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Extracellular pH: a fundamental regulator of bone cell function

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*A thesis submitted for the degree of
Doctor of Philosophy at the University of London*

2005

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ABSTRACT

Systemic acidosis is associated with bone loss and impaired bone mineralisation. The aim of this PhD project was to further investigate the action of extracellular pH on the function of osteoclasts and osteoblasts. I showed that blood-derived human osteoclasts exhibited a highly reproducible acid-activation response, with maximal activation close to pH 7.0, and little activity at blood pH (7.4). These experiments also provided strong evidence that accessory cells, such as osteoblasts or stromal cells, are not required for acid-activation of resorption. The pH-activation profile of human osteoclasts was similar to that of the recently discovered H⁺-sensing human G-protein-coupled receptor OGR1. Expression of OGR1 and TDAG8 (another GPCR) was detected in human osteoclasts and was upregulated by low pH. I obtained evidence that the multifunctional receptor TRPV1, which senses protons, heat and capsaicin, was expressed by human osteoclasts and was also upregulated at pH 7.0. Moreover, I showed that the alkaloid capsaicin strongly stimulated osteoclasts in non-acidified conditions. To date, only pertussis toxin has been reported to activate osteoclasts without co-stimulation by H⁺. Using mouse bone organ cultures I found that resorption-associated factors TRACP, cathepsin K and TRAF-6 were also upregulated by acidosis. The effect of PTH on human osteoclasts was also studied. I showed that PTH directly stimulates human osteoclasts in the absence of osteoblasts, but only when acid-activated. This finding suggests that the dogma that PTH stimulation of osteoclast is osteoblast-mediated may not be correct. Studies using primary rat osteoblast cultures showed that the formation of mineralised bone nodules is inhibited by acidosis. The same pH reduction, which increases Ca²⁺ and PO₄³⁻ solubility of hydroxyapatite by 2- and 4-fold respectively, did not alter collagen production or osteoblast proliferation but decreased alkaline phosphatase activity and expression. Thus, the primary effect of acidosis on osteoblast function is to cause a selective inhibition of bone mineralisation. In conclusion, this study showed that the important “double negative” action of acidosis on bone cells is consistent with a pathophysiological role of bone as a reserve of base to buffer excess protons when the kidneys and lungs are unable to maintain acid-base balance within narrow physiological limits.

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CHAPTER 1

General introduction

SKELETAL SYSTEM

The skeletal system is composed of bone and cartilage. Bone is a specialised connective tissue, which has a variety of functions. It provides a rigid framework for the body, protects vital organs, houses the bone marrow and it serves as a reservoir of ions (Ca^{2+} and PO_4^{3-}) for the maintenance of body homeostasis; the skeleton contains 99% of total body calcium (Baron 2003).

Macroscopically, bones can be classified as compact (cortical) or trabecular (cancellous). Compact bone, which is mainly found in the shafts of long bones and surfaces of flat bones, is much denser and less metabolically active than trabecular bone and comprises ~80% of the skeleton. This type of bone is arranged concentrically around central canals, the Haversian canals, which contain blood and lymphatic vessels, nerves and connective tissue. Trabecular bone is found mainly at the ends of long bones and in the inner parts of flat bones. Trabecular bone is composed of interconnecting plates within which lies the bone marrow (Baron 2003).

Microscopically, bone can be classified on the basis of its organisation into two types: woven or lamellar bone. Woven bone is an immature form with randomly arranged collagen fibres. This type of bone is generated when osteoblasts produce osteoid rapidly; *e.g.* during embryonic life, childhood and also in adults with pathological disorders characterised by rapid bone formation such as in Paget's disease (Meunier *et al.* 1980). Lamellar bone is composed of regular parallel bands of collagen arranged in sheets. Virtually all bone in a healthy adult is lamellar (Baron 2003).

Mature bone by weight is made up of 70% inorganic salts (mainly hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) crystals) and 30% organic matrix. Type I collagen represents the majority of bone collagen but trace amounts of types III, V and X and fibril-

associated collagen with interrupted triple helix (FACIT) which may function in regulating collagen fibril diameter during bone formation have also been reported (Robey and Boskey 2003). The remaining 10% of the organic matrix is composed of non-collagenous proteins such as osteocalcin (OCN), osteonectin, osteopontin (OPN) and bone sialoprotein (BSP) (Lian *et al.* 2003). The organic component of bone also contains traces of growth factors and cytokines that have an important local regulatory role in bone remodelling (Baron 2003). Bone is comprised of three main cell types: osteoblasts, osteocytes and osteoclasts; these are described below in detail.

OSTEOBLASTS

Phenotype

Osteoblasts are specialised bone-forming cells that work in groups to secrete osteoid and are involved in the subsequent process of mineralisation (Ducy *et al.* 2000b). Histologically, active osteoblasts appear as plump, cuboidal cells sitting on the bone surface and express large amounts of alkaline phosphatase (ALP), an enzyme involved in the mineralisation process (Fedde *et al.* 1999; Wennberg *et al.* 2000). At the ultrastructural level osteoblasts are characterised by the presence of a well-developed rough endoplasmic reticulum and Golgi complex. Cytoplasmic processes coupled with gap junctions are in contact with osteocyte processes and are also found between adjacent osteoblasts (Doty 1981). Quiescent bone surfaces are covered by a near-continuous single layer of flattened, inactive osteoblasts, often referred to as 'bone-lining cells' (Hughes and Aubin 1998).

Osteoblast origin and differentiation

Osteoblasts are derived from pluripotent mesenchymal cells (**Figure 1.1**) that also give rise to myocytes, under the transcriptional control of myogenic regulatory factors (MRFs) and myocyte enhancer factor 2 (MEF2), to adipocytes, under the control of CCAAT enhancer binding protein α, β, σ (C/EBP α, β, σ) and peroxisome proliferator activator receptor γ (PPAR γ), and to chondrocytes under the control of sex determining region Y box 5, 6 and 9 (Sox 5, 6, 9) and runt-related transcription

factor 2 (Runx2) (Arnold and Winter 1998; Akiyama *et al.* 2002; Lecka-Czernik *et al.* 2002).

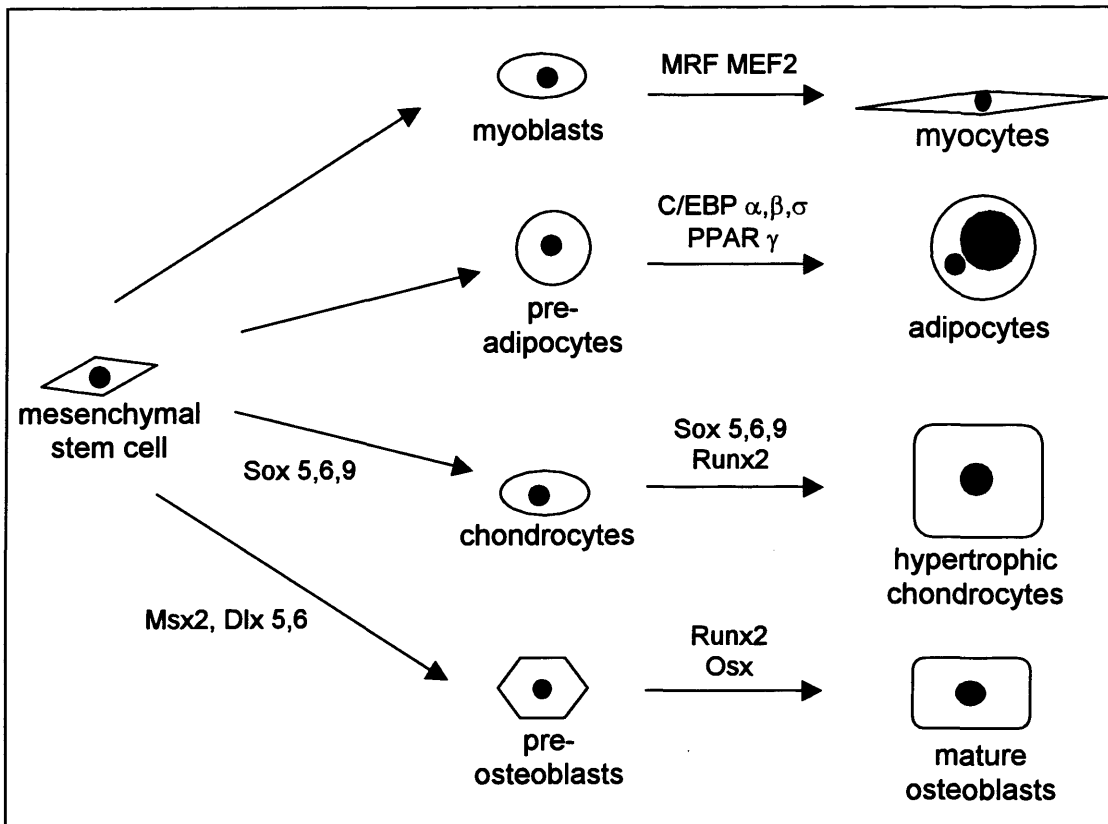


Figure 1.1. Transcriptional control of cells from mesenchymal origin. Osteoblasts differentiate from mesenchymal progenitor cells that also give rise to myocytes, adipocytes and chondrocytes. MRF = myogenic-regulatory factors, MEF2 = myocyte-enhancer factor 2, C/EBP α, β, σ = CCAAT-enhancer-binding protein α, β and σ , PPAR γ = peroxisome proliferator-activated receptor γ , Sox 5,6,9 = sex determining region Y-box 5,6 and 9, Runx2 = runt-related transcription factor 2, Msx2 = muscle-segment homologue 2, Dlx5 = homeobox containing transcription factor distal-less gene 5, Osx = osterix.

Differentiation and activity of osteoblasts is regulated by a variety of factors including transcription factors, hormones, cytokines, and growth factors (Ducy *et al.* 2000b). A summary of the developmental stages of the osteoblastic phenotype and characteristic features of each stage is shown in **Figure 1.2**.

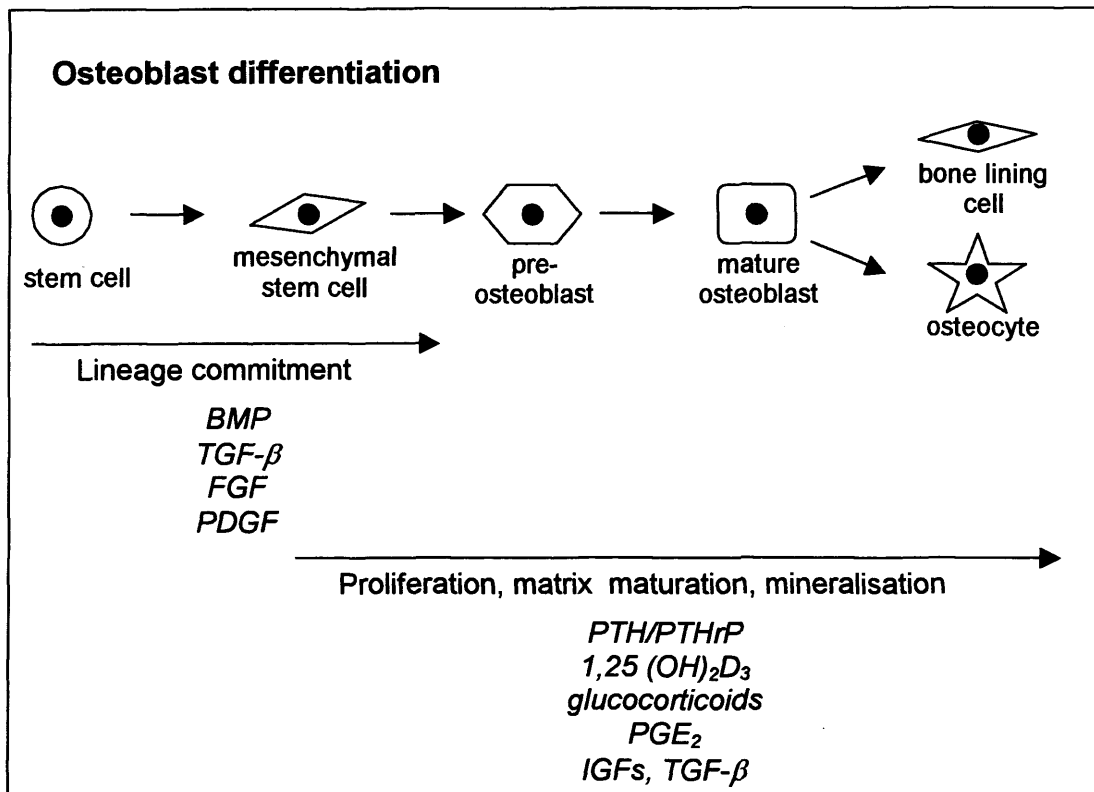


Figure 1.2. Regulation of osteoblast growth and differentiation. The diagram illustrates the morphological features of differentiating osteoblasts at each stage from stem cell to osteocyte and lists some of the signalling molecules that promote differentiation of the precursor cell populations (shown in italics).

There are many transcription factors involved in the regulation of osteoblast differentiation (Harada and Rodan 2003). Runt-related transcription factor 2 (Runx2) also known as core-binding factor 1 (Cbfa1), osteoblast-specific transcription factor 2 (Osf2) and acute-myeloid-leukaemia protein 3 (AML3), was first thought to be an osteoblast-specific transcription factor (Ducy *et al.* 1997). Runx2 can induce osteoblast-specific gene expression in fibroblasts and myoblasts *in vitro* (Ducy *et al.* 1997) and *Runx2*-deficient mice develop a skeleton made exclusively of cartilage with no osteoblasts present (Otto *et al.* 1997; Komori *et al.* 1997). However, further histological studies showed that *Runx2*-null mice have delayed chondrocyte maturation, suggesting that this factor is also involved in chondrogenesis (Inada *et al.* 1999). Most recently, another transcription factor was discovered: osterix (Osx). Like the *Runx2*-null mice, the *Osx*-knockout mice develop a skeleton composed entirely of cartilage, lacking osteoblasts and mineralised bone matrix. However, the hypertrophic chondrocytes in these mice are fully differentiated, indicating this is a

specific transcription factor of the osteoblast differentiation pathway. Moreover, *Runx2*-null mice do not express *Osx*, suggesting that this factor acts downstream from *Runx2* (Nakashima *et al.* 2002).

Muscle-segment homologue 2 (*Msx2*) and homeobox containing transcription factor distal-less gene 5 (*Dlx5*) are two transcription factors that are expressed at early stages of osteoblast differentiation (Bendall and Abate-Shen 2000). *Msx2* expression in osteoblast precedes the osteoblast marker osteocalcin (Newberry *et al.* 1997) and prevents terminal differentiation of osteoblasts, demonstrating that this factor maintains a balance between osteoblast proliferation and differentiation steps (Bidder *et al.* 1998). *Dlx5*, meanwhile, has both a positive and negative regulatory activity on the osteocalcin promoter by either inhibiting or stimulating *Msx2* expression (Newberry *et al.* 1998).

Transcription factors of the activating protein-1 (AP-1) complex are also involved in osteoblast differentiation. Overexpression of $\Delta FosB$, a *fosB* splice variant, and *fra-1* causes enhanced differentiation of osteoblasts, possibly as a result of altered progenitor cell differentiation, and increased osteoblast activity (Sabatakos *et al.* 2000; Jochum *et al.* 2000).

Endocrine factors involved in regulating osteoblast differentiation and activity include 1,25(OH)₂-vitamin D₃, parathyroid hormone (PTH), parathyroid hormone related protein (PTHrP), glucocorticoids and oestrogen. The role of these factors will be discussed in detail in the regulation of bone cell function section.

Members of all major families of growth factors have been implicated in the control of osteoblast differentiation (Lian *et al.* 2003). One family of growth factors that has been studied in detail is that of transforming growth factor β (TGF- β) and bone morphogenetic protein (BMP) superfamily, including TGF- β itself and BMP 2, 4 and 7 (Wang *et al.* 1990; Ahrens *et al.* 1993; Maliakal *et al.* 1994). TGF- β is produced by osteoblastic cells and functions in an autocrine manner as a potent chemotactic factor by recruiting osteoblast precursors to sites of bone formation (Pfeilschifter *et al.* 1990). This factor promotes the differentiation of osteoblasts and production of bone matrix, but it inhibits matrix mineralisation and expression of osteocalcin by osteoblasts (Pirkanen *et al.* 1994; Bonewald 1999). When injected

subcutaneously, TGF- β has been shown to induce rapid production of new bone matrix in mice (Marcelli *et al.* 1990). BMPs are secreted by osteoblasts into the extracellular matrix during bone formation. It is understood that BMPs regulate osteoblast and chondrocyte differentiation during skeletal development, as abnormal expression of BMP genes in mice and human is associated with skeletal defects in limb formation (Kingsley *et al.* 1992; Thomas *et al.* 1997).

TGF- β and BMPs bind to serine/threonine kinase membrane bound receptors, termed type I and II receptors (ten Dijke *et al.* 1996). These receptors, once activated, phosphorylate signal transducers called Smads. TGF- β stimulates Smad 2 and 3 whereas BMPs activate Smads 1, 5 and 8. Smad molecules form complexes among themselves that are capable of translocating to the nucleus where they regulate transcription of various target genes such as osteopontin and osteoprotegerin via interactions with Runx2 and Hox homeodomain proteins (Yang *et al.* 2000; Ito and Miyazono 2003). BMPs are also able to activate the MAPK signalling pathway, which is involved in the activation of alkaline phosphatase and osteocalcin expression in osteoblasts (Guicheux *et al.* 2003).

Recently leptin, a hormone produced by adipocytes, was shown to be involved in osteoblast differentiation and activity. Mice deficient in leptin (*ob/ob*) and its receptor (*db/db*) were found to have a higher bone mass compared to the wild-type. As both knockout models produced normal osteoblasts, it was believed at first that leptin acted on the hypothalamus to regulate body weight and fat mass (Ducy *et al.* 2000a). However, other investigators found that osteoblast precursors also express leptin receptors, thereby suggesting that this hormone has a central and peripheral action on bone (Thomas *et al.* 1999; Khosla 2002). These studies provide an explanation for the protection that obesity gives against osteoporosis (Ducy *et al.* 2000a).

Lipoprotein-receptor-related protein (LRP5) is another key molecule linked to bone mass disorders. Overexpression of this factor is responsible for the creation of high bone mass syndrome, whereas an inactivating mutation has been linked to osteoporosis-pseudoglioma syndrome, characterised by reduced bone mass (Gong *et al.* 2001; Little *et al.* 2002). Genetic studies on *LRP5*-knockout mice showed that this receptor is required for osteoblast proliferation and bone matrix deposition.

LRP5 is also a co-receptor of the Wnt signalling pathway, which regulates survival, proliferation and differentiation of osteoblasts (Kato *et al.* 2002).

Bone matrix and mineralisation

As mesenchymal cells become committed to the osteogenic lineage they start to express a number of proteins that characterise the osteoblast phenotype (Ducy *et al.* 2000b). Since the expression pattern of these proteins change as osteoblasts mature, they have become useful markers for osteoblast differentiation (**Table 1.1**). It is important to note that there may be significant variations in the temporal expression of osteoblast markers between different species, cell lines and even between different cells in the same culture conditions (Hughes and Aubin 1998).

Table 1.1: Changes in the expression of some phenotypic markers during osteoblast differentiation in culture

Markers	Pre-osteoblast	Osteoblast - early bone Formation	Osteoblast - late bone formation
type I collagen	++++	++++	+++
alkaline phosphatase	+++	++++	++
osteonectin	+	+++	++
BSP	++	++++	++
OPN	+	++++	++
OCN	-	++	++++

Bone is comprised of a number of extracellular matrix proteins that function as structural proteins. They are involved in matrix mineralisation and are also responsible for the recruitment and attachment of bone cells to specific sites. Some of these matrix proteins are type I collagen, alkaline phosphatase, osteonectin, osteocalcin, osteopontin and bone sialoprotein (Hughes and Aubin 1998).

The principle component of the organic matrix of bone is type I collagen, which functions both as a structural framework for bone and as a scaffold for mineral formation. Mature type I collagen are composed of three alpha-chains twisted in a rigid helix. Flanking the ends of the alpha chains are 32 amino acids forming the amino-terminal (-NH₂, NTX) and carboxy-terminal (-COOH, CTX) telopeptides (Eyre 1995). Crosslinking molecules are formed at the telopeptide ends of the collagen to provide a structural relationship between fibrils (Eyre 1995). To support mineral deposition, the post-translational modifications of bone collagen are significantly different from other type I collagen matrices (Knott and Bailey 1998). This fact has been exploited in the development of biochemical markers; for example, urine measurements of collagen cross-links are used clinically to assess the degree of bone resorption (Apone *et al.* 1997).

Alkaline phosphatase, is a homodimeric metalloenzyme that catalyses the hydrolysis of almost any phosphomonoester with the release of inorganic phosphate (P_i) and alcohol (Fernley 1971). Tissue non-specific *ALP*-knockout mice displays defect in bone mineralisation that mimic the phenotypic abnormalities of infantile hypophosphatasia (Fedde *et al.* 1999). Osteoblasts derived from these animals differentiate normally but are unable to initiate mineralisation *in vitro* (Wennberg *et al.* 2000). Osteonectin is a glycoprotein that is abundantly expressed in the skeleton. *In vitro* studies suggest that this protein binds to collagen and hydroxyapatite and can regulate cell proliferation and cell-matrix interactions (Young *et al.* 1992). Its exact function in bone remodelling remains to be determined, but osteonectin-deficient mice develop osteopenia with decreased bone formation (Delany *et al.* 2000).

Osteocalcin is widely used as a phenotypic marker for osteoblast differentiation due to its relative specificity (also expressed by hypertrophic chondrocytes) (Ducy *et al.* 1996). Osteocalcin and matrix-Gla protein are post-transcriptionally modified by the action of vitamin-K dependent γ -carboxylases. In the late-90s, these two proteins

were linked to an inhibitory effect on matrix mineral deposition. *Matrix-Gla*-knockout mice develop calcification in extraskeletal sites such as the aorta, and *osteocalcin*-deficient mice have increased bone mineral density (Ducy *et al.* 1996; Luo *et al.* 1997; Murshed *et al.* 2004). Moreover, osteocalcin may also be involved in recruiting and promoting attachment of osteoclasts at sites of resorption (Ducy *et al.* 1996).

Osteopontin and bone sialoprotein are members of the SIBLING family (small-integrin-binding ligand glycoproteins) and contain the RGD (arginine-glycine-aspartic acid) motif that binds to the integrin class of cell-surface molecules. However, there are also some cell attachment interactions that are not mediated via this motif (Baron 2003). Like MGP, OPN has been shown to be involved in inhibition of mineralisation (Speer *et al.* 2002).

There is also another class of proteins that can be considered non-collagenous bone matrix proteins. They consist of growth factors that are trapped in the bone matrix and may be released during bone resorption. These factors may not play a role in bone mineralisation as such, but are known to be very important in the bone remodelling process. These growth factors include TGF- β , and BMPs (Hughes and Aubin 1998).

The precise nature of bone mineral is still unclear but it is believed that it contains mainly hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2$). However, the crystal itself is not pure, making the mineral more like a carbonate apatite. This imperfect hydroxyapatite is more soluble than its geological counterpart facilitating the role of the skeleton as a reservoir for calcium, magnesium and phosphate ions (Glimcher 1998).

The first step of the mineralisation process happens inside of the extracellular matrix vesicles. Their formation occurs by polarized budding and ‘pinching-off’ from specific regions of the outer plasma membrane of osteoblasts. Polarised release of matrix vesicles into selected areas of developing matrix determines the non-random distribution of calcification (Boyan *et al.* 1988; Anderson 2003). These organelles contain a high concentration of Ca^{2+} and P_i , which creates an optimal environment for deposition of the initial amorphous mineral complexes (nucleation)

and formation of needle-like crystals of hydroxyapatite. The second step of the mineralisation process begins with the release of the mineral complex through the matrix vesicle membrane, exposing preformed hydroxyapatite crystals to the extracellular fluid. The extracellular environment normally contains sufficient Ca^{2+} and PO_4^{3-} to support continuous crystal proliferation, with preformed ones serving as templates for the formation of new crystals (Anderson 2003). The initial bone mineral deposition occurs at discrete sites throughout the collagenous matrix and as it matures the crystal becomes purer and larger in size; this increase in dimension results from the addition and aggregation of crystals already present (Landis 1995). Annexins, tissue non-specific alkaline phosphatase, osteonectin, osteocalcin and osteopontin have all been reported to be involved in the nucleation process (Glimcher 1989; Balcerzak *et al.* 2003).

OSTEOCYTES

Osteocytes are the most numerous cells in mature bone (Knothe-Tate *et al.* 2004). These cells originate from osteoblasts that became entrapped within the extracellular matrix. Osteocytes communicate with osteoblasts and each other by means of processes coupled by gap-junctions housed in little channels (canaliculi), of about 0.2-0.3 μm diameter (Doty 1981).

Osteocyte maturation and functional activity defines their morphology. Young osteocytes are similar to osteoblasts except for lower cell volume and protein synthesis machinery. Older osteocytes are even smaller and have an accumulation of glycogen in the cytoplasm (Knothe-Tate *et al.* 2004). Osteocytes are thought to mediate adaptive bone remodelling by responding to mechanical deformation (Burger and Klein-Nulend 1999). These cells respond *in vitro* and *in vivo* to increased loads by producing and modifying a number of molecules such as upregulation of nitric oxide (Pitsillides *et al.* 1995) and release of PGE_2 (Ajubi *et al.* 1996). Transmission of mechanical signals to the osteocyte cytoskeleton via cell surface receptors can occur directly through the solid matrix structure of the tissue as well as indirectly via fluid pressure and shear stresses imparted by fluid moving through the lacunocanalicular system due to load-induced fluid flow (Knothe-Tate 2003). Most recently, Noble and colleagues suggested that osteocyte apoptosis might trigger the

mechanism by which osteoclasts sense and remove damaged or redundant bone by releasing signals to attract osteoclasts to resorb bone (Noble *et al.* 2003).

OSTEOCLASTS

Phenotype

Osteoclasts are giant multinucleated cells responsible for bone resorption. They are usually located in resorbed cavities called Howship's lacunae. The cytoplasm of these cells is "foamy," with many vacuoles. Characteristic features of osteoclasts are the abundant Golgi complexes usually disposed around each nucleus, the mitochondria and transport vesicles loaded with lysosomal enzymes (Baron 2003).

Ultrastructural studies showed that resorbing osteoclasts are highly polarised cells and have four specialised membrane domains: 1) sealing zone also called clear zone, 2) ruffled border, 3) functional secretory domain and 4) basolateral (upper) membrane. The sealing zone is a region rich in filamentous actin (F-actin) organised as a ring surrounding the ruffled border. In the sealing zone the cell membrane forms a tight attachment to the bone surface, thereby isolating the resorption lacunae from the extracellular fluid and permitting the maintenance of a specific microenvironment in the lacunae. The pH of resorption lacunae has been shown to be very acidic (~ 4.0), therefore this space has generally been considered to be a 'extracellular lysosome'. (Fallon 1984; Baron *et al.* 1985). The ruffled border is formed by the fusion of intracellular acidic vesicles with the region of plasma membrane facing the bone (Blair *et al.* 1989), it is characterised by long-finger-like projections that penetrate the bone matrix. The degradation products from the resorption lacunae are released into the extracellular space at the functional secretory domain (FSD) (Nesbitt and Horton 1997) (**Figure 1.3**).

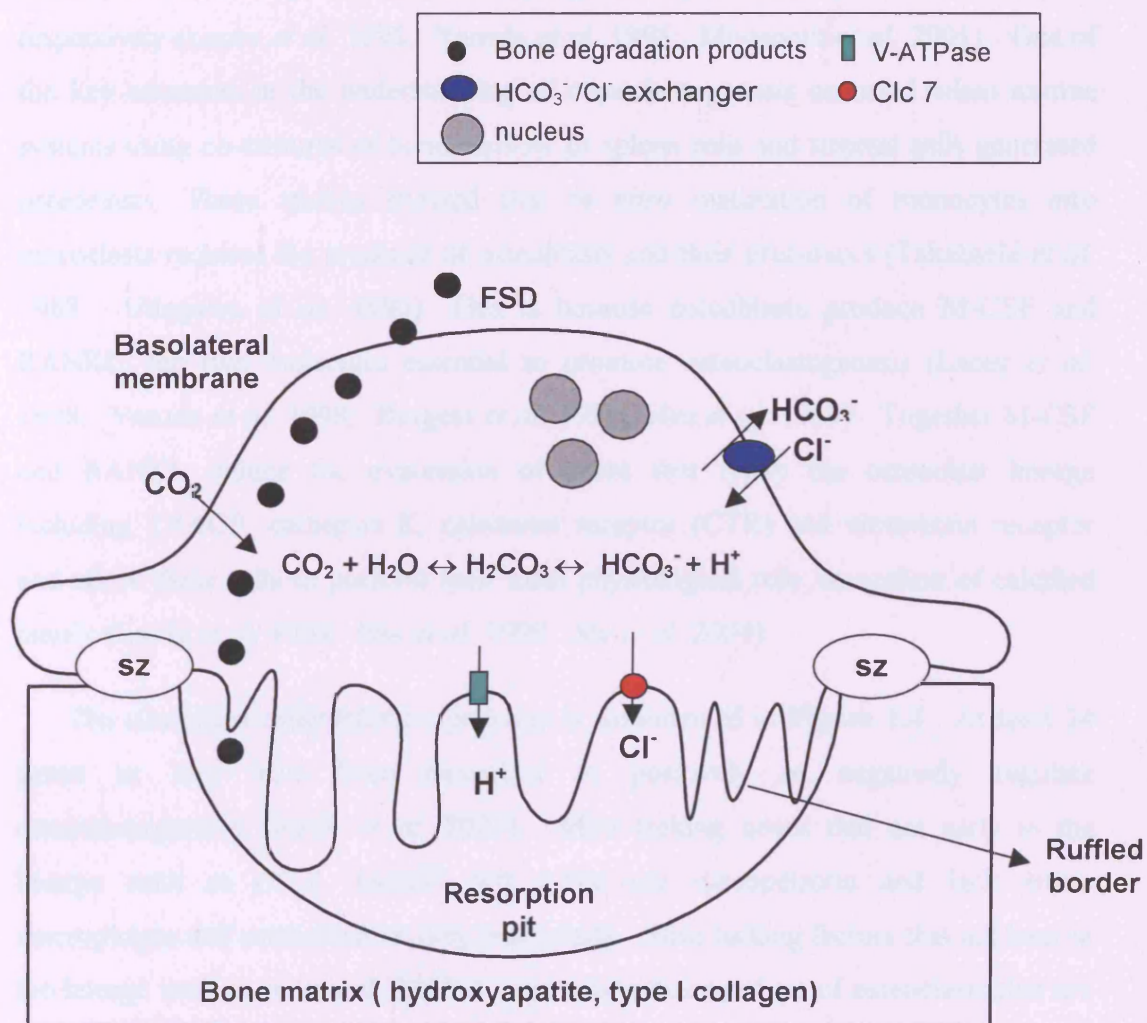


Figure 1.3. Schematic diagram of a resorbing osteoclast. SZ = sealing zone; FSD = functional secretory domain.

Osteoclast origin and differentiation

Osteoclasts are formed from the fusion of mononuclear precursors derived from haematopoietic stem cells of macrophage/monocyte lineage at or near the bone surface. This was first established by transplantation experiments *in vivo* in the 1970s (Walker 1975; Kahn and Simmons 1975). The pro-myeloid precursor can give rise to osteoclasts, macrophages or dendritic cells depending on whether they are exposed to receptor activator of NFκB ligand (RANKL), macrophage colony-stimulating

factor (M-CSF) or granulocyte macrophage colony-stimulating factor (GM-CSF), respectively (Lacey *et al.* 1998; Yasuda *et al.* 1998; Miyamoto *et al.* 2001). One of the key advances in the understanding of osteoclastogenesis occurred when murine systems using co-cultures of bone marrow or spleen cells and stromal cells generated osteoclasts. These studies showed that *in vitro* maturation of monocytes into osteoclasts requires the presence of osteoblasts and their precursors (Takahashi *et al.* 1988; Udagawa *et al.* 1990). This is because osteoblasts produce M-CSF and RANKL, the two molecules essential to promote osteoclastogenesis (Lacey *et al.* 1998; Yasuda *et al.* 1998; Burgess *et al.* 1999; Hsu *et al.* 1999). Together M-CSF and RANKL induce the expression of genes that typify the osteoclast lineage including TRACP, cathepsin K, calcitonin receptor (CTR) and vitronectin receptor and allow these cells to perform their main physiological role: resorption of calcified matrix (Lacey *et al.* 1998; Hsu *et al.* 1999; Shi *et al.* 2004).

The osteoclast differentiation pathway is summarized in **Figure 1.4**. At least 24 genes or loci have been described to positively or negatively regulate osteoclastogenesis (Boyle *et al.* 2003). Mice lacking genes that act early in the lineage such as *PU.1*, *M-CSF* and *c-Fos* are osteopetrotic and lack either macrophages and osteoclasts or only osteoclasts. Mice lacking factors that act later in the lineage such as *c-src* and *TRAF-6* have substantial numbers of osteoclasts that are not functional. Mutants of genes involved in the resorption process such as chloride channel 7 (*ClC-7*) and vacuolar ATPase (*V-ATPase*) have morphologically normal osteoclasts that are unable to resorb bone (Wagner and Karsenty 2001). A brief overview of the main factors involved in the osteoclast differentiation pathway will be discussed in more detail below.

PU.1 is a transcription factor that plays a pivotal role in the differentiation of a number of cell types such as macrophages, osteoclasts and B-cells (Simon 1998). The suggestion that PU.1 may regulate the expression of *c-fms* (the gene that encodes M-CSF receptor) has led to the investigation of the relationship between PU.1 and osteoclastogenesis. Mice lacking this gene show an osteopetrotic phenotype and lack macrophages and osteoclasts indicating that this factor is involved at the very early stages of myeloid differentiation (Tondravi *et al.* 1997).

Another transcription factor involved in osteoclast differentiation is *c-fos*, this is a cellular homologue of the *v-fos* oncogene and a key component of the AP-1 pathway. *c-fos* overexpression in different tissues and cell types cause mice to develop chondrosarcoma or osteosarcoma (a malignant tumour of mesenchymal origin with the ability to form bone tissue) and deletion studies produce osteopetrotic mice with a high number of macrophages (Wang *et al.* 1993; Grigoriadis *et al.* 1993; Grigoriadis *et al.* 1994). Therefore, *c-fos* was placed downstream from PU.1 in the differentiation pathway. This study was also very important as it confirmed that osteoclasts are derived from the same lineage as macrophages.

