number of TRAP+OC on the endocranial membrane of mouse parietal bones following one day of treatment with indomethacin (Fig 3.2) but those osteoclasts did not stain positively for β3 in the same circumstances (Fig 3.5). This confirms the work of Holt and Marshall (1998). The osteoclasts appear to detach from the bone and attach to the adjacent endocranial membrane. A recent study examining the disappearance of osteoclasts from the distal alveolar bone surface in force-induced remodelling in rat *in vivo* described an increase in "intact non-bone associated osteoclasts" one day into the experiment (Kobayashi *et al* 2000a). There was a simultaneous decrease in the number of "intact bone-associated osteoclasts". The authors presented evidence that this was partly due to increased apoptosis of osteoclasts. However, only 23% of osteoclasts were apoptotic after day one suggesting that the remaining osteoclasts were viable. The fate of TRAP+OC in my study that were incubated with indomethacin is not clear. Some TRAP negative OC were seen on the membrane but they are difficult to identify (Fig 3.3b). In a study of osteoclasts on parietal bones from mice of different ages, three different osteoclast morphologies were described with varying levels of acid phosphatase activity (Abe *et al*, 1990). It is likely that the type III osteoclasts described by these authors which were non-resorbing, low in acid phosphatase and of a stellate morphology, correspond to the TRAP negative osteoclasts seen following indomethacin treatment. No significant apoptosis could be detected in osteoclasts in response to OPG in the study reported

here. In support of this finding OPG did not cause apoptosis in isolated rabbit osteoclasts (Hakeda *et al*, 1998). However, in cultured stromal cells OPG had been reported to reduce mouse osteoclast survival and cause apoptosis (Akatsu *et al*, 1998). Apoptosis is known to occur in isolated cells but in indomethacin treated bones osteoclasts are not isolated from other cells but are in intimate contact with cells of the endocranial membrane. This may account for the discrepencies between these results.

Osteoclasts were able to re-adhere to the bone surface following culture with PTH, PGE_2, or $1,25D_3$, (Fig 3.4) which suggests that the effects of indomethacin are reversible. In support of this conclusion Marshall e*t al* (1995) showed that when parietal bones had been pre-incubated with indomethacin, the addition of PGE_2, PTH or $1,25D_3$ together with indomethacin for 20 hours, the number of TRAP+OC recovered to about their level in the absence of a pre-incubation (with indomethacin). Further support for the conclusion that the osteoclasts on the membrane could migrate to the bone surface came from the inclusion of 5-bromo-2'-deoxyuridine (BDU) in the culture media. This compound labels cells synthesizing DNA and can be detected with a specific antibody. Very few TRAP+OC nuclei were labelled in response to PTH-stimulated recovery of osteoclast numbers after indomethacin treatment *in vitro*. Thus, the TRAP+OC which form in response to PTH following incubation with indomethacin were not the result of cell proliferation *in vitro* but had been formed from pre-existing cells without DNA synthesis. As a positive control, many TRAP+OC became labelled when BDU was given to mice one day before excision of bones.

When parietal bones, which had been incubated with indomethacin for one day, had their endocranial membranes peeled back to the sagittal suture prior to culture in the presence of PGE_2, there was no subsequent increase in the number of TRAP+OC as was seen with intact bones (Fig 3.9). The osteoclasts seen on the bone following incubation with PGE_2 most probably have come from the endocranial membrane. Hence, intimate contact between the bone and the membrane is necessary for the osteoclasts to adhere to the bone surface. When the endocranial membrane was peeled back halfway to the sagittal suture prior to incubation with PGE_2, β3+OC were only seen on the unpeeled half of the bone surface (Fig 3.10). This also suggests that close cell to cell contact is needed

between the osteoblastic lining cells on bone and the osteoclasts on the endocranial membrane for the osteoclasts to become activated to adhere to the bone and begin resorption.

Although the osteoclast possesses several different integrins including $\alpha 2\beta 1$, $\alpha v\beta 1$ and $\alpha v\beta 3$, the most important integrin involved in bone resorption is $\alpha v\beta 3$. It is important in substrate recognition by the osteoclast but opinions differ as to whether it is involved in osteoclast adhesion to the bone surface and clear zone formation. This study looked at the role of the integrin subunit $\beta 3$ in the translocation of osteoclasts between the membrane and the bone in response to stimulators of bone resorption. When parietal bones which had been pre-treated for one day with indomethacin were incubated in the presence of PGE_2 (Fig 3.5) or sOPGL (Fig 4.6) followed by staining for the $\beta 3$ subunit, there was an increase in $\beta 3$+OC seen on the bone compared to the indomethacin control. This was a similar result to that seen when bones were stained for TRAP activity. However, in contrast to the expression of TRAP activity, when bones were incubated in the presence of indomethacin almost no $\beta 3$+OC were seen on the endocranial membrane. The $\beta 3$+OC phenotype was only associated with adhesion to the bone surface. This was in line with the observations of Holt *et al* (1998).

Osteoclasts on the parietal bone surface were strongly positive for $\beta 3$. When osteoclasts were seen in resorption lacunae, the distribution of the staining appeared to be more intense at the periphery of one end of the cell (Fig 4.7). It may be that this illustrates the direction in which the osteoclast is moving across the bone as the resorption lacuna was seen at the opposite end of the cell. Other workers have described the distribution of the integrin subunits as covering the surface of the rat osteoclast including the clear zone (Hultenby *et al*, 1993). Lakkakorpi *et al* (1991, 1993) saw a similar distribution of $\alpha v\beta 3$ but they did not observe the integrin in the clear zone of the rat osteoclast.

In order to elucidate the role of the $\alpha v\beta 3$ integrin in the translocation process of osteoclasts moving from the endocranial membrane to the bone surface, the RGD containing peptide echistatin was used in the culture system. Echistatin has a high affinity for integrins, in particular the $\alpha v\beta 3$ integrin and has been shown to inhibit bone resorption. Bones, pre-treated for one day with indomethacin, were cultured in the presence of PGE_2 together with echistatin and showed an increase in the number

of β3+OC seen on the endocranial membrane compared to the PGE_2 control (Fig 3.7). In contrast, few β3+OC were seen on the bone surface compared to the control (Fig 3.7). Thus echistatin blocked the translocation process of osteoclasts from the membrane to the bone surface. The presence of PGE_2 and echistatin allowed expression of the β3 subunit but the osteoclasts were unable to adhere to the bone surface presumably because the integrin binding site was blocked by echistatin. It may be that the echistatin causes the αvβ3 integrin to cluster at the surface of the osteoclast so that it becomes detectable when immunostained with an antibody, but does not allow the osteoclast to make contact with its natural substrate on the bone surface. Trusolino *et al* (1998) described the clustering of the αvβ3 integrin at focal contacts, which mediated attachment and spreading of human thyroid clones of papillary carcinoma cells. Conversely, in those cells in which the αvβ3 integrin was diffusely spread over the membrane and not connected to the cytoskeleton, there was no attachment. The binding of echistatin to the αvβ3 integrin on the osteoclast may mimic the attachment of the osteoclast to bone and so cause the integrin to cluster.

Echistatin is not only able to block the adhesion of osteoclasts to bone but is also able to block osteoclastic bone resorption both *in vivo* (Fisher *et al*, 1993) and *in vitro* (Sato *et al*, 1994). It has been shown to be the most potent of the RGD-containing peptides that inhibit isolated rat osteoclasts (Sato *et al*, 1990). Sato *et al* (1994) found that at a concentration of 10 nM echistatin could prevent the attachment of rat osteoclasts to bone slices. This was the same concentration of echistatin used in the study reported here to prevent the translocation of osteoclasts from the membrane to the bone. However, in order to stop the activity of resorbing osteoclasts, much higher concentrations (3000 fold) of echistatin were required (Sato *et al* 1994). Sato *et al* (1994) did not pre-incubate the cultures in the presence of indomethacin so there were probably already actively resorbing osteoclasts present on the bone. In the study reported here the bones were pre-incubated in indomethacin leaving very few osteoclasts on the bone surface. The few osteoclasts that remained on the bone were irregular in shape but did not become detached from the bone. Echistatin had the same effect when bones were incubated in the presence of sOPGL in that it blocked the translocation of osteoclasts from the membrane to the bone leaving TRAP+OC on the endocranial membrane.

Another study looked at the effects of a function-blocking anti-β3 antibody on the translocation of TRAP+OC from the endocranial membrane to the bone surface in mouse parietal bones. Holt and Marshall (1998) found that an anti-β3 antibody almost completely blocked the movement of TRAP+OC from the membrane to the bone surface. This was similar to the result seen with echistatin and these results imply that the β3 integrin subunit is needed for the translocation of osteoclasts from the membrane to the bone surface.

Evidence shows that the αvβ3 may not merely be involved in the recognition of the bone surface and attachment of the osteoclast to the bone, but may also act as a signal-transducing receptor. It has been suggested that when osteoclasts are exposed to RGD-containing peptides and proteins, there is a transient change in intracellular calcium (Zimolo *et al*, 1994). In addition when isolated osteoclasts are exposed to RGD-containing peptides, a wave of tyrosine phosphorylation occurs (Horne *et al*, 1992). Other workers have shown as association between αvβ3 and the signalling element $pp60^{c\text{-}src}$ in chicken osteoclasts (Rolnick *et al*, 1992). *Src* knockout mice have defective bone resorption and suffer from osteopetrosis. They do not form osteoclastic ruffled borders (Soriano *et al*, 1991) which suggests that the αvβ3 integrin acts as a signal transducer which when triggered by its cognate ligand on the bone surface leads to the formation of the sealing zone and ruffled border. Peptides which contain an RGD sequence are able to block the formation of both structures (Nakamura *et al*, 1996). Figure 6.1 depicts the hypothetical mechanism based on the evidence presented so far whereby indomethacin inhibits and PGE_2, PTH and $1,25D_3$ stimulate bone resorption in mouse parietal bones.

Soluble recombinant OPGL caused a dose-dependent increase in the number of TRAP+OC on the bone surface of freshly isolated parietal bones, that is without any pre-incubation in the presence of indomethacin (Fig 4.1). Following a one day incubation in the presence of indomethacin, sOPGL again brought about an increase in the number of TRAP+OC seen on the bone surface compared to the indomethacin control (Fig 4.3). A similar result was obtained to that seen following incubation in the presence of PGE_2. However, there was no significant decrease in the number of TRAP+OC seen on the membrane. This apparent difference between OPGL and PGE2 may due to soluble OPGL acting on pre-osteoclasts on the endocranial membrane that are not accessible to cell bound OPGL produced in response to

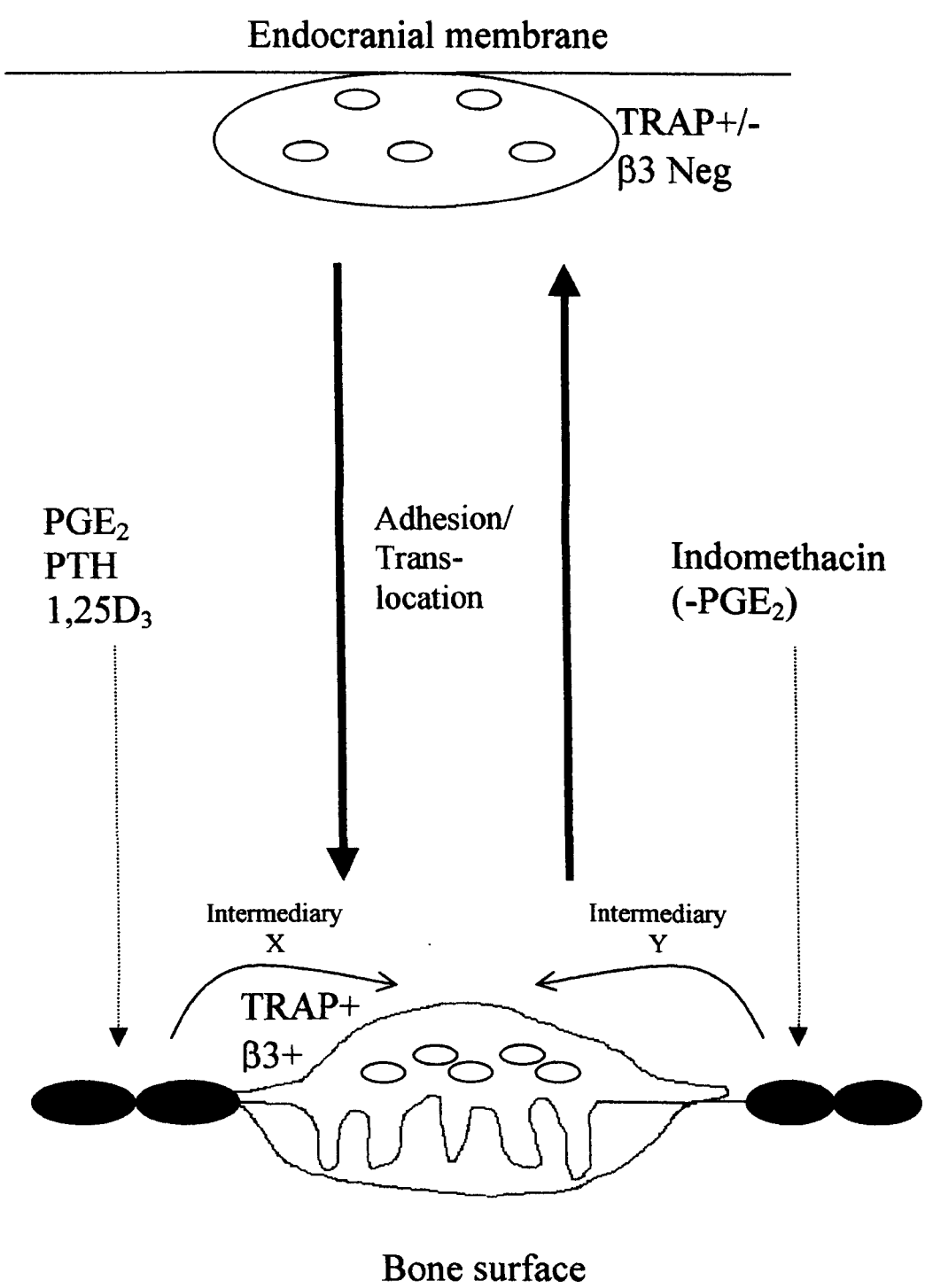


Figure 6.1 Diagrammatic representation of the possible mechanism of inhibition of bone resorption in the presence of indomethacin and the stimulation of bone resorption in the presence of PGE_2, PTH and $1,25D_3$. Two intermediaries, X and Y, are postulated to account for the actions of the effectors shown on osteoclast translocation between the bone and the endocranial membrane.

PGE2. Soluble OPGL may result in an increase in TRAP+ cells on the membrane formed from a pool of recently arrived monocytes. This may account for the variable data (Fig. 4.1) and the fact that no significant differences were seen in the number of TRAP+OC on the membrane. A recent report has shown that PGE_2 synergizes with OPGL to increase osteoclast formation and subsequent bone resorption in non-adherent mouse bone marrow cultures (Wani *et al*, 1999). In the study reported here sOPGL, in the presence of indomethacin, was sufficient for the translocation of osteoclasts from the membrane to the bone. However, this does not exclude the possibility of this synergism.

When parietal bones were pre-incubated in indomethacin for one day, the endocranial membranes peeled back to the sagittal suture and then incubated with sOPGL for a further 6 hours, there were significantly fewer β3+OC seen on the surface of the peeled bone compared to the intact bone (Fig. 4.4). At the same time there were significantly more β3+OC on the endocranial membrane of the peeled bone compare to the intact bone. This result shows that sOPGL is not simply causing the differentiation of pre-osteoclasts on the bone surface but that the increase in β3+OC on the bone is due to translocation of osteoclasts from the membrane. When the physical relationship between the bone and the membrane is disrupted by peeling back the membrane to the sagittal suture, few osteoclasts appear on the bone in response to sOPGL. This suggests that there is not a pool of osteoclast precursors on the bone surface and that osteoclasts seen on the bone surface of intact bones do indeed come from the endocranial membrane. This may either be by *de novo* differentiation from monocytes that arrived in the capillaries before excision of the bones or by re-activation of down-regulated osteoclasts, which are formed in the absence of bone resorptive stimuli.

When whole calvaria were cultured in the presence of PGE_2, PTH and $1,25D_3$, an increase in OPGL mRNA was seen (Fig 4.17. This increase occurred over a period of 3 to 6 hours. Yasuda *et al* (1998) showed an increase in mRNA in the presence of $1,25D_3$, PTH and PGE_2 in mouse primary osteoblasts *in vitro*. What appears to be happening in this system is that the addition of $1,25D_3$, PTH or PGE_2 brings about an increase in mRNA for OPGL which in turn causes the OPGL protein to be expressed on the surface of the osteoblastic bone lining cells. The cell-bound OPGL then makes contact with the receptor RANK on the osteoclasts on the

membrane. This brings about the transfer of signals to the inside of the cell, which causes the activation of the osteoclast and it becomes adhered to the bone surface where it commences bone resorption.

The signal transduction pathway *via* which osteoclasts are activated when OPGL binds to RANK is currently being elucidated. When OPGL binds to RANK the intracellular domain of RANK has been shown to interact with TRAF'S 1, 2, 3, 5 and 6 (Wong *et al.* 1998). A recent study had shown that this pathway can be used by other cytokines to induce activation and differentiation of osteoclasts independent of the OPGL/RANK pathway. Kobayashi *et al* (2000) have shown that TNF-α can cause osteoclast differentiation *via* TNFR1 and TNFR2 which are expressed by osteoclast precursors. This leads to the activation of NFκB and JNK, a common signalling pathway used by the OPGL/RANK interaction. It has previously been shown that IL-1α can activate NFκB in purified osteoclasts (Jimi *et al*, 1998). However, they were unable to induce osteoclast differentiation in mCSF-dependent bone marrow macrophages with IL-1α. It has also been shown that IL-1α receptors use TRAF6 as a signal transducing molecule (Cao *et al*, 1996). Kobayashi *et al* (2000) conclude that TRAF6 is a common signalling molecule used by both OPGL/RANKL and IL-1α in the activation of osteoclasts. They also state that both IL-1α and TNF-α can up-regulate the expression of mRNA for OPGL in osteoblast/stromal cells. However, OPG was only able partially to block the bone resorption induced by IL-1α. They suggest that the inflammatory cytokines TNF-α and IL-1α are involved in the bone resorption which occurs in bone diseases such as rheumatoid arthritis, periodontitis and post menopausal bone loss and that some of this could occur independently of the OPGL/RANK interaction.

When freshly isolated parietal bones were incubated in the presence of recombinant human OPG there was a reduction of TRAP+OC seen on the bone compared to the control (Fig 5.1). At the same time there was an increase in the number of TRAP+OC seen on the endocranial membrane compared to the control. This is the same result as was seen when bones were incubated in the presence of indomethacin.

A function-blocking antibody to OPG was found to block the translocation of osteoclasts from the bone surface to the endocranial membrane normally seen in the presence of indomethacin (Fig 5.6). Active osteoclasts were left on the bone

surface. A control antibody showed no effect. This implies that OPG is the mediator of the effects of indomethacin. The previous finding that sOPGL blocks the effects of indomethacin also suggests that OPG is involved in the adhesion of osteoclasts to the endocranial membrane.

OPG has been shown to inhibit osteoclastic bone resorption (Tsuda *et al* 1997) as well as osteoclastogenesis (Yasuda *et al* 1998). rhOPG was able to block the translocation of osteoclasts from endocranial membrane to bone that occurs in the presence of $1,25D_3$ (Fig 5.4) and PGE_2 (Fig 5.2). The osteoclasts on the endocranial membrane appear stellate in shape presumably due to a change in adhesive properties causing cytoplasmic retraction. OPG has been reported to affect osteoclast morphology, reducing the number of osteoclasts showing F-actin ring formation in untreated isolated rabbit osteoclasts (Hakeda *et al*, 1998) and in rat osteoclasts when treated with OPGL (Burgess *et al*, 1999). In mouse parietal bone cultures rhOPG caused a retraction of the cytoplasm of osteoclasts (Fig 5.3).

Osteoclasts were rapidly able to re-adhere to the bone surface following pre-incubation with indomethacin, in the presence of PTH, PGE_2, $1,25D_3$ or sOPGL (Fig 3.4 and Fig 4.6) which suggests that the effects of OPG are reversible. A possible mechanism for this has been suggested by studies of the effects of OPG on isolated osteoclasts on collagen gels (Hakeda *et al*, 1998) and bone slices (Burgess *et al*, 1999). They describe a reorganization of the cytoskeleton and a loss of the F-actin ring that is seen in resorbing osteoclasts. Other workers have illustrated the importance of the cytoskeleton in integrin avidity in a range of cell types (Trusolino *et al*, 1998). It has also been shown that when osteoclasts adhere to the endocranial membrane there is a reduction in the surface expression of the integrin β3 subunit whereas when osteoclasts adhere to bone there is an increase (Holt & Marshall, 1998). We may speculate that OPG, by neutralizing OPGL, may cause the osteoclast to default to a disruption of the cytoskeleton and the F-actin ring structure which in turn causes the clustered αvβ3 integrin to become dispersed throughout the membrane. As a result the osteoclast becomes detached from the bone surface and attaches to the adjacent endocranial membrane.

An enzyme-linked immunoassay was used to assay levels of OPG protein in culture media. Following 8 hours in culture, media from mouse parietal bones incubated in the presence of indomethacin contained significantly more OPG

activity than that incubated in the presence of PGE_2 (Fig 5.18). In another experiment over a culture period of 20 hours, lower levels of OPG activity were detected with PTH, PGE_2 and $1,25D_3$ and dexamethasone compared to indomethacin (Table 5.1). Only with dexamethasone were levels of OPG suppressed to that seen in the control medium. The assay will only detect free OPG and not that bound to OPGL which may be on the surface of the bone lining cells in response to the stimulators of bone resorption. It is not possible from these results to distinguish between two possible mechanisms of action of stimulators of bone resorption. These are: the down-regulation of OPG production together with the up-regulation of OPGL production, or the up-regulation of OPGL production coupled with a constant production of OPG. The continued production of some OPG even in the presence of some stimulators of bone resorption suggests incomplete suppression of OPG production. These results emphasize that it is the local balance between soluble OPG and cell-bound OPGL, which dictates whether an individual osteoclast will be actively resorbing.

Western blotting of the MG63 cell culture supernatents following incubation in the presence of indomethacin or dexamethasone showed OPG protein to be present in the medium from the cells incubated with indomethacin but not with dexamethasone (Fig 5.19). These results confirm those from the ELISA and imply that the secretion of OPG in mouse calvaria is regulated by effectors of bone resorption even though mRNA is not (*vide infra*).

In the results reported here no suppression of mRNA for OPG was found using either whole calvaria or calvarial cells cultured in the presence of PGE_2, PTH, $1,25D_3$ or dexamethasone (Fig 5.12, 5.13). Levels of mRNA for OPG could only be suppressed by the dexamethasone treatment of MG-63 cells (Fig 5.16). Many stimulators of bone resorption have been found by other workers to suppress mRNA for OPG. Murakami *et al* (1998) showed a down-regulation of mRNA for OPG with $1,25D_3$, PTH and IL-1α in mouse osteoblasts. Other studies have shown a decrease in mRNA for OPG in mouse stromal cell lines treated with $1,25D_3$ (Yasuda *et al* 1998; Horwood *et al*1998) but not in primary osteoblasts (Horwood *et al* 1998). Vidal *et al* (1998) showed that glucocorticoids could down-regulate mRNA for OPG in primary human osteoblast-like cells. However, dexamethasone treatment of primary osteoblasts showed an initial decrease in mRNA for OPG followed by an

increase (Murakami *et al* 1998). Hofbauer *et al* (1998) found an increase in mRNA levels for OPG in human foetal osteoblasts and normal trabecular osteoblasts in response to $1,25D_3$. It may be that the results reported here reflect the fact that this study used whole bone or bone cells from which mRNA was extracted, rather than cell lines or primary osteoblasts. Using whole bone in an organ culture system should give a picture closer to what occurs *in vivo* rather than cell lines which may be transformed or immortalized or at different stages of differentiation.

OPG appears to act by binding to OPGL with a high affinity and neutralizing its stimulatory action on the osteoclast (Lacey *et al*, 1998; Yasuda *et al*, 1998a; Yasuda *et al*, 1998b). However, a recent report has suggested that OPG may act directly on the osteoclast by binding to a receptor on the osteoclast to inhibit its activity (Hakeda *et al*, 1998). It has also been found to block bone resorption both *in vivo* (Simonet *et al*, 1997) and *in vitro* (Kwon *et al*, 1998). Akatsu *et al* (1998) reported that OPG inhibited osteoclast survival by interfering in the interaction between stromal cells and osteoclasts in mouse marrow cultures. It seems likely that an OPGL-like molecule brings about the translocation of osteoclasts which occurs in the presence of these stimulators of bone resorption. However, in our studies, OPG did not significantly affect the translocation of osteoclasts from the membrane to the bone surface stimulated by PTH. This may be due to either direct effects of PTH on the osteoclast or the induction of a cytokine which may itself act directly on the osteoclast. It had been reported that osteoclasts in a model of regenerating deer antler cartilage express PTH/PTHrP receptors so that PTH may have a direct effect on cells of the osteoclast lineage (Faucheux *et al* 2000).