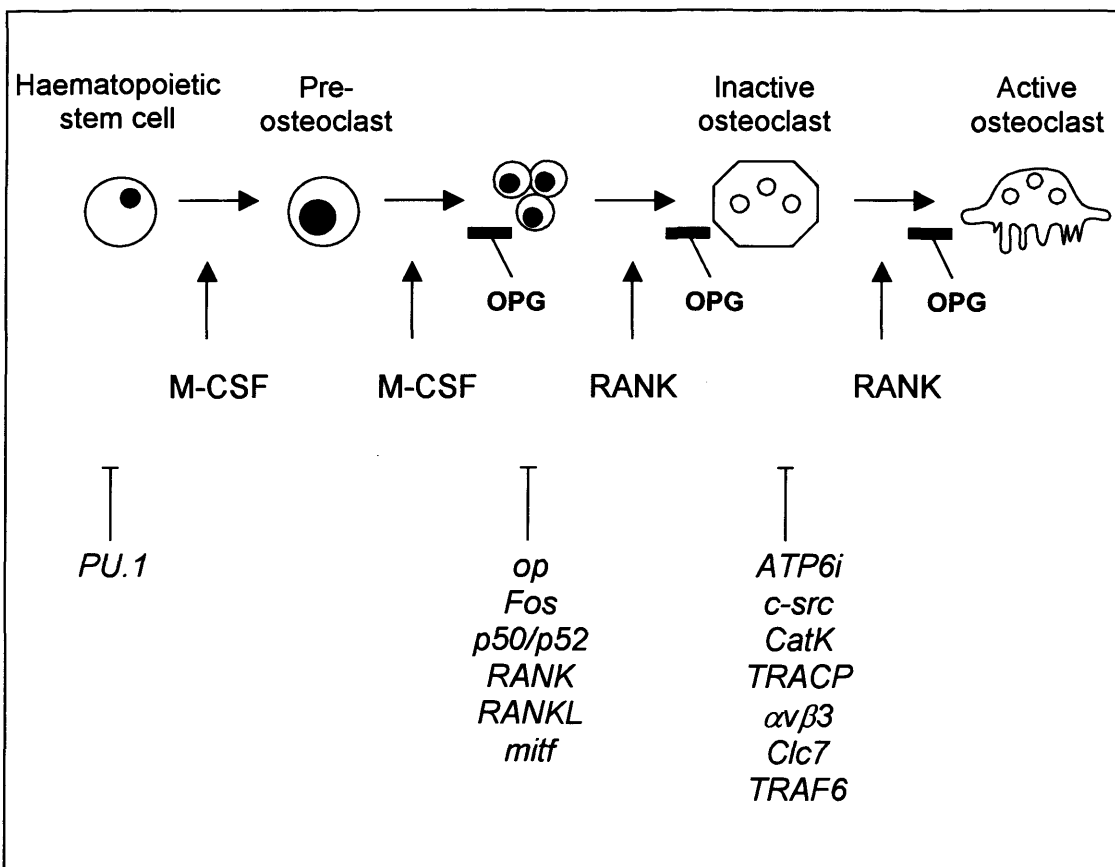


Figure 1.4. Osteoclast differentiation pathway. M-CSF and RANKL are essential molecules required for formation and activation of osteoclasts. OPG (osteoprotegerin) can bind to RANKL and regulate negatively both osteoclastogenesis and mature osteoclast activation. In the lower half of the diagram (in italics), shown are some of the single-gene mutations that block osteoclastogenesis and activation (adapted from Boyle *et al.* 2004).

Two other transcription factors that have been reported to play an important role in osteoclast differentiation are microphthalmic transcription factor (*mitf*) and NF κ B. Mice in which the *mitf* gene is disrupted exhibit an osteopetrotic phenotype caused by failure of mononuclear precursors to mature into multinucleated osteoclasts capable of bone resorption. This factor is also suggested to physically interact with PU.1 to regulate the expression of osteoclast genes such as TRACP and cathepsin K (Mansky *et al.* 2002). NF κ B is a dimer composed of various combinations of proteins: p50, p52, p65, c-Rel and Rel-B. The p50/52 double knockout shows an osteopetrotic phenotype due to arrested osteoclast differentiation (Franzoso *et al.* 1997). Further studies showed that it is a transcription factor involved in the RANK-RANKL pathway (Lomaga *et al.* 1999). Most recently, nuclear factor of activated T-cell 2 (NFAT2) was found to be the transcription factor that is most strongly induced during RANKL-stimulated osteoclast differentiation and it is also essential for osteoclastogenesis (Takayanagi *et al.* 2002; Ishida *et al.* 2002).

The requirement for secreted molecules to control osteoclast differentiation was first shown by studies of the osteopetrotic (*op/op*) mouse. These homozygous mutants lack osteoclasts and macrophages due to the failure of their haematopoietic stromal cells to release functionally active M-CSF (Yoshida *et al.* 1990; Takahashi *et al.* 1991). Further studies showed that the osteopetrotic phenotype of *op/op* mice could be partially reversed by overexpressing Bcl-2, a gene that prevents apoptosis in monocytes. These studies led to the conclusion that M-CSF binds to its receptor, c-fms, on osteoclast precursors to induce signals required for survival of osteoclast progenitors (Lagasse and Weissman 1997).

Another very important molecule involved in the osteoclast differentiation pathway is RANKL. This molecule, a member of the tumour necrosis factor family, is produced by osteoblasts, activated T-lymphocytes and endothelial cells. It is not only a stimulatory factor for the formation of osteoclasts but it is also essential for their survival (Wong *et al.* 1997). RANKL activates its specific receptor RANK that is present in osteoclasts and dendritic cells. The effects of RANKL are counteracted by OPG, which is secreted ubiquitously as an endogenous soluble receptor antagonist. OPG, which is a glycoprotein related to the TNF receptor superfamily, strongly

inhibits osteoclast differentiation by binding to RANK and therefore preventing the interaction between RANK and RANKL (Simonet *et al.* 1997; Yasuda *et al.* 1998).

RANKL is produced as a membrane bound protein on murine osteoblasts and stromal cells and is cleaved into a soluble form by a metalloprotease (Wong *et al.* 1997; Lacey *et al.* 1998). The discovery of RANKL constitutes a major advance in bone biology as it has greatly facilitated *in vitro* osteoclastogenesis. Purified cells of the monocyte/macrophage lineage, obtained from bone marrow or peripheral blood, can now be differentiated *in vitro* into mature osteoclasts by adding pure M-CSF and RANKL to the culture medium, replacing the requirement for stromal cells. (Yasuda *et al.* 1998; Suda *et al.* 1999).

RANK signalling is mediated by cytoplasmic factors, which control osteoclast function by activating downstream signalling pathways. There are at least six signalling cascades that are induced during osteoclastogenesis: inhibitor of NF κ B (IKK), c-Jun N-terminal kinase, p38, extracellular signal regulated kinase (ERK), calcineurin-calcium regulated NFAT2 and src pathways (Boyle *et al.* 2003). The preliminary step in RANK signalling is the binding to the TNFR-associated cytoplasmic factor or TRAFs. TRAF 2, 5 and 6 all bind to the cytoplasmic domain of RANK but only *TRAF-6* mutation results in osteopetrotic mice (Lomaga *et al.* 1999; Kobayashi *et al.* 2001). TRAFs in turn activate the signalling pathways described above. The transcription factors that are activated by the stimulation of RANK control the expression of resorption-associated genes such as TRACP, cathepsin k, c-src, integrin β 3 and CTR. The absence of these genes produces osteoclasts that are incapable of effective bone resorption (Boyle *et al.* 2003).

c-src, the cellular homologue of the *v-src* oncogene, is a cytoplasmic protein of the tyrosine kinase family, which is expressed ubiquitously. *c-src* deficient mice develop an osteopetrotic phenotype as osteoclasts present are unable to resorb bone (Boyce *et al.* 1992). This protein kinase is stimulated by a variety of signalling pathways, including RANK/RANKL. It binds to TRAF-6 to allow the RANK-mediated signalling to proceed through the lipid phosphatidylinositol 3-OH kinase (PI3K) and protein kinase AKT. These two molecules act downstream of *c-src* to induce cell survival, cytoskeletal rearrangement and osteoclast cell motility (Wong *et*

al. 1999). The function of the other factors mentioned above will be explained in detail below.

Recent studies have shown that cytokines such as TNF- α and IL-1 α can substitute for RANKL in generating osteoclasts from mouse marrow precursors and from peripheral blood mononuclear cells (Kobayashi *et al.* 2000; Kudo *et al.* 2002). Osteoclasts and their precursors express other receptors including TNF- α receptor, IL-1 α receptor, IL-6 receptor and TGF- β /BMP receptor superfamily. These receptors are most probably involved in signalling pathways that will eventually lead to regulation of osteoclast differentiation and/or activation, however, whether these receptor - ligand interactions augment or replace RANKL signalling is still controversial (Blair *et al.* 2005).

Resorption of mineralised matrix by osteoclasts

The sequence of cellular events needed for bone resorption can be divided into 5 main steps: 1) migration of osteoclasts to the resorption site, 2) attachment of osteoclasts to bone, 3) polarisation, 4) resorption, which includes the dissolution of the hydroxyapatite and removal and degradation of matrix products from the resorption lacunae and 5) osteoclast apoptosis or return of osteoclasts to the non-resorbing stage.

Cytokines, such as M-CSF, are likely to mediate osteoclast recruitment to the resorption site. In *in vitro* cultures, osteoclasts have been shown to migrate towards source of M-CSF (Fuller *et al.* 1993; Owens and Chambers 1993). Other cytokines suggested to be involved in this process are vascular endothelial growth factor (VEGF) and RANKL. It has been reported that the addition of antagonists of VEGF and RANKL in *ex vivo* cultures of embryonic bones reduces osteoclast recruitment. It has also been shown that both of these cytokines induce osteoclast recruitment in the Boyden chamber invasion assay in a dose dependent manner (Engsig *et al.* 2000; Henriksen *et al.* 2003).

Osteoclasts have been reported to attach preferentially to bone surfaces that have been exposed to collagenase. It is believed that this enzyme, expressed by osteoblasts, osteocytes, chondrocytes and bone lining cells, removes the non-

mineralised material lining the bone surfaces facilitating osteoclast attachment (Fuller and Chambers 1995). It has also been proposed that osteoid matrix degradation by this enzyme produces collagen fragments that are able to activate osteoclasts (Holliday *et al.* 1997). The molecular interactions that happen between the osteoclast plasma membrane and the bone matrix at the sealing zone are still unclear. The sealing zone is a region rich in filamentous actin (F-actin) organised as a ring (the actin ring) surrounding the ruffled border. The $\alpha_v\beta_3$ or vitronectin receptor (a member of the integrin family of adhesion molecule) has been established as an important molecule in osteoclast adhesion to bone (Davies *et al.* 1989). Osteoclast attachment to the bone matrix has been reported to occur due to interactions between vitronectin receptors on the osteoclast cell membrane with proteins in the bone matrix that contain the RGD amino acid sequence, such as osteopontin, fibronectin and bone sialoprotein (Miyachi *et al.* 1991; Flores *et al.* 1992). The integrin $\alpha_v\beta_3$ has been the main subject of research because antibodies against this integrin are effective inhibitors of bone resorption *in vitro* and *in vivo* (Horton *et al.* 1991). However, it has been shown that this molecule is not present in the sealing zone and consequently does not mediate the tight attachment of osteoclasts to the bone surface (Lakkakorpi *et al.* 1991). Other integrin adhesion molecule receptors that have been identified in osteoclasts are $\alpha_2\beta_1$ receptor, which binds to collagen, and $\alpha_v\beta_1$ that binds to collagen and fibronectin (Nesbitt *et al.* 1993; Helfrich *et al.* 1996). Other studies demonstrated that proteins of the cadherin family may also be involved in the tight attachment of osteoclasts to the bone matrix as pan-cadherin antibodies recognise the sealing zone membrane (Ilvesaro *et al.* 1998).

After migration and attachment to the bone surface, osteoclasts develop the sealing zone, form the ruffled border and start to extrude protons, Cl^- and proteolytic enzymes into the extracellular resorption zone between the ruffled border and the bone surface. The degradation of bone matrix is started by the action of carbonic anhydrase II (CAII); this enzyme is responsible for the production of protons in the cytoplasm which are pumped into acidic vesicles via the action of V-ATPase proton pump (Blair *et al.* 1989; Vaananen *et al.* 1990). The acidic vesicles then migrate and fuse to the cell membrane facing the bone matrix forming the ruffled border. The ruffled border formation and/or expansion seems to be mediated via $\alpha_v\beta_3$ integrin as β_3 -knockout mice develop an osteopetrotic phenotype due to abnormal ruffled border

formation (McHugh *et al.* 2000). Following fusion with the ruffled border the contents from the acidic vesicles (including H⁺) are pumped into resorption lacunae. The acid secreted into the lacunae has two main functions: 1) to solubilise the hydroxyapatite, revealing the organic matrix for degrading proteases, and 2) to provide the acidic pH required for protease activity (Vaananen *et al.* 2000).

Accumulating evidence shows that cathepsin K, the most abundantly expressed cysteine proteinase in osteoclasts, is a key protease required for bone degradation; this enzyme is able to cleave collagen at multiple sites within the triple helical structure (Drake *et al.* 1996; Garnero *et al.* 1998). Cathepsin K may be present in transcytotic vesicles that are responsible for transporting the products of bone degradation from the resorption lacunae to the functional secretory domain where they are released into the extracellular space (Salo *et al.* 1996; Salo *et al.* 1997; Nesbitt and Horton 1997). This enzyme is also responsible for TRACP activity by degrading it into two active subunits (Ljusberg *et al.* 1999). Matrix metalloproteinase-9 (MMP-9) or gelatinase B is another proteinase that appears to play an important role in the degradation process as it is highly expressed in the resorption lacunae. Its action does not seem to be as important as cathepsin K, since *MMP-9* knockout mice exhibit only transient disturbances of bone resorption (Vu *et al.* 1998).

Tartrate-resistant acid phosphatase (TRACP) also known as type 5 acid phosphatase or purple acid phosphatase is a widely used marker of osteoclasts in bone (Minkin 1982) but its function and subcellular localisation still remains unclear (Vaaraniemi *et al.* 2004). It has been suggested that TRACP is a lysosomal enzyme that is secreted through the ruffled border during bone resorption and may also play an important role in modulating intracellular vesicular transport (Hollberg *et al.* 2002). However, Halleen and colleagues (1999) showed that TRACP does not co-localise with known lysosomal markers and is not expressed at the ruffled border or in the resorption lacunae but to large cytoplasmic vesicles that have been identified as part of the transcytotic pathway. These investigators suggested that in osteoclasts, biosynthetic vesicles containing TRACP fuse to transcytotic vesicles containing degraded collagen fragments ingested by these cells during bone resorption. When these vesicles move towards the functional secretory domain their pH rises. This pH

change inhibits the action of cathepsin K and triggers the reactive oxygen species (ROS) activity of TRACP. The ROS generated by TRACP finalise the degradation of matrix components during transcytosis. The degraded material is then secreted into the extracellular space with active TRACP 5b (Halleen *et al.* 1999; Vaaraniemi *et al.* 2004).

The osteoclast ceases resorbing when its tight seal is broken and it is able to move away to a new location for resorption. *In vitro* studies have shown that osteoclasts can take part in multiple resorption cycles before they undergo apoptosis (Vaananen *et al.* 2000).

Maintenance of osteoclast intracellular pH

Acidic pH is essential for solubilisation of the hydroxyapatite crystals and digestion of the organic matrix by the proteases secreted by the osteoclasts in the resorption lacunae. As protons are secreted by the V-ATPase pump located to the ruffled border a load of base equivalents, mainly HCO_3^- , is generated in the osteoclast cytoplasm. The osteoclast intracellular alkalisation is compensated mostly by the action of $\text{HCO}_3^-/\text{Cl}^-$ exchanger in the basolateral membrane. This exchanger is responsible for the transfer of bicarbonate that accumulates in the cytoplasm, and also for the supply of a continuous inward current of Cl^- for the ruffled membrane chloride channel. (Rousselle and Heymann 2002).

The chloride channel is another important regulator of osteoclast intracellular pH (pH_i). The proton transport by V-ATPase is an electromagnetic process and acidification of resorption lacunae is accompanied by an interior positive membrane potential which is compensated by pumping anions, usually Cl^- , into the resorption lacunae (Blair *et al.* 1991). The combined activities of the V-ATPase and chloride transporter allow acidification of the resorption pit and regulation of pH_i but the chloride transporter is functionally and structurally dissociable from the proton pump (Schlesinger *et al.* 1997). Most recently knockout studies showed that animals deficient in *CIC-7*, a ubiquitously expressed chloride channel, develop an osteopetrotic phenotype and patients identified with infantile malignant osteopetrosis have mutated chloride channels, confirming that Cl^- conductance plays a role in resorption lacunae acidification and pH_i regulation (Kornak *et al.* 2001).

Osteoclasts appear to have different mechanisms of pH_i regulation depending on their resorptive state; during resorption osteoclast pH_i is mainly regulated by the activity of $\text{HCO}_3^-/\text{Cl}^-$ exchanger and V-ATPase pump, whereas during resting/migration phases, osteoclast pH_i is mainly regulated by the Na^+/H^+ exchanger (Hall *et al.* 1992; Lees *et al.* 2001). Osteoclasts may also recover their pH_i in a Na^+ -independent manner; Zn^{2+} -sensitive proton and K^+ -conductive pathways have been suggested to be involved in pH_i maintenance in osteoclasts, particularly when the Na^+/H^+ exchanger is inoperative (Arkett *et al.* 1992; Nordstrom *et al.* 1995; Rousselle and Heymann 2002).

BONE DEVELOPMENT AND GROWTH

In the foetus bone is formed from undifferentiated mesenchymal tissue by either intramembraneous or endochondral ossification. Intramembraneous ossification is the process by which bone is formed directly from the mesenchyme, whereas endochondral ossification involves the formation of cartilage before bone is formed (Provot and Schipani 2005). Intramembraneous bone formation is the dominant process in the formation of the calvarium and clavicle. In these bones, at the ossification centres, the mesenchymal cells proliferate and differentiate into osteoblasts. These cells secrete the osteoid, which becomes mineralised resulting in an island of woven bone. This type of bone is then extensively remodelled by resorption and appositional growth (*i.e.* the deposition of new layers of bony materials on preformed surfaces) to form the mature adult skeleton that is made up of lamellar bone (Baron 2003).

Most of the foetal skeleton is derived from cartilage, which forms minuscule scale models of bone. This process involves a slow degradation of hyaline cartilage structures that become transformed into bone by ossification (Provot and Schipani 2005).

Two major signalling pathways controlling endochondral ossification have been described: Indian hedgehog (Ihh) and parathyroid hormone related protein pathways. Ihh coordinates chondrocyte proliferation and differentiation as well as osteoblast differentiation. During endochondral ossification, Ihh is synthesised by chondrocytes and early hypertrophic chondrocytes. This molecule binds to its receptor Patched-1

(Ptc-1) leading to activation of Smoothed (Smo); active Smo triggers a cascade that leads to expression of osteopontin and other characteristic markers of chondrocytes. *Ihh*^{-/-} mice develop bone growth abnormalities after the condensation stage, they have a higher number of immature hypertrophic chondrocytes and do not have any osteoblasts (St Jacques *et al.* 1999; Long *et al.* 2001). Moreover, the cartilage of these knockout mice fails to synthesise PTHrP. This molecule seems to act primarily on halting the differentiation process by keeping the proliferating chondrocytes in the proliferative pool (Weir *et al.* 1996). Fibroblast growth factor (FGF) and BMPs also play important roles in the endochondral ossification process (Provot and Schipani 2005).

Runx2 and Sox9 are two transcription factors involved in the ossification process. Sox9 is essential to convert condensed mesenchymal cells into chondrocytes. This transcription factor in culture stimulates expression of cartilage matrix genes such as Col2a1, Col1a2 and aggrecan. Knockout studies showed that Sox9 is critical for all phases of chondrocyte lineage and is responsible for the expression of other Sox-family members (Sox5, 6) involved in chondrocyte differentiation (Bi *et al.* 1999; Akiyama *et al.* 2002).

BONE REMODELLING

Bone remodelling has been characterised by Frost (1969) as a three-step process that occurs in discrete locations in the skeleton by a population of cells called basic multicellular unit (BMU). The unit consists of a team of osteoclasts in front, forming the cutting cone, followed by a team of osteoblasts filling the resorption cavities excavated by the osteoclasts. Another component of the BMU is the capillary that lies along the whole length of the unit (Parfitt 1994). The first step in the remodelling process (origination) involves the digestion of the endosteal membrane by enzymes released from the bone lining cells, which expose the mineralised matrix. This is followed by osteoclast formation and assembly of these cells to form the cutting cone. The remodelling process then enters its second step: progression. During progression, the BMU travels in one direction for a finite period of time. Progression is sustained by the continual arrival of osteoclast precursors to replace those that have undergone apoptosis. Eventually the supply of osteoclast precursors is

halted; at this stage the BMU comes to the end of its lifespan and the cavity is refilled by the osteoblasts, this is the termination step (Parfitt 2002).

Under normal circumstances, in young adults (between the ages of about 20-45), remodelling activity keeps bone mass relatively constant. Certain physiological or pathological states can alter the balance of the turnover process, so that resorption begins to outstrip formation, leading to a net bone loss (see section on osteoporosis below). This could be due not only to enhanced osteoclast resorption but also to declining osteoblast function (Mosekilde 2001).

REGULATION OF BONE CELL FUNCTION

Bone cell function is regulated by a variety of endocrine, paracrine factors and mechanical stimuli (Raisz 1999). Many hormones affect bone remodelling via the production of local factors. In general, the pathophysiological roles of local factors are not well understood; these tend to be multifunctional and are not bone-specific (Raisz 1999). The action of some key local, hormonal and paracrine factors are discussed below in detail.

Parathyroid hormone and PTHrP

PTH, secreted by the parathyroid glands, is a single-chain polypeptide hormone consisting of 84 amino acids and its major role is the maintenance of calcium homeostasis. PTH is synthesised as a larger 116 amino acid precursor, which undergoes significant degradation to pro-PTH and finally to PTH, the secretory product. PTH in the circulation is cleaved into 2 subunits: a biologically active N-terminal fragment (1-34 fragment) and a biologically inactive C-terminal fragment (Gensure *et al.* 2005). The parathyroid gland maintains the plasma Ca^{2+} concentration within very tight limits by secreting PTH in response to small decreases in Ca^{2+} concentration. A negative feedback control inhibits the secretion of PTH when Ca^{2+} levels are elevated; this control is regulated principally at the level of Ca^{2+} receptors on the parathyroid glands. PTH raises Ca^{2+} levels directly by mobilizing calcium from bone (increased bone resorption) and decreasing renal excretion of Ca^{2+} by stimulating tubular reabsorption and indirectly by enhancing the renal formation of 1,25(OH)₂-vitamin D₃.

PTH-dependent regulation of mineral homeostasis is mediated via its receptor PTH type 1 receptor, which is highly expressed in bone and kidney (Juppner and Kronenberg 2003). This hormone has been shown to be involved in stimulating osteoblast differentiation and activity, leading to increased bone mass when intermittently administered at low doses to patients with osteoporosis and in ovariectomised rats (Sone *et al.* 1995; Dobnig and Turner 1995). PTH has also been shown to have an important role in bone development as *PTH*-knockout mice have impaired cartilage matrix mineralisation, reduced osteoblast numbers and trabecular bone (Miao *et al.* 2002). The prevailing view for many years has been that the PTH effect on osteoclast is mediated indirectly via osteoblasts (Rodan and Martin 1981; Rodan and Martin 1982; McSheehy and Chambers 1986a; McSheehy and Chambers 1986b). However, there have been several reports over the years indicating that osteoclasts from several species possess functional PTH receptors and most recently, I showed that isolated osteoclasts are sensitive to PTH in the absence of osteoblasts (Dempster *et al.* 2005). This will be discussed in more detail in Chapter 2.

PTHrP was first discovered as the product of cancer cells; it causes hypercalcemia in cancer patients mainly due to increased osteoclast activity (Stewart *et al.* 1980; Nakayama *et al.* 1996; Strewler 2000). Further work, showed that this molecule is expressed ubiquitously and it binds to the same receptor as PTH (PTH-1 receptor, also called PTH-PTHrP receptor) and activates the same transduction pathway with equal potency. Recently, short-term administration of PTHrP has been shown to increase bone density in post-menopausal women by 4-5% without stimulating bone resorption and it did not cause increases in blood calcium levels or other side effects, suggesting that this peptide may be an important tool in combating osteoporosis (Horwitz *et al.* 2003).

1,25(OH)₂-vitamin D₃

Vitamin D₃ is a secosteroid produced in the skin by the action of ultraviolet sunlight on precursors. Vitamin D₃ itself is an inert molecule until two successive hydroxylations in the liver and kidney turn it into the biologically active 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃, or calcitriol). Despite two hydroxylation steps, 1,25-(OH)₂D₃ is still very lipid soluble and acts like a steroid hormone: it binds to a nuclear vitamin D receptor (VDR) which exposes a DNA-binding region, resulting in

increased and/or inhibited transcription of certain mRNAs (*e.g.* increases the expression of RANK). VDRs are found mainly in the gut, kidney and bone. The main biological role of 1,25-(OH)₂D₃ is to maintain the serum Ca²⁺ levels within tight limits in the blood by increasing dietary uptake of Ca²⁺ in the gut, by facilitating Ca²⁺ reabsorption in the kidneys and by enhancing the mobilisation of Ca²⁺ stores from bone (Holick 1998). 1,25-(OH)₂D₃ increases the number of mature osteoclasts by inducing stem cells to differentiate into osteoclasts. However, this appears to be regulated indirectly through its action on osteoblasts, since mature osteoclasts do not express VDRs and also it is believed that the action of vitamin D₃ is also RANK/RANKL dependent (Suda *et al.* 2003). In osteoblasts, vitamin D₃ is known to act by increasing the expression of a number of osteoblast phenotypic genes at later stages of osteoblast differentiation such as osteocalcin (van Leeuwen *et al.* 2001).

Calcitonin

Calcitonin, a Ca²⁺ regulating hormone, is synthesised by the C-cells of the thyroid gland. This hormone is rapidly released in response to a moderate rise in circulating Ca²⁺ reflecting its physiological role as an “emergency” hormone to protect against development of hypercalcemia. The effects of calcitonin are mediated via its receptors, which are expressed on committed osteoclast precursors, and are used as a differentiation marker for mature osteoclasts (Hattersley and Chambers 1989; Lee *et al.* 1995). Calcitonin is a powerful inhibitor of osteoclast activity *in vitro* and *in vivo*. It has been shown to reduce osteoclast activity in all resorption assays studied to date and it also inhibits both basal and stimulated resorption in organ culture (Chambers and Magnus 1982; Arnett and Dempster 1987; Meghji *et al.* 2001). Calcitonin also inhibits osteoblast and osteocyte apoptosis (Plotkin *et al.* 1999). Clinical studies showed that decrease of urinary markers of bone resorption occurs within a few hours after subcutaneous or intramuscular injection of calcitonin, but rarely exceed 24 hours. Therefore, clinical usage of calcitonin for the treatment of osteoporosis is limited, due to its very short-term effect on bone resorption (Gonzalez *et al.* 1986; Zaidi *et al.* 2002).

Sex steroids (oestrogens & testosterone)

It has been known for a long time that oestrogen deficiency, such as that occurring at menopause, leads to skeletal depletion. Skeletal preservation by oestrogens in females may be related to the need of Ca^{2+} stores for embryonic skeletal development (Riggs *et al.* 2002). Oestrogen has been reported to inhibit bone resorption by reducing osteoclast numbers. *In vitro* and *in vivo* studies have demonstrated that oestrogen suppresses RANKL production by osteoblasts, T and B-cells (Eghbali-Fatourechi *et al.* 2003) and also increases OPG production (Hofbauer *et al.* 1999a). Additionally, bone resorbing cytokines such as IL-6, TNF- α and IL-1 have been shown to be potential candidates for mediating the bone loss associated with oestrogen deficiency (Krassas and Papadopoulou 2001). The finding that males with homozygous deletion of oestrogen receptor alpha (ER α) develop osteopenia and have unfused epiphysis, showed that this hormone is also important for male skeletal development (Syed and Khosla 2005).

Testosterone hormone can be converted via aromatase to oestrogen, which is known to inhibit bone resorption. However, *in vitro* and *in vivo* studies showed that testosterone can also act directly on bone cells. Gray and colleagues (1992) showed that primary rat osteoblast proliferation rate and collagen production are increased by testosterone and 5 α -dihydrotestosterone (DHT), a compound that cannot be converted to oestrogen. Long and short-term *in vivo* studies showed that androgen administration in ovariectomised rats reduced loss of cancellous bone in a dose dependent manner (Lea *et al.* 1998). Further studies using androgen antagonists showed that this effect is not oestrogen-mediated (Lea and Flanagan 1998).

Prostaglandins

Prostaglandins (PGEs) act on a variety of cells via cell-surface receptor subtypes called EP₁₋₄. PGE₂ has been shown to induce formation of mouse multinucleated osteoclasts from marrow progenitors and has also been reported to stimulate osteoclast formation in murine organ cultures but inhibits formation of human osteoclasts (Raisz 1995; Mundy 2003). In stromal cell and primary calvarial cell cultures, PGE₂ stimulates osteoblast differentiation (Flanagan and Chambers 1992; Weinreb *et al.* 1997). Systemic administration of PGE₂ to animals has been shown to

stimulate bone formation and increases bone mass, suggesting that prostaglandins have an anabolic effect *in vivo* (Ke *et al.* 1998).

Nucleotides

ATP and other nucleotides are recognised as important messenger molecules for cell-cell communication (Ralevic and Burnstock 1998). It has recently become evident that extracellular nucleotides play an important role in bone remodelling (Dixon and Sims 2000). ATP and other nucleotides can exert impressive stimulatory effects on formation and activity of osteoclasts in addition to inhibit bone formation by osteoblasts (Hoebertz *et al.* 2003). The effect of these nucleotides on bone seems to be mediated via P2Y or P2X receptor subtypes. Because both ADP and ATP are potent stimulators of bone resorption, and ATP and UTP are inhibitors of bone formation *in vitro*, this data suggest that nucleotides have an overall destructive catabolic effect on bone. Nucleotides are interesting targets for drug development in the future for several pathological bone loss conditions. The osteolytic activity of ADP could also be relevant to inflammatory conditions such as rheumatoid arthritis that lead to sustained systemic and localized bone loss (Hoebertz *et al.* 2003).

Oxygen

The vasculature in bone serves to provide oxygen and essential nutrients to the cells in the bone marrow and within the bone matrix. The oxygen tension in blood entering the bone via arteries is approximately 95 mmHg (12%). Within the marrow cavity of normal volunteers the interstitial pO₂ has been measured at 6.6% (Harrison *et al.* 2002), however, when the blood supply is reduced or disrupted; such as in bone fractures and inflammation; the pO₂ could drop much lower (< 1%) (Lewis *et al.* 1999). It has been proposed that hypoxia may be involved in a novel mechanotransduction pathway as the expression of hypoxia induced transcription factor 1 alpha (HIF1- α) by osteocytes increases in response to mechanical unloading (Gross *et al.* 2001). Osteoblasts and osteoclasts are also sensitive to the effect of hypoxia. Reducing pO₂ from 20% to 2% causes a 90% reduction in the area of bone nodules formed by osteoblasts *in vitro*, probably due to impaired collagen production and cell proliferation (Orriss *et al.* 2003). On the other hand, hypoxia strongly stimulates the formation of osteoclasts cultured *in vitro* in all species studied to date

and also in bone organ culture (Arnett *et al.* 2003; Utting *et al.* 2003). The effect of hypoxia emphasises the importance of the vasculature in bone.

Cytokines and growth factors

Under physiological conditions the immune and skeletal systems are regulated by a number of cytokines and growth factors, *e.g.* IL-1,2,3,4,6,10,11,13, vascular endothelial growth factor (VEGF), tumour necrosis factor- α (TNF- α), insulin growth factor (IGF), fibroblast growth factor (FGF) and RANKL. These molecules are produced by many different cell types and their receptors are widely distributed. The function of a some of the cytokines involved in bone metabolism is discussed below.

IL-1 is a family of two active peptides, α and β , which appears to have identical functions and bind to type I and II receptors (Mundy 2003). In the bone microenvironment, this molecule is produced by osteoclast precursors, stromal cells and osteoblasts and it has been shown to stimulate osteoclast function by inducing the expression of RANKL in osteoblasts (Hofbauer *et al.* 1999b). Conversely, IL-1 has also been reported to stimulate osteoclast activity in the absence of RANKL (Kudo *et al.* 2002).

IL-6 is a member of the gp130 cytokine family and appears to mediate the resorptive activity of osteoclasts directly and indirectly via the RANK/RANKL pathway (Kwan *et al.* 2004). IL-6 has also been reported to stimulate mesenchymal cells towards the osteoblastic lineage and to inhibit osteoblast apoptosis (Taguchi *et al.* 1998).

Vascular endothelial growth factor (VEGF) is expressed by osteoblasts, osteoclasts and hypertrophic chondrocytes. VEGF allele results in embryonic lethality; however, analysis of mice expressing only the VEGF 120 isoform have shown that this growth factor is involved in chondrocyte maturation and appears to have a direct role in regulating osteoblast activity (Zelzer *et al.* 2002). Upregulation of VEGF by bone cells have been associated with skeletal growth, bone healing and mechanical loading (Street *et al.* 2002).

Tumour necrosis factor (TNF) is produced by osteoblasts and consists of two related polypeptides (α and β). TNF- α acts by increasing bone resorption while

simultaneously decreasing bone formation. TNF- α has been shown to increase RANKL expression in osteoblastic cells and act synergistically with RANKL to induce osteoclastogenesis. Like IL-1 α , it remains controversial as to whether TNF- α can stimulate osteoclast formation in the absence of RANKL; some authors have demonstrated RANKL-independent formation (Kudo *et al.* 2002), while others have shown that RANKL is necessary for osteoclast formation even in the presence of TNF- α (Lam *et al.* 2000).

IGFs are polypeptides that enhance osteoblast proliferation, decrease osteoblastic apoptosis and stimulate bone matrix synthesis by mature osteoblasts. Two IGFs have been characterized: IGF1 and IGF2, both of which have similar biological activities in bone cells. It has been reported that *IGF-I*-null mice have decreased bone formation, whereas transgenic animals overexpressing this growth factor have increased bone formation (Zhao *et al.* 2000). PTH and growth hormones increase IGF expression, while glucocorticoids inhibits its expression (Sims *et al.* 2000; Canalis and Giustina 2001; Bikle *et al.* 2002)

BONE PATHOLOGY

Osteoporosis, the "classic" bone remodelling disorder, is defined as a reduction in bone mass associated with disruption of bone microarchitecture, resulting in increased bone fragility and increased fracture risk (Consensus development conference, 1993). It has been known for some time that oestrogen deficiency, such as that occurring during the menopause, leads to bone loss. Consequently, women account for most of the hip fractures in the UK (Eastell *et al.* 2001). However, there are other risks that should be taken into account such as genetic factors, sedentary lifestyle, excessive protein intake and chronic acidosis.

Osteoporosis treatment represents one of the great success stories of modern preventive medicine. Nowadays, there are a number of drugs and treatments used for the prevention and cure of osteoporosis. Hormone replacement therapy (HRT) in post-menopausal women effectively prevents osteoporosis (Compston 2004). However, recent studies showed that HRT increases the risk of both breast cancer and cardiovascular disease (Beral 2003). Bisphosphonates, which includes alendronate and risedronate, are orally-active synthetic analogues of pyrophosphate that inhibit

osteoclast action. These drugs are cost-effective and are thereby increasingly proving to be useful in treating a range of bone loss disorders. Teriparatide (1-34 amino acid fragment of PTH) has been shown to increase bone mass as explained above. This has become a very attractive option as intermittent PTH administration has been reported to increase bone mineral density due to an increase in osteoblast formation. Moreover, PTH has been shown to suppress osteoblast apoptosis (Jilka *et al.* 1999). It has been reported that oestrogen receptors on bone cells have a second mode of action that does not require their transport to the nucleus and this alternate pathway attenuates osteoblast apoptosis (Kousteni *et al.* 2001). Since then, a compound called estren has been identified that triggers the same receptor action. These drugs have been named ANGELS (activation of nongenotropic oestrogen-like signalling) and are also believed to promote osteoclast cell death (Kousteni *et al.* 2001; Moggs *et al.* 2003).

Osteomalacia is a disease characterised by impaired osteoid mineralisation resulting in soft bones with an increased tendency to fractures. In children this condition is known as rickets and is often caused by nutritional deficiency in vitamin D₃, Ca²⁺ or phosphate. (Prince and Glendenning 2004). Its causes will be discussed in detail in Chapter 4.

Inflammatory bone loss is a symptom of rheumatoid arthritis (RA) and periodontal disease. In RA, the inflamed synovium produces a variety of cytokines *e.g.* RANKL, IL-1 α and β , TNF- α , IL-6, M-CSF, IL-17 and PTHrP (Goldring and Gravalles 2000). These cytokines act by increasing osteoclast activation and formation from precursors present in the synovial fluid, leading to excessive osteoclastic resorption, *i.e.* bone loss (Goldring 2003).