In the mouse calvarial organ culture system prostaglandins, particularly PGE_2, brings about the translocation of osteoclasts from the endocranial membrane to the bone surface where they become activated to resorb bone. PGE_2 achieves this by inducing the expression of mRNA for OPGL, which acts directly on the osteoclast *via* the receptor RANK. OPGL provides the link between PGE_2 and other stimulators of bone resorption and the osteoclast. In this system mRNA for OPG is not down-regulated by stimulators of bone resorption but, OPG protein secretion is inhibited. When OPGL on the bone lining cell surface makes contact with RANK, several TRAF proteins act as signal transducers to activate NFκB and JNK (Wong *et al*, 1998; Dadgostar and Cheung, 1998). NFκB is then translocated to the cell

nucleus where it brings about the transcription of genes which allow the activation of osteoclasts and their movement from the endocranial membrane to the bone surface. The movement of the osteoclasts from the membrane to the bone is dependent on functioning αvβ3 integrin. Signalling *via* the αvβ3 integrin seems to bring about cytoskeletal rearrangement and the appearance of the F-actin ring associated with the resorbing osteoclast. During this cytoskeletal rearrangement, the αvβ3 integrin molecules probably become clustered allowing the osteoclast to recognize the bone surface. It is clear from these results with echistatin and function-blocking antibody to β3 that the αvβ3 integrin is involved in the adhesion of the osteoclast to the bone surface. When prostaglandin synthesis is blocked by indomethacin OPG protein is produced which causes the osteoclast to cease bone resorption and return to the endocranial membrane. OPG neutralizes OPGL and by default seems to cause disruption to the cytoskeleton and loss of the actin ring, which may cause the dispersal of the αvβ3 integrin throughout the membrane and the loss of recognition of the bone surface by the integrin (Hakeda *et al*, 1998). The osteoclast then adheres to the endocranial membrane by some unknown mechanism. The osteoclast can then be reactivated by OPGL if prostaglandin synthesis is allowed to recommence or if other stimulators of bone resorption are produced. This mechanism is summarized in Fig 6.2.

Conclusion

A mechanism is described whereby bone resorption is acutely controlled by rapidly modulating the adhesion of osteoclasts to the bone surface. This mechanism is controlled by the bone lining cells which integrate a range of local and systemic influences and convert them to an appropriate level of OPGL on the cell surface. When the osteoclast or its precursor is in contact with this OPGL final differentiation and activation of the osteoclast occurs. At least part of this activation involves the production of adhesive molecules including the integrin αvβ3 on the surface of the osteoclast. When these make contact with their ligands on the bone surface the enzymes and structures necessary for bone resorption are produced. The process is reversible. When the resorptive stimulus disappears OPG is secreted

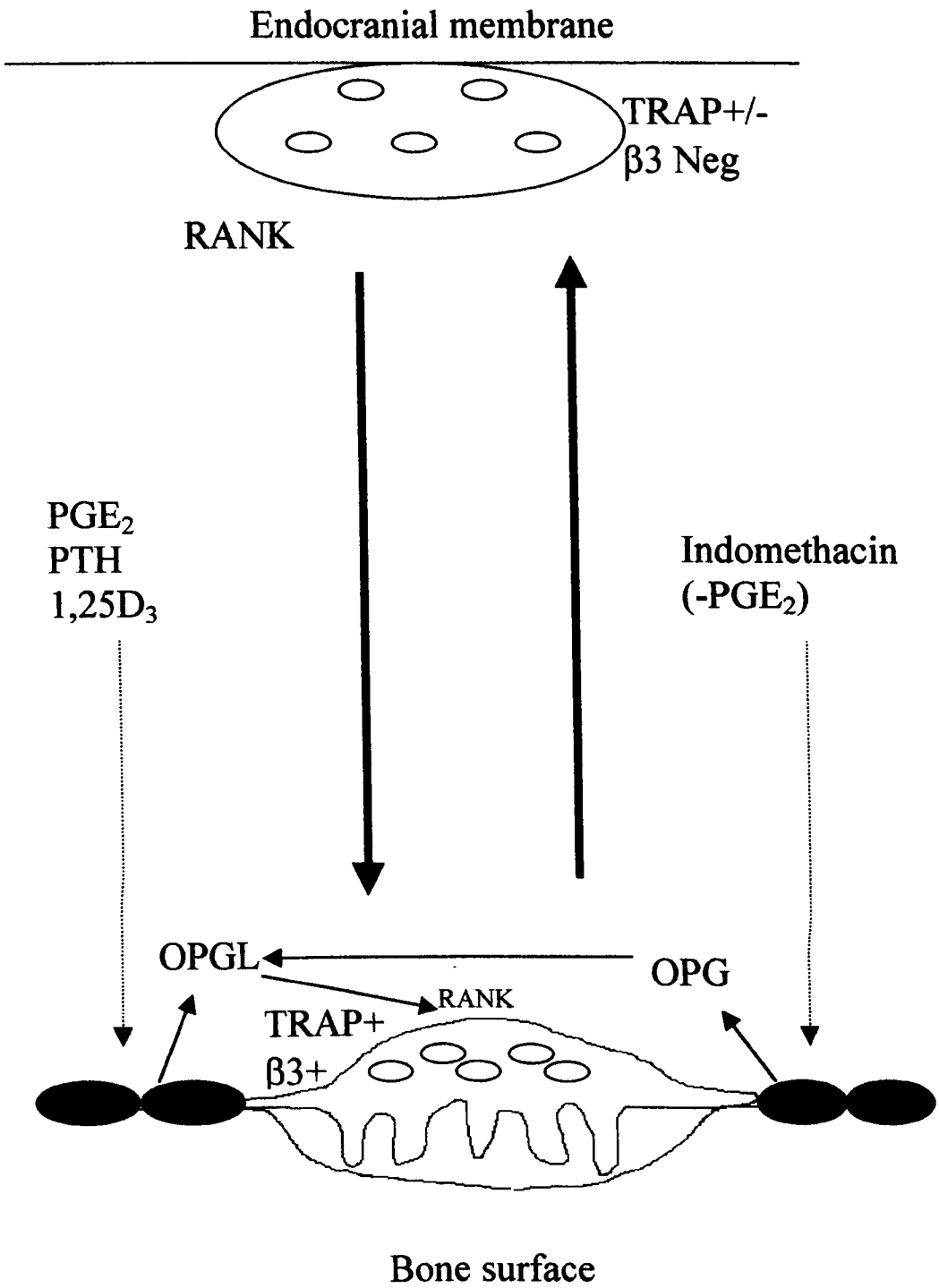


Figure 6.2 Diagrammatic representation of the possible mechanism of the inhibition of bone resorption in the presence of OPG and the stimulation of bone resorption in the presence of OPGL. PGE_2, PTH and $1,25D_3$ stimulate the bone lining cells to produce OPGL and indomethacin causes them to produce OPG. These intermediaries stimulate or inhibit osteoclast adhesion to bone and they control bone resorption.

which blocks OPGL and the osteoclast returns to its quiescent state. Apart from ensuring that bone resorption is exquisitely sensitive, for instance, to the calcium status of the host and to mechanical demands, this mechanism is fail-safe. Osteoclasts cannot differentiate in the absence of mCSF, OPGL and bone, and revert to a non-resorptive state without OPGL so that aberrant bone resorption is less likely. The importance of this mechanism can be seen from the reports of the use of OPG in the control of bone resorption *in vivo*. OPG has been found to block bone loss due to estrogen deficiency in mice (Simonet *et al*, 1997). The first human clinical trials of OPG involving 52 postmenopausal women showed a dose-dependent decrease in the bone turnover marker N-telopeptide/creatinine. Levels were reduced by 80% following a single injection of the highest dose tested (Bekker *et al*, 1999). OPG has been found to block osteolysis in transplantable tumours in mice (Honore *et al*, 2000). They found an almost total elimination of tumour induced bone destruction and osteoclasts at sites of tumour. It was also found by the same group that OPG could ameliorate the pain associated with osteolysis in mice (Honore *et al*, 2000). The mice with bone cancer showed a significant reduction in pain score when treated with OPG compared to control mice. It remains to be determined whether OPG can inhibit osteolysis and reduce bone cancer pain in humans but it may lead to improved quality of life for patients suffering from primary or metastatic bone cancer.

Publications related to this work

Marshall MJ, O'Brien EA Holt I, (1998). Integrins are involved in the movement of osteoclasts between the periosteum and the bone surface. *Bone* 22 (Suppl. 3 abstract C115) . Seventh Workshop on Cell Biology of Bone and Cartilage in Health and Disease).

O'Brien EA, Williams JHH, Marshall MJ (1999). Osteoclast differentiation factor controls the translocation of osteoclasts from the periosteum to the bone surface in mouse calvaria. *Calcified Tissue International* 64 (Suppl 1abstract O-35) XXVIth European Symposium on Calcified tissues, Maastricht, Holland).

O'Brien EA, Williams JHH, Marshall MJ (1999). Osteoprotegerin causes osteoclasts to detach from the surface of mouse parietal bone and adhere to the adjacent periosteum. *Journal of Bone and Mineral Research*14 (6) abstract P04. Bone & Tooth Spring Meeting June 1999). Winner of prize for Best Poster Presentation.

O'Brien EA, Rowlands MN, Marshall MJ (2000). Differential adhesion of the osteoclast between the periosteum and the bone surface is regulated by osteoclast differentiation factor and osteoprotegerin. *Bone* 26 (Suppl 3 abstract C62) Eighth Workshop on Cell Biology of Bone and Cartilage in Health and Disease Davos.

5) Rowlands MN, O'Brien EA, Sharp CA, Marshall MJ (2000) Formulation of tartrate-resistant-acid-phosphatase positive osteoclast-like cells in response to osteoclast differentiation factor and macrophage colony stimulating factor. *Calcified Tissue International* 66 (Suppl 1 P222). XXVII European Symposium on Calcified Tissues, Tampere Finland.

O'Brien EA, Marshall MJ (2000) Osteoprotegerin is produced when prostaglandin synthesis is inhibited causing osteoclasts to detach from the surface of bone and attach to the periosteum. *Journal of Bone and Mineral Research* 15 (6) (Abstract P77) Bone and Tooth Spring Meeting June Cambridge.

O'Brien EA, Williams JHH, Marshall MJ. (2000) Osteoprotegerin ligand regulates osteoclast adherence to the bone surface in mouse calvaria. *Biochemical and Biophysical Research Communications* 274: 281-290.

O'Brien EA, Williams JHH, Marshall MJ. (2001) Osteoprotegerin is produced when prostaglandin synthesis is inhibited causing osteoclasts to detach from the surface of mouse parietal bone and attach to the endocranial membrane. *Bone* (in press).

References

Abe, K., Ohno, K., & Hasegawa, K. (1990). Morphological relationships between osteoclasts and bone resorption surfaces on mouse parietal bones. *Archives of Histology and Cytolology*, 53, 95-102.

Abu-Amer, Y. and Tondravi, M. M. (1997). NF-κB and bone: The breaking point. *Nature Medicine*, 3, 1189-1190.

Adachi, K., Chole, R. A., & Yee, J. (1991). Indomethacin inhibition of middle ear bone resorption. *Archives of Otolaryngology Head and Neck Surgery*, 117, 267-269.

Agarwala, N. and Gay, C. V. (1992). Specific binding of parathyroid hormone to living osteoclasts. *Journal of Bone and Mineral Research*, 7, 531-539.

Akamine, T., Jee, W. S. S., Ke, H. Z., Li, X. J., & Lin, B. Y. (1992). Prostaglandin E_2 prevents bone loss and adds extra bone to immobilized distal femoral metaphysis in female rats. *Bone*, 13, 11-22.

Akatsu, T., Murakami, T., Nishikawa, M., Ono, K., Shinomiya, N., Tsuda, E., Mochizuki, S., Yamaguchi, K., Motoyoshi, K., & Nagata, N. (1998). Osteoclastogenesis inhibitory factor suppresses osteoclast survival by interfering in the interaction of stromal cells with osteoclast. *Biochemical and Biophysical Research Communications*, 250, 229-234.

Akatsu, T., Takahashi, N., Debari, K., Morita, I., Murota, S., Nagata, N., Takatani O., & Tsuda, T. (1989). Prostaglandin promote osteoclast-like cell formation by a mechanism involving cyclic adenosine 3',5'-monophosphate in mouse bone marrow cell cultures. *Journal of Bone and Mineral Research*, 4, 29-35.

Ammann, P., Rizzoli, R., Bonjour, J.P., Bourrin, S., Meyer, J.M., Vassalli, P., & Garcia, I. (1997). Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency. *Journal of Clinical Investigation*, 99, 1699-1703.

Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., Teepe, M. C., DuBose, R. F., Cosman, D., & Galibert, L. (1997). A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature*, 390, 175-179.