Several cancers have profound effects upon the skeleton, causing an increase in osteoclast formation and activity either systemically, as in humoral hypercalcaemia of malignancy, or locally, as in bone metastases, *e.g.* breast cancer derived. One major product of breast cancer cells is PTHrP, which promotes osteoclast formation (Guisse *et al.* 1996). Tumours are poorly vascularised areas and as a result severe tissue acidosis develop in human malignancies; the pH levels in mammary carcinomas (measured by the use of a pH-sensitive electrode) for example, has been found to be

as low as 6.4 (Jirtle 1988). Acidosis by itself is a powerful stimulator of bone resorption, a process that contributes to cancer pain (Mantyh *et al.* 2002).

ACID-BASE BALANCE

Buffering of hydrogen ions

The importance of the acid-base homeostasis in the maintenance of normal cellular responses and physiological integrity has long been recognised. The mechanisms regulating the intracellular and extracellular H^+ concentration are important as many cellular responses are diminished by acidosis including cytosolic and membrane associated enzyme activities (Lardner 2001). The activity of phosphofructokinase, for example, the key enzyme controlling the glycolytic pathway of mammals, is inhibited by H^+ .

Mechanisms exist for buffering H^+ loads both inside and outside of cells. In the extracellular fluid, H^+ buffering is largely accomplished by the CO_2/HCO_3^- system, which roughly accounts for 80-90% of total metabolic acid buffering. In the intracellular space, however, the pH is mainly controlled by protein- and phosphate-buffer systems (Swenson 2001). In addition to passive chemical buffering, it is clear that cells have a capacity to regulate their own intracellular pH levels. Most cells appear to have intracellular pH values that are appreciably more alkaline than would be predicted if H^+ and HCO_3^- were passively distributed across the plasma membrane. Transmembrane pH differences vary among tissues but range between 0.2 and 0.5 units in contrast to the 0.8 and 1.5 unit difference that would occur if the intracellular pH regulatory mechanism was not present (Roos and Boron 1981). Transmembrane ion exchanges not only help maintain intracellular pH above that dictated by passive electrochemical forces but are also stimulated selectively in the face of endogenous or exogenous acidosis. These include exchange of extracellular Na^+ for intracellular H^+ via the Na^+/H^+ membrane antiporter and active H^+ extrusion via V-ATPase (Swenson 2001).

Two types of acids are produced in the body as a result of metabolism: carbonic acid (H_2CO_3) and non-volatile or fixed acids. Ultimately, the lungs and kidneys together maintain the overall acid-base balance. However, the extracellular fluid

needs to be protected against sudden changes in H^+ concentration. The blood pH, for example, must be maintained between 7.35 and 7.45 (35-45 nmol/l H^+) (Green and Kleeman 1991); pH values greater than 7.70 (20nmol/l H^+) or less than 7.00 (100nmol/l H^+) are usually incompatible with life. In order to maintain the H^+ concentration within such tight limits in the blood, the body has 4 major buffering systems: haemoglobin buffer with its imidazole groups from the histidine residues in the erythrocytes, the phosphate buffer, the plasma proteins and most importantly the carbonic acid-bicarbonate system.

In the peripheral tissue, CO_2 produced as a result of metabolism, diffuses freely down a concentration gradient across the cell membrane into the red blood cells. This gradient is maintained because the red blood cells do not produce any CO_2 due to their anaerobic metabolism. The ionisation of carbonic acid occurs spontaneously but CAII is required for the production of H_2CO_3 (Tashian 1992). As CAII is located mainly in erythrocytes, these cells are the principal site of H^+ and HCO_3^- formation in the blood. The haemoglobin protein is a more effective buffer when deoxygenated, so as it passes through the capillary beds and gives up oxygen to the tissues, it collects the H^+ formed from the dissociation of carbonic acid. The HCO_3^- , meanwhile, pass from the erythrocytes down their concentration gradient into the plasma, in exchange for Cl^- ions, to maintain electrical neutrality (Ganong 2001).

In the lungs, the PCO_2 in the alveoli is maintained at low levels due to ventilation. Therefore, the PCO_2 of the pulmonary capillaries is higher than the PCO_2 in the alveoli so the gradient is reversed. CAII is required as a catalyst to convert H_2CO_3 into H_2O and CO_2 , the latter diffuses into the alveoli and is excreted by the lungs and the oxygen is transported back to the tissues.

The non-volatile acids are excreted via the kidneys where the phosphate buffer plays a major role in the elimination of H^+ in the urine. Most of the buffer is present as HPO_4^{2-} , which can combine with H^+ to form $H_2PO_4^-$, which is excreted in the urine. Ammonia can also act as a urinary buffer. It is formed by the deamination of glutamine under the influence of glutaminase. Ammonia readily diffuses across the cell membrane into the tubular lumen, where it combines with H^+ to form NH_4^+ . This molecule is then excreted in the urine. In order to maintain the acid-base

homeostasis, the different buffering systems are in constant equilibrium with each other (Ganong 2001).

Disturbances of acid-base status

Disturbance of the body's acid-base balance results in the plasma containing either too many or too few hydrogen ions resulting in acidosis or alkalosis, respectively. These disturbances may be due to respiratory causes (where the primary defect is change in CO₂ levels) or metabolic (where the primary defect is change in HCO₃⁻ concentration). Acid-base status can be understood on the basis of the relationship represented in the following reaction:



Accumulation of H⁺ in the system shifts the equilibrium to the left as the extra H⁺ combine with HCO₃⁻ to form H₂CO₃. However, since there is no ventilatory abnormality, any related increase in plasma H₂CO₃ is transient, as the related increase in dissolved CO₂ is immediately excreted by the lungs (Adroque and Adroque 2001).

A common cause of chronic metabolic acidosis in the Western countries is a protein-rich diet (New 2003). The average Western diet generates the equivalent of 1mmol of acid /kg body weight per day (equivalent of 8 ml of concentrated HCl) and the more acid precursors a diet contains the greater the extent of systemic acidity (Kurtz *et al.* 1983); food products such as cola drinks, which contain phosphoric acid and have a pH of 3, are very popular within these societies (Barzel 1995).

Ageing and the menopause are also contributory factors to acidosis (Orr-Walker *et al.* 1999). As individuals age, their overall renal function declines, and so does their ability to excrete acid (Frassetto and Sebastian 1996). Hence, older individuals are slightly but significantly more acidic (Frassetto *et al.* 1996; New 2003). Mild, acute or chronic metabolic acidosis has also been reported in connection with gastroenteritis, where it is associated with increases in bone resorption indices (Yildizdas *et al.* 2004), fasting (ketoacidosis) and heavy exercise. In renal disease (the major cause of chronic severe systemic acidosis), the ability of the kidneys to secrete H⁺ into the urine is impaired or lost. The arterial blood pH in these situations may be as low as 7.2.

Addition of CO_2 to the system without changes in HCO_3^- levels causes a decrease in pH and results in respiratory acidosis. During acute respiratory acidosis, a rise in PCO_2 shifts the equilibrium in the carbonic acid reaction to the right resulting in increased $[\text{H}^+]$ and $[\text{HCO}_3^-]$. Equilibration of H^+ with body buffer systems limits its potential rise and a new steady state is achieved in minutes. This type of acidosis may occur due to chronic respiratory diseases such as emphysema and acutely, due to prolonged exercise. For example, it has been reported that the blood pH of men after 10 second sprints in a cycle ergometer separated by 30-second recovery intervals decreased from pH ~ 7.4 to pH ~ 7.2 (Ratel *et al.* 2002).

In metabolic alkalosis the equilibrium in carbonic acid reaction is shifted to the right due to loss of H^+ . This is mainly caused due to prolonged vomiting (loss of gastric juice). Other causes of severe or mild chronic metabolic alkalosis are due to serious medical problems such as congenital chloride diarrhoea, hyperparathyroidism and various neoplasms; calcitonin administration for the treatment of osteoporosis, vitamin D_3 excess or a vegetarian diet, however the latter notion is still controversial (Escanero *et al.* 1991; New 2004).

Respiratory alkalosis is the most common acid-base disorder among seriously ill patients. It is brought about by hyperventilation. Humans and other mammals hyperventilate at high altitudes and during physical activity; the hyperventilation partially compensates for the lower tension of oxygen in inhaled air. This leads to a lower concentration of carbon dioxide and carbonic acid in the blood resulting in higher pH and respiratory alkalosis.

Acidosis can also occur locally in sites of low blood flow such as in initial stages of fracture healing, as a result of inflammation, infection, wounds and tumours (Arnett 2003). As individuals age, the general quality of the vascular supply around the body tends to decline. It has been reported that ageing results in a progressive loss of the medullary blood supply which eventually leads to marrow ischaemia (Bridgeman and Brookes 1996). The likely consequence of such ischaemia is hypoxia, which causes local acidosis (Arnett 2003).

Effect of acidosis on the skeleton

The negative effect of acidosis on the skeleton has been known for many years. Early in the twentieth century, *in vivo* studies showed that skeletal depletion (Goto 1918) and calcium and phosphate loss (Shohl and Sato 1923) were associated with experimental acidosis. In humans with normal renal function, the provision of additional dietary acids leads to an increase in urine calcium excretion without a parallel increase in intestinal calcium absorption, resulting in a net loss of calcium from the body, suggesting that the source of the additional urinary calcium is the bone mineral. Besides serving as a Ca^{2+} reservoir, the skeleton contains 80% of the body's carbonate, which, in response to a metabolic acid challenge, can be acutely released to buffer protons in an acellular physicochemical reaction (Barzel 1995). Bone is also relatively well vascularised, which allows a rapid mobilisation of the bone ions (Green and Kleeman 1991). Patients with a defect in renal acid excretion, distal renal tubular acidosis, have osteopenia that is substantially corrected by long-term administration of base (Domrongkitchaiporn *et al.* 2002). Treatment of postmenopausal women with oral potassium bicarbonate to neutralize endogenous acid production leads to improved calcium retention, reduced bone resorption, and increased bone formation (Sebastian *et al.* 1994). It is possible that calcium supplements may be favourable to bone, not just through the additional mineral that they provide but also through their provision of additional alkali. This notion is in agreement with previous work that showed the anti-osteoporotic effect of calcium supplementation is due to an inhibition of osteoclastic bone resorption and that elderly individuals who are mildly acidotic may benefit most from ingestion of calcium salts (Dawson-Hughes *et al.* 1990; Reid *et al.* 1995).

Although high protein intake has often been associated with low bone mass, strong data also exist for the opposite effect. Randomised controlled trials showed that protein supplements reduce bone loss at the contralateral hip in patients with upper femoral fracture, possibly due to protein-induced increase in insulin-like growth factor I, which is known to be osteogenic (Bonjour *et al.* 1996; Schurch *et al.* 1998). Moreover, it has been found that bone loss over a 4-year period was greatest in individuals with the lowest protein intake (Hannan *et al.* 2000). It must be borne in mind that vegetable proteins deliver high levels of sulphur and grain products, which

is consumed by many vegetarians and vegans, have a high potential renal acid load (Remer and Manz 1995).

It had long been thought that buffering of acid loads by bone was only due to short-term physicochemical reactions (Barzel 1995); however, as will be discussed in the subsequent chapters, it is now widely accepted that acidosis predominantly activates more long-term cellular mechanisms, involving both osteoclasts and osteoblasts.

Effect of acidosis on other cell types

In contrast with the response of osteoclasts, generally cell function appears to be impaired by acidosis (Lardner 2001). However, studies on the effect of acidosis on the behaviour of immunocompetent cells show that this condition may have a 'positive' effect depending on the function analysed. Lymphocyte motility, for example, has been shown to increase at pH 6.5 compared to pH 7.1 (Ratner 1992). Extracellular acidosis also increases the expression of β_2 integrin, CD18 and delay the rate of spontaneous apoptosis in human neutrophils (Trevani *et al.* 1999). Most recently, it has been reported that lowering pH markedly increases the antigen presenting capacity of dendritic cells, the professional antigen-presenting cells in immune responses (Vermeulen *et al.* 2004). This is a potentially important, as dendritic cell and osteoclasts are derived from myeloid precursors. Oxidisation of low density lipoprotein (LDL) by macrophages also appears to be increased by low pH (Morgan and Leake 1993). This could be relevant to the pathogenesis of atherosclerosis since LDL oxidation occurs in atherosclerotic plaques.

HYPOTHESES

In this PhD project I tested the following hypotheses:

- 1) Are normal human osteoclasts activated by low pH in a similar manner to rodent and chick osteoclasts in vitro? Is this effect mediated via osteoblasts?
- 2) Does acidosis alter the expression of resorption-associated factors such as cathepsin K and TRACP?
- 3) Does chronic metabolic acidosis affect the function of normal osteoblasts in the pH range that is critical for modulating osteoclast function, i.e. pH 7.5 – 6.8?
- 4) Are known proton-sensing receptors (e.g. TRPV1, ASIC, OGR1, TDAG8 and GPR4) expressed by bone cells?

CHAPTER 2

Effect of acidosis on the formation and activity of osteoclasts

INTRODUCTION

Acute effects of pH on mature osteoclasts

The direct effect of pH on osteoclasts was first discovered by Arnett and Dempster in 1986. They reported that resorption pit formation by rat osteoclasts increases remarkably as medium pH is reduced, *i.e.* lowering the pH from 7.4 to 6.8 causes a 14-fold increase in resorption. In these experiments, culture media were buffered by HEPES in the absence of bicarbonate so that the main independent variable was the proton concentration. They also found that the low pH effect was completely abolished by physiological concentrations of human calcitonin, suggesting that the acid response is mediated by living osteoclasts.

Further studies showed that in media buffered physiologically with $\text{HCO}_3^-/\text{CO}_2$, resorption pit formation by rat osteoclasts is stimulated when pH is reduced either by increasing PCO_2 or by decreasing HCO_3^- concentration (Arnett *et al.* 1994). The issue whether metabolic or respiratory acidosis are more potent stimulators of bone resorption remains controversial. In calvarial cultures, metabolic acidosis is more potent than respiratory acidosis in stimulating resorption, suggesting that decreased medium HCO_3^- , and not just a fall in pH, is necessary to enhance net Ca^{2+} efflux from calvaria (Bushinsky 1989; Meghji *et al.* 2001). Interestingly, the resorption response curve of mouse calvarial bones and that of mature rat osteoclasts to metabolic acidosis are very similar (Arnett and Spowage 1996; Meghji *et al.* 2001). This will be discussed in more detail in Chapter 3.

Investigations using physiological $\text{HCO}_3^-/\text{CO}_2$ -buffered media showed that rat osteoclasts are particularly sensitive to pH changes in the region close to pH 7.1. In this region, small shifts in extracellular pH (between pH 7.15 and 7.25) were

sufficient to produce a six-fold increase in the rat osteoclast resorptive activity; below 7.0 this effect begins to plateau (Arnett and Spowage 1996).

The stimulatory effect of low pH has also been observed in osteoclasts derived from mouse (Hoebertz and Arnett 2003), rabbit (Shibutani and Heersche 1993) and chick (Teti *et al.* 1989b; Walsh *et al.* 1990; Morrison and Arnett 1997). Chick osteoclasts, however, have a basal rate of resorption in non-acidified conditions (pH ~7.40) that is generally much higher than that of neonatal rat osteoclasts (Arnett and Dempster 1987; Morrison and Arnett 1997).

Surprisingly, osteoclasts are able to resorb bone efficiently even at pH ~6.3 (Murrills *et al.* 1993). These authors carried out a time-course experiment over a 72-hour period on the effect of pH on bone resorption and cytotoxicity in both rat and chick osteoclasts. Their results showed a consistent stimulatory effect of low pH on bone resorption in both species, which was enhanced remarkably in the first 24 hours of the assay. They also showed that a significant cytotoxic effect of low pH was only observed in the accessory cell population suggesting that osteoclasts are more resistant than other cell types to the cytotoxic effect of low pH.

Longer-term effects of pH on osteoclasts and osteoclast formation

Osteoclast activation by low pH is not merely a short-term effect from which tachyphylaxis (“escape”) occurs, as can be the case for some hormone-mediated phenomena. Longer-term cultures of mature rat osteoclasts show that H⁺-activated cells continue to resorb over periods of 7 days, magnifying effects of modest pH differences (Hoebertz and Arnett 2003). Long-term stimulatory effects of low pH on osteoclastic resorption may be pertinent to *in vivo* situations such as rheumatoid arthritis, where the local pH is reduced and bone resorption is increased (Steen 1992).

Paradoxically, the formation of murine osteoclasts from marrow precursors is slightly inhibited by reducing the extracellular pH. However, osteoclasts formed in long-term mouse marrow cultures at blood pH (7.4) show no resorptive activity until stimulated by acidification (Hoebertz and Arnett 2003). Osteoclast activation can then be considered as a two-step process. The initial “switching on” of resorption requires acidification of the extracellular environment of the osteoclast below pH 7.2.

Further stimulation by other osteolytic agents such as RANKL (Hoebertz *et al.* 2000; Hoebertz and Arnett 2003), PTH (Arnett and Dempster 1986), 1,25-dihydroxy-vitamin D₃ (Murrills *et al.* 1998) and purine nucleotides (Hoebertz and Arnett 2003), can then occur.

PTH and bone cells

The parathyroid hormone type 1 receptor (PTH1R) is a G-protein-coupled receptor that is highly expressed in bone and kidney. It mediates the PTH-dependent regulation of mineral homeostasis, as well as the actions of PTH-related peptide - PTHrP (Mannstadt *et al.* 1999). The prevailing view for many years has been that osteoclasts do not express PTH receptors and that PTH effects on osteoclasts are mediated indirectly via osteoblasts (McSheehy and Chambers 1986a; McSheehy and Chambers 1986b). However, there have been several reports indicating that osteoclasts from several species possess functional PTH receptors. For example, immunocytochemical analysis in fixed rat bone tissue showed that osteoclasts express higher levels of PTH compared to osteoblasts and osteocytes (Rao *et al.* 1983). It has also been reported that bovine PTH binds specifically to avian osteoclasts *in vitro* and this binding occurs in a manner that displays the properties of receptor-dependent hormone binding, *ie*: saturability, time-dependence, temperature-dependence, and hormone specificity (Teti *et al.* 1991; Agarwala and Gay 1992). Osteoclast expression of the PTH1R was demonstrated in deer antler (Faucheux *et al.* 2002), in sections of rat bone (Gay *et al.* 2003) and in human bone biopsies of patients with secondary hyperparathyroidism (Langub *et al.* 2001) and most recently on human osteoclasts derived from peripheral blood mononuclear cells (PBMCs) (Dempster *et al.* 2005).

Previous reports left open a possibility that the stimulatory effect of acidosis on osteoclast was osteoblast mediated. Therefore, in this chapter the effect of acidosis on osteoclast formation and activation was studied in a system devoid of osteoblasts (Flanagan and Massey 2003). Additionally, I investigated the interaction of the effects of PTH and acidosis on osteoclasts in the absence of osteoblasts.

MATERIAL AND METHODS

Materials

Culture media and buffers were purchased from Invitrogen (Paisley, UK). Bulk liquids (organic solvents, acids and bases) were purchased from VWR International Ltd (Dorset, UK). Mouse recombinant RANKL was purchased from R&D Systems (Abingdon, UK) or obtained from Dr Colin Dunstan (Amgen, Thousand Oaks, USA) and mouse recombinant M-CSF was a kind gift of the Genetics Institute (Boston, USA). Human peripheral blood was obtained from healthy adult volunteers (age range 20-55; males and females), with approval from the UCL Ethics of Human Research Committee. Elephant ivory, a homogeneous, resorbable mineralised tissue with a composition similar to bone, was kindly donated by HM Customs and Excise (Heathrow, London, UK). Whole bovine femurs were obtained from a local butcher. Cylinders containing custom mixtures of O₂, CO₂ and N₂ were purchased from BOC (London, UK).

Human osteoclast assay

Prior to cultures, dentine discs or bone chips were prepared as per Hoebertz and Arnett (2003) and Murrills *et al.* (1998) protocols, respectively. Elephant ivory was prepared by cutting 200-300 µm thick transverse sections on a low speed saw with a diamond-wafering blade (Buehler, Coventry, UK). The ivory wafers produced were soaked for 2 hours in distilled water to reduce brittleness and 5 mm diameter discs were then punched out using a standard, single paper hole punch (Rexel, Aylesbury, UK). These discs fit neatly into the wells of 96-multiwell plates. Bone cylinders were cut (50-75 mm) from the diaphyseal region where cortical bone is thickest, using a hacksaw. Surplus adherent tissue was stripped off the periosteal surface using a scalpel. The cylinders were then cut radially to create 3-5 'arcs' of bone and 'defatted' by sonicating in 10-minute periods in acetone until bone is clean. Transverse slices (4.0 mm² x 0.2 mm) were cut using a low speed diamond saw (Buehler, Evanston, USA). The dentine discs/bone chips were cleaned by sonication in multiple changes of distilled water and stored dry at room temperature. Before use, dentine discs/bone chips were graphite pencil-numbered, sterilised by brief

immersion in ethanol, allowed to air-dry completely, and finally rinsed in sterile phosphate-buffered saline (PBS).

Mononuclear cells were isolated from 20 ml of human peripheral blood as described previously (Massey *et al.* 2001; Flanagan and Massey 2003). Blood was collected from normal volunteers by standard venipuncture into heparinized tubes and diluted in 15 ml of PBS. Diluted peripheral blood was gently layered over 15 ml of Ficoll-Paque (Amersham International, Bucks, UK) and centrifuged for 30 minutes at 400 x g at 25°C with the brake set “off”. The mononuclear cell layer was removed from the Ficoll-Paque/plasma interface and washed in 10 ml of PBS (10 minutes at 400 x g, 4°C). The cells were resuspended in MEM with 15% heat-inactivated fetal calf serum (FCS) and plated onto 5 mm diameter ivory discs or 4 mm² bone chips (2 x 10⁵ cells per disc) in 96-multiwell plates. Discs were washed gently after 4 hours, then cultured in minimum essential medium with 15% heat-inactivated FCS, 5 or 10 ng/ml M-CSF (depending on the age of the batch) and 5 mmol/l OH⁻ (as NaOH) in 5% CO₂ / 95% atmospheric air. After 4 days, discs were transferred to 25 cm² flasks with polyethylene ‘plug-seal’ caps (Falcon, Becton Dickinson, Oxford, UK), containing 1 ml of medium per disc plus 1 ng/ml RANKL (R&D Systems) or 5 ng/ml RANKL (Amgen). Flasks were purged via a needle inserted through the loosened cap with gas mixture containing 5% O₂, with 5% CO₂ (balance N₂) in order to increase osteoclast yield as hypoxia stimulates osteoclast formation (Arnett *et al.* 2003). The sealed flasks were incubated up to 16 days at 37°C in 5% CO₂ / 95% atmospheric air. Culture medium was replaced every 2-3 days; the final replacement was with control or test medium modified by the addition of 15, 10, 5, 0 mmol/l H⁺ (as HCl), or 5 mmol/l OH⁻ and 0-100ng/ml hPTH (Sigma-Aldrich, Poole, UK). In order to study the effect of acidosis on osteoclastogenesis, the cells were cultured from day 1 in the presence of 10 mmol/l OH⁻ (as NaOH) or 15 mmol/l H⁺ (as HCl), depending on total media volume.

Culture medium pH, PCO₂ and PO₂ were monitored using a blood gas analyser (ABL 705, Radiometer, Crawley, UK). The blood gas analyser automatically equilibrates two buffer solutions (high and low pH) by means of air and pure CO₂. The equilibrated solutions are used for electrode calibration, which was set to occur every 2 hours. Moreover, the high degree of accuracy of the blood gas analyser is

achieved via internal calibration; the regular use of known quality control standards is also necessary. The results obtained are within 0.2% of the expected values. Measured PCO₂ typically drops for each subsequent reading, causing pH values to rise accordingly. The pH readings for each group were then back-corrected to the pH value associated with the initially measured PCO₂ value, by using pH – PCO₂ calibration curves previously recorded for the appropriate media (**Figure 2.1**).

Experiments were terminated by fixing bone chips or ivory discs in 2% glutaraldehyde. Discs were then stained for TRACP by using leucocyte acid phosphatase kit (Sigma kit 387-A) according to the manufacturer's protocol. Osteoclasts were defined as TRACP-positive with two or more nuclei and /or clear evidence of resorption pit excavation (since functional mononuclear osteoclasts are often observed in human PBMC cultures). The total number of these cells and area resorbed on each discs were assessed "blind" by transmitted/reflected light microscopy. Area of resorption was assessed by reflected light microscopy via output from a Sony CCD colour video camera (DXC-151A; Sony Corporation, Japan) using standard 'dot-count' morphometry.

Statistics

Comparison between pairs of means was performed by using t-test and multiple comparisons were performed by one-way analysis of variance using 'InStat' (Version 1.13, GraphPAD software) with use of Bonferroni correction for multiple comparisons; representative data are presented as means \pm SEM for 5 or 6 replicates. Results are presented for representative experiments that were each repeated at least 2-3 times.

RESULTS

Relationship between pH and PCO₂ in tissue culture media

Figure 2.1 demonstrates the relationship between pH and PCO₂ in tissue culture media. At equilibrium with 5% CO₂ (38 mmHg), unmodified MEM with Earle's salts and 10% FCS has an operating pH of ~ 7.2. However, unmodified DMEM (used for culturing osteoblasts), at equilibrium with 5% CO₂ has an operating pH of ~ 7.4. Addition of 82 μ l of 11.5 M HCl to 100 ml of MEM or 205 μ l of 11.5 M HCl to 100 ml of DMEM provides 10 and 25 mmol/l H⁺ respectively and results in an operating pH of ~ 7.0 in a 5% CO₂ atmosphere (metabolic acidosis).

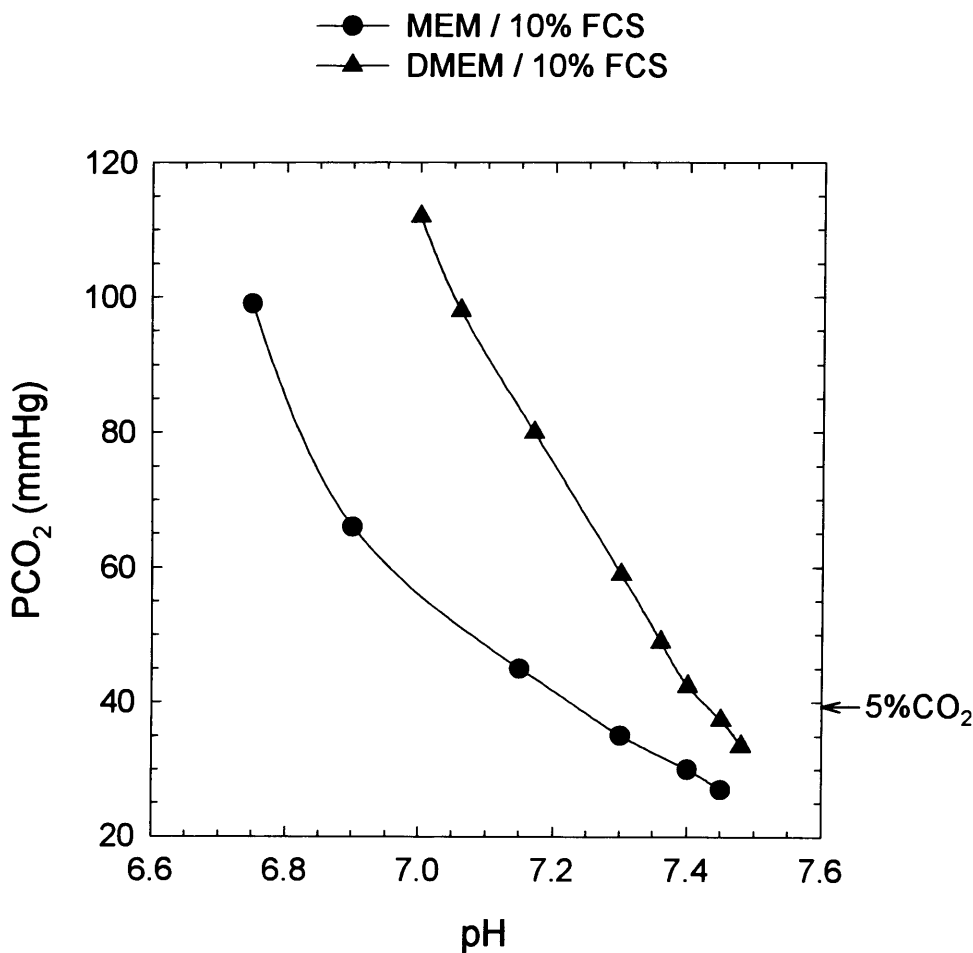


Figure 2.1. Relationship between pH and PCO₂ in tissue culture media.

Acid-activation of blood-derived human osteoclasts

Osteoclast resorptive activity was markedly increased by a reduction in extracellular pH from 7.42 to pH 7.01 (**Figure 2.2**). Note that human osteoclasts cultured at non-acidified conditions (pH 7.42) still retained some resorptive function (**Figure 2.2 B**), unlike rat osteoclasts which are almost completely inactive at pH 7.4 (Arnett and Dempster 1986; Arnett and Spowage 1996).

Reducing extracellular pH for the final 3 days of culture caused a striking, progressive increase in resorption pit formation by blood-derived osteoclasts cultured on bone chips. The acid-response was highly reproducible with peak stimulation (5-6 fold) of resorption close to pH 7.0 and half-maximal activation at pH ~7.3 (**Figure 2.3**). Osteoclasts behaved similarly when cultured on dentine or bone chips (data not shown).

As shown in **Figure 2.4**, at physiological pH (~7.4), basal level of resorption of osteoclasts generated from PBMCs obtained from different donors was low. These results are representative of experiments carried out using PBMCs from 8 healthy adult volunteers (age range 20-55; males and females). Reducing pH from ~7.4 to ~7.0 increased the area resorbed/osteoclast by ~9-fold for donors 1 and 3 and by ~5-fold for donors 2 and 4 (**Figure 2.4 A**). The effect of pH treatment on the total number of osteoclasts per dentine disc was either not significant between the treatment groups or only modestly different (**Figure 2.4 B**). However, even modest changes in osteoclast number could not account for the marked increase in resorption due to acidosis. For example, osteoclast obtained from donor 3 showed a 25% increase in the total number of osteoclasts when these cells were cultured at low pH, however the increase in resorption activity/osteoclast was 9-fold.

Functional, mononuclear osteoclasts were often observed in hPBMC cultures as shown in **Figure 2.5**.

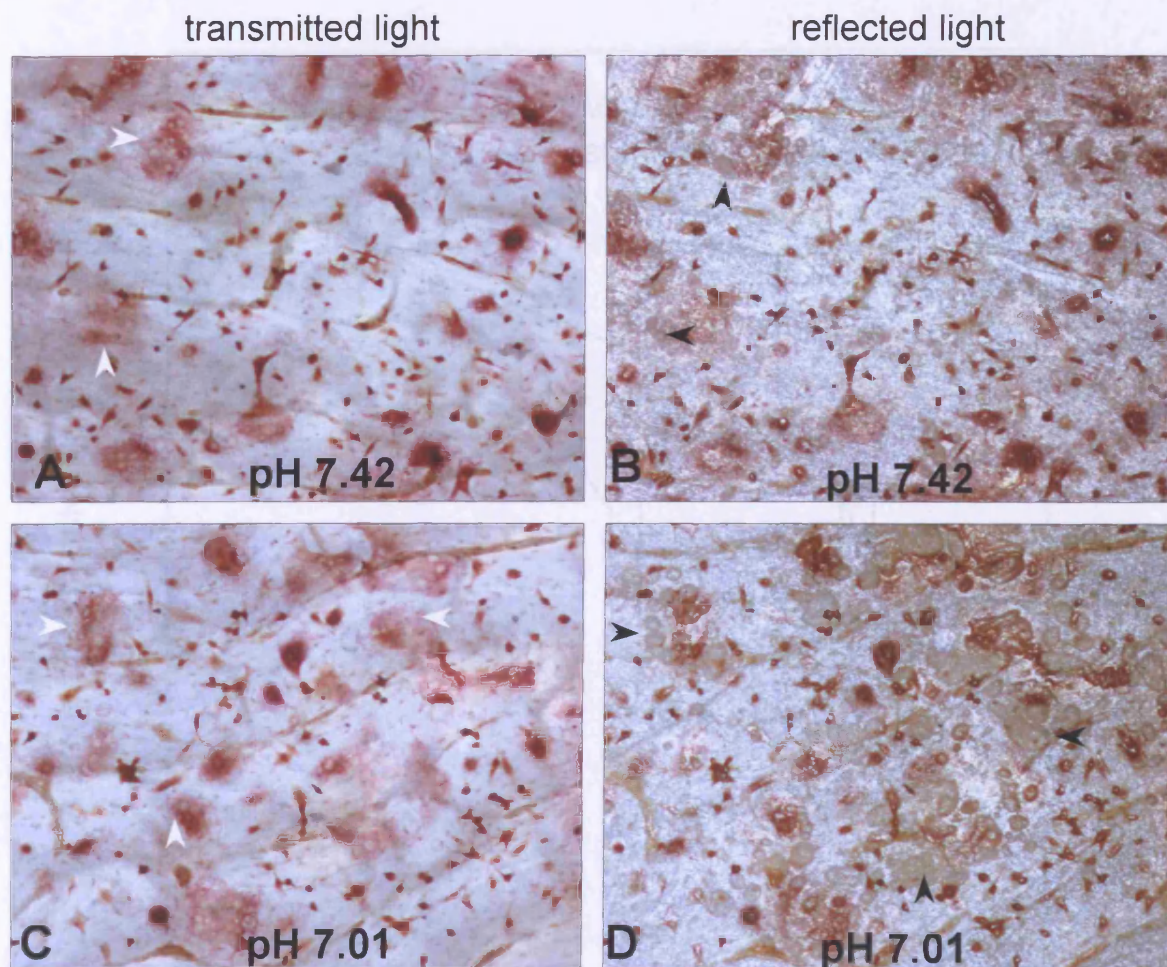


Figure 2.2. Stimulatory effect of acidosis on osteoclast activation in human PBMC cultures. Representative micrographs (10x objective) of cells cultured on 16 mm² bone chips in MEM for 12 days with RANKL (1ng/ml), M-CSF (5ng/ml) and 5 mmol/l OH⁻ to generate osteoclasts, followed by 2 days in MEM modified by the addition of 15 mmol/l H⁺ or 5 mmol/l OH⁻. A. transmitted light image of osteoclasts cultured at pH 7.42. B. corresponding reflected light image. C. transmitted light image of osteoclasts cultured at pH 7.01 for the last two days of culture. D. corresponding reflected light image. White arrows point to examples of TRACP-positive osteoclasts and black arrows to resorption pits. Note the presence of very few resorption pits at pH 7.42 (B) whereas abundant pits (dark grey areas) are seen when cells are cultured at pH 7.01 (D).