Arch, R. H., Gedrich, R. W., & Thompson, C. B. (1998). Tumor necrosis factor receptor-associated factors (TRAFs)-a family of adapter proteins that regulates life and death. *Genes and Development*, 12, 2821-2830.

Arend, W. P., Welgus, H. G., Thompson, R. C., & Eisenberg, S. P. (1990). Biological properties of recombinant human monocyte-derived interleukin 1 receptor antagonist. *Journal of Clinical Investigation*, 85, 1694-1697.

Arita, K., Kato, K., Tojo, T., & Nishino, M. (1989). Studies on factors causing tooth resorption. 1. Role of prostaglandins as a chemical mediator on resorption of deciduous teeth in rabbits. *Japanese Journal of Periodontology*, 27, 621-628.

Ash, P., Loutit, J. F., & Townsend, K. M. S. (1981). Osteoclasts derive from hematopoietic stem cells according to marker giant lysosomes of beige mice. *Clinical Orthopaedics*, 155, 249-258.

Barnicot, N. A. (1948). The local action of the parathyroid and other tissues on bone in intracerebral grafts. *Journal of Anatomy*, 82, 233-248.

Baron, R., Neff, L., Louvard, D., & Courtoy, P. J. (1985). Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. *Journal of Cell Biology*, 101, 2210-2222.

Bekker, P. J., Holloway, D., Nalanishi, A., Arrighi, H. M., & Dunstan, C. R. (1999). Osteoprotegerin (OPG) has a potent and sustained anti-resorptive activity in postmenopausal women. *Journal of Bone and Mineral Research*, 14, S180.

Bell, N. H., Hollis, B. W., Shary, J. R., Eyrre, D. R., Eastell, R., Colwell, A., & Russell, R. G. (1994). Diclofenac sodium inhibits bone resorption in postmenopausal women. *American Journal of Medicine*, 96, 349-346.

Bitgood, M.J. and McMahon, A.P. (1995). *Hedgehog* and *Bmp* genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Developmental Biology*, 172, 126-138.

Blumenkrantz, N.and Sondergaard, J. (1972). Effects of prostaglandin E_1 and $F_{2\alpha}$ on biosynthesis of collagen. *Nature New Biology*, 239, 246-251.

Bonewald, L. F. and Mundy, G. R. (1990). Role of transforming growth factor-beta in bone remodeling. *Clinical Orthopaedics and Related Research*, 250, 261-276.

Bord, S., Horner, A., Beeton, C. A., Hembry, R. M., & Compston, J. E. (1999). Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) distribution in normal and pathological human bone. *Bone*, 24, 229-235.

Boyce, B. F., Windle, J. J., Reddy, S. V., Wright, K., Leach, R. J., & Roodman, G. D. (1993). Targeting SV-40 T antigen to the osteoclast in transgenic mice causes osteopetrosis, transformation and apoptosis of oseoclasts. *Journal of Bone and Mineral Research*, 8, S118 (abstract).

Boyce, B. F., Yoneda, T., Lowe, C., Soriano, P., & Mundy, G. R. (1992). Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice. *Journal of Clinical Investigation*, 90, 1622-1627.

Boyce, B. F., Aufdemorte, T. B., Garrett, I. R., Yates, A. J. P., & Mundy, G. R. (1989). Effects of interleukin-1 on bone turnover in normal mice. *Endocrinology*, 125, 1142-1150.

Boyde, A., Ali, n. N., & Jones, S. J. (1984). Resorption of dentine by isolated osteoclasts *in vitro*. *British Dental Journal*, 156, 216-220.

Brandstrom, H., Jonsson, K. B., Vidal, O., Ljunghall, S., Ohlsson, C., & Ljunggren, O. (1998a). Tumor necrosis factor-α and -β upregulate the levels of osteoprotegerin mRNA in human osteosarcoma MG-63 cells. *Biochemical and Biophysical Research Communications*, 248, 454-457.

Brandstrom, H., Jonsson, K. B., Ohlsson, C., Vidal, O., Ljunghall, S., & Ljunggren, O. (1998b). Regulation of osteoprotegerin mRNA levels by prostaglandin E_2 in human bone marrow stroma cells. *Biochemical and Biophysical Research Communications*, 247, 338-341.

Bromley, M., and Woolley, D. E. (1984). Chondroclasts and osteoclasts at subchondral sites of erosion in the rheumatoid joint. *Arthritis and Rheumatism*, 27, 968-975.

Bucay, N., Sarosi, I., Dunstan, C. R., Morony, S., Tarpley, J., Capparilli, C., Scully, S., Tan, H. L., Xu, W., Lacey, D.L., Boyle, W. J., & Simonet, W. S. (1998). *Osteoprotegerin*-deficient mice develop early onset osteoporosis and arterial calcification. *Genes and Development*, 12, 1260-1268.

Buckwalter, J.A., Glimcher, M.J., Cooper, R.R., & Recker, R. (1995). Bone biology. *Journal of Bone and Joint Surgery*, 77-A, 1256-1275.

Burgess, T.L., Qian, Y., Kaufman, S., Ring, B. D., Van, G., Capparelli, C., Kelley, M., Hsu, H., Boyle, W.J., Dunstan, C.R., Hu, S., & Lacey, D. (1999). The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *Journal of Cell Biology*, 145, 527-538.

Canalis, E. (1996). Mechanisms of glucocorticoid action in bone: implications to glucocortocoid –induced osteoporosis. *Journal of Clinical Endocrinology and Metabolism*, 81, 3441-3447.

Cao, Z., Xiong, J., Takeuchi, T., Kurama, T., & Goeddel, D.V. (1996). TRAF6 is a signal transducer for interleukin-1. *Nature*, 383, 443-446.

Carmeliet, G., Verstuyf, A., Daci, E., & Bouillon, R. (1999). The vitamin D hormone and its nuclear receptor: genomic mechanisms involved in bone biology. In M. J. Siebel, S. P Robins, & J. P. Bilezikian (Eds), *Dynamics of Bone and Cartilage Metabolism* (pp. 217-231). London: Academic Press.

Chambers, T.J. (1985). The pathobiology of the osteoclast. *Journal of Clinical Pathology*, 38, 241-252.

Chambers, T. J., Revell, P. A., Fuller, K., & Athanasou, N. A. (1984). Resorption of bone by isolated osteoclasts. *Journal of Cell Science*, 66, 383-399.

Chambers, T. J., and Magnus, C. J. (1982). Calcitonin alters behavior of isolated osteoclasts. *Journal of Pathology*, 136, 27-40.

Chaudhary, L.R., Spelsberg, T.C., & Riggs, B.L. (1992). Production of various cytokines by normal human osteoblast-like cells in response to interleukin-1β and tumor necrosis factor-α: lack of regulation by 17β-estradiol. *Endocrinology*, 130, 2528-2534.

Chyun, Y. S. and Raisz, L. G. (1984). Stimulation of bone formation by prostaglandin E_2. *Prostaglandins*, 27, 97-103.

Clark, I. M. and Murphy, G. (1999). Matrix proteinases. In M. Siebel, S. Robins, & J. Bilezikian (Eds), *Dynamics of Bone and Cartilage Metabolism* (pp 137-150). London: Academic Press.

Collins, D.A. and Chambers, T.J. (1992). Prostaglandin E_2 promotes osteoclast formation in murine hematopoietic cultures through an action on hematopoietic cells. *Journal of Bone and Mineral Research*, 7, 555-561.

Coopper, R. R., Milgram, J. W., & Robinson, R. A. (1966). Morphology of the osteon. *Journal of Bone and Joint Surgery*, 48-A, 1239-1240.

Dadgostar, H. and Cheng, G. (1998). An intact zinc finger is required for tumor necrosis factor receptor-associated factor-mediated nuclear factor-κB activation but is dispensible for c-Jun N-terminal kinase signaling. *Journal of Biological Chemistry*, 273, 24775-24780.

Delaisse, J-M., Boyde, A., Macconnachie, E., Ali, N. N., Sear, C. H. Jr., Eeckhout, Y., Vaes, G., & Jones, S. J. (1987). The effects of inhibitors of cysteine-proteinases and collagenase on the resorptive activity of isolated osteoclasts. *Bone*, 8, 305-313.

Duong, Le. T. Lakkakorpi, P., Nakamura, I., & Rodan, G. A. (2000). Integrins and signaling in osteoclast function. *Matrix Biology*, 19, 97-105.

Duong, Le. T. and Rodan, G. A. (1999). The role of integrins in osteoclast function. *Journal of Bone and Mineral Metabolism*, 17, 1-6.

Duong, Le. T. Lakkakorpi, P. T., Nakamura, I., Machwate, M., Nagy, R. M., & Rodan G. A. (1998). PYK2 in osteoclasts is an adhesion kinase, localized in the sealing zone activated by ligation of αvβ3 integrin, and phosphorylated by Src kinase. *Journal of Clinical Investigation*, 102, 881-892.

El Haj, A.J., Minter, S. L., Rawlinson, S. C. F., Suwillo, R., & Lanyon, L. E. (1990). Cellular responses to mechanical loading in vitro. *Journal of Bone and Mineral Research*, 5, 923-932.

Eriksen, E. F., Colvard, D. S., Berg, N. J., Graham, M. L., Mann, K. G., Spelsberg, T. C., & Riggs, B. L. (1988). Evidence of estrogen receptors in normal human osteoblast-like cells. *Science*, 241, 84-86.

Everts, V., Korper, W., Jansen, D. C., Steinfort, J., Lammerse, I., Heera, S., Docherty, A. J. P., & Beerston, W. (1999). Functional heterogeneity of osteoclasts: matrix metalloproteinases participate in osteoclastic resorption of calvarial bone but not in resorption of long bone. *FASEB*, 13, 1219-1230.

Everts, V., Delaisse, J-M., Korper, W., Niehof, A., Vaes, G., & Beertsen, W. (1992). Degradation of collagen in the bone-resorbing compartment underlying the osteoclasts involves both cysteine-proteinases and matrix metalloproteinases. *Journal of Cell Physiology*, 150, 221-231.

Facheux. C., Nicholls, B., Horton, M.A., &Price, J.S. (2000). A role for PTHrP and RANKL in the osteoclast formation in regenerating deer antler cartilage. *Journal of Bone and Mineral Research. Twenty second annual meeting of the American Society for Bone and Mineral Research, Ontario, Canada, (Abstract SA254).*

Fan, X., Biskobing, D. M., Fan, D., Hofstetter, W., & Rubin, J. (1997). Macrophage colony stimulating factor downregulates M-CSF receptor expression and entity of progenitors into the osteoclast lineage. *Journal of Bone and Mineral Research*, 12, 1387-1395.

Felix, R., Cecchini, M. G., & Fleisch, H. (1990). Macrophage colony stimulation factor restores in vivo bone resorption in the op/op osteopetrotic mouse. *Endocrinology*, 127, 2592-2594.

Fiers, W. (1993). Tumor necrosis factor. In E. Sim (Ed.), *The Natural Immune System: Humoral Factors* (9965-119). Oxford: Oxford University Press.

Filipponi, P., Mannarelli, C., Pacifici, R., Grossi, E., Moretti, I., Tini, S., Carloni, C., Blass, A., Morucci, P., Hruska, K. A., & Avioli, L. V. (1988). Evidence for a prostaglandin-mediated bone resorptive mechanism in subjects with fasting hypercalciuria. *Calcified Tissue International*, 43, 61-66.

Fiore, C. E., Falcidia, E., Foti, R., Motta, M., & Tamburino, C. (1994). Differences in the time course of the effects of oophrectomy in women on parameters of bone metabolism and interleukin-1 levels in the circulation. *Bone Mineral*, 20, 79-85.

Fischman, D. A., and Hay, E. D. (1962). Origin of osteoclasts from mononuclear leucocytes in regenerating newt limbs. *Anatomical Record*, 143, 329-334.

Fisher, J. E., Caulfield, M. P., Sato, M., Quartuccio, H. A., Gould, R. J., Garsky, V. M., & Rodan, G. A. (1993). Inhibition of osteoclastic bone resorption in vivo by echistatin, an "Arginyl-Glycyl-Aspartyl" (RGD)-containing protein. *Endocrinology*, 132, 1411-1413.

Franchi-Miller, C. and Saffar, J. L. (1995). The 5-lipoxygenase inhibitor BWA4C impairs osteoclastic resorption in a synchronized model of bone remodeling. *Bone*, 17, 185-191.

Friedenstein, A. J. (1973). Determined and inducible osteogenic precursor cells. In Hard tissue growth repair and remineralization *Ciba Foundation Symposium 11 (New Series)* (pp 169-185) Amsterdam: Elsevier.

Friedenstein, A. J., Petrakova, K.V., Kurolesova, A. I., & Frolova, G. P. (1968). Heterotopic transplants of bone marrow. Analysis of precursor cells for osteogenic and haematopoietic tissues. *Transplantation*, 6, 230-247.

Friedenstein, A. J., Lalykina, K. S., & Tolmacheva, A. A. (1967). Osteogenic activity of peritoneal fluid cells induced by transitional epithelium. *Acta. Anatomica*, 68, 532-549.

Friedenstein, A. J., Piatetzky-Shapiro, I. I., & Petrakova, K. V. (1966). Osteogenesis in transplants of bone marrow cells. *Journal of Embryology and Experimental Morphology*, 16, 381-390.

Fujikawa, Y., Sabokbar, A., Neale, S., & Athanasou, N. A. (1996). Human osteoclast formation and bone resorption by monocytes and synovial macrophages in rheumatoid arthritis. *Annals of Rheumatic Disease*, 55, 816-822.

Fuller, K., Wong, B., Fox, S., Choi, Y., & Chambers, T. (1998). TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *Journal of Experimental Medicine*, 188, 997-1001

Fuller, K. and Chambers, T. J. (1989). Effects of arachidonic acid metabolites on bone resorption by isolated osteoclasts. *Journal of Bone and Mineral Research* 4, 209-215.

Galvin, R. J., Bryan, P., Horn, J. W., Rippy, M. K., & Thomas, J. E. (1996). Development and characterization of a porcine model to study osteoclast differentiation and activity. *Bone*, 19, 271-279.

Garcia, C., Boyce, B. F., Gilles, J., Dallas, M., Qiao, M., Mundy, G. R., & Bonewald, L. F. (1996). Leukotriene B4 stimulates osteoclastic bone resorption both in vitro and in vivo. *Journal of Bone and Mineral Research*, 11, 1619-1627.

Garrett, I.R. and Mundy, G.R. (1989). Relationship between interleukin-1 and prostaglandins in resorbing neonatal calvaria. *Journal of Bone and Mineral Research*, 4, 789-794.

Gay, G. V. and Mueller, W. J. (1974). Carbonic anhydrase and osteoclasts: Localization by labeled inhibitor autoradiography. *Science*, 183, 432-434.

Girasole, G., Passeri, G., Jilka, R. L., & Manolagas, S. C. (1994). Interleukin-11: A new cytokine critical for osteoclast development. *Journal of Clinical Investigation*, 93, 1516-1524.

Girasole, G., Jilka, R. L.,Passeri, G., Boswell, S., Boder, G., Williams, D. C., & Manolagas, S. C. (1992). 17β-Estradiol inhibits interleukin-6 production by bone marrow derived stromal cells and osteoblasts in vitro: a potential mechanism for the anti-osteoporotic effects of estrogens. *Journal of Clinical Investigation*, 89, 883-891.

Glowacki, J., Cox, K. A., O'Sullivan, J., Wilkie, D., & Deftos, L. J. (1986). Osteoclasts can be induced in fish having an acellular bony skeleton. *Proceedings of the National Acadameyof Science*, 83, 4104-4107.

Gothlin, G. and Ericsson, J. L. E. (1976). The Osteoclast. *Clinical Orthopaedics and Related Research*, 120, 201-231.

Gothlin, G. and Ericsson, J. L. E. (1972). On the histogenesis of the cells in fracture callus. *Virchows Archive B Cell Pathology*, 12, 318-329.

Greenfield, E. M., Horowitz, M. C., & Lavish, S. A. (1996). Stimulation by parathyroid hormone of interleukin-6 and leukaemia inhibitory factor expression in osteoblasts is an immediate-early gene response induced by cAMP signal transduction. *Journal of Biological Chemistry*, 271, 10984-10989.

Grigoriadis, A. E., Wang, Z. Q., Cecchini, M. G., Hofstetter, W., Felix, R., Fleisch, H. A., & Wagner, E. F. (1994). c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science*, 266, 443-448.

Hakeda, Y., Kobayashi Y., Yamaguchi, K., Yasuda, H., Tsuda, E., Higashio, K., Miyata, T., & Kumegawa, M. (1998). Osteoclastogenesis inhibitory factor (OCIF) directly inhibits bone-resorbing activity of isolated mature osteoclasts. *Biochemical and Biophysical Research Communications*, 251, 796-801.

Hall, T. J. and Chambers, T. J. (1989). Optimal bone resorption by isolated rat osteoclasts requires chloride/bicarbonate exchange. *Calcified Tissue International*, 45, 378-380.

Halleen, J, M., Raisanen, S., Salo, J. J., Reddy, S. V., Roodman, G. D., Hentunen, T.A., Lehenkari, P. P., Kaija, H., Vihko, P., & Vaananen, H. K. (1999). Intracellular fragmentation of bone resorption products by reactive oxygen species generated by tartrate-resistant acid phosphatase. *Journal of Biological Chemistry*, 274, 22907-22910.

Hancox, N. M. (1972). The Osteoclast. In Bourne (Ed), The Biochemistry and Physiology of Bone. New York: Academic Press.

Hancox, N. M. and Boothroyd, B. (1961). Motion picture and electron microscope studies in the embryonic avian ostoeclast. *Journal of Biophysical and Biochemical Cytology*, 11, 651-661.

Harell, A., Dekel, S., & Binderman, I. (1977). Biochemical effect of mechanical stress on cultured bone cells. *Calcified Tissue Research*, 22, 202-207.

Harris, M., Jenkins, M. V., Bennet, A., Wills, M. R. (1973). Prostaglandin production and bone resorption by dental cysts. *Nature*, 45, 213-215.

Hofbauer, L.C., Khosla, S., Dunstan, C.R., Lacey, D L., Spelsberg, T.C., & Riggs, B.L. (1999a). Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. *Endocrinology*, 140, 4367-4370.

Hofbauer, L.C., Gori, F., Riggs, B.L., Lacey, D.L., Dunstan, C.R., Spelsberg, T.C., & Khosla, S. (1999b). Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. *Endocrinology*, 140, 4382-4389.

Hofbauer, L. C., Dunstan, C., Spelsberg, T.C., Riggs, B.L., & Khosla, S. (1998). Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines. *Biochemical and Biophysical Research Communications*, 250, 776-781.

Hoffbrand, A. V. and Pettit, J. E. (1995). Blood cell formation. (Haemopoiesis). In *Essential Haematology*, (pp1-11). London: Blackwell Science.

Holt, I. and Marshall, M.J. (1998). Integrin subunit β3 plays a crucial role in the movement of osteoclast from the periosteum to the bone surface. *Journal of Cellular Physiology*, 175, 1-9.

Holt, I., Davie, M. W. J., Braidman, I. P., & Marshall, M. J. (1994). Interleukin-6 does not mediate the stimulation by prostaglandin E_2, parathyroid hormone, or 1,25dihydroxyvitamin D_3 of osteoclast differentiation and bone resorption in neonatal mouse parietal bones. *Calcified Tissue International*, 55, 114-119.

Honore, P., Luger, N. M., Sabino, M. A., Schwei, M. J., Rogers, S. D., Mach, D. B., O'Keefe, P. F., Ramnaraine, M. L., Clohisy, D. R., & Mantyh, P. W. (2000). Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. *Nature Medecine*, 6, 521-528.

Horne, W. C., Neff, L., Chatterjee, D., Levy, J. B., & Baron, R. (1992). Osteoclasts express high levels of $pp60^{c\text{-}src}$ in association with intra-cellular membranes. *Journal of Cell Biology*, 119, 1003-1013.

Horton, M. A. (1997). The αvβ3 integrin "Vitronectin Receptor". *International Journal of Biochemistry and Cell Biology*, 29, 721-725.

Horton, M. A., Taylor, M. L., Arnett, T. R., & Helfrich, M. H. (1991). Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. *Experimental Cell Research*, 195, 368-375.

Horwood, N.J., Elliot, J., Martin, T.J., & Gillespie, M.T. (1998). Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells. *Endocrinology*, 139, 4743-4746.

Hsu, H., Lacey, D. L., Dunstan, C. R., Solovyev, I., Colombero, A., Timms, E., Tan, H., Elliot, G., Kelley, M. J., Sarosi, I., Wang, L., Xia, X., Elliot, R., Chiu, L., Black, T., Scully, S., Capparelli, C., Morony, S., Shimamoto, G., Bass, M. B., & Boyle, W. J. (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proceecings of the National Academy of Sciences USA*, 96, 3540-3545.

Hughes, D. E., Dai, A., Tiffee, J. C., Li, H. H., Mundy, G. R., & Boyce, B. F. (1996). Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-β. *Nature Medecine*, 2, 1132-1136.

Hultenby, K., Reinholt, F. P., & Heinegard, D. (1993). Distribution of integrin subunits on rat metaphyseal osteoclasts and osteoblasts. *European Journal of Cell Biology*, 62, 86-93.

Inaoka, T., Bilbe, G., Ishibashi, O., Tezuka, K., Kumegawa, M., & Kokubo, T. (1995). Molecular cloning of human cDNA for cathepsin K: novel cysteine proteinase predominantly expressed in bone. *Biochemical and Biophysical Research Communications*, 206, 89-96.

Inui, T., Ishibashi, O., Inaoka, T., Origane, Y., Kumegawa, M., Kokubo, T., & Yamamura, T. (1997). Cathepsin K antisense oligodeoxynucleotide inhibits osteoclastic bone resorption. *Journal of Biological Chemistry*, 272, 8109-8112.

Jaworski, Z.F.J., Duck, B., & Sekaly, G. (1981). Kinetics of osteoclasts and their nuclei in evolving secondary Haversian systems. *Journal of Anatomy*, 133, 397-405.

Jaworski, Z.F.J. and Hooper, C. (1980). Study of cell kinetics within evolving secondary Haversian systems. *Journal of Anatomy*, 131, 91-102.

Jilka, R. L., Hangoc, G., Girasole, G., Passeri, G., Williams, D. C., Abrams, J. S., Boyce, B., Broxmeyer, H., & Manolagas, S. C. (1992). Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science*, 257, 88-91.

Jimi, E., Akiyama, S., Tsurukai, T., Okahashi, N., Kobayashi, K., Udagawa, N., Nishihara, T., Takahashi, N., & Suda, T. (1999). Osteoclast differentiation factor acts as a multifunctional regulator in murine osteoclast differentiation and function. *Journal of Immunology*, 163, 434-442.

Jimi, E., Nakamura, I., Ikebe, T., Akiyama, S., Takahashi, N., & Suda, T. (1998). Activation of NFκB is involved in the survival of osteoclasts promoted by interleukin-1. *Journal of Biological Chemistry*, 273, 8799-8805.

Jones, S. J., and Boyde, A. (1977). Some morphological observations on osteoclasts. *Cell and Tissue Research*, 185, 387-397.

Jorgensen, H. R. I., Svanholm, H., & Host, A. (1988). Bone formation induced in an infant by systemic prostaglandin E_2 administration. *Acta Orthopaedica Scandinavica*, 59, 464-466.

Karsenty, G. (1999). The genetic transformation of bone biology. *Genes and Development*, 13, 3037-3051.

Kassem, M., Harris, S. A., Spelsberg, T. C., & Riggs, B. L. (1996). Estrogen inhibits interleukin-6 production and gene expression in a human osteoblastic cell line with high levels of estrogen receptors. *Journal of Bone and Mineral Research*, 11, 193-199.

Kawaguchi, H., Pilbeam, C., Harrison, J. & Raisz, L.G. (1995). The role of prostaglandins in the regulation of bone metabolism. *Clinical Orthopaedics and Related Research*, 313, 36-46.

Keeting, P. E., Rifas, L., Harris, S. A., Colvard, D. S., Spelsberg, T. C., Peck, W. A., & Riggs, B. L. (1991). Evidence for interleukin-1 beta production by cultured normal human osteoblast-like cells. *Journal of Bone and Mineral Research*, 6, 827-833.

Khosla, S., Spelsberg, T. C., & Riggs, B. L. (1999). Sex steroids effects on bone metabolism. . In M. J. Siebel, S. P Robins, & J. P. Bilezikian (Eds), *Dynamics of Bone and Cartilage Metabolism* (pp. 217-231). London: Academic Press.

Kimble, R. B., Bain, S., & Pacifici, R. (1997). The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice . *Journal of Bone and Mineral Research*, 12, 935-941.

Kimble, R. B., Srivastava, S., Ross, P.F., Matayoshi, A., & Pacifici, R. (1996). Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1 and tumor necrosis factor-mediated stimulation of macrophage colony stimulating factor production. *Journal of Biological Chemistry*, 271, 28890-28897.

Kimble, R. B., Bain, S. D., Kung, V., & Pacifici, R. (1995). Inhibition of IL-6 activity in genetically normal mice does not prevent ovariectomy-induced bone loss. *Journal of Bone and Mineral Research*, 10, S160 (abstract).