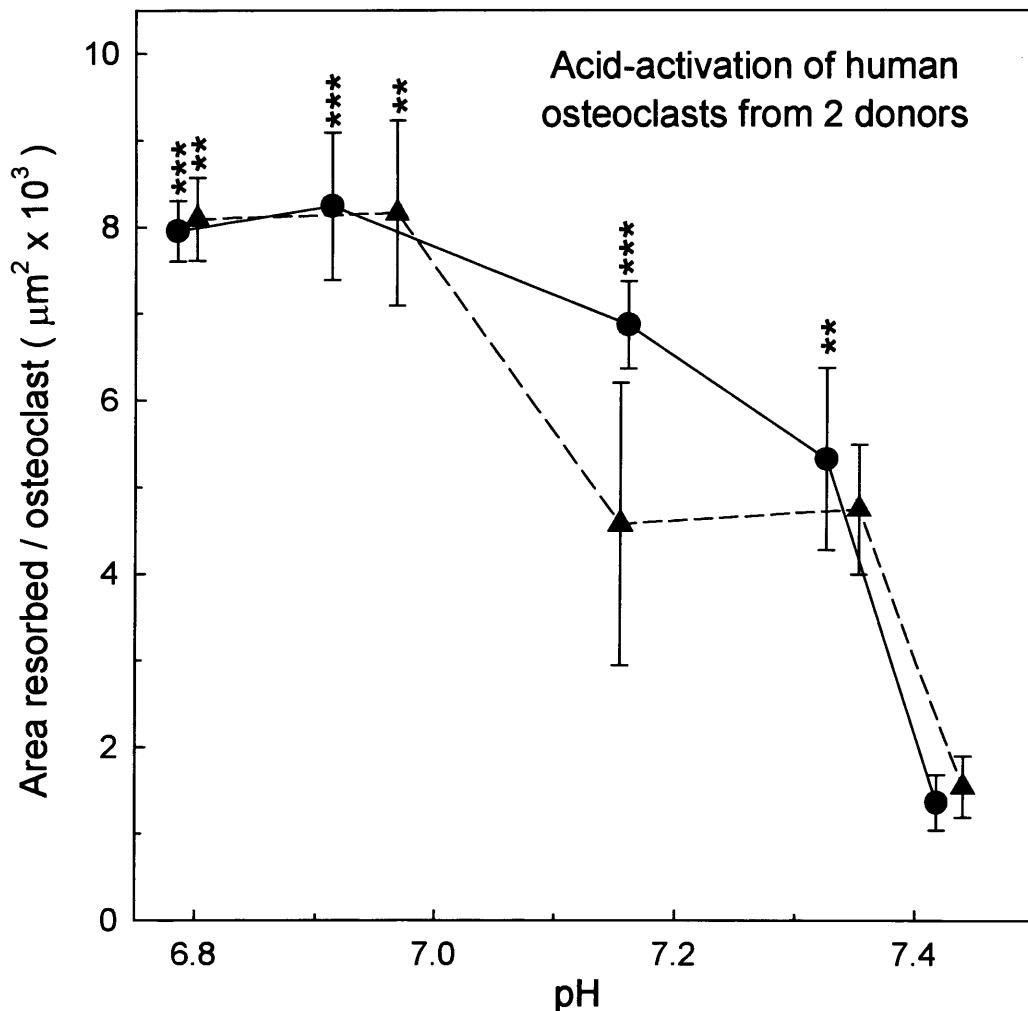


Figure 2.3. Acidosis stimulates resorption pit formation by human osteoclasts formed from PBMCs. Cells were cultured on 16mm² bovine bone chips in MEM for 16 days with RANKL (5ng/ml), M-CSF (10ng/ml) and 5 mmol/l OH⁻ to generate osteoclasts, followed by 3 days in MEM modified by the addition (from left to right) of 15, 10, 5, 0 mmol/l H⁺, and 5 mmol/l OH⁻. Significantly different from respective control (pH 7.42 and pH 7.40): *p<0.05, ** p <0.01, ***p<0.001. Values are means ± SEM (n = 6).

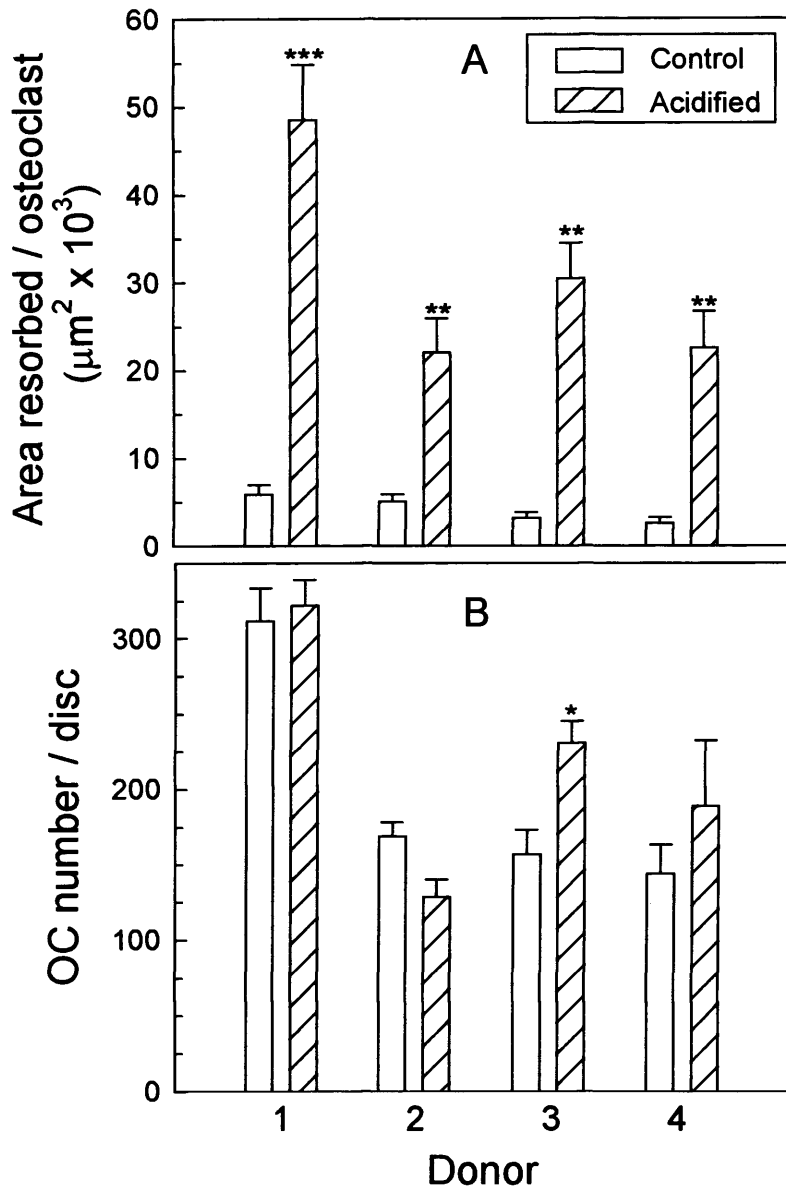


Figure 2.4. Effect of acidosis on osteoclast number and resorption pit formation by human osteoclasts from four healthy donors. Cells were cultured on 5 mm diameter dentine discs in MEM for 14 days with RANKL (1ng/ml), M-CSF (5ng/ml) and 5 mmol/l OH^- to generate osteoclasts, followed by 2 days in MEM modified by the addition of 15 mmol/l H^+ or 5 mmol/l OH^- . pH values of control (*i.e.* blood pH) groups for donors 1-4 were 7.41, 7.38, 7.43, 7.39, respectively, and those of acidified groups for donors 1-4 were 6.97, 7.01, 6.95 and 6.98, respectively. These results are representative of experiments carried out using PBMCs from 8 healthy adult volunteers (age range 20-55; males and females). Significantly different from the respective control group: * $p < 0.05$, ** $p < 0.01$. Values are means \pm SEM ($n = 8$).

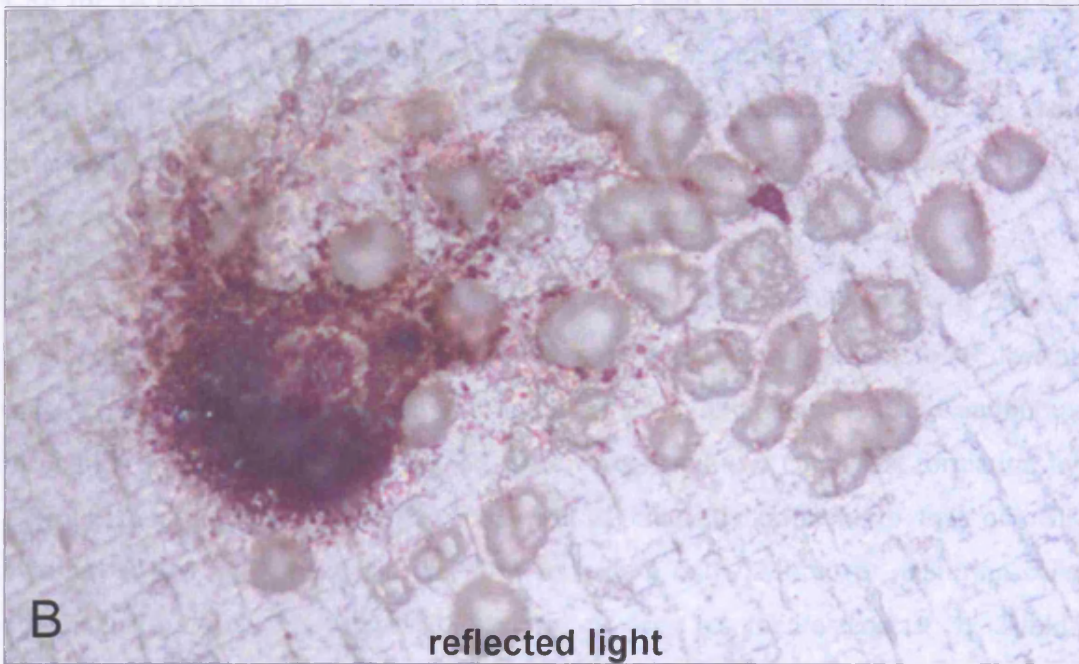
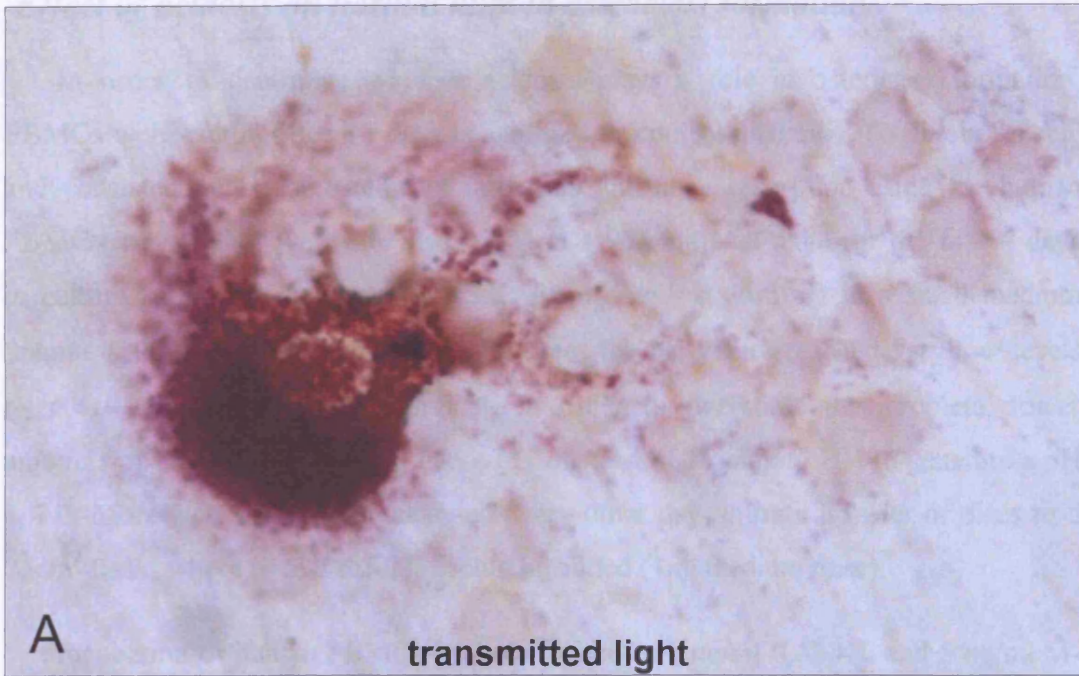


Figure 2.5. Typical appearance of a TRACP-positive mononuclear cell derived from human PBMC cultures able to resorb dentine substrate (40x objective). A. transmitted light image of a typical mononuclear osteoclast. B. corresponding reflected light image, showing multiple small resorption pits (grey, cloud-like features).

Effect of acidosis on normal human osteoclast formation

In order to determine whether acidosis plays a role in osteoclast formation, PBMCs were cultured for 14 days in acidified or control medium. To obtain reliable and consistent levels of osteoclast formation and bone resorption using the human PBMC assay, cells have to be maintained in a 96-multiwell plate for the first 4 days in culture. I found that culturing cells from day 1 at low pH in a small medium volume (200 μ l) was difficult as at many times the pH values reached very low levels (\sim 6.4), which affected cell survival. In order to overcome this problem, lower amounts of HCl were added (10 mmol/l H⁺ instead of 15 mmol/l H⁺) to generate a pH \sim 7.0. Moreover, media were changed every other day up until transfer of discs to a 25cm² flask, where excess medium could be added (1ml medium /disc).

Incubation of human PBMCs in the presence of 1 ng/ml RANKL and 5 ng/ml M-CSF for 14 days at pH 7.43 resulted in abundant TRACP-positive multinucleated cell formation (171 \pm 6 osteoclasts/disc), but almost no resorption (0.50 \pm 0.05 mm²). These cells were still able to resorb at pH \sim 7.45, contrary to rodent and osteoclastoma-derived cells that are completely “switched off” above pH 7.30 and 7.40 respectively (Arnett and Spowage 1996; Hoebertz *et al.* 1999).

In cultures maintained at control pH (7.45) for 12 days followed by 2 days in acidified medium (pH 6.99), formation of human osteoclasts was similar to 14 days at pH 7.46, but resorption pit formation was increased 11-fold (**Figure 2.6**). Continuous incubation in acidified media (pH 7.01 for 14 days) reduced osteoclast formation by \sim 2-fold, but the area resorbed/disc was not significantly different to that of cells cultured at low pH for the last two days in culture only. Moreover, culturing cells continuously at low pH increased the ratio of area resorbed/osteoclast by 2-fold: 0.072 \pm 0.008 mm² compared to 0.036 \pm 0.007 mm² when cells were acidified for the last two days in culture.

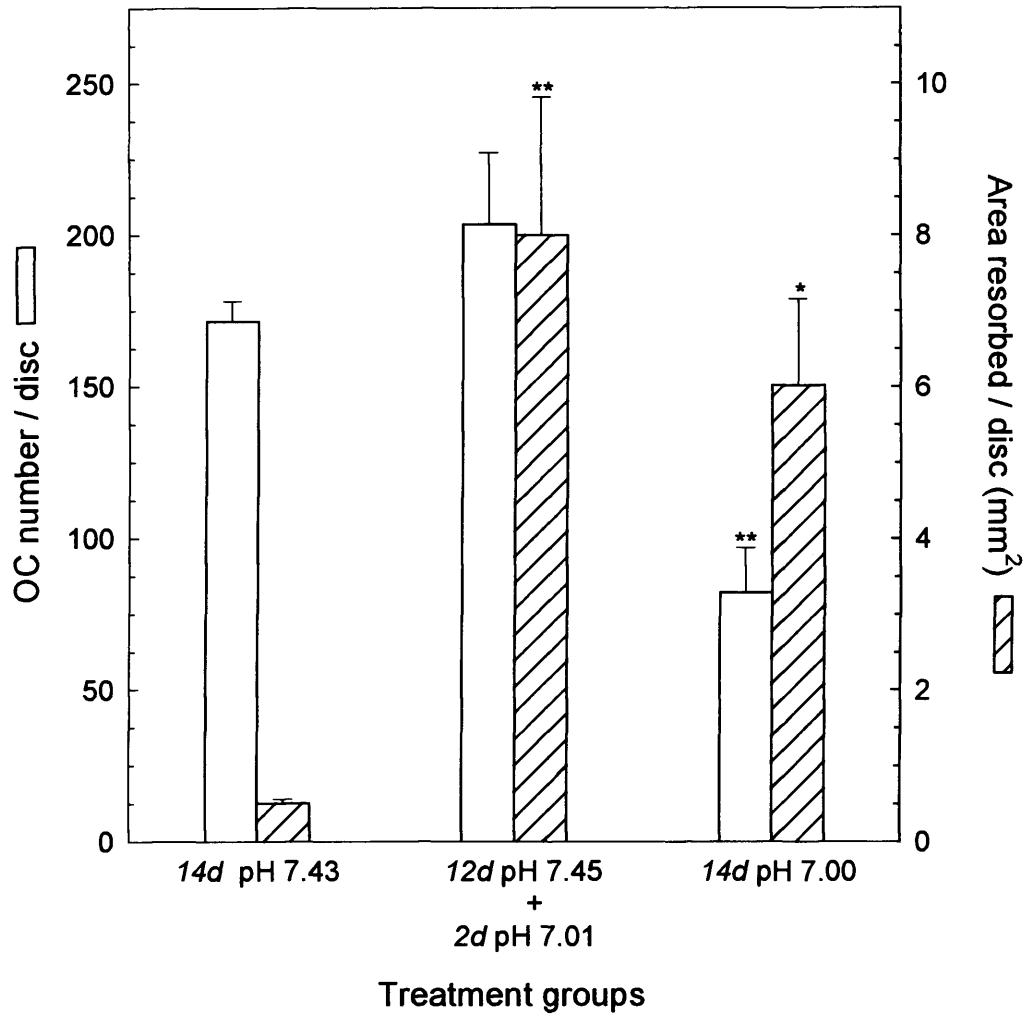


Figure 2.6. Effect of extracellular pH on osteoclast formation and activity. Cells were cultured on 5mm diameter dentine disc in MEM for 14 days with RANKL (1ng/ml), M-CSF (5ng/ml) in the presence of 5 mmol/l OH⁻ or 15mmol/l H⁺ and for 12 days in the presence of 5 mmol/l OH⁻ followed by 2 days in the presence of 15mmol/l H⁺. Significantly different from control (14d at pH 7.43): *p<0.5, ** p <0.01. Values are means ± SEM (n = 8).

Interaction between PTH and pH

To test whether PTH influences osteoclast function in the absence of osteoblasts, PBMCs were cultured for 12 days in the presence of 1 ng/ml RANKL and 5 ng/ml M-CSF and 7.5 mmol/l OH⁻, followed by 2 days in medium modified by the presence of 50 and 100 ng/ml hPTH at control or acidified conditions. In this experiment, a larger amount of NaOH was added to the culture media to increase the pH levels of the control group from ~7.40 (generated when 5 mmol/l H⁺ is added) to ~7.46 in order to “switch off” the cells almost completely. I found that CO₂ levels must be maintained at constant levels throughout the experiment otherwise even very small fluctuations due to opening the incubator door too many times, for example, increased the pH levels up to 7.50, which adversely affected cell survival. Surprisingly, even when medium pH reached 7.52, osteoclasts were still present, but the number of cells was greatly reduced (data not shown). The final pH measurements for the groups were as follows: 7.46, 7.45 and 7.48 for 0, 50 and 100 ng/ml PTH respectively for the control group, and 7.01, 7.02 and 7.00 for 0, 50 and 100 ng/ml PTH respectively for the acidified group. **Figure 2.7** shows clearly that the effect of PTH on these cells was dependent on extracellular acidification. Culturing cells in the presence of 50 ng/ml PTH at low pH increased osteoclast resorptive activity by 1.9-fold, whereas no effect was seen when these cells were cultured at control pH (~ 7.48) in the presence of 50 or 100 ng/ml PTH. Note that the combined effect of acidosis and PTH (100 ng/ml) caused a 45-fold increase in resorption compared to control group (pH 7.46, no PTH). The lack of PTH effect at non-acidified cultures was only observed when osteoclasts were completely “switched off” which only happened pH ~ 7.45, however, the effect was always upmost at pH ~7.0. There were no significant changes in the number of osteoclasts due to PTH treatment for the last 2 days in culture.

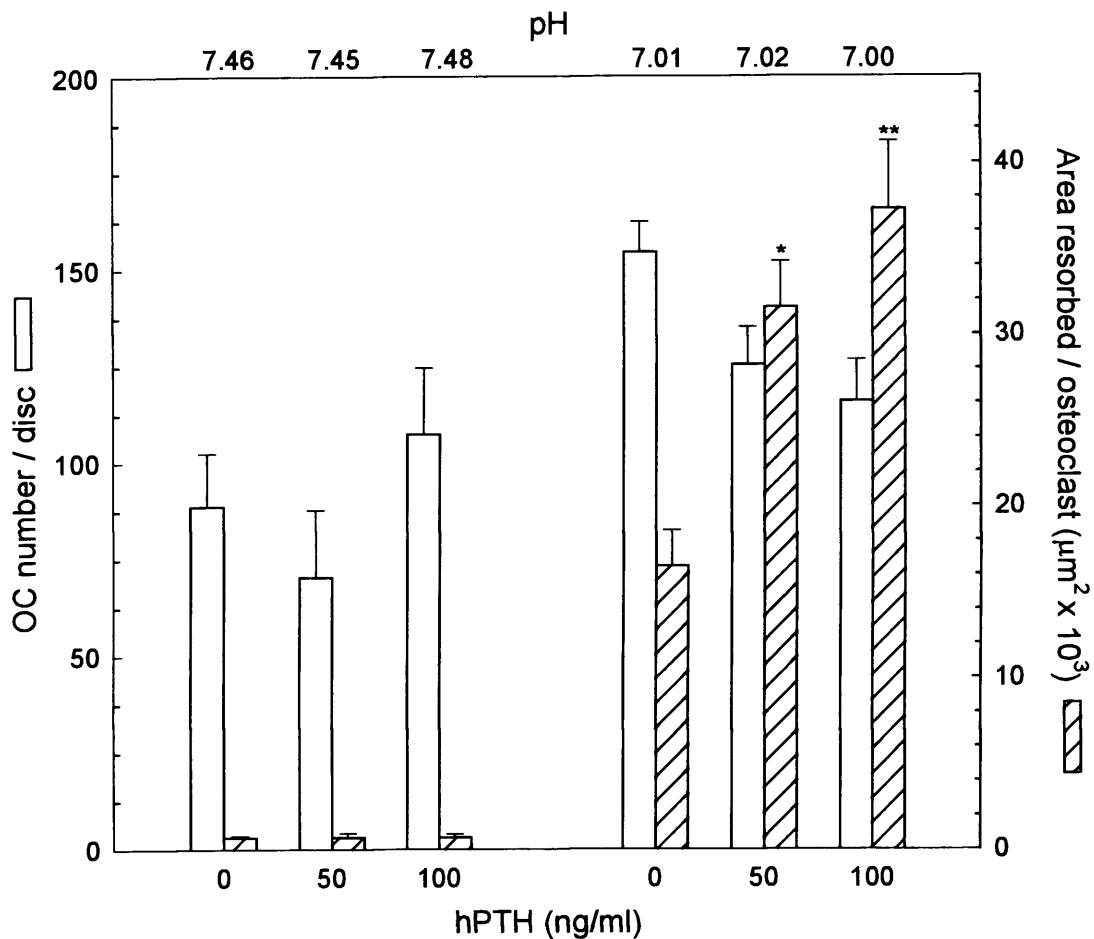


Figure 2.7. Effect of PTH on human osteoclasts derived from PBMCs. Cells were cultured on 5 mm dentine discs in MEM for 12 days with RANKL (1ng/ml), M-CSF (5ng/ml) and 5 mmol/l OH⁻ to generate osteoclasts, followed by 2 days in MEM modified by the addition of 0, 50 or 100 ng/ml PTH in the presence of 15 mmol/l H⁺ or 5 mmol/l OH⁻. Significantly different from the respective control (no PTH added): *p<0.5, **p <0.01. Values are means ± SEM (n = 8).

DISCUSSION

The results presented in this chapter show that mature normal human osteoclasts are activated by acidosis. However, osteoclast formation was optimal at around pH 7.40 and was inhibited by low pH. In a second series of experiments, the interaction between PTH and pH on human osteoclasts was studied. The results obtained showed PTH is able to regulate the resorptive function of human osteoclasts directly when cells were cultured at acidified conditions.

Reducing extracellular pH for the final 2-3 days of culture caused striking, progressive increases in resorption pit formation by normal human osteoclasts, with peak stimulations up to 9-fold at pH ~6.9-7.0. The acid-activation curve of human osteoclasts differed from that of rat or mouse osteoclasts, in that it was shifted markedly in the alkaline direction, with half-maximal activation at pH ~7.3 as opposed to pH ~7.1 for rodents (Arnett and Dempster 1986; Arnett and Spowage 1996). Most surprisingly, the pH-activation profile of human osteoclasts corresponds with that of the recently discovered H⁺-sensing human G-protein-coupled receptor reported to be present on bone cells (Ludwig *et al.* 2003; Komarova *et al.* 2005). Such pH sensors will be discussed in detail in Chapter 5.

The stimulatory response of normal human osteoclasts to acid was not donor-specific. Studies carried out in osteoclasts obtained from individuals from different genetic backgrounds, sex and age showed that despite different basal levels of resorption, lowering the pH for the final 2 days in culture from ~7.4 to ~6.9 was enough to cause at least 5-8 fold increases in osteoclast resorptive activity.

In contrast to the stimulatory effect of pH on osteoclast activity, the formation of osteoclast from PBMCs is optimal at around pH 7.40 and is somewhat inhibited at low pH (~2-fold inhibition at pH 7.0). This result is similar to that obtained for mouse osteoclasts (Hoebertz and Arnett 2003). However, the resorptive activity of these cells was not decreased by continuous incubation at acidified medium. On the contrary, the osteoclasts present were more active compared to those cultured in acidified medium for the last two days in culture.

In the course of this study, TRACP-positive, human mononuclear cells were found to be capable of bone and dentine resorption. These findings are in agreement

with previous reports that showed mononuclear cells *in vitro* from chick (Prallet *et al.* 1992), murine and human sources are capable of resorbing bone (Sarma and Flanagan 1996; Nicholson *et al.* 2000). Analysis of resorption cavities in human iliac crest biopsies has shown that as much as two thirds of the cavity may be excavated by mononucleated rather than multinucleated osteoclasts (Eriksen 1986). Most recently, it has been demonstrated that CD14⁺ cultures give rise to a mixed population of mononucleated and multinucleated cells capable of resorption, suggesting heterogeneity in the maturity of the CD14⁺ osteoclast precursors. These authors suggested that with longer incubation periods the mononucleated cells might become multinucleated (Nicholson *et al.* 2000).

In a second series of experiments, the interaction between PTH and pH on mature normal human osteoclasts was studied. This is the first study to show a direct effect of PTH on osteoclast activity and supports the findings of our collaborators who showed that human osteoclasts express functional PTH receptors (Dempster *et al.* 2005). This is evidence against the belief that PTH effect is osteoblast-mediated (Rodan and Martin 1981; McSheehy and Chambers 1986a; McSheehy and Chambers 1986b); this will be discussed in more detail in Chapter 6. Consistent with my results, it has been reported that PTH caused a rapid and dramatic increase in superoxide anion production (an index of cell activity) by rat osteoclasts, whereas treatment of osteoblast-like cells with PTH had no such effect (Datta *et al.* 1996). PTH also appears to activate CAII activity and consequently regulates the acid production in avian and rat osteoclasts (Anderson *et al.* 1985; Hunter *et al.* 1988). It has also been shown that PTHrP stimulates deer antler osteoclast formation via a mechanism that only partially involves the RANKL pathway (Faucheux *et al.* 2002). Recently, Gay and colleagues (2003) have proposed that PTHrP, which is produced by both osteoblasts and osteoclasts, may play a role in the communication between these two cells, i.e., PTHrP produced by osteoblasts may regulate osteoclast activity via PTH1R in osteoclasts, and vice versa.

Osteoclast activation by slight extracellular acidification (pH ~7.0) is the key initial requirement for the stimulatory action of PTH on resorption to occur. The stimulatory action of PTH on osteoclastic resorption is broadly in line with earlier reports of the activation of mature rat osteoclasts by this molecule at low pH in low

density mixed cultures containing cells of the osteoblast lineage (Arnett and Dempster 1986). The actions of other osteolytic agents *e.g.* 1,25-(OH)₂D₃, RANKL, ATP and ADP are also enhanced by acidification (Morrison *et al.* 1998; Murrills *et al.* 1998; Hoebertz and Arnett 2003). Conversely, slight alkalisation markedly attenuates the osteolytic action of PTH, 1,25-(OH)₂D₃ and PGE₂ in calvarial cultures (Meghji *et al.* 2001). Thus, osteoclast activation can be regarded as a 2-step process, with acidification the obligatory first step. Moreover, the weight of evidence now indicates strongly that extracellular protons can be considered to represent the long sought “osteoclast activation factor” (OAF).

My observations on the direct action of PTH on osteoclasts reinforce the necessity for studies of the effect of many pro-resorptive agents using assays such as PBMCs or CD-14⁺ cells (Flanagan and Massey 2003). It is clear that in the presence of cells other than osteoclast precursors, molecules can exert different effects. For example, TGF β is known to enhance osteoclast formation from PBMCs (Massey *et al.* 2001) but inhibits osteoclastogenesis in human bone marrow cultures, in which there is a stromal cell population (Sarma and Flanagan 1996).

In summary, the results presented in this chapter give evidence for the important role of acidosis on human osteoclasts, and also provides formal proof that accessory cells, such as osteoblasts or stromal cells, are not required for the acid-activation response of mature osteoclasts. Extracellular pH is clearly an important influence on bone homeostasis and must additionally be considered a critical factor in the design, monitoring and interpretation of all bone resorption experiments. Moreover, the findings presented here suggest that there seems to be a dual regulatory effect of PTH on bone cells. This molecule acts directly on human osteoclasts and also, indirectly, via osteoblasts. Appreciation of this dual regulatory mechanism may ultimately advance our understanding of the complex effects of PTH on the human skeleton.

CHAPTER 3

Effect of acidosis on resorption-associated factors *in vitro*

INTRODUCTION

Bone organ culture and acidosis

Organ culture of neonatal mouse calvaria studies have been used to investigate whether the Ca^{2+} efflux from bone by acidosis occurs due to physicochemical dissolution of bone or by cell-mediated means. This model offers key advantages. First, the bone cells exist in their normal histological and anatomical relationship. Second, the release of cold Ca^{2+} gives a more valid representation of bone resorption since it reflects changes in total mineral dissolution, whereas measurement of $^{45}\text{Ca}^{2+}$ release, as used previously by other investigators (Dominguez and Raisz 1979; Bushinsky *et al.* 1992), only demonstrate changes in resorption of relatively immature mineral (Meghji *et al.* 1998).

Bushinsky and colleagues showed that in short-term neonatal mouse calvarial cultures (3 hours), the net Ca^{2+} efflux observed when the pH was decreased from ~7.40 (either by reducing HCO_3^- or by increasing CO_2) appeared to be due to physicochemical dissolution of bone calcium carbonate (Bushinsky *et al.* 1983; Bushinsky *et al.* 1985; Bushinsky and Lechleider 1987). However, after 24-48 hours, cell mediated Ca^{2+} release predominated in response to metabolic acidosis (Bushinsky 1989; Bushinsky *et al.* 1992; 1995). More recently, it has been reported that the steep response of mouse calvarial bones to metabolic acidosis is very similar to that of isolated mature rat osteoclasts in which resorption is essentially “switched off” above pH 7.20 and stimulated maximally at pH ~6.90 (Meghji *et al.* 2001). Critically, acid-stimulated Ca^{2+} release was not observed in dead bones and was completely blocked from live bones by calcitonin (Meghji *et al.* 2001). These findings confirmed and amplified the earlier work of Goldhaber and Rabadjija who

suggested that H^+ stimulated Ca^{2+} release from bone is osteoclast mediated (Goldhaber and Rabadjija 1987).

Contrary to the reported behaviour of disaggregated rat osteoclast cultures (Arnett *et al.* 1994), stimulation of resorption in mouse calvarial cultures are more sensitive to metabolic than respiratory acidosis (Bushinsky *et al.* 1992; Meghji *et al.* 2001). The reasons why a reduction in bicarbonate concentration should have a greater effect on osteoclastic bone resorption than an increase in the partial pressure of carbon dioxide in calvaria are unclear. However, a low extracellular HCO_3^- concentration (as in metabolic acidosis) might be expected to facilitate the exchange of intracellular HCO_3^- for Cl^- , leading to enhanced H^+ availability for increased resorption. Increasing CO_2 levels (as in respiratory acidosis) could promote calcium carbonate accretion and the formation of carbonated apatite (Bushinsky 1995; Meghji *et al.* 2001). This may explain the apparent reduction in calcium efflux seen in calvarial cultures incubated in medium acidified by increased PCO_2 . During metabolic acidosis however, the low HCO_3^- concentration would be expected to favour the dissolution of bone calcium carbonate and carbonated apatite.

Goldhaber and Rabadjija (1987) were the first to suggest that the stimulatory effect of acidosis on bone resorption in whole organ cultures might involve prostaglandin synthesis, as addition of indomethacin (100ng/ml) to the cultures strongly inhibited the Ca^{2+} release by added protons. Indomethacin acts as a non-selective inhibitor of cyclooxygenase activity (*i.e.* COX-1 and COX-2) and thus, prostaglandin production (Vane 1971). These findings are in agreement with the observation that ibuprofen, another inhibitor of prostaglandin synthesis, produces the same effect in whole organ cultures (Meghji *et al.* 2001). Acid stimulated Ca^{2+} release in mouse calvaria was also shown to be blocked by the 5-lipoxygenase inhibitors, MK 886 and BW 70C (Meghji *et al.* 2001). This finding suggests that leukotrienes, stimulators of bone resorption in a number of systems, may also be involved in mediating the acidosis effect in mouse calvarial cultures (Meghji *et al.* 1988; Garcia *et al.* 1996; Meghji *et al.* 2001)

The effect of acidosis on bone organ culture is not restricted to osteoclasts. The expression of collagen type 1 and alkaline phosphatase decrease when bone cells are cultured at low pH (~6.80) compared to control (~7.40) suggesting that osteoblast

activity is suppressed by acidosis (Krieger *et al.* 1992). RANKL mRNA expression has been shown to increase when calvarial bones are cultured at low pH (Frick and Bushinsky 2003). This finding led to the conclusion that acidosis stimulates prostaglandin production by osteoblasts, which acting in a paracrine manner increases osteoblastic RANKL synthesis. RANKL then stimulates osteoclastic activity and osteoclastogenesis to promote bone resorption and buffering of proton load (Frick and Bushinsky 2003).

Moreover, it has been demonstrated that PTH and insulin-like growth factor 1 and 2 (IGF-1 and 2) enhance medium acidification by primary rat osteoblasts and osteoblast-like UMR-106 and SaOS-2 cells. These authors measured real time changes in the pH beneath osteoblasts after stimulation with either IGF-1, IGF-2 or PTH; the drop in medium pH could conceivably explain the indirect effect of these factors on osteoclast function (Barrett *et al.* 1997; Santhanagopal and Dixon 1999). It has also been shown that treatment of calvarial bones with PTH, $1,25(\text{OH})_2\text{D}_3$ and PGE_2 is associated with medium acidification which could by itself stimulate resorption to some extent in bone organ cultures (Belinsky and Tashjian, Jr. 2000; Meghji *et al.* 2001).

Regulation of osteoclast gene expression by metabolic acidosis

The mechanism by which extracellular acidification stimulates osteoclasts to resorb bone is still not clear. However, since the discoveries of H^+ -sensing receptors present on bone cells it seems reasonable to hypothesise that acidosis would alter the specific pattern of gene expression in osteoclasts (Ludwig *et al.* 2003; Komarova *et al.* 2005).

Studies carried out in mature rat osteoclasts showed that upregulation of osteoclastic expression of mRNA for calcitonin receptor (CTR) and carbonic anhydrase II (CAII) occurs within 4 hours of acidification (Biskobing and Fan 2000). CTR is an osteoclast marker and it has been reported that calcitonin binding to its receptor results in rearrangement of the osteoclast cytoskeleton and decreased bone resorption (Baron *et al.* 1990; Komarova *et al.* 2003). It has been suggested that CTR upregulation occurs as the osteoclasts become activated by acidosis to allow a rapid downregulation of the cell once the resorptive stimulus is removed, thereby

CTR works as negative regulator (Biskobing and Fan 2000). CAII catalyses the production of protons, which are actively secreted by osteoclasts into the resorption lacunae for the dissolution of bone mineral. In addition to increased CAII mRNA expression, the vacuolar ATPase (V-ATPase) pump activity has also been reported to be increased by low extracellular pH (Nordstrom *et al.* 1997). The upregulation of these two factors augments the capacity of bone resorption by osteoclasts (Biskobing and Fan 2000). Other investigators also showed by RT-PCR that CAII mRNA expression increased in individual rabbit osteoclasts in response to lowered pH (Asotra *et al.* 1994).