Kimble, R. B., Vannice, J. L., Bloedow, D. C., Thompson, R. C., Hopfer, W., Kung, V. T., Brownfield, C., & Pacifici, R. (1994). Interleukin-1 receptor antagonist decreased bone loss and bone resorption in ovariectomized rats. *Journal of Clinical Investigation*, 93, 1959-1967.

King, K. L., D'Anza, J. J., Bodary, S., Pitti, R., Siegel, M., Lazarus, R. A., Dennis, M. S., Hammonds, R. G. Jr., & Kukreja, S. C. (1994) Effects of kistrin on bone resorption in vitro and serum calcium in vivo. *Journal of Bone and Mineral Research*, 9, 381-387.

Kitazawa, R., Kimble, R. B., Vannice, J. L., Kung, V. T., & Pacifici, R. (1994). Interleukin-1 receptor antagonist and tumour necrosis factor binding protein decrease osteoclast formtion and bone resorption in ovariectomized rats. *Journal of Clinical Investigation*, 94, 2397-2406.

Klein, D. C., and Raisz, L. G. (1970). Prostaglandins: stimulation of bone resorption in tissue culture. *Endocrinology*, 86, 1436-1440.

Kobayashi, Y., Hashimoto, F., Miyamoto, H., Kanaoka, K., Miyazaki-Kawashita, Y., Nakashima, T., Shibata, M., Kobayashi, K., Kato, Y., & Sakai, H. (2000a). Force- induced osteoclast apoptosis in vivo is accompanied by elevation in transforming growth factor β and osteoprotegerin expression. *Journal of Bone and Mineral Research*, 15, 1924-1934.

Kobayashi, Y., Takahashi, N., Jimi, E., Udagawa, N., Takami, M., Kotake, S., Nakagawa, N., Kinosaki, M., Yamaguchi, K., Shima N., Yasuda, H., Morinaga, T., Higashio, K., Martin T.J., & Suda, T. (2000b). Tumor necrosis factor α stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *Journal of Experimental Medecine*, 191, 275-285.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y-H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., & Kishimoto, T. (1997). Targeted disruptiom of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*, 89, 755-764.

Kong, Y.Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., Capparelli, C., *Li, J., Elliott, R., McCabe, S., Wong, T., Campagnuolo, G.*, Moran, E., Bogoch, E.R., Van, G., Nguyen, L.T., Ohashi, P.S., *Lacey, D.L., Fish, E., Boyle, W.J., &* Penninger, J.M. (1999). Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature*, 402, 304-309.

Kurihara, N., Civin, C., & Roodman, G. D. (1991). Osteotropic factor responsiveness of highly purified populations of early and late precursors for human multinucleated cells expressing the osteoclast phenotype. *Journal of Bone and Mineral Research*, 6, 257-261.

Kurihara, N., Chenu, C., Civin, C. I., & Roodman, G. D. (1990a). Identification of committed mononuclear precursors for osteoclast-like cells formed in long-term marrow cultures. *Endocrinology*, 126, 2733-2741.

Kurihara, N., Bertolini, D., Suda, T., Akiyama, Y., & Roodman, G. D. (1990b). IL-6 stimulates osteoclast-like multinucleated cell formation in long-term human marrow cultures by inducing IL-1 release. *Journal of Immunology*, 144, 4226-4230.

Kwon, B.S., Wang, S.A., Udagawa, N., Haridas, V., Lee, Z. H., Kim, K.K., OH, K., Greene, J., Li, Y., Su, J., Gentz, R., Aggarwal, B.B., & Ni, J. (1998). TRI, a new member of the tumor necrosis factor receptor superfamily, induces fibroblast proliferatiion and inhibits osteoclastogenesis and bone resorption. *FASEB*, 12, 845-854.

Lacey, D.L., Timms, E., Tan, H-L., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliot, R., Colombero, A., Elliot, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y-X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Gou, J., Delaney, J., & Boyle, W. J. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*, 93, 165-176.

Lakkakorpi, P. T., Helfrich, M. H., Horton, M. A., & Vaananen, H. K. (1993). Spatial organization of microfilaments and vitronectin receptor, αvβ3, in osteoclasts. A study using confocal laser scanning microscopy. *Journal of Cell Science*, 104, 663-670.

Lakkakorpi, P. T., Horton, M. A., Helfrich, M. H., Karhukorpi, E. K., & Vaananen, H. K. (1991). Vitronectin receptor has a role in bone resorption but does not mediate the tight sealing zone attachment of osteoclasts to the bone surface. *Journal of Cell Biology*, 115, 1179-1186.

Lasfargues, J. J., and Saffar, J. L. (1983). Effects of indomethacin on bone destruction during experimental periodontal disease in the hamster. *Journal of Periodontal Research*, 18, 110-117.

Lee, S. K. and Lorenzo, J. A. (1999). Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. *Endocrinology*, 140, 3552-3561.

Lee, B., Thirunavukkarasu, K., Zhou, L., Pastore, L., Baldini, A., Hecht, J., Geoffroy, V., Ducy, P., & Karsenty, G. (1997). Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF*2/CBFA1* in cleidocranial dysplasia. *Nature Genetics*, 16, 307-310.

Lerner, U. H. (2000). Osteoclast formation and resorption. *Matrix Biology*, 19, 107-120.

Lerner, U. H. and Grubb, A. (1992). Human cystatin C, a cysteine proteinase inhibitor inhibits bone resorption in vitro stimulated by parathyroid hormone-related peptide of malignancy. *Journal of Bone and Mineral Research*, 7, 433-440.

Lerner, U. H. (1987). Modification of the mouse calvarial technique improve responsiveness to stimulators of bone resorption. *Journal of Bone and Mineral Research*, 2, 375-383.

Lerner, U. and Fredholm, B.B. (1985). Prostaglandin E_2 and 2-chloroadenosine act in concert to stimulate bone resorption in cultured murine calvarial bones. *Biochemical Pharmacology*, 34, 937-940.

Lomaga, M. A., Yeh, W., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie, A., Wakeham, A., Khoo, W., Sasaki, T., Cao, Z., Penniger, J. M., Paige, C. J., Lacey, D. L., Dunstan, C. R., Boyle, W. J., Goeddel, D. V., & Mak, T. W. (1999). TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signalling. *Genes and Development*, 13, 1015-1024.

Lorenzo, J. A. and Raisz, L. G. (1999). Cytokines and prostaglandins. In M. J. Siebel, S. P Robins, & J. P. Bilezikian (Eds), *Dynamics of Bone and Cartilage Metabolism* (pp. 217-231). London: Academic Press.

Lorenzo, J. A., Naprta, A., Rao, Y., Alander, C., Galccum, M., Widmer, M., Gronowicz, G., Kalinowski, J., & Pilbeam, C. C. (1998). Mice lacking the type 1 interleukin-1 receptor do not lose bone mass after ovariectomy. *Endocrinology*, 139, 3022-3025.

Lorenzo, J. A., Sousa, S., Alander, C., Raisz, L. G., & Dinarello, C. A., (1987). Comparison of the bone resorbing activity in the supernatents from phytohemagglutinin-stimulated human peripheral blood mononuclear with that of cytokines through the use of an anti-serum to interleukin-1. *Endocrinology*, 121, 1164-1170.

Loutit, J. F. and Nisbet, N. W. (1982). The origin of osteoclasts. *Immunobiology*, 161, 193-203.

Lowe, C., Yoneda, T., Boyce, B. F., Chen, H., Mundy, G. R., & Soriano, P. (1993). Osteopetrosis in src-deficient mice is due to an autonomous defect of osteoclasts. *Proceedings of the National Academy of Science*, 90, 4485-4489.

Manolagas, S. C. and Jilka, R. L. (1995). Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *New England Journal of Medecine*, 332, 305-311.

Marks, S. C. Jr., Seifert, M. F., & McGuire, J. L. (1984). Congenitally osteopetrotic *(op/op)* mice are not cured by transplants of spleen or bone marrow cell from normal littermates. *Metabolic Bone Disease and Related Research*, 5, 183-183.

Marshall, M.J., Holt, I., & Davie, M.W.J. (1996). Inhibition of prostaglandin synthesis leads to a change in adherence of mouse osteoclasts from bone to periosteum. *Calcified Tissue International*, 59, 207-213.

Marshall, M.J., Holt, I., & Davie, M.W.J. (1995). The number of tartrate-resistant acid phosphatase-positive osteoclasts on neonatal mouse parietal bones is decreased when prostaglandin synthesis is inhibited and increased in response to prostaglandin E_2, parathyroid hormone, and 1,25dihydroxyvitamin D_3. *Calcified Tissue International*, 56, 240-245

Marshall, M. J., and Davie, M. W. J. (1991). An immunocytochemical method for studying the kinetics of osteoclast nuclei on intact mouse parietal bones. *Journal of Histochemistry*, 23, 402-408.
Marshall, M. J., Nisbet, N. W., & Green, P. M. (1986). Evidence for osteoclast production in mixed bone cell culture. *Calcified Tissue International*, 38, 268-274.

Masarachia, P., Yamamoto, M., Leu, C-T., Rodan, G., and Duong, L. (1998). Direct demonstration of the inhibitory effect of echistatin on bone resorption in mice with secondary hyperparathyroidism. *Endocrinology*, 139, 1401-1410.

Mbalaviele, G., Orcel, P., Morieux, C., Nijweide, P. J., & De Vernejoul, M. C. (1995). Osteoclast formation from human cord blood mononuclear cell co-cultured with mice embryonic metatarsals in the presence of M-CSF. *Bone*, 16, 171-177.

McHugh, K., Hodivala-Dilke, K., Zheng, M-H., Namba, N., Lam, J., Novack, D., Feng, X., Ross, P., Hynes, R.O., & Teitelbaum, S. (2000). Mice lacking β3 integrins are osteosclerotic because of dysfunctional osteoclasts. *Journal of Clinical Investigation*, 105, 433-440.

McSheehy, P. M. J., and Chambers, T. J. (1986). Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. *Endocrinology*, 118, 824-828.

McSheehy, P. M. J., and Chambers, T. J. (1987). 1,25-dihydroxyvitamin D_3 stimulates rat osteoblastic cells to release a soluble factor that increases osteoclastic bone resorption. *Journal of Clinical Investigation*, 80, 425-429.

Meena, C.,and Roodman, G. D. (1997). Increased sensitivity to $1,25(OH)_2D_3$ of osteoclast precursors in Paget's disease is not due to differences in expression levels of the 1,25-D receptor. *Journal of Bone and Mineral Research*, 12, S330.

Miller, S. C. (1981). Osteoclast cell-surface specializations and nuclear kinetics during egg-laying in Japanese quail. *American Journal of Anatomy*, 162, 35-43.

Minkin, C., Fredricks, R. S., Pokress, S, Rude, R. K., Sharp, C. F. Jr., Tong, M., & Singer, F. R. (1981). Bone resorption and humoral hypercalcaemia of malignancy-stimulation of bone resorption in vitro by tumor extracts is inhibited by prostaglandin synthesis inhibitors. *Journal of Clinical Endocrinology and Metabolism*, 53, 941-947.

Miyaura, C., Kusano, K., Masuzawa, T., Chaki, O., Onoe, Y., Aoyagi, M., Sasaki, T., Tamura, T., Koishihara, Y., Oshugi, Y., & Suda, T. (1995). Endogenous bone-resorbing factor in estrogen deficiency: Cooperative effects of IL-1 and IL-6. *Journal of Bone and Mineral Research*, 10, 1365-1373.

Mizuno, A., Amizuka, N., Irie, K., Murakami, A., Fujise, N., Kanno, T., Sato, Y., Nakagawa, N., Yasuda, H., Mochizuki, S., Gomibuchi, T., Yano, K., Shima, N., Washida, N., Tsuda, E., Morinaga, T., Higashio, K., & Ozawa, H. (1998). Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. *Biochemical and Biophysical Research Communications*, 247, 610-615.

Mohan, S., Jennings, J.C., Linkhart, T.A., & Baylink, D.J. (1988) Primary structure of human skeletal growth factor: homology with human insulin-like growth factor-II. *Biochimica et Biophysica Acta*, 966, 44-55.
Muller-Newen, G., Kohne, C., & Heinrich, P. C. (1996). Soluble receptors for cytokines and growth factors. *International Archives of Allergy and Immunology*, 111, 99-109.

Murakami, T., Yamamoto, M., Yamamoto, M., Ono, K., Nishikawa, M., Nagata, N., Motoyoshi, K., & Akatsu, T. (1998). Transforming growth factor-β1 increases mRNA levels of osteoclastogenesis inhibitory factor in osteoblastic/stromal cells and inhibits the survival of murine osteoclast-like cells. *Biochemical and Biophysical Research Communications*, 252, 747-752.

Nakagawa, N., Kinosaki, M., Yamaguchi, K., Shima, N., Yasuda, H., Yano, K., Morinaga, T., & Higashio, K. (1998). RANK is the essential signalling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochemical and Biophysical Research Communications*, 253, 395-400.