In order to understand better the response of osteoclasts to acidosis, I studied the expression of mRNAs for the resorption associated genes cathepsin K, TRACP, TRAF-6 and c-src at control (pH ~7.4) and acidified (pH ~6.9) conditions in mouse organ bone cultures. The protein expression of TRACP and cathepsin K was also investigated in normal human osteoclast cultures when exposed to the same conditions.

MATERIALS AND METHODS

Materials

Culture media and buffers were purchased from Invitrogen (Paisley, UK). Bulk liquids (organic solvents, acids and bases) were purchased from VWR International Ltd (Dorset, UK). Molecular biology reagents were purchased from Promega (Southampton, UK).

Mouse calvarial bone resorption assay

The method, which measures bone resorption as Ca^{2+} release from neonatal mouse calvaria, is similar to that described in detail by Meghji *et al.* (1998). Calvaria were dissected from five-day-old mice (MF1-strain), which had been killed by cervical dislocation. The calvaria were pooled and washed in phosphate-buffered saline (PBS), before being divided along the sagittal suture and placed in fresh PBS. Half-calvaria were cultured individually on 1 cm² stainless steel grids (Minimesh, FDP quality) at the air-liquid interface in 6-well plates with 1.5 ml of Biggers, Gwatkin and Heyner medium (BGJb), 5% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, in a humidified 5% CO₂ incubator. After an initial 24-hour pre-incubation period, the medium was removed and replaced with control or test media, modified by the addition of 15 mmol/l H⁺, 1µM PGE₂ and 2µg/ml osteoprotegerin (OPG). Each experimental group consisted of five individual cultures. The bones were maintained for an extra 72-hour period without further medium changes, and without opening the incubator door, so as to ensure constant CO₂ levels and to minimise pH fluctuations.

After 72 hours, experiments were terminated by withdrawing culture medium. Bones were either transferred to Eppendorf tubes containing 1ml of RNAlater (Sigma-Aldrich Co., Dorset, UK) and stored at -20°C until use. Incubator CO₂ was determined by immediately measuring a culture medium sample using the blood gas analyser. The mean final pH of each treatment group was determined by removing and pooling a 100 µl sample from each replicate; the pooled samples were then re-equilibrated with CO₂ in the incubator before measurement; slight differences in CO₂

tension between groups were normalised to the initially measured value using pH-CO₂ calibration curves constructed as previously described (Murrills *et al.* 1998).

Ca²⁺ measurement

Resorption was assessed as Ca²⁺ release into the culture medium over the 72-hour period. Ca²⁺ concentrations in culture medium at the end of experiments were measured colorimetrically by autoanalyser (Chem Lab Instruments, UK) using the following procedure. Samples were acidified with excess 1 M HCl and subjected to continuous flow dialysis against the metal complexing agent, cresolphthalein complexone (CPC), to separate Ca²⁺ from proteins; 2.5 g/l of 8-hydroxyquinoline was added to samples to eliminate Mg²⁺ interference. Dialysed Ca²⁺ bound to CPC was then determined following reaction with 2-amino-2-methylpropano-1-ol; the absorbance of the resultant purple-coloured solution was measured at 570 nm.

Dr Sajeda Meghji (Eastman Dental Institute, UCL) performed the calvarial assay and Ca²⁺ measurements.

Human osteoclast formation assay

Human osteoclasts derived from PBMCs were obtained as per protocol detailed in Chapter 2. At the end of the experiment, the cells were either lysed for RNA extraction or immunostained as explained below.

RNA extraction

RNA from bone tissue or osteoclast cells was extracted using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Paisley, UK). The bone tissue was immersed in an Eppendorf tube containing 1ml of RNAlater or TRIzol reagent and stored at -70°C until cell lysis. When using TRIzol, the Eppendorf tubes were covered with tin foil, immersed in liquid nitrogen and then smashed by using a hammer. The frozen tissue was transferred to a chilled mortar and samples were ground. Care was taken to remove any plastic from the mortar. The bone tissue immersed in RNAlater was transferred to a chilled mortar and ground. The ground sample was then transferred to an Eppendorf tube and 1ml of TRIzol reagent added. Cells were lysed in TRIzol reagent for 5 minutes prior to commencement of

extraction. After lysis, chloroform (200µl) was added and tubes were mixed by inversion for 20 seconds and incubated at room temperature for 3 minutes. At the end of incubation period the samples were centrifuged at 12500 x g for 15 minutes and after centrifugation, the aqueous layer was transferred to a clean tube containing 5 µg of glycogen (RNA carrier) and 500 µl of isopropanol. At this point the samples were stored at -20°C overnight to increase nucleic acid precipitation. The samples were then spun for 10 minutes at 12500 x g, the supernatant discarded and 1ml of 75% ethanol added. The samples were centrifuged at 7500 x g for 5 minutes and after centrifugation, the supernatant was discarded and the pellet was allowed to air dry. RNA was resuspended in 50 µl of DEPC-water and it was quantified by measuring the absorbance at 260nm using a spectrophotometer. At 260nm, one absorbance unit is equivalent to 40 ng/ml RNA.

DNase treatment

This was carried out by using RQ1 RNase-free DNase (Promega) according to the manufacturer's protocol. The DNase digestion reaction was set up as follows:

- Up to 8µl RNA
- 1 µl RQ1 DNase 10x reaction buffer
- 1u/µg RNA of RQ1 RNase-free DNase
- Nuclease-free water to a 10 µl final volume

The sample was incubated for 30 minutes at 37°C and 1 µl RQ1 DNase stop solution added to terminate the reaction, followed by a 10-minute incubation period at 65°C to inactivate the DNase. At the end of the experiment, the total RNA was quantified as explained above. Samples were stored at -70°C until use.

cDNA reaction

This was performed by using ImProm-II™ reverse transcriptase according to the manufacturer's protocol. The DNase-treated RNA reaction was set up as follows:

- 100 ng/reaction RNA template
- 0.1 µg oligo (dT)₁₂₋₁₈
- Up to 5 µl of double distilled water

The reaction was incubated at 70°C for 5 minutes and quick chilled at 4°C for at least 5 minutes. The reaction mixture was then transferred to a tube containing the following reagents:

- ImProm-II™ 5x reaction buffer
- 3mM MgCl₂
- 0.5mM dNTP mix;
- 20 U recombinant RNasin ribonuclease inhibitor
- 1 µl ImProm-II™ reverse transcriptase
- Up to 15 µl double-distilled water

The final 20µl volume reaction was incubated for 5 minutes at 25°C (annealing cycle) followed by incubation for 60 minutes at 42°C (elongation cycle) and the reverse transcriptase was heat-inactivated by incubating the reaction for 15 minutes at 70°C. At the end of the experiment the cDNA was stored at -20°C until use.

Polymerase chain reaction

PCR was carried out on cDNA generated from total RNA. RT-PCR reactions were set up as follows:

- 2.5 µl 10 x PCR buffer
- 1.5mM MgCl₂
- 1mM dNTP mix
- 1mM forward primer
- 1mM reverse primer
- 0.2 µl Taq polymerase (5U/µl)
- 1 µl cDNA
- Up to 25 µl double distilled water

Amplification was performed in an Eppendorf master cycler gradient PCR machine programmed for the following cycles:

- One cycle of two minutes at 94°C
- 28-35 cycles of 30seconds at 94°C, 30sec at annealing temperature, 30 seconds at 72°C
- One cycle for 7minutes at 72°C

The oligonucleotide primers (MWG, Ebersberg, Germany) and the annealing temperature used to amplify the different genes under study are shown in **Table 3.1 and 3.2**. The annealing temperature and the elongation period were determined empirically based on the melting temperature (T_m) of each primer and size of PCR fragments, respectively. The optimal number of PCR cycles for each set of primers was established to ensure that the analysis was done on the linear phase of DNA amplification

GAPDH served as control for the bone organ culture and human osteoclast cultures. β -actin was not used as a control for these cultures as β -actin expression has been shown to vary depending on the osteoclast position in the bone tissue and highly active osteoclasts have been shown to express higher levels of β -actin compared to less active ones (Kukita *et al.* 2003). The oligonucleotide primers were designed in such a manner that they either span the exon/exon boundaries to avoid amplification of genomic DNA or anneal to sequences in exons on both sides of an intron so if genomic DNA was amplified the product would be much larger than the product obtained from the intronless mRNA.

Table 3.1: Primers used for RT-PCR studies in human osteoclasts

Factor	Forward primer	Reverse primer	Ta
GAPDH	gtg aac ggg aag ctc act gg	tga ggt cca cca ccc tgt tg	57°C
Cat K	ctt gtg gac tgt gtg act	aac act gca tgg ttc aca	57°C
TRACP	aga tgg aca tgt gga cgg	ggg cct caa agg tct cct gg	55°C

Ta = annealing temperature

Table 3.2: Primers used for RT-PCR studies in mouse calvaria

Factor	Forward primer	Reverse primer	Ta
GAPDH	gtg aac ggg aag ctc act gg	ctt gtg gac tgt gtg act	57°C
B-actin	gtt cgc cat gga tga cga t	tct ggg tca tct ttt cac gg	53°C
Cat K	ctt gtg gac tgt gtg act	gga tgg att cat ggg tgg tg	57°C
c-src	ggg caa caa agt tgc agt c	acg ttg tct tca gac acc	55°C
TRAF-6	atc ctg agc aga tgc act	ctt gtg ccc tgc atc cct	55°C
TRACP	aga tgg aca tgt ggc cgg	gtt gta cgt ctt gga gag	55°C

Ta = annealing temperature

Agarose gel electrophoresis of DNA

Agarose gels were generally made up as 1.5% multi purpose agarose (Roche, Lewes, UK) in 1 x TAE buffer (0.04M Tris-acetic acid, 10mM EDTA pH 8.0). The agarose/TAE solution was heated in a microwave oven on full power (750 W) for ~ 1 minute and cooled to about 50°C. Then, 2µl of a 10mg/ml solution of ethidium bromide was added and the gel solution poured into a gel former and a comb inserted. When set, the gel was placed into an electrophoresis tank, the comb removed and 1 x TAE added. Care was taken to cover the surface of the gel with the running buffer. Samples to be electrophoresed were first mixed with 4 x sample loading buffer (40g/l sucrose, 2.5g/l bromophenol blue in distilled water) and loaded into the wells with a micropipette. The gel was electrophoresed at 5-10 volts/cm for approximately half hour until the bromophenol blue dye had migrated two-thirds of the length of the gel. The PCR products were visualised on an UV transilluminator.

Sequencing of PCR products

Sequencing was carried out by Sequiserve (Germany). The double stranded DNA to be sequenced was diluted to a concentration of at least 100 ng/ μ l and the forward and reverse primers to a concentration of 10 pmol/ μ l.

Immunocytochemistry

Immunocytochemistry was carried out on osteoclasts obtained from peripheral blood mononuclear cells cultured for either dentine discs or bone chips as detailed in Chapter 2. Monoclonal cathepsin K antibody was a kind gift of Glaxo Smith Kline (Pennsylvania, PA, USA). At the end of the experiment, dentine discs or bone chips bearing osteoclasts were transferred to 24-well plates containing MEM, no more than 2 discs were placed in each well. The cells were washed twice in MEM and then fixed in 60:40 mixture of paraformaldehyde fixation buffer (3.5% paraformaldehyde, 2% sucrose in PBS) with MEM at room temperature for 10 minutes. The discs were then washed twice in PBS, transferred to a clean well containing wash buffer (5% normal calf serum, 0.05% sodium azide in PBS) and stored at 4°C before immunostaining. Discs were then rinsed twice in PBS and cells permeabilized with 1ml of ice-cold Triton X-100 permeabilization buffer (20mM HEPES, 300mM sucrose, 50mM NaCl, 3mM MgCl₂, 0.5% Triton X-100, 0.05% sodium azide in PBS at pH 7.0). After permeabilization discs were rinsed twice in PBS and soaked in wash buffer for 30 minutes. The excess buffer from the surface of the discs was drained by touching their edge onto a tissue paper. The discs were then placed on the lid of a 24 well plate on 20 μ l of 5 μ g/ml primary antibody then the remaining 30 μ l of primary antibody was added to the top of the discs; the cells were probed for 30 minutes. After probing, the discs were washed twice in PBS and allowed to soak for 30 minutes in wash buffer. The cells were then probed in 50 μ l of goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (DakoCytomation Ltd., Denmark) at 1:40 dilution in wash buffer for 30 minutes. After incubation the cells were washed twice in PBS and stained for phalloidin-rhodamine conjugate (Invitrogen Paisley, UK) at 5 U/ml in wash buffer for 30 minutes. At the end of the immunostaining procedure, the discs were transferred to a clean well containing wash-buffer. The osteoclasts on dentine discs were monitored with a Leica TCS NT confocal laser scanning microscope (Heidelberg, Germany). Fluorescent images

were collected in sequential 16 steps through the osteoclasts for FITC (488nm filter) and rhodamine (568 nm filter) fluorochromes and displayed in xy planes at constant photomultiplier tube voltage threshold (PMT) and laser intensity. The negative control was scanned prior to samples in order to adjust the PMT of each channel to gate out any background signals from non-specific staining and autofluorescence from the cells and dentine discs. The fluorescence staining was quantified by mapping the area for analysis and measuring the fluorescence intensity/ μm^2 of the compressed file (stack of images) using Leica Simulator software (Heidelberg, Germany). The sample preparation and confocal microscopy was demonstrated by Dr Steve Nesbitt (Bone and Mineral Centre, UCL).

Tartrate-resistant acid phosphatase assay

Tartrate-resistant acid phosphatase activity of conditioned medium was determined as follows. Human osteoclasts were cultured in medium supplemented with RANKL and M-CSF for 12 days at pH ~7.4, followed by a further 48 hour incubation in the presence of 5 mmol/l NaOH or 15 mmol/l HCl. At the end of the experiment, conditioned medium was collected and TRACP activity measured colorimetrically by using tartrate-resistant acid phosphatase reagent kit (Biotron Diagnostics, Hemet, California, USA) according to the manufacturer's protocol.

Statistics

Comparison between pairs of means was performed by using t-test and multiple comparisons were performed by one-way analysis of variance using 'InStat' (Version 1.13, GraphPAD software) with use of the Bonferroni correction for multiple comparisons; representative data are presented as means \pm SEM for 5 or 6 replicates. Results are presented for representative experiments that were each repeated at least 2-3 times.

RESULTS

Effect of acidosis on osteoclast-mediated Ca^{2+} release from mouse calvaria

Basal levels of Ca^{2+} release in non-acidified control bones (pH 7.20 ± 0.02) were very low after 3 days of culture (~ 0.2 mmol/l). Acidification of culture medium to pH 7.05 ± 0.02 by addition of 15 mmol/l H^+ as HCl resulted in 10-fold increase in Ca^{2+} release from bones and was equivalent to that of a maximal dose of PGE_2 (1 μM). HCO_3^- acidosis stimulated Ca^{2+} release was completely blocked by the addition of 2 $\mu\text{g/ml}$ osteoprotegerin (**Figure 3.1**).

Effect of acidosis on regulation of resorption-associated factors in mouse calvarial cultures

The mRNA expression of resorbing associated factors TRACP, cathepsin K, c-src and TRAF-6 was investigated on mouse calvarial cultures. Bones were cultured for 72 hours in control (pH 7.20 ± 0.02) or test medium modified by the addition of 1 μM PGE_2 (pH 7.26 ± 0.01), 15 mmol/l H^+ (pH 7.05 ± 0.02) or 2 ng/ml OPG (pH 7.05 ± 0.03). PGE_2 was used as a positive control.

Regulation of mRNA for cathepsin K (an enzyme involved in organic matrix degradation by osteoclasts), TRAF-6 (an intracellular factor associated with osteoclast activation) and TRACP (required for optimal resorption) were elevated strikingly by acidosis and PGE_2 , a known stimulator of resorption in mouse calvarial cultures (Meghji *et al.* 2001). Surprisingly, mRNA for c-src, thought to be required for osteoclast ruffled border formation was inhibited by acidosis (**Figure 3.2**). All four factors studied were downregulated when bones were cultured in the presence of OPG at low pH, however, c-src downregulation by acidosis was greatest when OPG was not present in the culture. In one series of cultures, the expression of cathepsin K mRNA was not detected when bones were cultured at pH ~ 7.20 (control group) and was only evident in acidified groups (data not shown).

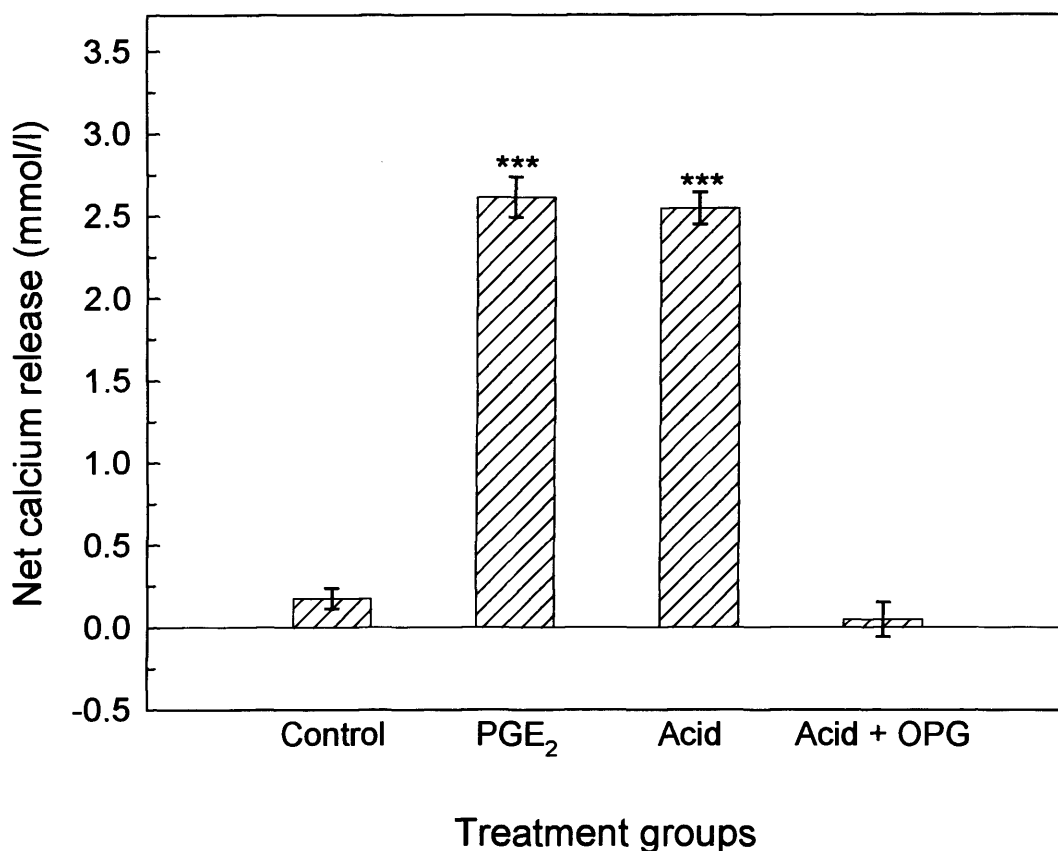


Figure 3.1. Effect of acidosis on Ca²⁺ release by mouse calvaria. Mouse calvarial bones were cultured for 3 days in control (pH 7.20 ± 0.02) or test media modified by the addition of 1µM PGE₂ (pH 7.26 ± 0.01), 15 mmol/H⁺ (pH 7.05 ± 0.02) or 15 mmol/H⁺ and 2µg/ml osteoprotegerin (OPG) (pH 7.05 ± 0.03). Stimulation of Ca²⁺ release from mouse calvaria by metabolic acidosis was equivalent to the maximal effect elicited by PGE₂ (1µM). The stimulatory effect of acidosis on Ca²⁺ release was completely blocked by OPG (2µg/ml), indicating this is an entirely cell-mediated action. Values are means ± SEM (n = 5). Significantly different from control: *** p<0.001.

Effect of acidosis on regulation of resorption-associated factors in human osteoclast cultures

The effect of acidosis on the regulation of resorption-associated genes were carried out in normal human osteoclast-cultured from TRACP⁺ cultured on dentine data in bone cells by using RT-PCR analysis. The expression of cathepsin K and calcitonin receptor-like receptor (CLR) mRNA were strongly upregulated by acidosis in human osteoclasts when pH was lowered from 7.41 to 7.01.

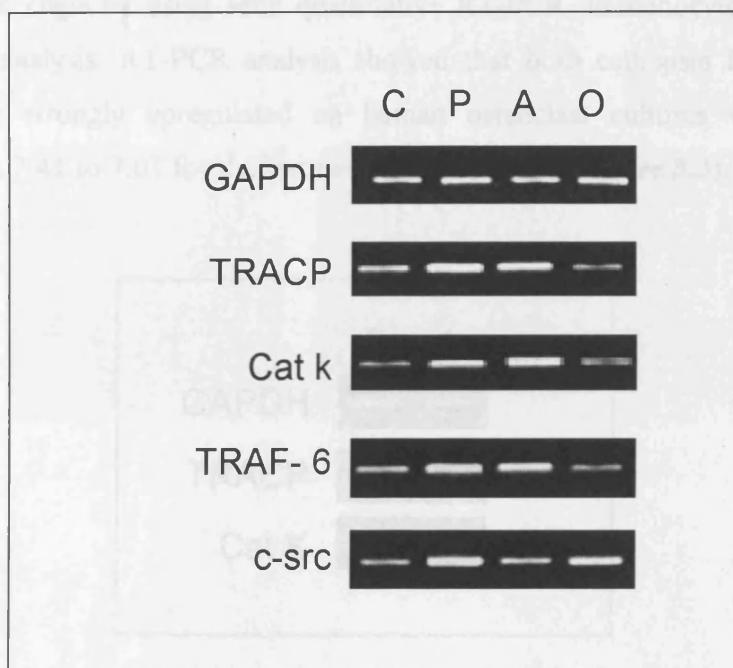


Figure 3.3. Effect of acidosis on expression of TRACP and osteopontin mRNA in human osteoclast cultures. Cells were cultured for 12 days in the presence of RANKL (1 ng/ml) and M-CSF (50 ng/ml) and 5 μg/ml β₂M, followed by a

Figure 3.2. Effect of extracellular pH on expression of mRNAs for resorption associated factors in mouse calvarial cultures. Cells were cultured for 3 days at pH 7.20 ± 0.02 (C), at pH 7.26 ± 0.01 in the presence PGE₂ (P), at pH 7.05 ± 0.02 (A) or at pH 7.05 ± 0.03 in the presence 2 ng/ml OPG (O). RT-PCR was performed on DNase-treated total RNA using GAPDH as control.

Using confocal microscopy analysis I found that cathepsin K intensity increased in reducing pH from 7.41 to 7.01 for the last two days in culture (Figure 3.4 A). It is noteworthy that staining intensity is defined by the immunofluorescence acid staining, very weak at both control and acidified cultures. Staining intensity for cathepsin K was increased 10-fold based on fluorescence intensity measurements of cultures maintained for the last two days at pH 7.01 (Figure 3.4 B).

A pH reduction from 7.41 to 7.01 caused a 2-fold increase in the levels of TRACP activity in the culture medium as assessed colorimetrically (Figure 3.5).

Effect of acidosis on regulation of resorption-associated factors in human osteoclast cultures

The effect of acidosis on the regulation of resorption associated genes were carried out in normal human osteoclasts obtained from PBMCs cultured on dentine discs or bone chips by using semi quantitative RT-PCR, immunocytochemistry and colorimetric analysis. RT-PCR analysis showed that both cathepsin K and TRACP mRNA were strongly upregulated on human osteoclast cultures when pH was lowered from 7.41 to 7.01 for the last two days of culture (**Figure 3.3**).

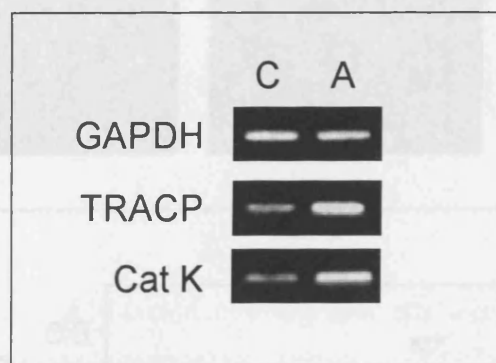


Figure 3.3. *Effect of acidosis on expression of TRACP and cathepsin K mRNAs in human osteoclast cultures.* Cells were cultured for 12 days in the presence of RANKL (1ng/ml) and M-CSF (5ng/ml) and 5 mmol/l H⁺, followed by a further 48-hour in medium at pH 7.41 (control, C) or pH 7.01 (acid, A). RT-PCR was performed on DNase-treated total RNA using GAPDH as control.

Using confocal microscopy analysis I found that cathepsin K intensity increased by reducing the pH from 7.41 to 7.01 for the last two days in culture (**Figure 3.4 A**). It is noteworthy that resorbing osteoclasts, identified by the immunofluorescent actin ring staining, were seen at both control and acidified cultures. Staining intensity for cathepsin K was increased ~5-fold based on fluorescence intensity measurements of cultures maintained for the last two days at pH 7.01 (**Figure 3.4 B**).

A pH reduction from 7.41 to 7.01 caused a 2-fold increase in the levels of TRACP activity in the culture medium as assessed colorimetrically (**Figure 3.5**).

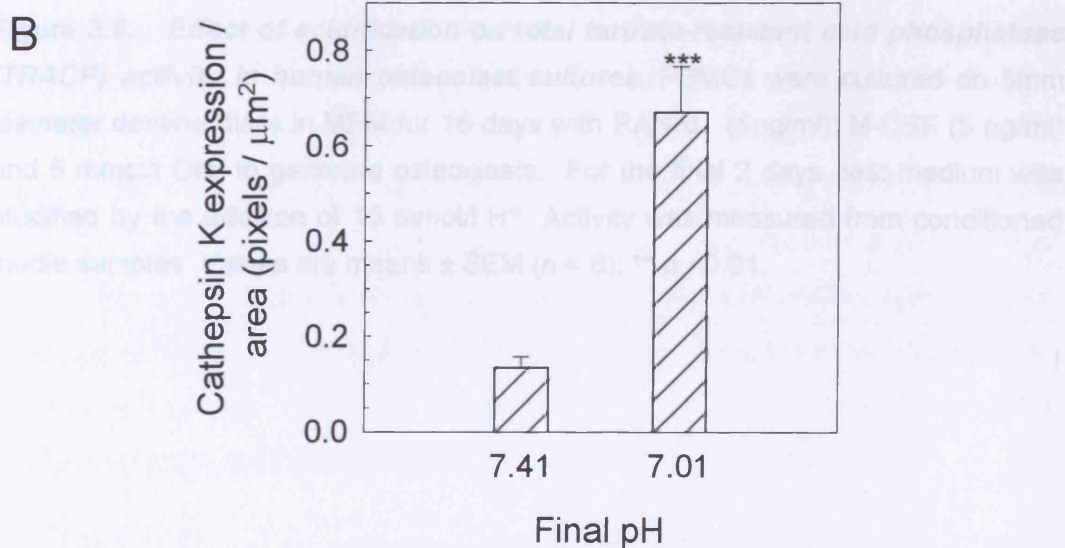
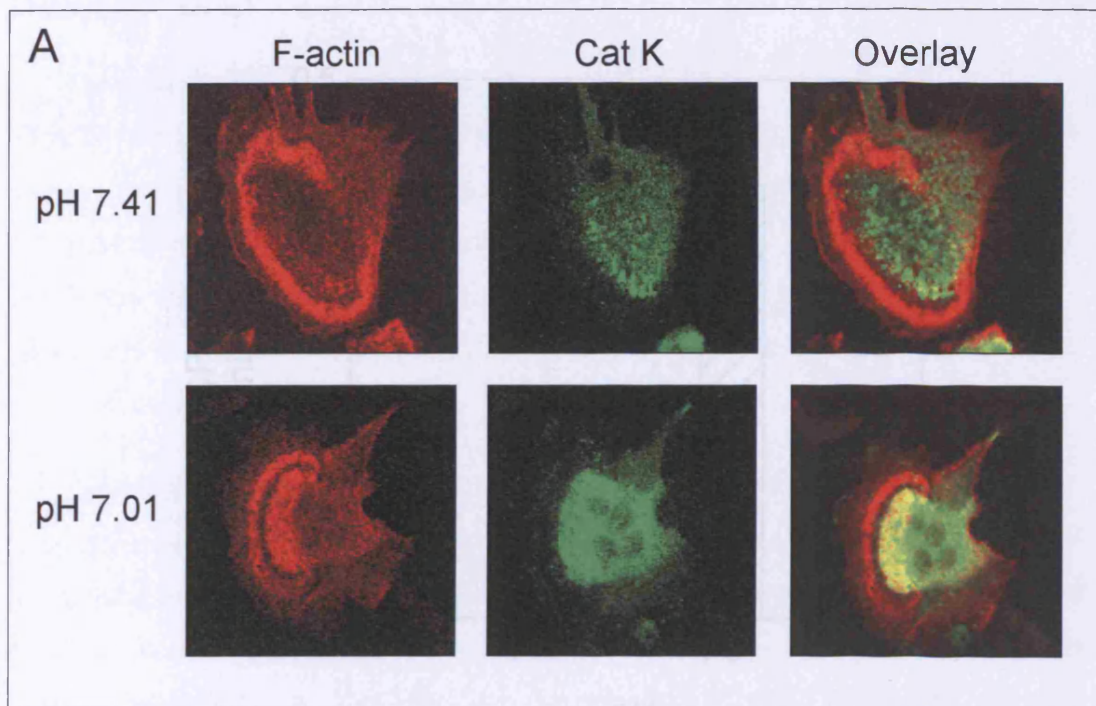


Figure 3.4. Acidosis upregulates expression of cathepsin K by human osteoclasts. A. Resorbing osteoclast immunostained for cathepsin K (green) cultured on bone chip, imaged by confocal microscopy. Rhodamine-phalloidin staining was used to demonstrate F-actin distribution (red). B. Quantitative immunocytochemical analysis of human osteoclasts generated from PBMCs shows a five-fold increase on cathepsin K protein expression in response to acidification for 2 days. Significantly different from control (pH 7.41): *** $p < 0.001$. Values are means \pm SEM ($n = 15$).

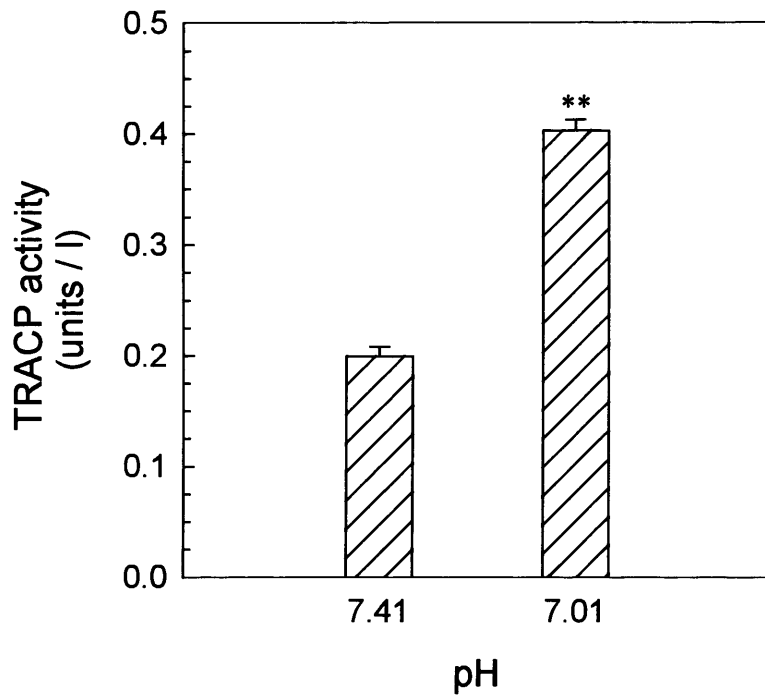


Figure 3.5. Effect of acidification on total tartrate-resistant acid phosphatase (TRACP) activity in human osteoclast cultures. PBMCs were cultured on 5mm diameter dentine discs in MEM for 16 days with RANKL (1ng/ml), M-CSF (5 ng/ml) and 5 mmol/l OH⁻ to generate osteoclasts. For the final 2 days, test medium was modified by the addition of 15 mmol/l H⁺. Activity was measured from conditioned media samples. Values are means ± SEM (n = 6); ** p <0.01.

DISCUSSION

The results presented in this chapter show that the resorption-associated factors TRACP and cathepsin K were upregulated by acidosis in mouse calvarial and normal human osteoclast cultures. Moreover, the adaptor protein TRAF-6 was also upregulated at acidic conditions in mouse calvarial cultures. However, c-src mRNA expression was surprisingly downregulated when bone was cultured at low pH. I also found that the effect of acidosis on mouse calvaria was completely blocked by osteoprotegerin.

The experimental system used to study the mRNA expression of resorption-associated genes by normal human osteoclasts had the advantage that RNA was extracted from cells cultured on natural, resorbable substrates, such as dentine discs or bone chips. These conditions mimic the *in vivo* situation better than the use of glass or plastic surfaces. The latter lack the organic matrix and mineral that plays an important role in the regulation of osteoclastic function. It has been shown that special structural features of active osteoclasts such as ruffled border and the sealing zone are not formed on an artificial surface (Lakkakorpi *et al.* 1989; Lakkakorpi *et al.* 1996; Lehenkari *et al.* 1997). In order to obtain sufficient RNA for RT-PCR analysis, the cells were cultured at low oxygen (5%), which is known to increase osteoclast formation in cells derived from murine and human sources (Arnett *et al.* 2003). Moreover, during RNA extraction the isopropanol incubation was performed overnight at -20°C , which increased the nucleic acid precipitation and consequently the final RNA yield. The use of glycogen as a carrier during the RNA extraction facilitated the visualisation of the RNA pellet during the procedure.

Both TRACP and cathepsin K expression were upregulated when mouse calvaria and isolated human osteoclasts were cultured at low pH compared to the control group. The function and subcellular localisation of TRACP is still controversial although this factor is widely used as a histological marker for osteoclasts (Vaaraniemi *et al.* 2004). It has been suggested that TRACP role may be linked to the main physiological function of osteoclasts: degradation of the mineralised bone matrix. TRACP knockout mice develop mild osteopetrosis and transgenic mice overexpressing TRACP develop mild osteoporosis (Hayman *et al.* 1996; Angel *et al.* 2000). This phosphatase has been reported to be involved in intracellular vesicular

transport as deletion of this factor generates osteoclasts with increased ruffled border and accumulation of cytoplasmic vesicles (Hollberg *et al.* 2002). TRACP also appears to play an important role in dephosphorylation of osteopontin, a phosphoprotein of bone that binds to osteoclast cell surface integrins via a RGD motif, thereby mediating attachment of osteoclasts to bone as well as activating various signalling pathways involving c-src and Akt/PI-3 kinase. When dephosphorylated OPN can no longer promote osteoclast RGD-dependent attachment (Flores *et al.* 1992; Andersson *et al.* 2003). In contrast, Väänänen and colleagues showed that TRACP is co-localised with collagen fragments in the transcytotic compartments of resorbing osteoclasts, not at the ruffled border. They showed that TRACP may help in destructing the large amounts of matrix-degradation products transported by these vesicles as explained in detail in Chapter 1 (Vaaraniemi *et al.* 2004).

Cathepsin K is a cysteine proteinase expressed abundantly and selectively in osteoclasts (Drake *et al.* 1996). This enzyme plays an important role in osteoclast function as it degrades the type I collagen bone matrix after the demineralisation of the matrix by protons pumped into the extracellular resorption zone. Cathepsin K is able to cleave interstitial collagen within multiple sites of the triple helix structure (Garnero *et al.* 1998). Recent evidence suggests that cathepsin K may be present in transcytotic vesicles of resorbing osteoclasts and may be responsible for the proteolytic activation of the TRACP enzyme into TRACP 5b, which is a specific and sensitive marker of bone resorption (Halleen *et al.* 2000; Vaaraniemi *et al.* 2004).