Nakamura, I., Jimi, E., Duong, L.T., Sasaki, T., Takahashi, N., Rodan, G. A., & Suda, T. (1998). Tyrosine phosphorylation of $p130^{cas}$ is involved in actin organization in osteoclasts. *Journal of Biological Chemistry*, 273, 11144-11149.

Nakamura, I., Gailit, J., & Sasaki, T. (1996). Osteoclast integrin αvβ3 is present in the clear zone and contributes to cellular polarization. *Cell and Tissue Research*, 286, 507-515.

Nesbitt, S. A. and Horton, M. A. (1997). Trafficking of matrix collagens through bone resorbing osteoclasts. *Science*, 276, 266-269.

Nesbitt, S., Nesbit, A., Helfrich, M., & Horton, M. (1993). Biochemical characterization of human osteoclast integrins. Osteoclasts express αvβ3, α2β1, and αvβ1 intcgrins. *Journal of Biological Chemistry*, 268, 16737-16745.

O'Brien, E. A., Williams, J. H. H., & Marshall, M. J. (2001). Osteoprotegerin is produced when prostaglandin synthesis is inhibited causing osteoclasts to detach from the surface of mouse parietal bones and attach to the endocranial membrane. *Bone*, (In press).

Ohsaki, Y., Takahashi, S., Scarcez, T., Demulder, A., Nishihara, T., Williams, R., & Roodman, G. D. (1992). Evidence for an autocrine/paracrine role for IL-6 in bone resorption by giant cell tumors of bone. *Endocrinology*, 131, 2229-2934.

O'Keefe, R. J., Teo, L. A., Singh, D., Puzas, J. E., Rosier, R. N., & Hicks, D. G. (1997). Osteoclasts constitutively express regulators of bone resorption: an immunohistochemical and in situ hybridization study. *Laboratory Investigation*, 76, 457-465.

Oursler, M. J., Pederson, L., Fitzpatrick, L., Riggs, B.L., and Spelsberg, T. (1994) Human giant cell tumors of the bone (osteoclastomas) are estrogen target cells. *Proceedings of the National Academy of Science*, 91, 5227-5231.

Oursler, M. J., Cortese, C., Keeting, P., Anderson, M. A., Bonde, S. K., Riggs, B. L., & Spelsberg T. C. (1991). Modulation of transforming growth factor-beta production in normal human osteoblast-like cells by 17β-estradiol and parathyroid hormone. *Endocrinology*, 129, 3313-3320.

Pacifici, R., Brown, C., Puscheck, E., Friedrich, E., Slatopolsky, E., Maggio, D., McCracken, R., & Avioli, L. (1991). Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. *Proceedings of the National Academy of Science*, 88, 5134-5138.

Pacifici, R., Rifas, L., McCracken, R., Vered, I., McMurtry, C., Avioli, L. V., & Peck, W. A. (1989). Ovarian steroid treatment blocks a postmenopausal increase in blood monocyte interleukin 1 release. *Proceedings of the National Academy of Science USA*, 86, 2398-2402.

Parfitt, A. M. (2000). The mechanism of coupling: a role for the vasculature. *Bone*, 26, 319-323.

Parfitt, A. M. (1994). Osteonal and hemi-osteonal remodelling: the spatial and temporal framework for signal traffic in adult human bone. *Journal of Cellular Biochemistrt*, 55, 273-286.

Paul, S. R., Bennett, F., Calvetti, J. A., Kelleher, K., Wood, C. R., O'Hara, R. M. Jr. Leary, A. C., Sibley, B., Clark, S. C., Williams, D. A., & Yang, Y-C. (1990). Molecular cloning of a cDNA encoding interleukin-11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proceedings of the National Academy of Sciences, USA*,,87, 7512-7516.

Pead, M. J. and Lanyon, L. E. (1989). Indomethacin modulation of load-related stimulation of new bone formation in vitro. *Calcified Tissue International*, 45, 34-40.

Pfeilschifter, J., Chenu, C., Bird, A., Mundy, G. R., & Roodman, G. D. (1989). Interleukin-1 and tumor necrosis factor stimulate the formation of human osteoclast-like cells in vitro. *Journal of Bone and Mineral Research*, 4, 113-118.

Pfeilschifter, J., Seyedin, S. M., & Mundy. (1988). Transforming growth factor beta inhibits bone resorption in fetal rat long bone cultures. *Journal of Clinical Investigation*, 82, 680-685.

Pilam, N. B., Nyiredy, K. O., & Arnaud, C. D. (1982). Parathyroid hormone receptors in avian bone cells. *Proceedings of the National Academy of Sciences, USA, 79, 2061-2063.*

Poli, V., Balena, R., Fattori, E., Markatos, A., Yamamoto, A., Tanaka, H., Ciliberto, G., Rodan, G. A., & Costantini, F. (1994). Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. *Journal of European Molecular Biology*, 13, 1189-1196.

Quinn, J.M. W., Elliot, J., Gillespie, M.T., & Martin, T.J. (1998) A combination of osteoclast differentiation factor and macrophage-colony stimulating factor is sufficient for both human and mouse osteoclast formation in vitro. *Endocrinology*, 139, 4424-4427.

Raisz, L. G. and Fall, P. M. (1990). Biphasic effects of prostaglandin E_2 on bone formation in cultured fetal rat calvariae: Interaction with cortisol. *Endocrinology*, 126, 1654-1659.

Raisz, L. G., Alander, C. B., & Simmons, H. A. (1989). Effects of prostaglandin E_3 and eicosapentaenoic acid on rat bone in organ culture. *Prostaglandins*, 37, 615-625.

Raisz, L. G., Vanderhoek, J. Y., Simmons, H. A., Kream, B. E., & Nicolaou, K. C. (1979). Prostaglandin synthesis by fetal rat bone in vitro: Evidence for a role of prostacyclin. *Prostaglandins*, 17, 905-914.

Raisz, L. G.and Koolemans-Beynen, A. R. (1974). Inhibition of bone collagen synthesis by prostaglandin E_2 in organ culture. *Prostaglandins*, 8, 377-385.

Raisz, L. G. and Niemann, I. (1967). Early effects of parathyroid hormone and thyrocalcitonin on bone in organ culture. *Nature*, 214, 486-487.
Reddy, S. V. and Roodman, G, D. (1998). Control of osteoclast differentiation. *Critical Reviews in Eukaryotic Gene Expression*, 8, 1-17.

Reich, K. M. and Fangos, J. A. (1991). Effects of flow on prostaglandin E_2 and inositol triphosphate levels in osteoblasts. *American Journal of Physiology*, 261, C428-431.

Reynolds, J.J. and Dingle, J.T. (1969). A sensitive *in vitro* method for studying the induction and inhibition of bone resorption. *Calcified Tissue Research*, 4, 339-349.

Rickard, D., Russell, G., & Gowen, M. (1992). Oestradiol inhibits the release of tumor necrosis factor but not interleukin 6 from adult human osteoblasts in vitro. *Osteoporosis International*, 2, 94-102.

Riggs, B. L. and Melton, L. J. (1992). The prevention and treatment of osteoporosis. *New England Journal of Medecine*, 327, 620-627.

Robinson, D. R., Tashjian, A. H. Jr., & Levine, L. (1975). Prostaglandin-stimulated bone resorption by rheumatoid synovia-possible mechanism for bone destruction in rheumatoid arthritis. *Journal of Clinical Investigation*, 56, 1181-1187.

Robinson, J. A., Riggs, B. L., Spelsberg, T. C., & Oursler, M. J. (1996). Osteoclasts and transforming growth factor-beta: Estrogen-mediated isoform specific regulation of production. *Endocrinology*, 137, 615-621.

Rodan, G. A. and Martin, T. J. (2000). Therapeutic approaches to bone disease. *Science*, 289, 1497-1514.

Rodan, G. A. and Martin, T. J. (1981). Role of osteoblasts in hormonal control of bone resorption: A hypothesis. *Calcified Tissue International*, 33, 349-351.

Rolnick, F., Huskey, M., Gupta, A., & Hruska, K. A. (1992). The signal generating complex of the occupied αvβ3 integrin includes *src*, phosphatidylinositol 3 kinase (P13) kinase and phospholipase Cγ [abstract] . *Journal of Bone and Mineral Research*, 7, (Suppl 1), S52.

Romas, E. and Martin, T. J. (1997). Cytokines in the pathogenesis of osteoporosis. *Osteoporosis International*, 7, S47-S53.

Roodman, G. D. (1999). Cell biology of the osteoclast. *Expermental Hematology*, 27, 1229-1241.

Sabatini, M., Boyce, B., Aufdemorte, T., Bonewald, L., & Mundy, G. R. (1988). Infusions of recombinant human interleukins 1 alpha and 1 beta cause hypercalcemia in normal mice. *Proceedings of the National Academy of Science USA*, 85, 5235-5239.

Saffar, J.L. and Leroux, P. (1988). Role of prostaglandins in bone resorption in a synchronized remodeling sequence in the rat. *Bone*, 9,141-145.

Sarma, U. and Flanagan, A. M. (1996). Macrophage colony-stimulating factor induces substantial osteoclast generation and bone resorption in human bone marrow cultures. *Blood*, 88, 2531-2540.

Sato, M., Garsky, V., Majeska, R.J., Einhorn, T.A., Murray, J., Tashjian, A.H. Jr., & Gould, R.J. (1994). Structure-Activity studies of the s-Echistatin inhibition of bone resorption. *Journal of Bone and Mineral Research*, 9, 1441-1449.

Sato, M., Sardana, M. K., Grasser, W. A., Garsky, V. M., Murray, J. M., & Gould, R.J. (1990) Echistatin is a potent inhibitor of bone resorption in culture. *Journal of Cell Biology*, 111, 1713-1723.

Sato, M., Fujii, Y., Kasono, K., Saji, M., Tsushima, T., & Shizume, K. (1986). Stimulation of prostaglandin E_2 and bone resorption by recombinant human interleukin 1 alpha in fetal mouse bones. *Biochemical and Biophysical Research Communications*, 138, 618-624.

Seckinger, P., Klein-Nulend, J., Alander, C., Thompson, R. C., Dayer, J. M., & Raisz, L. G. (1990). Natural and recombinant human Il-1 receptor antagonists block the effects of IL-1 on bone resorption and prostaglandin production. *Journal of Immunology*, 145, 4181-4184.

Silverberg, S.J., Fitzpatrick, L.A., & Bilezikian, J.P. (1995). Primary hyperparathyroidism. In K.L. Becker (Ed), *Principles and Practice of Endocrinology and Metabolism* (pp.512-519). Philadelphia: Lippincott.

Simonet, W.S., Lacey, D.L., Dunstan, C.R., Kelley, M., Chang, M-S., Luthy, R., Nguyen, H.Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliot, R., Colombero A., Tan, H-L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L., Hughes, T.M., Hill, D., Pattison, W., Campbell, P., Sander, S., Van, G., Tarpley, P., Derby, P., Lee, R., Amgen EST Programme, & Boyle, W.J. (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell*, 89, 309-319.

Soriano, P., Montgomery, C., Geske, R., & Bragley, A. (1991). Targeted disruption of the *c-src* proto-oncogene leads to osteopetrosis in mice. *Cell*, 64, 693-702.

Speck, N. A. and Stacy, T. (1995). A new transcription factor family associated with human leukemias. *Critical Reviews in Eukaryotic Gene Expression*, 5, 337-364.

Steingrimsson, E., Moore, K. J., Lamoreux, M. L., Ferre-D'Amare, A. R., Burley, S. K., Sanders Zimring, D. C., Skow, L. C., Hodgkinson, C. A., Arnheiter, H., Copeland, N. G., & Jenkins, N. A. (1994). Molecular basis of mouse *micropthalmia (mi)* mutations helps explain their developmental and phenotypic consequences. *Nature Genetics*, 8, 256-263.

Stenbeck, G. and Horton, M.A. (2000). A new specialized cell-matrix interaction in actively resorbing osteoclasts. *Journal of Cell Science*, 113, 1577-1587.

Stewart, A. F. (1996). Humoral hypercalcemia of malignancy. In M. J. Favus (Ed), *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism* (pp198-203) Philadelphia: Lippincott-Raven.

Suda, T., Takahashi, N., Udagawa, N., Jimi, E., Gillespie, M., & Martin, J. M. (1999). Modulation of osteoclast differentiation and function by the new member of the tumor necrosis factor receptor and ligand families. *Endocrine Reviews*, 20, 345-357.

Suda, T., Udagawa, N., Nakamura, I., Miyaura, C., & Takahashi, N. (1995). Modulation of oseoclast differentiation by local factors. *Bone*, 17, 87S-91S.

Sundquist, K. and Marks, S. (1994). Bafilomycin A1 inhibits bone resorption and tooth eruption in vivo. *Journal of Bone and Mineral Research*, 9, 1575-1582.