The finding that TRAF-6 is upregulated in mouse organ cultures by lowering the pH to ~ 7.0 fits well with the stimulatory action of acidosis on the resorptive activity of bone-resorbing cells in human osteoclast and mouse calvarial cultures (**Figure 2.2 and 3.1**). TRAF-6 is one of the essential genes necessary for osteoclast cytoskeletal organisation and resorptive function since *TRAF-6* knockout mice exhibit severe osteopetrosis due to abnormal ruffled border formation (Lomaga *et al.* 1999; Naito *et al.* 1999; Armstrong *et al.* 2002).

The downregulation of c-src mRNA expression in calvaria was most surprising as this molecule is one of the essential genes necessary for osteoclast activation, like TRAF-6, c-src is reportedly involved in cytoskeletal arrangement and osteoclast

resorptive activity (Soriano *et al.* 1991; Boyce *et al.* 1992). There are many explanations for this discrepant result. First, this kinase is regulated by a variety of factors including RANKL and interleukin-1 (IL-1), which may or not be affected by acidosis. It is known that osteoclast stimulation by both RANKL and IL-1 induces the association of TRAF-6, with c-src, leading to the formation of a huge molecular complex and resulting in actin-ring formation and osteoclast activation (Armstrong *et al.* 2002; Nakamura *et al.* 2002). Second, osteoclast function was studied in a complex (whole bone) environment, many other cell types normally present in bone such as endothelial cells, nerves and osteoblasts may influence the response of osteoclasts to an acidotic state by releasing a number of local factors, which could eventually regulate c-src expression (Meghji *et al.* 1998). Therefore, further studies will have to be carried out using single cell techniques such as immunocytochemistry in bone organ and disaggregated osteoclast cultures at different time points in order to elucidate the expression pattern of this kinase to acidosis.

The observation that OPG blocked the effect of acidosis on mouse calvaria is remarkably interesting as this compound is being tested by Amgen as a treatment for bone cancer (Garber 2003). The experimental conditions used in this study mimics that of a tumour microenvironment, which is acidic, supporting the notion that OPG may be a powerful treatment for the pain associated with bone cancer (Gerweck and Seetharaman 1996; Mantyh *et al.* 2002). The differentiation of osteoclasts requires the interaction of the receptor RANK, which is expressed by precursor osteoclasts to RANKL. Sequestration of this compound by OPG prevents the binding of RANKL to its receptor (RANK) preventing osteoclast differentiation and activation (Lacey *et al.* 1998; Yasuda *et al.* 1998). OPG is likely to be used in cancer patients as the tumour microenvironment induces formation and activation of osteoclasts (Mantyh *et al.* 2002). The inhibitory action of OPG may also suggest that the effect of acidosis on bone may be mediated via RANK and RANKL interaction, supporting previous findings by Bushinsky and colleagues who showed that RANKL synthesis is increased on mouse calvaria when bones are exposed to low pH (Frick and Bushinsky 2003).

The work presented in this chapter illustrates that the response of osteoclasts to extracellular pH is a cell-mediated process, which is tightly regulated by the

osteoclasts. The earlier suggestion that low extracellular pH may simply aid resorption by reducing the gradient against which osteoclasts must pump H^+ is unlikely to account for all of the effect (Arnett and Dempster 1986). Extracellular protons is most probably perceived primarily by H^+ -sensing receptors such as OGR1, TDAG8 or TRPV1, which are expressed by bone cells as shown in Chapter 5.

CHAPTER 4

Acidosis inhibits mineralised bone formation by osteoblasts *in vitro*

INTRODUCTION

Osteoblast and acidosis

Extracellular acidification (pH 6.9-7.0) is now recognised as a key initial requirement for activation of osteoclasts to form resorption pits on bone (see Chapter 2). However, the effects of acidosis on osteoblast function are less well understood.

Bushinsky and colleagues were the first to suggest that the calcium efflux during acidosis in mouse calvarial cultures, results from a combination of inhibited osteoblastic and stimulated osteoclastic activity (Krieger *et al.* 1992). They showed that bone organ cultures, osteoblastic collagen synthesis and alkaline phosphatase activity are both decreased by lowering the extracellular pH (Krieger *et al.* 1992). Further studies using *in vitro* bone nodule formation as a model system suggested that acidosis inhibits nodule formation, with metabolic acidosis causing a greater effect than respiratory acidosis (Sprague *et al.* 1994; Frick *et al.* 1997). Changes in extracellular pH have also been reported to alter the expression of genes critical to the function of mouse osteoblasts (Frick *et al.* 1997). These authors found that decreasing pH from 7.4 to 6.8 leads to a parallel reduction in mRNA for *egr-1* (an immediate early response gene) and type 1 collagen 40 minute after medium change. Expression of other matrix protein mRNAs, including osteopontin and matrix Gla protein, was also reported to be downregulated by acidosis in long-term cultures (Frick and Bushinsky 1998).

Ramp and co-workers found that glycolysis, alkaline phosphatase activity and collagen production by primary chick and human osteoblasts are reduced at low pH, and hypothesised that pH 7.2 may be the optimal pH for osteoblast function (Ramp *et al.* 1994; Kaysinger and Ramp 1998). Moreover, it has been demonstrated that

increasing extracellular pH from 6.9 to 7.6 increases gap junction connections between osteoblast-like MC3T3-E1 cells (Yamaguchi *et al.* 1995). *In vivo* studies of low blood flow, that occurs in disuse, ageing and initial stages of fracture healing, showed that activity of osteoblasts were decreased most probably due to the reduced local pH surrounding these cells (Reeve *et al.* 1988).

The work presented in this chapter was aimed at investigating the direct effect of chronic metabolic acidosis on the function of normal osteoblasts, paying particular attention to the pH range that is critical for modulating osteoclast function, *i.e.* pH 7.5 – 6.8.

Mineral deposition in vitro

Wong and Cohn (1975) pioneered the use of mouse calvaria to obtain cells of the osteoblastic lineage by sequential collagenase digestion. The cells obtained based on this method are either osteoblasts or osteoblast precursors, which are able to form bone nodules *in vitro* when cultured in the presence of dexamethasone, beta-glycerophosphate and ascorbic acid (Bellows *et al.* 1986). The synthetic glucocorticoid dexamethasone increases bone nodule numbers in a dose dependent manner in human and rat osteoblasts (Bellows *et al.* 1987; Ishida *et al.* 1996; Cheng *et al.* 1996). It is noteworthy that the circulating levels of glucocorticoids in both humans and rats (10^{-8} – 10^{-7} M) are similar to the maximal stimulatory responses of osteoblasts to dexamethasone *in vitro* (Bellows *et al.* 1987; Cheng *et al.* 1996). Beta-glycerophosphate acts as a substrate for alkaline phosphatase, presumably resulting in an increased local concentration of inorganic phosphate and subsequent mineral deposition (Bellows *et al.* 1992). The amount of beta-glycerophosphate added to the culture media is very critical as higher concentrations of this compound have been reported to promote non-physiological mineral deposition; therefore, its concentration should never exceed 2 mM in culture (Bellows *et al.* 1992). Ascorbic acid promotes post-translational modifications of collagen, which are required for its maturation (Prockop and Kivirikko 1995). Treatment of post-confluent cells with ascorbic acid also increases the expression of osteoblast differentiation markers such as alkaline phosphatase and also osteocalcin; this vitamin is essential for nodule production and thus matrix mineralisation in long-term cultures (Harada *et al.* 1991; Franceschi *et al.* 1994). As osteoblasts mature *in vitro* they sequentially exhibit a

well-characterised pattern of gene and protein expression, which mimics bone formation *in vivo* (see **Table 1.1**). About 8 days after reaching confluence, small white mineralised nodules can be seen macroscopically in living unstained primary osteoblast cultures. Subsequently these nodules increase in size and organised cell layers around the nodules can be observed. The nodules formed have a characteristic trabecular morphology (see **Figure 4.1**).

The bone nodule formation assay used in this study has a number of advantages. First, it allows the ultimate function of osteoblasts, namely bone formation, to be studied quantitatively. Second, cells are exposed directly to environments of precisely controlled known pH, in a manner that is not possible using bone organ cultures, or *in vivo*. Third, osteoblast function is studied in a simple environment that is relatively free from the influence of the other cell types normally present in bone such as endothelial cells, nerves and cells of haematopoietic origin including osteoclasts.

MATERIALS AND METHODS

Materials

Tissue culture media and sera were purchased from Invitrogen (Paisley, UK). Bulk liquids (organic solvents, acids and bases) were purchased from VWR International Ltd (Gillingham, Dorset, UK). Radiochemicals were purchased from Amersham International (Bucks, UK). All other reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless stated otherwise.

Bone nodule assay

The effects of acid on bone nodule formation by primary rat osteoblasts were studied using modifications of an assay described previously (Hoebertz *et al.* 2002). Cells were obtained from calvaria of 2-day-old neonatal Sprague-Dawley rats by sequential digestion at 37°C in 0.25% trypsin in PBS for 10 minutes, 0.2% collagenase type II in HBSS for 30 minutes, then 0.2% collagenase type II in HBSS for 60 minutes, rejecting the first two digests. The cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) + 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, and cultured in a humidified atmosphere of 5% CO₂ – 95% air until confluence (2 - 4 days). At confluence, cells were subcultured into 24-well plates at a density of 10⁴/well in DMEM. After a 24-hour pre-incubation period, medium was replaced with DMEM supplemented with 50 µg/ml ascorbic acid, 2 mM β-glycerophosphate and 10⁻⁸ M dexamethasone (mixture abbreviated 'supplemented DMEM'). Test media were acidified by adding 5 - 30 mmol/l H⁺ as concentrated HCl, to give an effective pH range from ~ 7.35 to ~ 6.90. Cells were cultured for up to 21 days, with half-medium changes every 3 days.

At the end of the experiment, cultures were fixed in 2% glutaraldehyde for 5 minutes, washed with 70% ethanol and left to air-dry. Mineralised bone nodules were visualised by staining with alizarin red (1% solution w/v in water) for 5 minutes, rinsed with 50% ethanol to remove excess stain, then air-dried. The plates were imaged at 800 dpi using a high-resolution flat-bed scanner (Epson Perfection 3200). Binary images of each individual well were then subjected to automated analysis (Scion Image software, Scion Corporation; <http://www.scioncorp.com>), at constant

threshold level and minimum particle size, to determine the number and plan surface area of mineralised bone nodules.

Some cultures were stained to demonstrate collagen using Masson's trichrome method. Briefly, this entailed fixation in Bouin's fluid, and staining in 1% Weigert's iron haematoxylin solution for 10 minutes, then 0.5% acid fuchsin, 0.5% glacial acetic acid for 5 minutes, followed by treatment with 1% phosphomolybdic acid solution for 5 minutes and staining for 5 minutes in 2% methyl blue, 2.5% glacial acetic acid solution.

Proliferation assay

Cell proliferation was determined as ^3H -thymidine incorporation into DNA, essentially as described previously (Gray *et al.* 1992). Briefly, calvarial osteoblasts were cultured up to 15 days in supplemented DMEM in the presence of 0-30 mmol/l HCl and labelled with 1 $\mu\text{Ci/ml}$ of [$6\text{-}^3\text{H}$]thymidine (22.0 Ci/mmol; 814 GBq/mmol) for the final 6 hours of culture. Cell layers were washed three times with PBS containing 1mM unlabelled thymidine, then detached using 0.25% trypsin for 5 minutes at 37°C, and precipitated with 7.5% trichloroacetic acid (TCA) in the presence of 0.2% (w/v) bovine serum albumin (BSA), followed by centrifugation at 1500 x g. Pellets were washed with 7.5% TCA / 0.2% BSA, recentrifuged, then digested overnight at 37°C in 0.2 M NaOH and mixed with scintillation cocktail (Wallac Optiphase Hisafe II, Fisher Scientific, Loughborough, UK) for scintillation counting.

^3H -proline incorporation assay

Collagen production was estimated as ^3H -proline incorporation into pepsin-resistant protein, essentially as described previously (Webster and Harvey 1979; Gray *et al.* 1992). Briefly, L-[2,3, ^3H] proline (42 Ci/mmol; 1.55 TBq/mmol) was added to osteoblast cultures to a final concentration of 1 $\mu\text{Ci/ml}$, together with 50 $\mu\text{g/ml}$ β -aminopropionitrile monofumarate and 50 $\mu\text{g/ml}$ ascorbate for the final 24 hours of culture. Experiments were terminated by addition of an equal volume of cold 1mM acetic acid containing 1mg/ml of pepsin (EC 3.4.23.1; 2500-3200 U/mg protein), followed by incubation for 4 hours at 20°C with gentle shaking.

Subsequent steps were carried out at 4°C. Carrier rat-tail collagen (100 µg in 1ml 0.5 mM acetic acid) was added and the mixture was allowed to precipitate for 2 hours, and then centrifuged for 20 minutes at 4000 x g. The pellet was discarded and pepsin-resistant protein was precipitated from the supernatant by the addition of NaCl (0.5 ml of 25% w/v solution in 0.5 mM acetic acid) for 2 hours. The mixture was centrifuged for 30 minutes at 4000 x g and the precipitation step was repeated. The final pellet was dissolved in 0.5 ml of 0.5 mM acetic acid, and mixed with scintillation cocktail for counting as described above.

Alkaline phosphatase assay

The alkaline phosphatase (ALP) activity of cell lysates from parallel cultures was determined as described previously (Bakker and Klein-Nulend 2003). Briefly, primary osteoblasts were cultured in supplemented medium up to 6 days in the presence of 0-30 mmol/l HCl. The cells were then washed once in PBS, incubated on ice and 1 ml of cold ultrapure water added to the monolayer. The cells were then harvested by using a cell scraper and the cell suspension transferred to a 1.5ml Eppendorf tube and sonicated on ice for 10 minutes, cell debris was then precipitated for 10 minutes at 500 x g. The supernatant was used for the determination of ALP activity and total protein content. The ALP activity was measured colorimetrically by using alkaline phosphatase reagent kit (Biotron Diagnostics, Hemet, California, USA) and the protein content of the cell lysates measured using Protein Assay Reagent Kit (Sigma, Dorset, UK) according to the manufacturer's protocol based on the modified Lowry method (Peterson 1977); BSA was used as standard.

RNA isolation and RT-PCR

Osteoblasts cultured as described above were lysed in TRIzol solution at day 3, 5, 10 and 17 of culture (Invitrogen, Paisley, UK), and total RNA was extracted according to the manufacturer's instructions. RT-PCR was performed as per protocol detailed in Chapter 3. The primers used for this experiment were purchased from MWG (Ebersberg, Germany) and are shown in **Table 4.1**.

Table 4.1: Primers used for RT-PCR studies in osteoblast cultures

Factor	Forward primer	Reverse primer	Ta
β -actin	gtt cgc cat gga tga cga t	tct ggg tca tct ttt cac gg	53°C
OPN	cgg tga aag tgg ctg agt	gac tcg gga tac tgt tca	50°C
MGP	ccc tgt gct atg aat ctc	gac tcc gta aca aag cga	50°C
OCN	gca gac acc atg agg acc ct	gca gct gtg ccg tcc ata c	59°C
ALP	ctc att tgt gcc aga gaa	gtt gta cgt ctt gga gag	50°C

Ta = annealing temperature

Detection of apoptosis

Osteoblast apoptosis was assessed using a kit (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics Ltd, Lewes, East Sussex, UK) according to the manufacturer's protocol. This assay measures cytoplasmic histone DNA fragments (mono and oligonucleosomes) produced after cell death using mouse monoclonal antibodies against histones and DNA. Briefly, cells were lysed *in situ* after being cultured in supplemented medium, for 3, 7 and 10 days in the presence of 0 or 30 mmol/l HCl. Mono and oligonucleosomes released into the cytoplasm were detected by measuring the absorbance at 450 and 490 nm; duplicate determinations were made. The results were normalised to osteoblast numbers in parallel wells, assessed using a haemocytometer, after sequential treatment with 0.2% collagenase type II for 30 minutes, and 1% trypsin for 10 minutes to release cells.

Electron Microscopy

Osteoblasts were seeded onto 1 cm diameter Melanex polymer (ICI, Dumfries, UK) discs (10^4 cells / disc) and cultured in 24-well plates in supplemented DMEM for up to 21 days in the presence of 0 or 30 mmol/l HCl. Cultures were terminated by fixation in 2.5% glutaraldehyde for 1 hour, followed by treatment with 1% osmium tetroxide solution for 30 minutes, washing and immersion in 1% uranyl acetate. Samples were then dehydrated through a graded ethanol series, cleared in propylene oxide and embedded in Araldite resin. Ultrathin (80-90 nm) planar sections were collected on 200 mesh grids, counterstained with lead citrate and viewed in a JEOL 1010 series transmission electron microscope. Mr Mark Turmaine (Department of Anatomy and Developmental Biology, UCL) prepared the samples for electron microscopy analysis.

Statistical analysis

Multiple comparisons were made by one-way analysis of variance (ANOVA) with use of the Bonferroni correction for multiple comparisons ('Instat' version 1.3, Graph pad software); representative data are presented as means \pm SEM. Significance was assumed at $p < 0.05$. Results presented were for a representative experiment that was repeated at least three times unless stated otherwise.

RESULTS

Effect of acidosis on bone nodule formation and morphology in vitro

The effect of acidosis was tested on bone nodule formation by cultured rat osteoblasts. Reducing the extracellular pH from 7.43 to 6.90 had a strong inhibitory effect on mineralised bone nodule formation by these cells; at low pH, mineralisation was almost completely abolished. This inhibitory effect was easily visible to the naked eye as illustrated in **Figure 4.1**. In spite of the lack of mineralisation at pH 6.90, the matrix-containing nodules were still formed with distinct edges and a defined shape, suggesting that the collagen production/deposition was relatively unaffected by lowering the environmental pH. Quantitative analysis (**Figure 4.2**), as assessed by total nodule area and number of nodules/well, shows that lowering pH from 7.43 to 6.90 inhibited bone nodule formation in a dose dependent manner. A pH reduction from 7.43 to 7.32 was sufficient to halve mineralisation, with complete abolition at pH 6.93.

To determine whether the pH effect on mineralised bone nodule formation was reversible, primary rat osteoblasts were cultured for 20 days at pH 7.43 or 6.96 then incubated for a further 10 days at pH 6.99 or 7.42, respectively. The results show that switching medium pH from 7.01 to 7.44 for the final 10 days allowed mineralisation of the matrix-containing nodules present in culture (**Figure 4.3 A**). The opposite effect was observed when medium pH was switched from 7.43 to 6.96 at day 20; *i.e.* the mineral deposited onto the matrix was completely dissolved by day 30 (**Figure 4.3 B**).

In order to check the role of pH on crystal forming independent of cellular effects, osteoblasts were cultured for 20 days at pH 7.44 or 7.03, killed by glutaraldehyde fixation, then incubated for a further 10 days at pH 6.97 or 7.42, respectively. Decreasing medium pH from ~7.4 to ~6.9 for the final 10 days, after glutaraldehyde fixation, caused mineral dissolution (**Figure 4.3 C**); the morphology of the matrix-containing nodules was similar to those shown in **Figure 4.3 B** at day 30, suggesting that mineral dissolution by acidosis is independent of cellular effects. Increasing medium pH from 7.03 to 7.42, in the absence of living osteoblasts, was sufficient to allow mineral deposition onto the matrix-containing nodules. The

mineral, however, precipitated randomly onto the matrix; the mineralised nodules formed had a disorganised morphology with less distinct edges in comparison to those formed when the pH was increased in the presence of living cells (**Figure 4.3. A**). Quantitative analysis (**Figure 4.4**), as assessed by total nodule area and number of nodules/well, shows that decreasing the pH of the medium from ~ 7.40 to ~7.00 for the last 10 days in culture, in the presence or absence of living osteoblasts, decreased the area of bone nodules formed by ~8-fold compared to those cells cultured at physiological pH for 20 days. When osteoblasts were switched from acidified (~7.00) to neutral (~7.40) conditions, the area of bone nodules formed increased by ~20-fold compared to those cells cultured at pH ~7.00 for 20 days.

As there were no apparent differences on the morphology of matrix-containing nodules when rat osteoblasts were cultured at low pH (6.90) compared to control (pH 7.43), the collagen structure was examined using Masson's trichome staining and transmission electron microscopy (**Figure 4.5**). Histological staining showed that abundant collagen fibres were deposited by the osteoblasts at both pH 7.43 and 6.90 (**Figure 4.5 A, B**) and there were no significant differences on collagen ultrastructure when cells were cultured at control and acidified media (**Figure 4.5 C, D**).

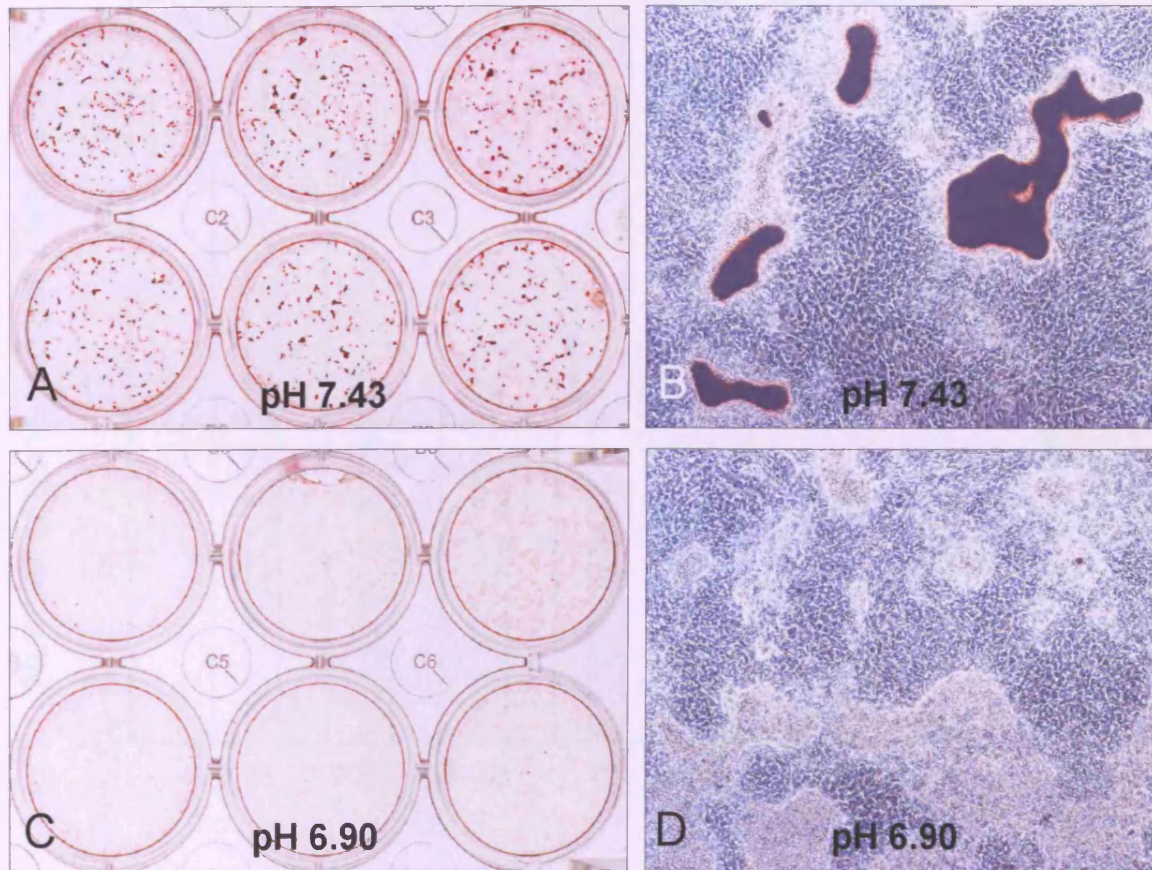


Figure 4.1. Inhibitory effect of acidosis on bone nodule formation. Primary rat osteoblasts were cultured in plastic wells for 16 days in control medium at pH 7.43 or in acidified medium at pH 6.90, with 10^{-8} M dexamethasone, 2 mM β -glycerophosphate and 50 μ g/ml ascorbate. **A, C.** Bone nodules consisting of mineralised extracellular matrix, visualised by alizarin red staining, are evident only in control wells at pH 7.43. **B, D.** Appearance of control and acidified cultures at higher magnification (phase contrast microscopy, 10x objective); the failure of matrix to mineralise at pH 6.90 is clearly evident.

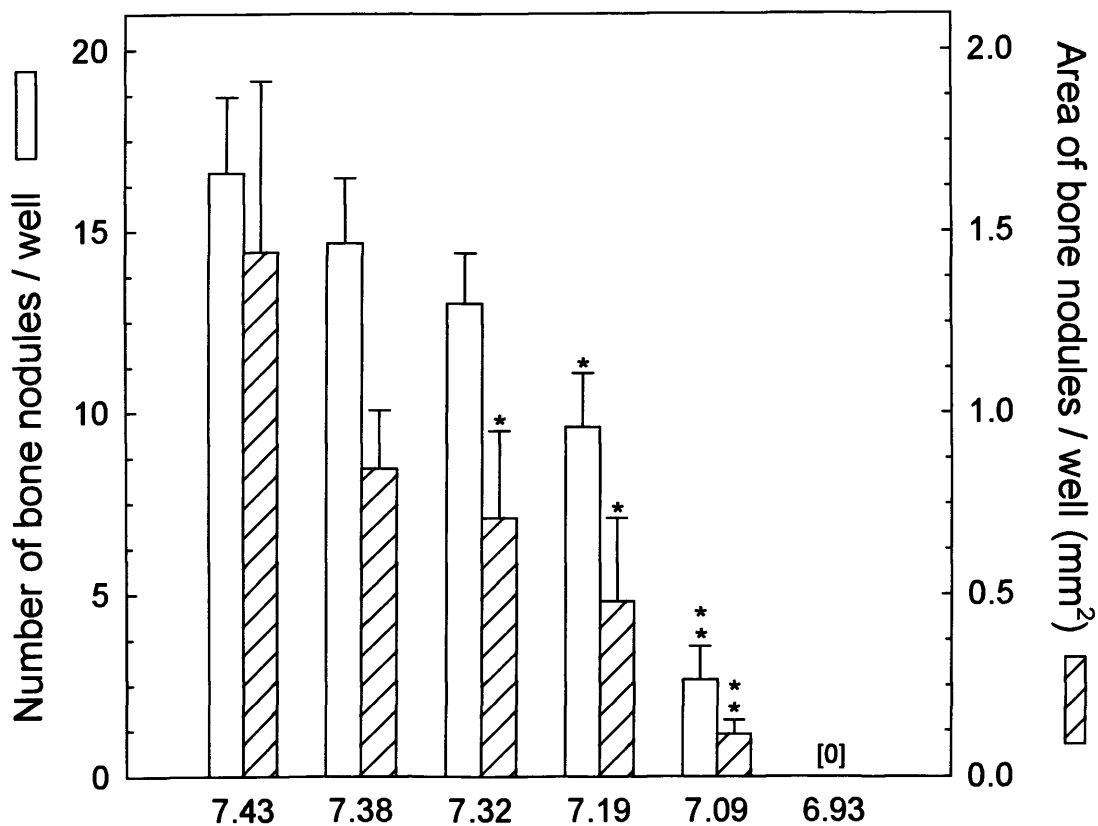
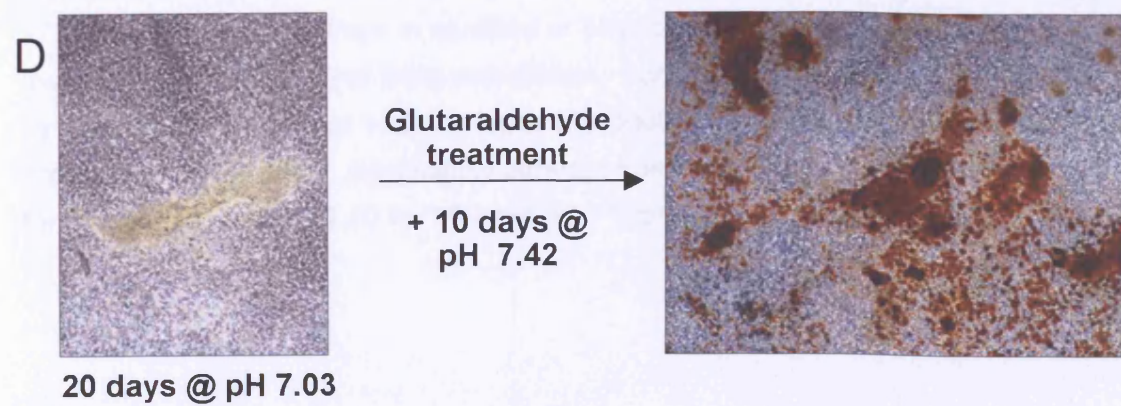
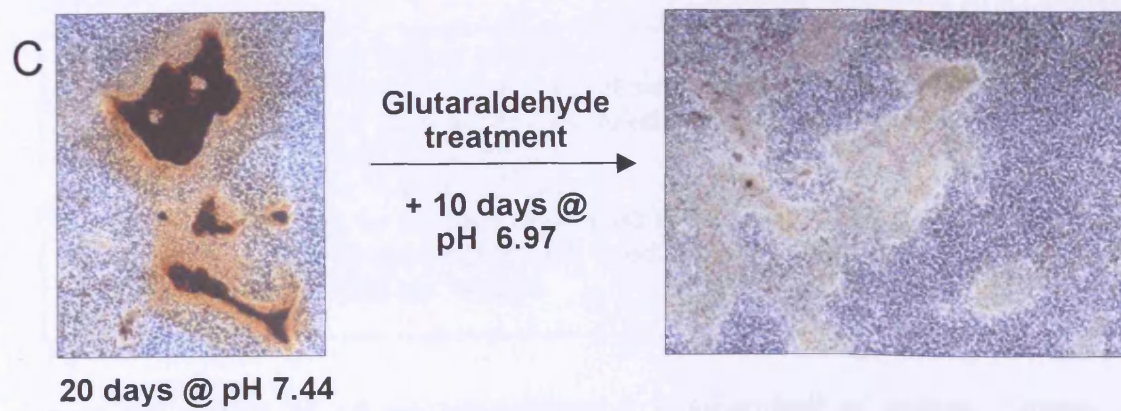
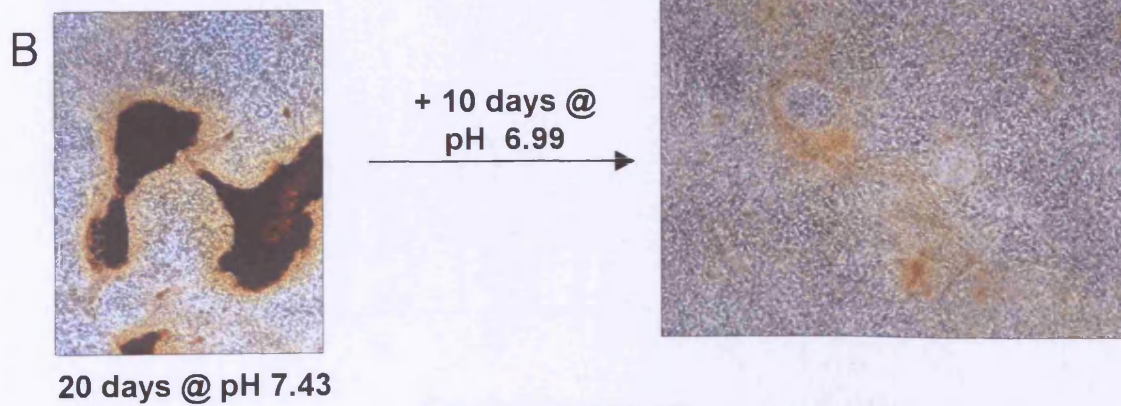
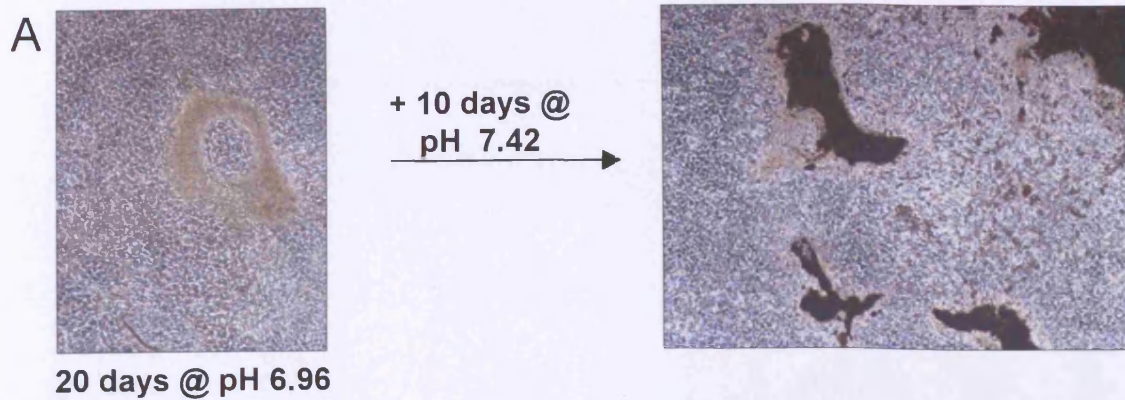
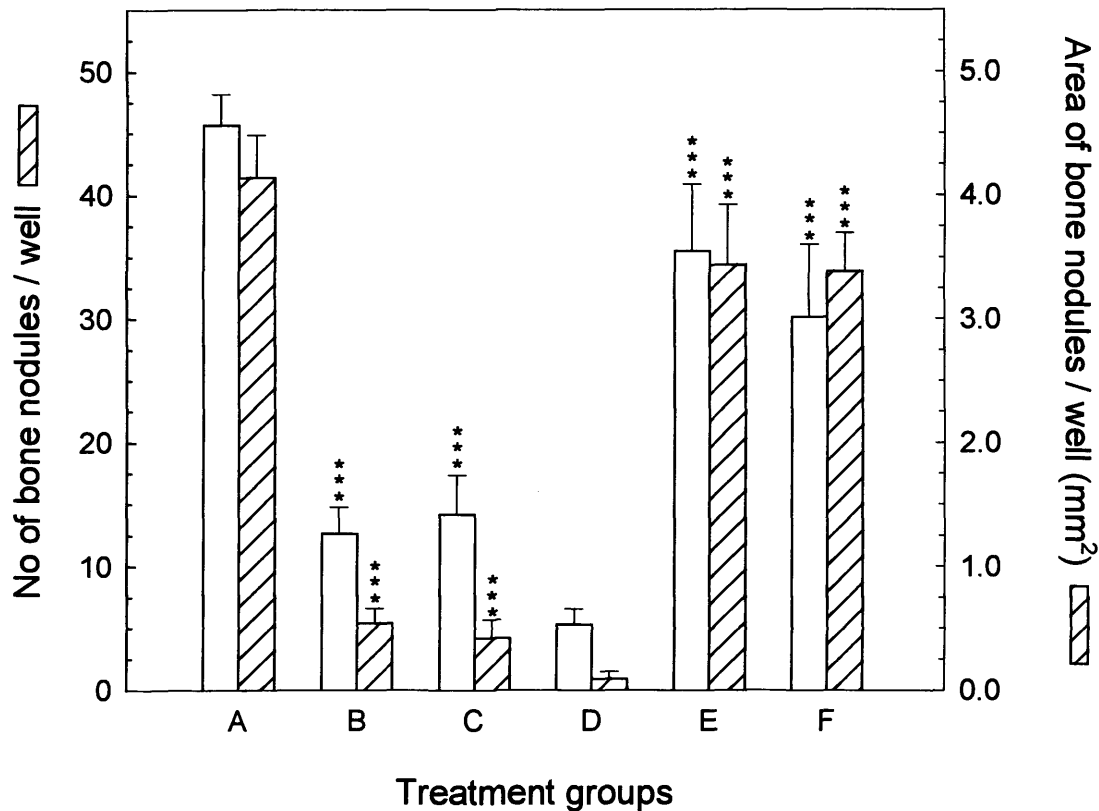


Figure 4.2. Inhibitory effect of acidosis on bone nodule formation by cultured rat osteoblasts. In 16-day cultures, decreasing pH caused progressive reduction in formation of mineralised nodules stained by alizarin red, with complete inhibition at pH 6.93. Values are means \pm SEM (n=4); significantly different from control (pH 7.43) value, * $p < 0.05$, ** $p < 0.01$.

Figure 4.3. Role of pH on crystal forming independent of cellular effects.

Primary rat osteoblasts were cultured in bone nodule-forming medium at control (~7.40) or acidified (~6.90) pH for 20 days and then switched to different test media for the remainder of the experiment. **A.** Cells were cultured for 20 days at pH 6.96, then switched to neutral medium (pH 7.42) for the final 10 days in culture. **B.** Osteoblasts were cultured in neutral medium (pH 7.43) prior to incubation in acidified medium (pH 6.99) for the final 10 days in culture. **C.** Cells were incubated at pH 7.44 for 20 days, killed by glutaraldehyde fixation, then incubated in acidified medium (pH 6.97) for the last 10 days in culture. **D.** At day 20, cells were killed by glutaraldehyde fixation and switched from acidified (pH 7.03) to neutral medium (pH 7.42), for the remainder of the experiment. The presence of mineralised bone nodules was determined by alizarin red staining.





A = cells cultured for 20 days at pH 7.45
B = cells cultured for 20 days at pH 7.43 followed by 10 days at pH 6.99
C = cells cultured for 20 days at pH 7.44, fixed in glutaraldehyde and further incubated at pH 6.97 for 10 days
D = cells cultured at pH 7.02 for 20 days
E = cells cultured cells for 20 days at pH 6.96 followed by 10 days at pH 7.42
F = cells cultured for 20 days at pH 7.03, fixed in glutaraldehyde and further incubated at pH 7.42 for 10 days

Figure 4.4. Role of pH on mineralisation independent of cellular effects. Osteoclasts were cultured for 20 days at control (pH ~7.40) or acidified medium (pH ~7.00), followed by 10 days in acidified or physiological conditions, respectively, in the presence or absence of living osteoblasts. Bone nodule formation was quantified by automated analysis of binary images of nodules formed in plastic wells. Values are means \pm SEM (n=4); significantly different from respective control value: pH 7.45 for 20 days (A) and pH 7.02 for 20 days (B); *** p<0.001.

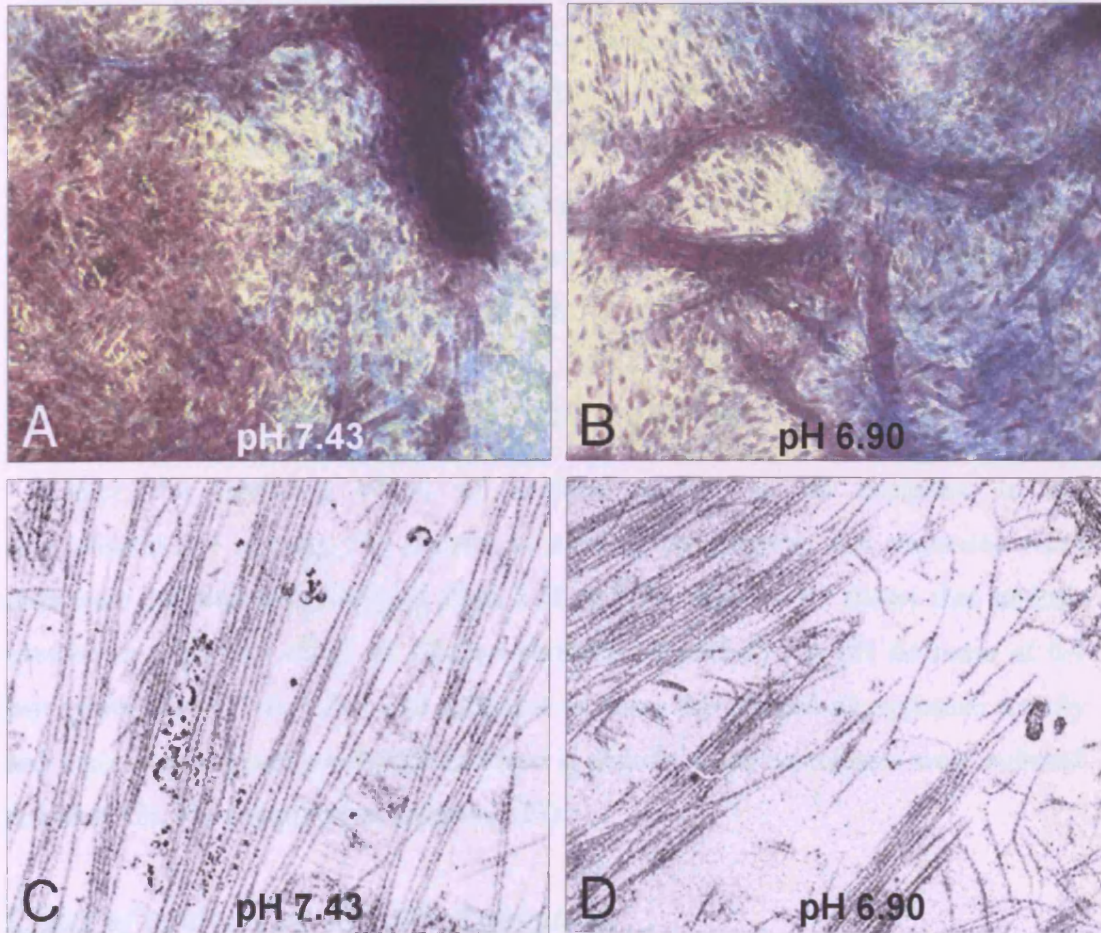


Figure 4.5. Effect of acidosis on collagen structure. A, B. Representative images showing collagen fibres (stained blue-purple using Masson's trichrome) laid down in 21-day cultures of rat calvarial osteoblasts maintained in both control medium at pH 7.43 and acidified medium at pH 6.90 (phase contrast microscopy, 20x objective). C, D. Transmission electron microscopy showed that collagen fibril formation and organisation was similar in cultures at pH 7.43 and 6.90 (3000 x original magnification).

Effect of acidosis on ³H-thymidine and ³H-proline incorporation in osteoblast cultures

The effect of acidosis on the proliferation rate of osteoblasts cultured at pH levels ranging from 7.44 to 6.87 for 5, 9 and 15 days was assessed by using an assay that measures ³H-thymidine incorporation into DNA. As expected, cell proliferation rate was slightly reduced with time in culture, but it was unaffected by pH reduction from 7.44 to 6.87 (**Figure 4.6 A**). The same pH reduction had no effect on collagen production (**Figure 4.6 B**) tested as ³H-proline incorporation into pepsin resistant protein and did not affect protein measured by Lowry method (data not shown).

Effect of acidosis on alkaline phosphatase activity

Since the inhibitory effect of acidosis seemed to be exclusive to the mineralisation of nodules, the activity of alkaline phosphatase was measured when cells were cultured at pH ranging from 7.78 to 6.79. **Figure 4.7** shows that acidosis exerted an inhibitory effect on alkaline phosphatase activity. A pH decrease of 0.1 unit (from pH 7.37 to 7.29) was sufficient to halve the alkaline phosphatase activity and there was a significant 8-fold decrease in activity when osteoblasts were cultured at low pH (6.90) compared to control (7.37).

Effect of acidosis on osteoblast apoptosis

To determine whether the inhibitory effect of acidosis on mineralised nodule formation by osteoblasts might involve increased apoptosis, I used an immunochemical assay to measure levels of mono and oligonucleosomes in the cytoplasm of osteoblasts cultured for 3, 10 and 20 days at pH 7.41 and pH 6.93. Apoptosis rate increased with time in culture but no effect of pH was evident (**Figure 4.8**).

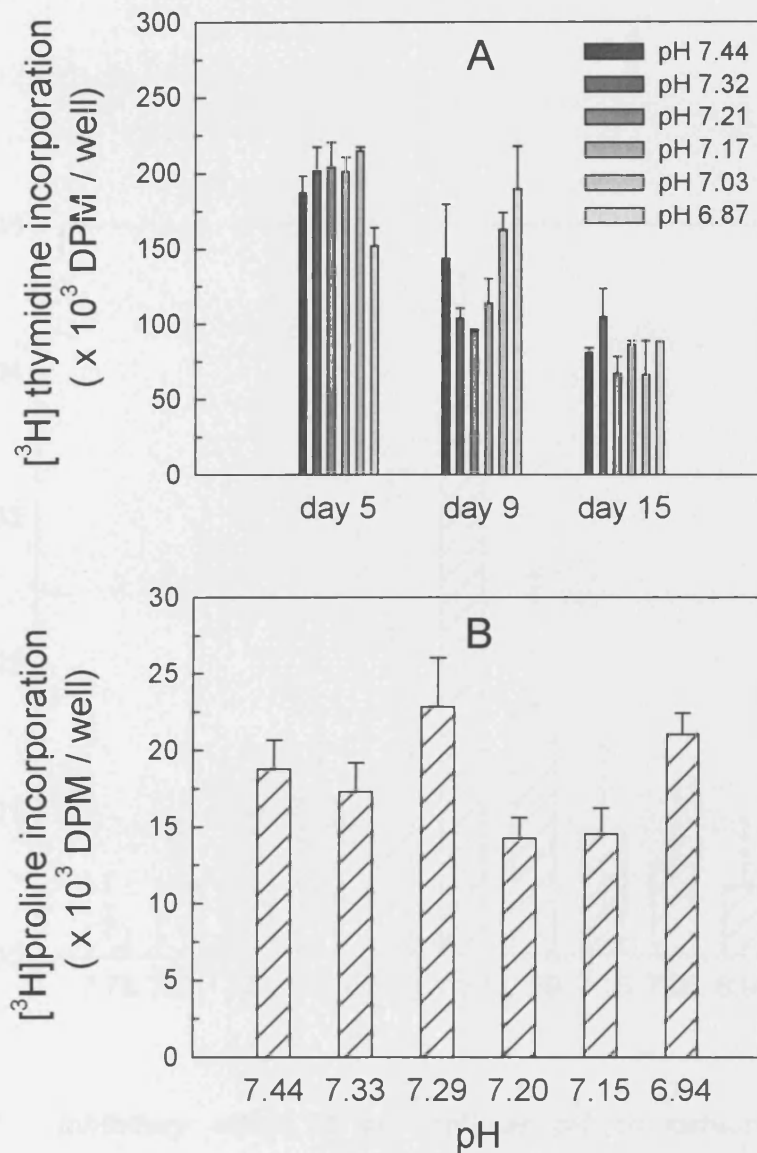


Figure 4.6. Effect of acidosis on cell proliferation and collagen synthesis in primary rat calvarial osteoblasts. **A.** Progressive acidification to pH 6.87 did not affect osteoblast proliferation, assessed by [³H]-thymidine incorporation. Values are means ± SEM (n = 4); differences between treatment groups were not significant. **B.** Acidification to pH 6.93 caused no reduction in collagen synthesis in 16-day osteoblast cultures, assessed as [³H]-proline incorporation in the presence of β-aminopropionitrile monofumarate. Values are means ± SEM (n = 8); differences between treatment groups were not significant.

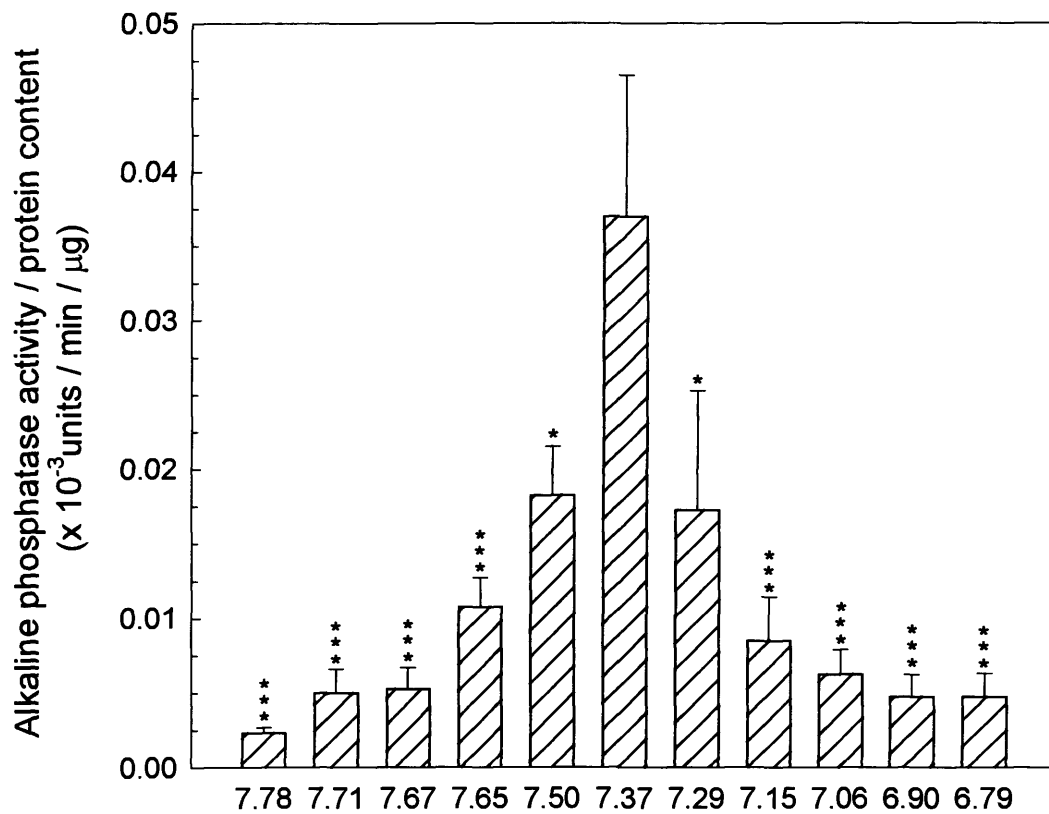


Figure 4.7. Inhibitory effect of extracellular pH on osteoblast alkaline phosphatase activity. Confluent cells were cultured for 6 days in bone nodule-forming medium at indicated extracellular pH levels. Alkaline phosphatase activity was measured colorimetrically from cell lysates and normalised to total Lowry protein content. Values are means \pm SEM (n = 6); significantly different from control (pH 7.37) value, * p<0.05, *** p<0.001.

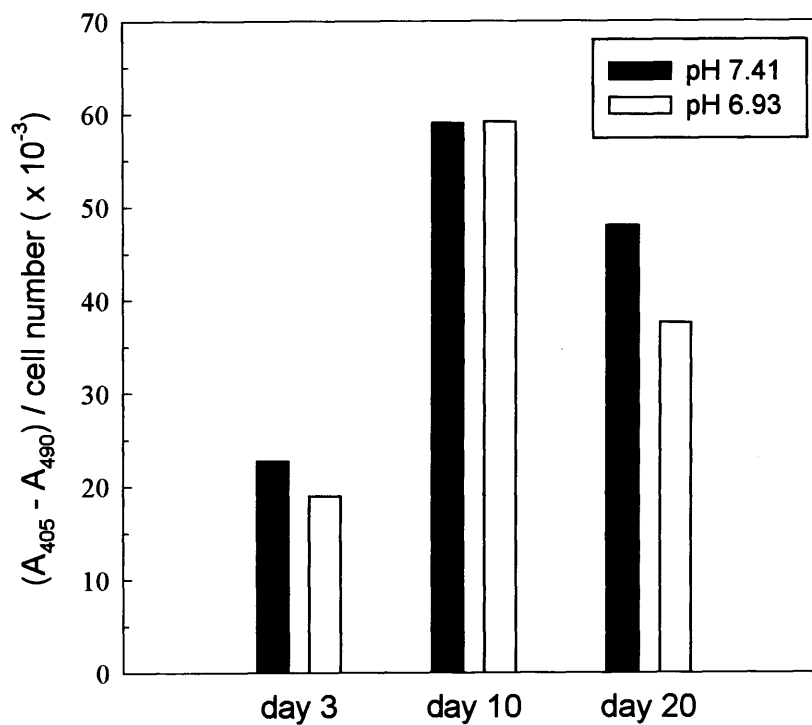


Figure 4.8. Acidosis does not induce apoptosis in rat osteoblasts. Cells were cultured at low (6.93) or 'physiological' pH (7.41) for 3, 10 and 20 days. Apoptosis was determined colorimetrically in pooled cell lysates using an ELISA kit that detects cytoplasmic histone DNA fragments; results are normalised to cell number. Values are means of duplicate determinations.

Effect of acidosis on osteoblast mRNA expression

RT-PCR experiments showed that expression of mRNA for alkaline phosphatase (ALP) by primary osteoblasts increased progressively with time in culture at pH 7.42 but was strongly inhibited at all time points at pH 6.92. Osteopontin (OPN) mRNA was also slightly downregulated at low pH but only at the early time point (day 3). In contrast, matrix Gla protein (MGP) mRNA was clearly upregulated at all time points at pH 6.92. OCN mRNA was upregulated at later time points (days 10 and 17), but its expression was not affected by culturing cells at low pH, suggesting that acidosis does not impair osteoblast differentiation (**Figure 4.9**).

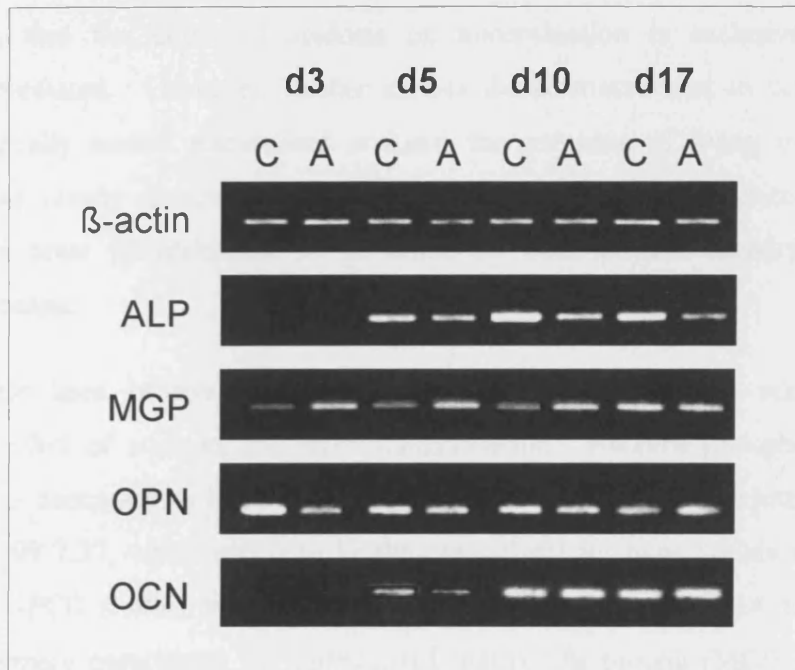


Figure 4.9. *Effect of extracellular pH on expression of mRNAs for alkaline phosphatase (ALP), matrix Gla protein (MGP), osteopontin (OPN) and osteocalcin (OCN) by rat primary calvarial osteoblasts.* Cells were cultured for 3, 5, 10 or 17 days in bone nodule-forming medium at pH 7.42 (control, C) or pH 6.92 (acid, A). RT-PCR was performed on DNase-treated total RNA using β-actin as control. The PCR products were confirmed by sequencing analysis.

DISCUSSION

The study described in this chapter shows that acidosis inhibits mineralised bone nodule formation by cultured rat osteoblasts. This effect appears to be primarily due to decreased mineralisation at low pH.

In spite of the complete inhibition of bone nodule mineralisation by acidosis (pH 6.90), cultures stained using the Masson's trichrome method showed that characteristic nodules containing type 1 collagen fibrils were still deposited by osteoblasts, and TEM analysis indicated that collagen ultrastructure and organisation was not significantly altered by acidosis. These data, together with the observation that lowering the extracellular pH is sufficient to dissolve bone nodules once mineralised, in the presence or absence of living osteoblasts, could lead to the conclusion that the effect of acidosis on mineralisation is exclusively physico-chemical mediated. However, further studies demonstrated that in order to form morphologically normal mineralised nodules, the presence of living osteoblasts is essential, as clearly illustrated in **Figure 4.3**. Therefore, the inhibitory effect of acidosis in bone mineralisation is governed by both physico-chemical and cell-mediated means.

Separate lines of evidence help to explain the mechanisms underlying the inhibitory effect of acidosis on matrix mineralisation. Alkaline phosphatase (ALP) activity, was decreased by lowering pH levels from 7.37 to 6.80. The enzyme activity peaked at pH 7.37, which seems to be the optimal pH for bone nodule formation to occur. RT-PCR studies showed that acidosis downregulated mRNA for ALP and OPN in primary osteoblasts but upregulated matrix Gla protein (MGP) mRNA and did not affect the expression of osteoblast differentiation marker OCN. The upregulation of MGP by acidosis is consistent with the report that *MGP*-knockout mice exhibit extensive lethal ectopic calcification and the notion that MGP functions as an inhibitor of mineralisation (Murshed *et al.* 2004). Osteopontin is also considered to be an inhibitor of extracellular matrix mineralisation (Speer *et al.* 2002) but OPN mRNA was downregulated at low pH, most notably at the earliest time point (3 days).

My results are in line with an earlier report that mouse osteoblasts cultured in acidified medium produces fewer nodules over a 3-week incubation period compared with cells incubated in neutral medium (Sprague *et al.* 1994). However, my findings fit less well with subsequent reports from the same group that acidosis inhibits mRNA for matrix Gla protein, osteopontin and type 1 collagen (Frick *et al.* 1997; Frick and Bushinsky 1998).

Surprisingly, the osteoblast proliferation rate and apoptosis levels were unaffected when cells were incubated in acidified medium compared with cells incubated at control pH. These data together with the collagen analysis suggest that the primary effect of acidosis on osteoblasts is to reduce mineral deposition. Earlier reports showed that a reduction in medium pH produced a marked increase in hydroxyapatite solubility. The effect has been reported to be particularly pronounced near pH 7.0, where a small drop in pH (0.1 unit) causes a 10-fold increase in Ca^{+2} and PO_4^{-3} solubility (Neuman and Neuman 1958). More recently, Larsen and Jensen (1989) studied the effect of pH on the hydroxyapatite solubility of human dental enamel in the pH range of 4.6 and 7.6. They reported that Ca^{+2} and PO_4^{-3} solubilities increased 2 and 4-fold respectively when the pH was decreased from 7.40 to 6.90 (Figure 4.10).

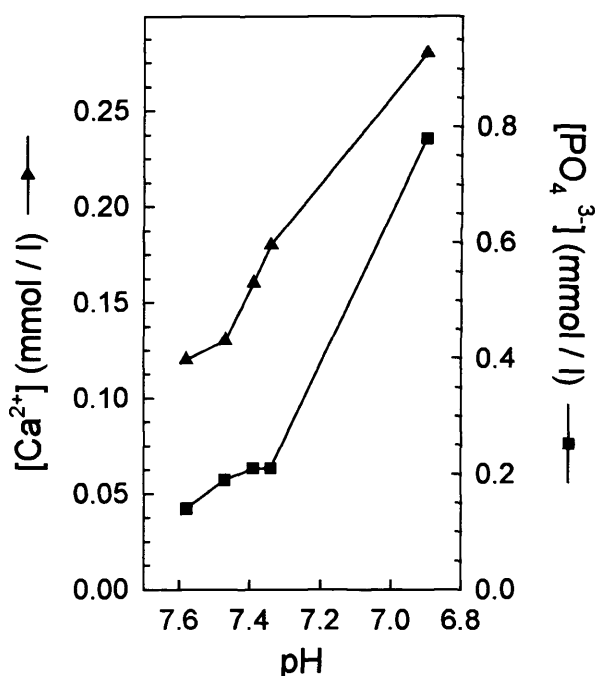


Figure 4.10. The solubility of calcium and phosphate in hydroxyapatite at different pH levels. Adapted from Larsen and Jensen, 1989.

The powerful inhibition of ALP (and the upregulation of MGP) at low pH may conceivably involve cell-mediated H⁺-sensing mechanisms. Proton-sensing G-protein-linked receptors have recently been identified to respond sensitively to pH changes in the range of 7.5 – 6.8 with increases in inositol phosphate or cyclic AMP; one of these novel receptors, OGR1 is functionally expressed on osteoblasts (Ludwig *et al.* 2003).

The data presented here may help explain why some patients with chronic renal metabolic acidosis develop osteomalacia, a condition in which the lag time between osteoid deposition and mineralisation is increased, (Cochran and Nordin 1969; Cunningham *et al.* 1982). This disorder has been reported in 20-30% of patients with renal tubular acidosis, an effect that has been assigned to reduced synthesis of 1,25(OH)₂ vitamin D₃. However, my results indicate that acidosis might also directly contribute to this condition by reducing mineralisation via increased hydroxyapatite solubility and decreased ALP activity. This notion is supported by previous *in vivo* reports that showed alkaline treatment improved this condition (Richards *et al.* 1972; Disthabanchong *et al.* 2004).

It is also important to bear in mind that the normal pH of the extracellular fluid bathing cells is likely to be below 'physiological' (*i.e.* blood) pH and subject to complex, dynamic gradients, depending on the metabolic activity of the cells and their distance from the nearest capillary. Non-invasive pH measurements has shown that in normal subcutaneous tissue, pH decrease by ~0.32 units over a distance of 50 µm away from a blood vessel (pH ~7.40), a distance equivalent to approximate five cell diameters (Martin and Jain 1994). These authors also showed the pH levels in normal skin is ~7.20. My own results suggest for optimal mineralised bone formation to occur *in vivo*, extracellular pH in the immediate osteoblast environment should be ≥ 7.20. My data emphasise the importance of the vasculature for bone health and suggest a rationale for the histological observation that bone formation normally takes place in proximity to blood vessels (Brandao-Burch *et al.* 2005).

In summary, my results indicate that low pH causes an “osteomalacic” condition *in vitro* due to increased CaPO_4^{3-} solubility, decreased alkaline phosphatase activity, and possibly increased MGP expression. These effects will be compounded *in vivo* by the strong stimulatory action of acidosis on osteoclast activity shown in Chapter 2.

CHAPTER 5

Mechanism of pH sensing by bone cells

INTRODUCTION

H⁺-sensing receptors and bone cells

In vitro, small reductions in extracellular pH close to the physiological range result in large stimulation of osteoclast activity both in the absence (Arnett and Dempster 1986; Arnett and Dempster 1987) and in the presence of bicarbonate (Carano *et al.* 1993; Shibutani and Heersche 1993; Arnett *et al.* 1994; Arnett and Spowage 1996; Brandao-Burch and Arnett 2004). The mechanisms by which extracellular acidification stimulates osteoclasts to resorb bone are still not very well understood. Most simply, a low ambient pH may favour resorption by reducing the gradient against which osteoclasts must pump H⁺ across the ruffled border. However, given the steepness of the osteoclast response to small changes in extracellular pH (Chapter 2), this physicochemical explanation seems unlikely to account for all of the effect. Moreover, acidosis leads to an increase in sealing zone formation, indicating increased adhesion to the substrate (Teti *et al.* 1989a; Murrills *et al.* 1993) and upregulates the expression of resorption associated factors such as TRACP and cathepsin K (Chapter 3). Therefore, it is reasonable to suppose that a “pH receptor” coupled to some signalling/ amplification system is present on or in the osteoclast.

Previous work carried out by members of our laboratory showed that lowering extracellular pH, in the absence of bicarbonate, does not lead to a parallel intracellular acidification and results in prompt and reversible increases in membrane conductance. Therefore, a small drop in pH is sufficient to activate osteoclast membrane ion transport (Dunina-Barkovskaya and Arnett 1999). This work mimicked the conditions that mature rat osteoclasts were exposed to in the original experiment performed by Arnett and Dempster (1986), where they showed a reduction in medium pH from 7.4 to 6.8 results in a 14-fold increase in resorption.

Low pH is a key initial requirement for a number of pro-resorptive factors including PTH (Chapter 2) ATP, vitamin D₃ and RANKL (Morrison *et al.* 1998; Murrills *et al.* 1998; Hoebertz *et al.* 2000), to be able to stimulate osteoclasts to resorb bone. The interaction with ATP is mostly interesting because the receptor P2X₂, a subtype of the P2 receptor family, has been shown to require extracellular acidification to show its full sensitivity to ATP (King *et al.*, 1996). Further work showed that acid-induced bone resorption, in the absence of ATP, could be partly inhibited by the general P2 receptor antagonist suramin and by apyrase, an ecto-nucleotidase ATP scavenger, suggesting that the P2X₂ receptor might be the putative 'pH receptor' on osteoclasts (Morrison *et al.*, 1998). An unusual feature of the P2X₂ receptor is the presence of 8 histidyl residues in the extracellular loop domain (Brake *et al.*, 1994); histidine is exceptional in having an imidazole side chain with a pK_b of 6.8 (Creighton 1993). Thus, small pH shifts in the physiological range will change the charge and protonation of histidine side chains, with possible alterations in conformation of this domain. However, work using P2X₂ knockout animals suggested that this receptor does not seem to represent the putative 'pH receptor' on osteoclasts, since cells derived from these mice were still strongly stimulated by acid alone (Hoebertz 2001).

Acidosis accompanies many painful conditions such as cancer (Vaupel *et al.* 1989; Gerweck and Seetharaman 1996). The finding that afferent sensory neurons can be directly excited by protons has generated considerable interest among basic and clinical researchers (Sutherland *et al.* 2000). The two main classes of acid sensing ion channels expressed by nociceptors are the acid sensing ion channels (ASIC) and transient receptor potential vanilloid subtype 1 receptors (TRPV1) also known as vanilloid receptor 1 (VR1) (Waldmann *et al.* 1997; Caterina *et al.* 1997). These receptors are of particular relevance to bone cancer pain as most neurons that innervate the bone express ASIC and/or TRPV1 (Olson *et al.* 1998; Guo *et al.* 1999). Since bone cancer has been associated with increased osteoclast activity, and this cell is known to be directly activated by acidosis, it is reasonable to suggest that osteoclasts themselves may also express ASIC and TRPV1 receptors. Moreover, TRPV1 receptor has been shown to be expressed in macrophages; these cells evolve from the same cell lineage and therefore it is likely that they may share similar signaling mechanisms with osteoclasts.

ASICs are members of the amiloride-sensitive Na^+ channel/degenerin superfamily of ion channels (Waldmann *et al.* 1999). A rapid drop in extracellular pH induces opening of this cation channel. ASIC1 has a pH activation profile that strongly resembles the pH response of osteoclasts (it is activated below 7.0), whereas ASIC2, or mammalian neuronal homologue of degenerins (MDEG1) requires a more acidic pH (Lingueglia *et al.* 1997). A third channel, dorsal root ganglion ASIC (DRASIC) or ASIC3, is activated by $\text{pH} < 4.5$ (Price *et al.* 2001). Immunocytochemical studies showed that despite being specifically expressed in osteoclasts, ASIC1 is not expressed in the membrane to serve as an extracellular pH-sensing receptor (Hoebertz 2001).

TRPV1 receptors are non-selective cation channels that detect heat, extracellular protons and capsaicin, the irritant alkaloid from plants in the Capsicum family (chilli peppers) (Caterina *et al.* 1997; Benham *et al.* 2003). The convergence of these stimuli on TRPV1 channels expressed in peripheral sensory nerves underlies the common perceptual experience of pain due to hot temperatures, tissue damage and exposure to capsaicin. Protons interact with the channel directly by increasing the probability of channel opening and also to potentiate the effects of capsaicin or heat, therefore working as activators and modulators of TRPV1 (Tominaga *et al.* 1998; Sprague *et al.* 2001). Structure–function analyses have shown that protons modulate TRPV1 activity by interacting with a glutamate residue (E600) on the extracellular surface of this channel protein. This residue serves as an important regulatory site for proton modulation of TRPV1 responses to heat over a physiologically relevant pH range of 6–8. On the other hand, capsaicin, which is lipophilic, binds to a site close to the cytoplasmic side of the receptor. Therefore, protons and vanilloids apparently exert their actions on TRPV1 from opposite sides of the membrane, consistent with an allosteric mechanism of proton-mediated potentiation (Jordt and Julius 2002). Most recently, Mantyh and colleagues reported that administration of TRPV1 antagonist JNJ-17203212 or blockade of TRPV1 gene in a mouse model of bone cancer attenuates the pain associated with this condition. These authors suggest that this is because TRPV1 is present on sensory neuron fibres that innervate the mouse femur (Ghilardi *et al.* 2005).

Knockout studies have revealed an important role in bone metabolism for another subtype of the TRPV family: TRPV5. These knockout mice display significant disturbances in bone structure, including reduced trabecular and cortical bone thickness, and dysfunctional Ca^{2+} homeostasis (Hoenderop *et al.* 2003). Moreover, TRPV6, also known as epithelial calcium channel 2 (EcaC2) has been reported to be expressed by osteoblasts (Weber *et al.* 2001).

Recent work has described a novel class of human proton-sensing G-protein-coupled receptors (GPCRs) that respond to reduction in extracellular pH by eliciting rises in intracellular cyclic AMP (TDAG8 and GPR4) or inositol phosphate (OGR1) (Ludwig *et al.* 2003; Wang *et al.* 2004; Ishii *et al.* 2005). These proton-sensing GPCRs are of particular interest because their pH response range (pH 7.6-6.8) and their half-maximal activation points at 37°C (pH ~7.3) correspond closely with that of normal human osteoclasts (see Chapter 2). Like the P2X_2 receptor, the OGR1 has clusters of histidyl residues in its extracellular loop domain (Ludwig *et al.* 2003). Initial localisation studies indicated that OGR1 is expressed by osteoblasts and osteocytes but GPR4 was not detected on rat osteoblasts by RT-PCR analysis (Ludwig *et al.* 2003). It has been recently reported that OGR1 is also expressed by rat osteoclasts and that acidosis increases the accumulation of the transcription factor NFAT2 in the nuclei (Komarova *et al.* 2005). They also showed that activation of this transcription factor involves calcineurin stimulation and induced transient elevation of intracellular Ca^{2+} , which they speculated to be due to OGR1 activation.

The work presented in this chapter investigates the effect of the alkaloid capsaicin (the TRPV1 agonist) on osteoclast formation and activation using normal human osteoclasts formed from PBMCs. Additionally, expression of different H^+ -sensing G-protein-coupled receptors on bone cells was studied.

MATERIALS AND METHODS

Cell culture

Capsaicin and capsazepine were purchased from Sigma-Aldrich (Dorset, UK). The effects of capsaicin (0 - 20 μ M) were studied in osteoclasts derived from PBMCs, as described in Chapter 2. The effect of capsazepine was tested on osteoclast activation by adding 10 μ M of this TRPV1 antagonist for the last two days in culture in the presence of 5 mmol/l NaOH and 15 mmol/l H⁺ as HCl, resulting in an operating pH close to pH 7.4 and 7.0 respectively.

Bone nodule assay

Primary rat osteoblasts were obtained as described previously in Chapter 4. These cells were cultured in plastic wells for 10, 12 or 15 days in control or acidified medium modified by the presence of 0 or 25 mmol/l HCl.

Reverse transcriptase PCR

RT-PCR analysis on the expression of OGR1, TDAG8, GPR4, ASIC1a and TRPV1 mRNA was carried out as detailed in Chapter 3. The primers used were purchased from MWG (Ebersberg, Germany) and are shown in **Table 5.1 and 5.2**.