Sundquist, K., Lakkakorpi, P., Wallmark, B., & Vaananen, H. K. (1990). Inhibition of osteoclast proton transport by bafilomycin A1 abolishes bone resorption. *Biochemical and Biophysical Research Communications*, 168, 309-313.

Sunyer, T., Lewis, J., & Osdoby, P. (1997). Estrogen decreases the steady state levels of the IL-1 signaling receptor (Type 1) while increasing those of the IL-1 decoy receptor (Type II) mRNAs in human osteoclast –like cells. *Journal of Bone and Mineral Research*, 12, S135 (abstract)

Takahashi, N., Udagawa, N., Tanaka, S, Murakami, H., Owan, I., Tamura, T., & Suda, T. (1994). Postmitotic osteoclast precursors are mononuclear cells which express macrophage-associated phenotypes. *Developmental Biology*, 163, 212-221.
Takahashi, N., Udagawa, N., Akatsu, T., Tanaka, H., Isogai, Y., & Suda, T. (1991). Deficiency of osteoclasts in osteopetrotic mice is due to a defect in the local microenvironment provided by osteoblastic cells. *Endocrinology*, 128, 1792-1796.

Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J. M., Martin, T. J., & Suda, T. (1988). Osteoblastic cells are involved in osteoclast formation. *Endocrinology*, 123, 2600-2602.

Takai, H., Kanematsu, M., Yano, K., Tsuda, E., Higashio, K., Ikeda, K., Watanabe, K., & Yamada, Y. (1998). Transforming growth factor-β stimulates the production of osteoprotegerin/osteoclastogenesis inhibitory factor by bone marrow stromal cells. *Journal of Biological Chemistry*, 273, 27091-27096.

Takeda, S., Yoshizawa, T., Fukumoto, S., Nagai, Y., Murayama, H., Matsumoto, T., Kato, S., & Fujita, J. (1997). Bone metabolism and in vitro osteoclast formation in VDR knock-out mice. *Journal of Bone and Mineral Research*, 12, S110.

Takeshita, S., Kaji, K., & Kudo, A. (2000). Identification and characterization of the new osteoclast progenitor with macrophage macrophage phenotypes being able to differentiate into mature osteoclasts. *Journal of Bone and Mineral Research*, 15, 1477-1488.

Tamura, T., Udagawa, N., Takahashi, N., Miyaura, C., Tanaka, S., Yamada, Y., Koishihara, Y., Ohshigi, Y., Kumaki, K., Taga, T., Kishimoto, T., & Suda, T., (1993). Soluble interleukin-6 receptor triggers osteoclast formation by interleukin-6. *Proceedings of the National Academy of Sciences USA*, 90, 11924-11930

Tan, K. B., Harrop, J., Reddy, M., Young, P., Terrett, J., Emery, J., Moore, G., & Truneh, A. (1997). Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells. *Gene*, 204, 35-46.

Tanaka, S., Amling, M., Neff, L., Peyman, A., Uhlmann, E., Levy, J. L., & Baron, R. (1996). c-Cbl is downstream of c-Src in a signaling pathway necessary for bone resorption. *Nature*, 383, 528-531.

Tanaka, S., Takahashi, N., Udagawa, N., Tamara, T., Akatsu, T., Stanley, E.R., Kurokawa, T., & Suda, T. (1993). Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors. *Journal of Clinical Investigation*, 91, 257-263.

Teitelbaum, S. (2000). Bone resorption by osteoclasts. *Science*, 289, 1504-1508.

Teti, A., Rizzoli, R., & Zambonin-Zallone, A. (1991). A parathyroid hormone binding to cultured avian osteoclasts. *Biochemical and Biophysical Research Communications*, 174, 1217-1222.

Thompson, D.D. and Rodan G.A. (1988). Indomethacin inhibition of tenotomy-induced bone resorption in rats. *Journal of Bone and Mineral Research*, 4, 409-414.
Thomson, B. M., Mundy, G. R., & Chambers, T. J. (1987). Tumor necrosis factors alpha and beta induce osteoblastic cells to stimulate osteoclastic bone resorption. *Journal of Immunology*, 138, 775-779.

Tinkler, S. M. B., Linder, J. E., Williams, D. M., & Johnson, N. W. (1981). Formation of osteoclasts from blood monocytes during 1 alpha-OH Vit D-stimulated bone resorption in mice. *Journal of Anatomy*, 133, 389-396.

Tondravi, M. M., McKercher, S. R., Anderson, K., Erdmann, J. M., Quiroz, M., Maki, R., & Tietelbaum, S. L. (1997). Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature*, 386, 81-84.

Tong, H., Lin, H., Wang, H., Sakai, D., & Minkin, C. (1995). Osteoclasts respond to parathyroid hormone and express mRNA for its receptor. *Journal of Bone and Mineral Research*, 10, S322.

Trusolino, L., Serini, G., Cecchini, G., Besati, C., Ambesi-Impiombato, F.S., Marchisio, P.C., & De Filippi, R. (1998). Growth factor-dependent activation of $\alpha v\beta 3$ integrin in normal epithelial cells: implication for tumor invasion. *Journal of Cell Biology*, 142, 1145-1156.

Tsuda, E., Goto, M., Mochizuki, S., Yano, K., Kobayashi, F., Morinaga, T., & Higahsio, K. (1997). Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochemical and Biophysical Research Communiations*, 234, 137-142.

Tsukii, K., Shima, N., Mochizuki, S., Yamaguchi, K., Kinosaki, M., Yano, K., Shibata, O., Udagawa, N., & Yasuda, H. (1998). Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1α,25-dihydroxyvitamin D_3, prostaglandin E_2, or parathyroid hormone in the microenvironment of bone. *Biochemical and Biophysical Research Communications*, 246, 337-341.

Turner, C.H., Forwood, M.R., & Otter, M. W. (1994). Mechanotransduction in bone: Do bone cells act as sensors of fluid flow ? *FASEB J*, 8, 875-878.

Udagawa, N., Chan, J., Wada, S., Findlay, D. M., Hamilton, J. A., & Martin, T. J. (1996). C-fos antisense DNA inhibits proliferation of osteoclast progenitors in osteoclast development but not macrophage differentiation *in vitro*. *Bone*, 18, 511-516.

Uy, H. L., Guise, t. A., De La Mata, J., Taylor, S. D., Story, B. M., Dallas, M. R., Boyce, B. F., Mundy, G. R., & Roodman, G. D. (1995). Effects of parathyroid hormone (PTH)-related protein and PTH on osteoclasts and osteoclast precursors *in vivo*. *Endocrinology*, 136, 3207-3212.

Vaananen, H. K. (1996). Osteoclast function: Biology and Mechanisms. In J. Bilizekian, J. Raisz, & G. Rodan (Eds.), *Principles of Bone Biology* (pp 103-115). New York: Academic Press.
Vaananen, H. K., Zhao, H., Mulari, M., & Halleen, J. M. (2000). The cell biology of osteoclast function. *Journal of Cell Science*, 113, 377-381.

Vaananen, H. K. and Horton, M. (1995). The osteoclast clear zone is a specialized cell-extracellular matrix adhesion structure. *Journal of Cell Science*. 108, 2729-2732.

Vaes, G. (1988). Cellular biology and biochemecal of bone resorption. *Clinical Orthopaedics and Related Research*, 231, 239-271.

Vane, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. *Nature*, 231, 232-235.

Vidal, O.N.A., Brandstrom, H., Jonsson, K.B., & Ohlsson, C. (1998a). Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: down-regulation by glucocorticoids. *Journal of Endocrinology*, 159, 191-195.

Vidal, O.N.E., Sjogren, K., Eriksson, B.I., Ljunggren, O., & Ohlsson C. (1998b). Osteoprotegerin mRNA is increased by interleukin-1α in the human osteosarcoma cell line MG-63 and in human osteoblast-like cells. *Biochemical and Biophysical Research Communications*, 248, 696-700.

Votta, B. J., Levy, M. A., Badger, A., Bradbeer, J., Dodds, R. A., James, I. E., Thompson, S., Bossard, M. J., Carr, T., Connor, J. R., Tomaszek, T. A., Szewczuk, L., Drake, F. H., Veber, D. F., & Gowen, M. (1997). Peptide aldehyde inhibitors of cathepsin K inhibit bone resorption both in vivo and in vitro. *Journal of Bone and Mineral Research*, 12, 1396-1406.

Walker, D. G. (1975). Bone resorption restored in osteopetrotic mice by transplants of normal bone marrow and spleen cells. *Science*, 190, 784-785.

Walker, D. G. (1972). Congenital osteopetrosis in mice cured by parabiotic union with normal siblings. *Endocrinology*, 91, 916-920.

Wani, M.R., Fuller, K., Kim, N.S., Choi, Y., & Chambers, T. (1999). Prostaglandin E_2 cooperates with TRANCE in osteoclast induction from hemopoietic precursors: synergistic activation of differentiation, cell spreading and fusion. *Endocrinology*, 140, 1927-1935.

Wiktor-Jedrzejczak, W., Ahmed, A., Szczylik, C., & Skelly, R. R. (1982). Hematological characterization of congenital osteopetrosis in *op/op* mouse. *Journal of Experimental Medecine*, 156, 1516-1527.

Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., & Choi, Y. (1999). TRANCE, a TNF family member, activates Akt/PKB through a signalling complex involving TRAF6 and c-Src. *Molecular Cell*, 4, 1041-1049.

Wong, B. R., Josien, R., Lee, S. Y., Vologodskaia, M., Steinman, R. M., & Choi, Y. (1998). The TRAF family of signal transducers mediates NF-κB activation by the TRANCE receptor. *Journal of Biological Chemistry*, 273, 28355-28359.

Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F. S. III., Frankel, W, N., Lee, S. Y., & Choi, Y. (1997). TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *Journal of Biological Chemistry*, 272, 25190-25194.

World Health Organization. (1994). Assessment of fracture risk and it's application to screening for postmenopausal osteoporosis. *W.H.O. Tech. Rep. Ser.*, 843.

Xing, L., Franzoso, G., Siebenlist, U., & Boyce, B. F. (1997). Targeted disruption of the p50 and p52 subunits of NF-κB transcription factor leads to failure of osteoclast formation and osteopetrosis. *Journal of Bone and Mineral Research*, (abstract) 12, S136.

Yamada, K. M. and Miyamoto, S. (1995). Integrin transmembrane signalling and cytoskeletal control. *Current Opinions in Cell Biology*, 29, 721-725

Yamane, T., Kunisada, T., Yamazaki, H., Era, T., Nakano, T., & Hayashi, S. I. (1997). Development of osteoclasts from embryonic stem cells through a pathway that is c-fms but not c-kit dependent. *Blood*, 90, 3516-3523.

Yasuda,H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomayasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higsahio, K., Udagawa, N., Takahashi, N., & Suda, T. (1998a). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proceedings of the National Academy of Science*, 95, 3597-3602.

Yasuda, H., Shima, N., Nakagawa, N., Mochizuki, S., Yano, K., Fujise, N., Sato, Y., Goto, M., Yamaguchi, K., Kuriyama, M., Kanno, T., Murakami, A., Tsuda, E., Morinaga, T., & Higashio, K. (1998b). Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro*. *Endocrinology*, 139, 1329-1337.

Yoshida, H., Hayashi, S. I., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Schultz, L. D., & Nishikawa, S. I. (1990). The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature*, 345, 442-445.

Young, R. W. (1962). Cell proliferation and specialization during endochondral ossification in young rats. *Journal of Cell Biology*, 14, 357-

Zambonin Zallone, A., Teti, A., Gaboli, M., & Marshisio, P. C. (1989). $Beta_3$ subunit of vitronectin receptor is present in osteoclast adhesion structures and not in other monocyte-macrophage derived cells. *Connective Tissue Research*, 20, 143-149.

Zambonin Zallone, A., Teti, A., & Primavera, M. V. (1984). Monocytes from circulating blood fuse *in vitro* with purified osteoclasts in primary culture. *Journal of Cell Science*, 66, 335-342.

Zhang, D., Udagawa, N., Nakamura, I., Murakami, H., Saito, S., Yamasaki, K., Shibasaki, Y., Morii, N., Narumiya, S., Takahashi, N., & Suda, T. (1995). The small GTP-binding protein, *rho* p21, is involved in bone resorption by regulating cytoskeletal organization in osteoclasts. *Journal of Cell Science*, 108, 2285-2292.

Zimolo, Z., Wesolowski, G., Tanaka, H., Hyman, J. L., Hoyer, J. R., & Rodan, G. A. (1994). Soluble αvβ3-integrins ligands raise $[Ca^{2+}]i$ in rat osteoclasts and mouse-derived osteoclast-like cells. *American Journal of Physiology (Cell Physiology)*, 266, C376-C381.