Table 5.1: Primers used for rat RT-PCR analysis

Factor	Forward primer	Reverse primer	Ta
β actin	gtt cgc cat gga tga cga t	tct ggg tca tct ttt cac gg	53°C
OGR1	ctg agc cca tga gga gtg tg	ggt agg acg cca gca gca aa	52°C

Ta = annealing temperature

Table 5.2: Primers used for human RT-PCR analysis

Factor	Forward primer	Reverse primer	Ta
GAPDH	gtg aac ggg aag ctc act gg	tga ggt cca cca ccc tgt tg	57°C
OGR1	tgg gga aca tca ctg cag	atg ttc tcg tac agg agg	52°C
TDAG8	ccg gaa gaa ata tgg aag g	cac aga tat cag cag ttg g	52°C
GPR4	acc acc tct ttc cgc cat	cag cac agg aag gcg at	53°C
ASIC1a	gca gat cct gct ctg gac ttc c	aat gac ctc gta ggc gta gtc	57°C
TRPV1	gca aga aca tct gca agc tgc	gct gac aga gca ctg gtg ttc	55°C

Ta = annealing temperature

Immunocytochemistry and confocal analysis

Immunocytochemical procedures were performed as described in Chapter 3. The OGR1 and TDAG8 antibodies used were purchased from LifeSpan Biosciences (Seattle, WA, USA) and Insight Biotechnology Ltd. (Wembley, Middlesex, UK), respectively. The fluorescein isothiocyanate (FITC)-conjugated secondary antibodies used were purchased from DakoCytomation Ltd., Ely, Cambridgeshire, UK.

Western blot analysis

Isolated osteoclasts or osteoblasts were lysed in ice-cold radio immunoprecipitation (RIPA) lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1mg/ml aprotinin, 1 mM Na₃VO₄ and 2.5mg/ml deoxicollic acid). Cell homogenates were sonicated for 5 minutes and stored in the -80°C freezer for at least half-hour before use. Protein concentrations from lysates were determined using the Bradford assay (Sigma-Aldrich, Gillinham, Dorset, UK). Prior to loading total protein samples were denatured by incubating samples at 100°C for 5 minutes in the presence of 5x reducing sample buffer (60mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 10% β-mercaptoethanol and 0.1% bromophenol blue). Protein samples (20 µg/ lane) were loaded onto SDS-PAGE (10%) gels and transferred onto a polyvinylidenedifluoride

(PVDF) membrane (Amersham) by the use of a wet tank blotter (Bio-Rad, Hercules, CA, USA) at 150V for 1 hour. Membrane was afterwards blocked with 5% non-fat milk and incubated with OGR1 antibody (1:200) or TDAG8 (1:1000) overnight at room temperature. After washing, blots were incubated in a horseradish peroxidase-conjugated secondary antibodies (for 1 hour at room temperature). The peroxidase-detection system (1.25mM luminol, 0.2 mM coumaric acid, 0.1M Tris pH 8.5, 0.032% hydrogen peroxide) was used for the visualization of the immunoreactivity. Western blotting performed without primary antibody served as negative control.

RESULTS

Effect of capsaicin on normal human osteoclast activation

The effect of capsaicin on the activation of blood-derived osteoclasts at control and acidified pH was tested by culturing these cells for 12 days at control (pH ~7.40) pH followed by a further 48 hours in medium modified by the addition of 5 mmol/l NaOH or 15 mmol/l HCl in the presence of 0, 2, 20, 200, 2000 and 20000 nM capsaicin (TRPV1 agonist). The results shown in **Figure 5.1** indicate that capsaicin at hormonal concentrations (≥ 2 nM) strongly activates osteoclasts to resorb dentine in non-acidified conditions, but does not enhance the resorption activity of these cells at low pH. Capsaicin treatment increased osteoclast resorptive activity by approximately 4-fold, with maximal activation at 200nM capsaicin at physiological pH. Capsaicin did not appear to affect osteoclast survival: cell numbers were not significantly altered among treatment groups in either control or acidified media. To study the involvement of the TRPV1 receptor on the acid response by osteoclasts further, the antagonist capsazepine was tested. The stimulatory effect of low pH on osteoclast resorption was halved by treatment with 10 μ M capsazepine (**Figure 5.2**). This compound may be marginally toxic to osteoclasts at 10 μ M as cell numbers were somewhat reduced, although osteoclast morphology was not obviously affected (**Figure 5.2 A and B**).

Effect of capsaicin on normal human osteoclast formation

PBMCs were treated with 0, 2, 20, 200, 2000 and 20000 nM of capsaicin in the presence of 5 mmol/l NaOH or 15 mmol/l HCl from day 4 of culture. The effect of capsaicin on the formation of human osteoclasts at control or acidified pH is shown in **Figure 5.3**. Capsaicin treatment inhibited osteoclastogenesis at control pH; an increase in capsaicin concentration led to a decrease in osteoclast number, with maximal inhibition of 4-fold occurring at 20000nM capsaicin. This was accompanied by a 2-4-fold increase in osteoclast resorptive activity with maximal activation at 200nM capsaicin. Osteoclast formation was not affected by capsaicin when cells were cultured at low pH (~7.0).

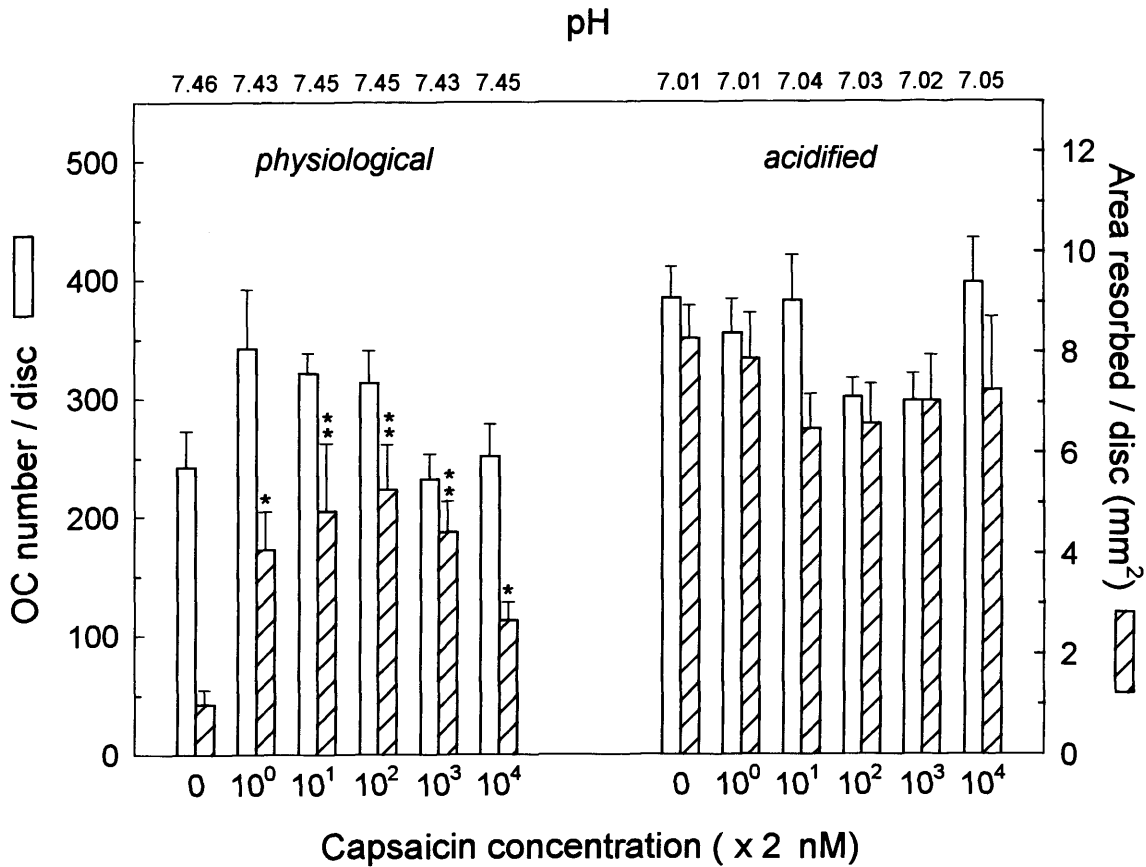


Figure 5.1. Effect of capsaicin on the activity of human osteoclasts derived from PBMCs. Cells were cultured on dentine discs for 12 days in the presence of RANKL (1ng/ml), M-CSF (5ng/ml) and 5 mmol/l OH⁻, followed by a further 48-hour incubation in culture medium modified by the addition of 5 mmol/l OH⁻ or 15 mmol/l H⁺ and 0, 2, 20, 200, 2000 or 20000 nM capsaicin. Significantly different from each respective control (pH 7.46 or 7.01), * p<0.05, ** p <0.01. Values are means ± SEM (n = 8).

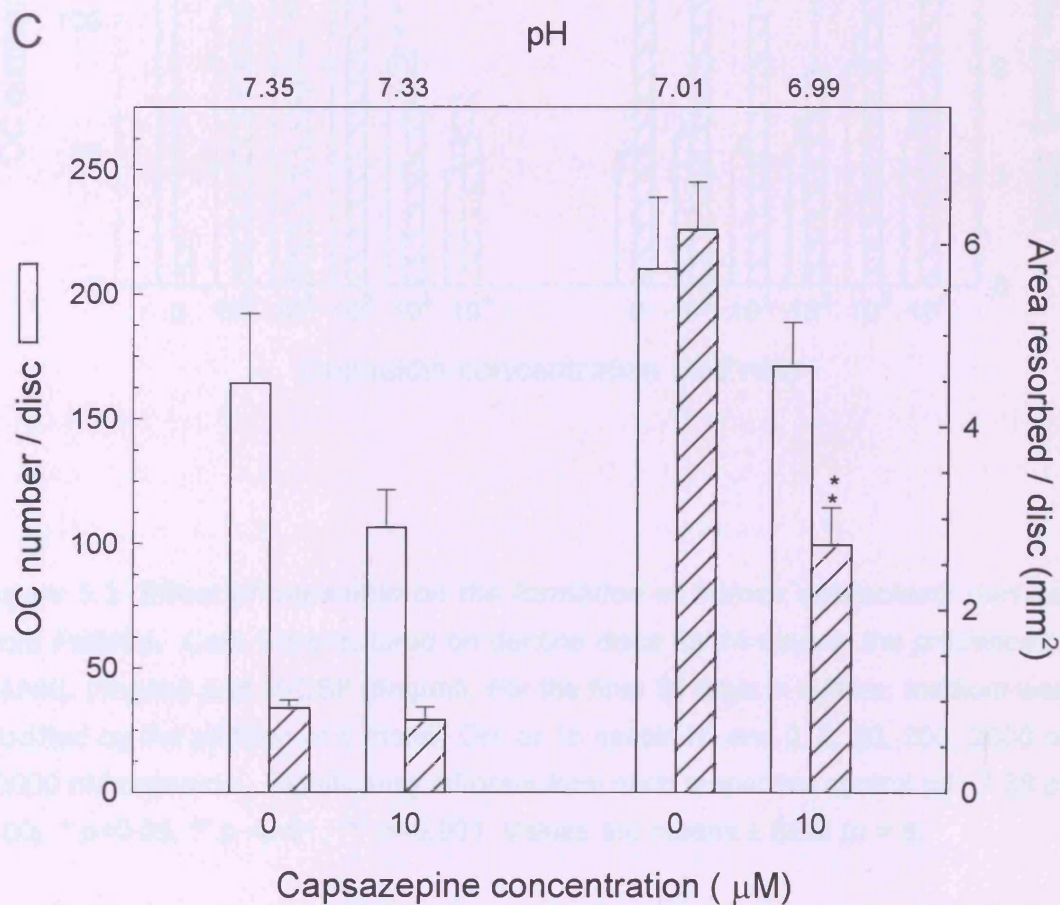
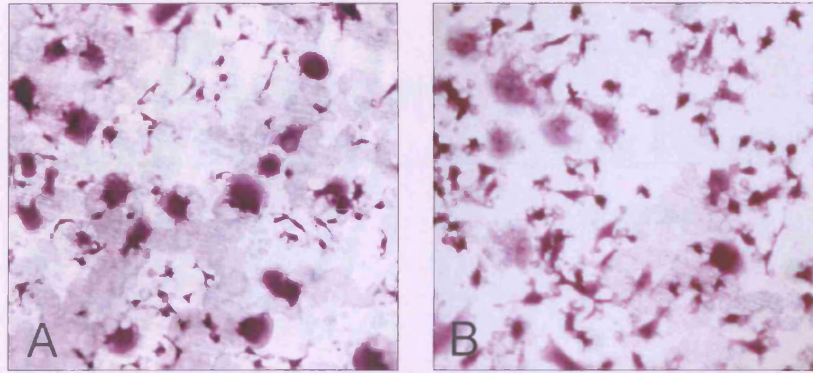


Figure 5.2. Inhibitory effect of capsazepine on osteoclast activation in human PBMC cultures. Cells were cultured for 12 days in the presence of 5 mmol/l OH⁻ to generate osteoclasts, followed by 2 days in MEM modified by the addition 5 mmol/l OH⁻ or 15 mmol/l H⁺ and 0 or 10 μM capsazepine. A. Transmitted light image of osteoclasts cultured at pH 7.01 B. Osteoclasts cultured at pH 6.99 in the presence of 10 μM capsazepine; cells were stained with toluidine blue. C. Quantification analysis show that capsazepine inhibits the acid effect by osteoclasts. Significantly different from each respective control (pH 7.35 or 7.01), ** p < 0.01. Values are means ± SEM (n = 8).

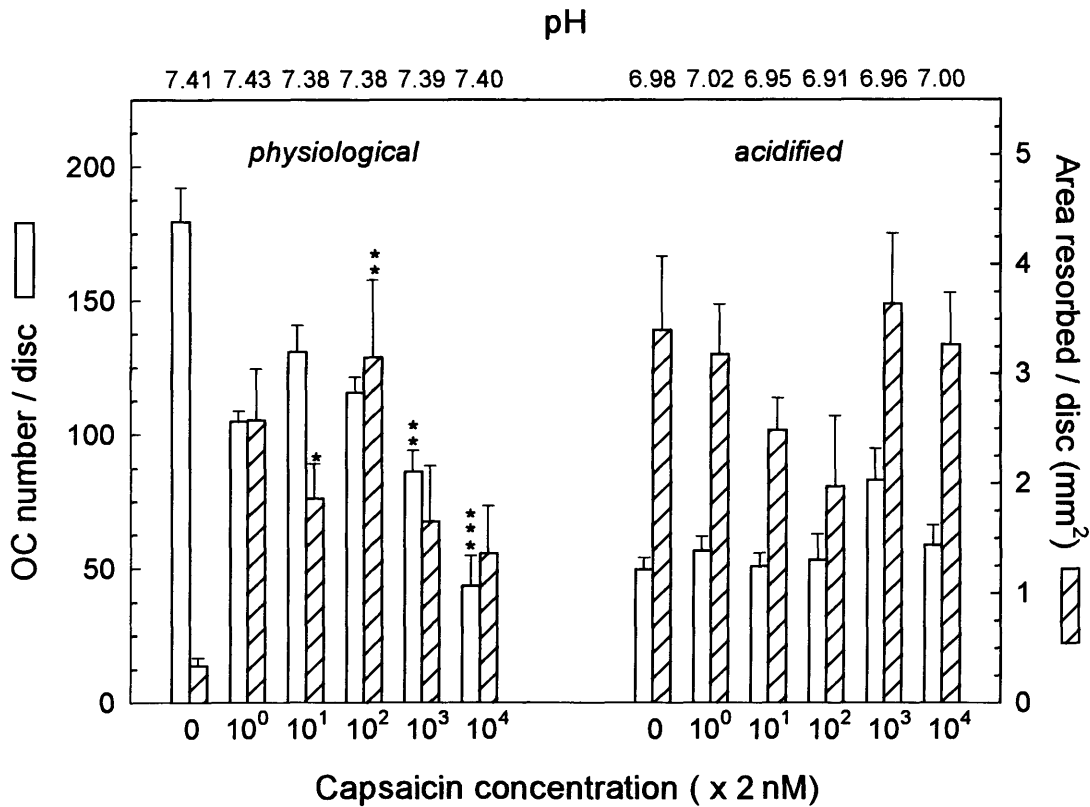


Figure 5.3. Effect of capsaicin on the formation of human osteoclasts derived from PBMCs. Cells were cultured on dentine discs for 14 days in the presence of RANKL (1ng/ml) and M-CSF (5ng/ml). For the final 10 days in culture, medium was modified by the addition of 5 mmol/l OH⁻ or 15 mmol/l H⁺ and 0, 2, 20, 200, 2000 or 20000 nM capsaicin. Significantly different from each respective control (pH 7.38 or 7.00), * p<0.05, ** p <0.01, *** p<0.001. Values are means ± SEM (n = 8).

Effect of acidosis on H⁺-sensing receptors expression

The mRNA expression of different H⁺-sensing receptors was investigated in primary rat osteoblasts and human osteoclast cultures. Primary rat osteoblasts were cultured for 10, 12 or 15 days at pH 7.41 or 6.90. The expression of mRNA for OGR1 by primary rat osteoblasts did not change with time in culture at pH 7.41 but was upregulated at all time points at pH 6.90 (**Figure 5.4 A**). PBMCs were cultured on dentine discs for 12 days at ~ pH 7.40, followed two days at pH 7.45 or 6.92. RT-PCR analysis (**Figure 5.4 B**) shows that the multifunctional receptor TRPV1 and the G-protein-coupled receptors OGR1 and TDAG8 were both expressed by human osteoclasts and were upregulated by acidosis. ASIC1a was also detected by RT-PCR but its expression was downregulated by lowering the pH from 7.45 to 6.92. The expression of GPR4 was not detected in human osteoclast cultures by RT-PCR (data not shown).

Expression of TDAG8 and OGR1 receptors was also studied on blood-derived osteoclasts cultured on dentine discs for the final two days at pH 7.38 or 6.98 using immunocytochemistry. No membrane staining was detected for TDAG8 receptor; however confocal analysis revealed cytoplasmic staining which appeared to be upregulated when these cells were cultured at low pH (**Figure 5.5**). OGR1 receptor was also identified in cultured human osteoclasts and at acidified conditions it was expressed in the membrane. OGR1 staining was also observed at the sealing zone when cells were cultured at both control (pH 7.38) or acidified (pH 6.98) conditions (**Figure 5.6**). Immunoblotting was performed using total protein samples obtained from cultured human osteoclasts and rat osteoblasts (**Figure 5.7 and 5.8**). Immunoblot with OGR1 primary antibody generated a single reactive band of the expected weight (43 kDa), which was upregulated at acidified conditions in both cultures. A single TDAG8-reactive band at approximately 90 kDa was detected and it was overexpressed at acidified conditions in both cultures. This band, however, was not in the expected weight range (43 kDa). Protein lysate obtained from rat spleen was used as a positive control.

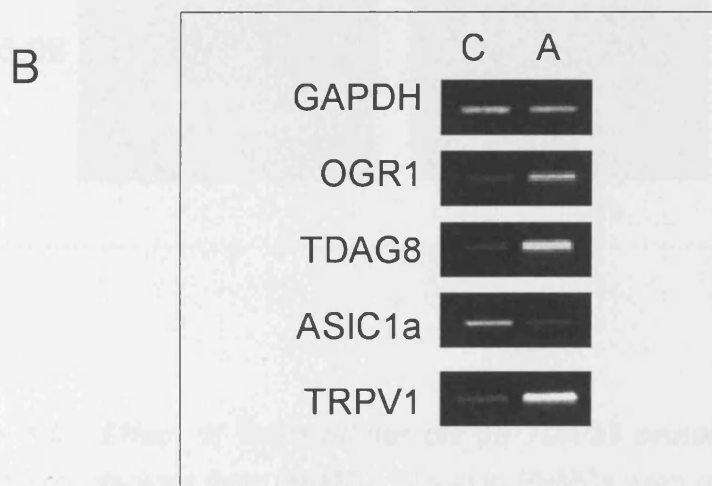


Figure 5.4. Effect of extracellular pH on expression of mRNA for H^+ -sensing receptors by rat primary calvarial osteoblasts and blood-derived human osteoclasts. **A.** Cells were cultured in plastic wells for 10, 12 or 15 days in control (C) medium at pH 7.41 or in acidified medium (A) at pH 6.90. RT-PCR was performed on DNase-treated total RNA using β -actin as control. **B.** PBMCs were cultured for 12 days in the presence of RANKL (1ng/ml), M-CSF (5ng/ml) and 5 mmol/l H^+ , followed by a further 2 days in medium at pH 7.45 (control, C) or pH 6.92 (acid, A). RT-PCR was performed on DNase-treated total RNA using GAPDH as control. PCR products were confirmed by sequencing.

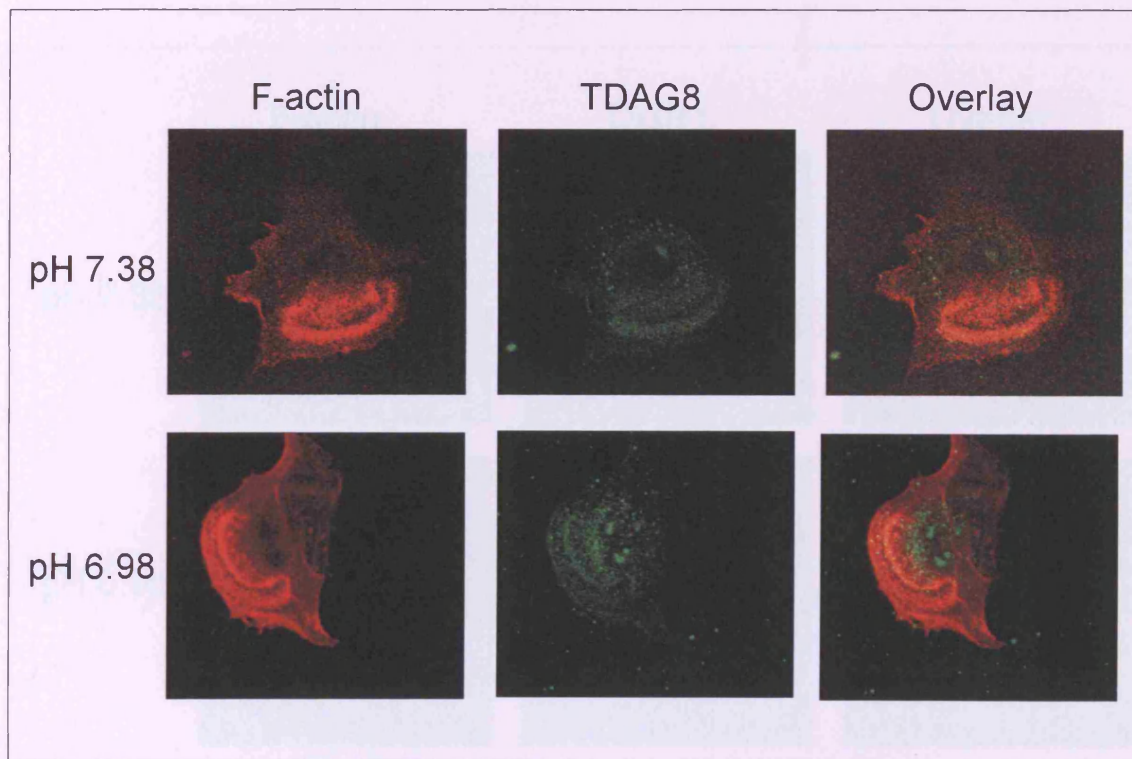


Figure 5.5. Effect of extracellular pH on TDAG8 protein expression by human osteoclasts derived from PBMCs. Human PBMCs were maintained at pH ~ 7.40 for 14 days, then at the indicated pH for a further 2 days. Resorbing osteoclast immunostained for TDG8 receptor (green) cultured on dentine disc, imaged by confocal microscopy. Rhodamine-phalloidin staining was used to demonstrate the F-actin 'ring' (shown in red). TDAG8 protein expression was upregulated in human osteoclast cultures by acidosis but this receptor does not appear to be expressed in the membrane.

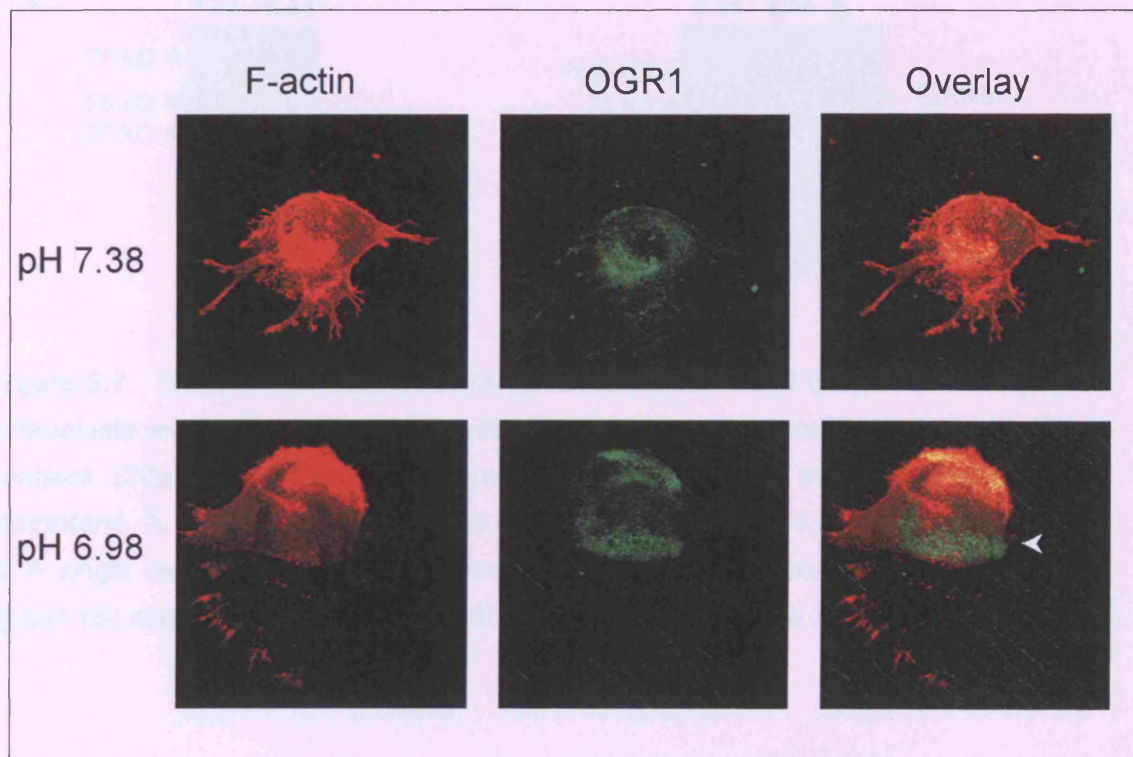


Figure 5.6. Effect of extracellular pH on OGR1 protein expression by human osteoclasts derived from PBMCs. Human PBMCs were maintained at pH ~ 7.40 for 14 days, then at the indicated pH for a further 2 days. Resorbing osteoclast immunostained for OGR1 receptor (green) cultured on dentine disc, imaged by confocal microscopy. Rhodamine-phalloidin staining was used to demonstrate the F-actin 'ring' (shown in red). OGR1 appears to be expressed in the membrane in acidified conditions (see white arrow). Note that it is also localised to the sealing zone regardless of pH levels (yellow).

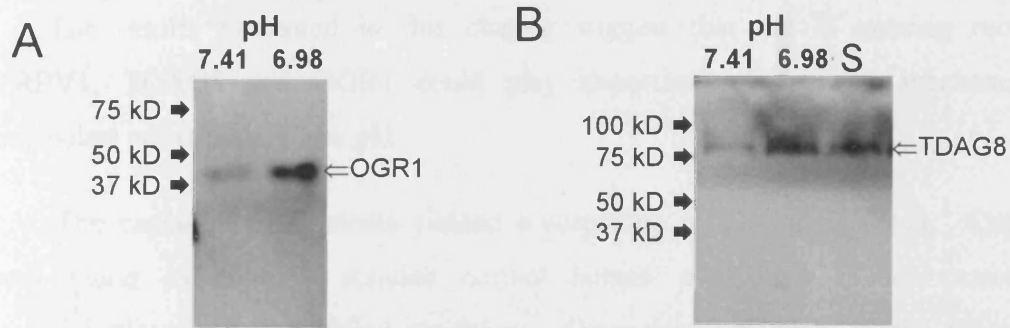


Figure 5.7. Western blotting of primary rat osteoblast cell lysates. Primary rat osteoblasts were cultured in plastic wells for 15 days at indicated pH. Total protein samples (20 μ g/well) were loaded onto 10% SDS gel and transferred to PDVF membrane. **A.** A single OGR1-reactive band of approximately 43 kDa was detected. **B.** A single reactive band of approximately 90 kDa was detected. Cell lysate from spleen (S) was used as positive control. Molecular markers are indicated on the left.

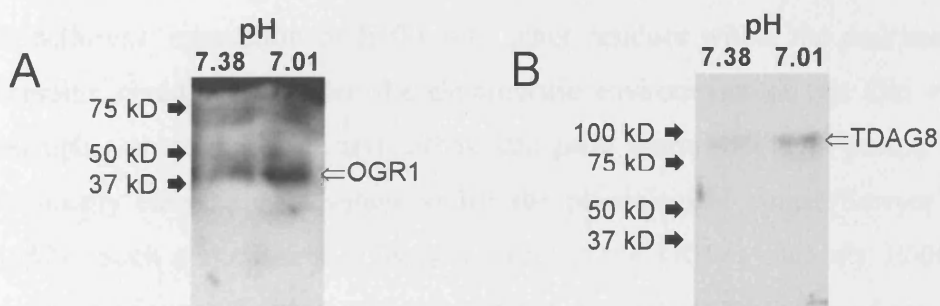


Figure 5.8. Western blotting of blood-derived osteoclast cell lysates. PBMCs were cultured for 14 days at pH ~ 7.40, then for a further 2 days at indicated pH. Total protein samples (10 μ g/well) were loaded onto 10% SDS gel and transferred to PDVF membrane. **A.** The blot was probed with polyclonal antibody to OGR1 receptor. A single reactive band of approximately 43 kDa was detected. **B.** The blot was probed with a polyclonal antibody to TDAG8 receptor. A single reactive band of approximately 90 kDa was detected. Molecular markers are indicated on the left.

DISCUSSION

The results presented in this chapter suggest that the H⁺-sensing receptors TRPV1, TDAG8 and OGR1 could play important roles in the mechanism of osteoclast activation by low pH.

The capsaicin experiments yielded a surprising and dramatic result. Capsaicin was found to strongly activate normal human osteoclasts at low nanomolar concentrations in non-acidified conditions. Osteoclast formation, on the other hand, was decreased by capsaicin at pH ~ 7.4 although this reduction in osteoclast numbers was accompanied by a greater increase in resorption. My results showed that osteoclast formation was affected in a similar manner by acidosis (see Chapter 2). To date, only pertussis toxin (PTX), which inactivates the inhibitory G protein (G_i), has been reported to 'switch on' osteoclasts without co-stimulation by H⁺ (Spowage and Arnett 1995). Protons have been reported to bind to a glutamate residue (E600) on the extracellular domain of TRPV1 channels (Jordt *et al.* 2000). Titration of free Glu residues by protons usually occurs at pKa 4.3 (Creighton 1993), which is well below the half-maximal proton concentration of pH 7.3 that I found to be effective to stimulate osteoclast resorption (Chapter 2; Brandao-Burch and Arnett 2004). Nonetheless, interaction of E600 with other residues within the multimeric channel complex could greatly alter the electrostatic environment of this Glu residue. For example, amino acid carboxyl-carboxylate pairs sharing a single proton can display drastically elevated pKa values within the physiological range (Sawyer and James 1982). Such a mechanism is likely to occur in the TRPV1 channels; E600 may form similar pairings with other proton-accepting or -donating amino acid residues across the outer surface of the channel. This pH-dependent process could lead to the channel opening at higher pH or influence the response of the channel complex to other agonists such as heat (Jordt *et al.* 2000).

My results also showed that the acid-activation effect was marginally inhibited by capsazepine, a synthetic analogue of capsaicin itself. The inhibitory effect of capsazepine was not obviously due to cell toxicity. However, there are problems associated with the use of capsazepine as an antagonist for TRPV1. First, it is a relatively weak antagonist which appears to show species-specific activity (McIntyre *et al.* 2001; Oh *et al.* 2001) and second, at similar concentrations to which it acts on

TRPV1, it inhibits other voltage gated Ca^{2+} channels (Docherty *et al.* 1997). It is noteworthy, however, that capsazepine has been reported to block the response of capsaicin and heat most strongly to human TRPV1 compared to other species (McIntyre *et al.* 2001). Therefore, further work will need to be carried out with the use of a more selective TRPV1 antagonist such as SB-366791 (Gunthorpe *et al.* 2004)

Work by members of our laboratory showed that changes in ambient temperature within the pathophysiological range affect the formation and activity of osteoclasts. The effect of decreasing rodent osteoclast activity by increasing temperature to 40°C was upmost when cells were cultured at physiological pH compared to pH 7.00 (Ms Jennifer Utting, UCL, London, personal communication). Since TRPV1 respond to heat as well as protons, these results are consistent with my own data as they suggest that TRPV1, which responds to heat and is modulated by protons may play a crucial role in osteoclast pathophysiology (Andersson *et al.* 2004).

Preliminary results demonstrated that both TDAG8 and OGR1 were expressed by bone cells and were upregulated at acidified conditions suggesting that these receptors may be involved in the activation of osteoclasts by acidosis. Additional evidence for OGR1 expression on osteoclasts is provided by recent work of Komarova and colleagues (2005) who reported that OGR1 is expressed on rat osteoclasts and RAW 264.7 cells using immunocytochemistry. Surprisingly, western blotting results suggest that TDAG8 receptor might be present on bone cells as a dimer as a single reactive band of ~ 90 KDa (twice as big as expected) was detected. Further work is needed to detect the receptor as a monomer, this can be achieved by using harsher denaturing conditions (*e.g.* increasing the concentration of beta-mercaptoethanol and/or boiling the protein for longer periods). Also, the use of a different commercially available TDAG8 antibody would also be necessary to check for antibody-antigen specificity.

ASIC1a mRNA expression was downregulated by acidosis. This is in accordance with previous work performed by members of our laboratory who demonstrated that despite this receptor being specifically expressed on osteoclasts, it was not expressed in the membrane to serve as an extracellular pH-sensing receptor (Hoebertz 2001). I was not able to detect the presence of GPR4 mRNA in osteoclast-forming human

PBMC cultures; however, this result will have to be confirmed by other methods, such as immunocytochemistry. Additionally, TRPV1 receptor mRNA was also upregulated by low pH. Further work will be needed to confirm the presence of TRPV1 on the surface of human osteoclasts and also to check whether these receptors are specifically present on these cells.

As capsaizine did not completely inhibit the acid-response by human osteoclasts, it is reasonable to hypothesise that other receptors such as P2X₂, TDAG8 and OGR1 may also be involved in this mechanism. It has been reported that GPCRs are co-localised with TRPV1 on primary afferent sensory neurons (Julius and Basbaum 2001). GPCRs are also known to increase TRPV1 activity indirectly by stimulation of intracellular second messengers such as protein kinase A (PKA) and protein kinase C (PKC) (Julius and Basbaum 2001). Most recently, Komarova and colleagues (2005) showed that in osteoclast-like cells, acidosis leads to activation of phospholipase kinase (PLC), which in turn, activates the hydrolysis of inositol phosphate and the subsequent stimulation of PKC, known to activate TRPV1. It is therefore reasonable to speculate that in osteoclasts TDAG8, OGR1 and TRPV1 (and perhaps other putative pH receptors) act together to ensure that bone cells can perform the critical pathophysiological role of maintenance of acid-base balance. These discoveries could be an important step towards identifying new classes of compounds that modulate osteoclast, and also osteoblast activity.

CHAPTER 6

General discussion and future work

The work presented in this thesis has focused on the actions of extracellular pH on the function of normal osteoclasts and osteoblasts. Evidence is also presented for the mechanism by which acidosis regulates bone cell function.

This project was planned based on the pioneering work of Arnett and Dempster (1986) which demonstrated a direct positive effect of low pH on osteoclast resorptive activity, and studies reporting that osteoblasts are also able to respond to small changes in culture medium pH (Krieger *et al.* 1992). Despite these observations the mechanism by which low pH affects bone cell function is still not fully understood.

The most basic function of the vasculature is to deliver food and O₂ to cells and remove waste, such as H⁺ and CO₂. As individuals age, this ability decreases due to decline of the vascular supply and renal insufficiency; the consequence of such a decline is likely to be a reduction in blood pH. It has been reported that in normal individuals, blood [H⁺] decreases by 6-7% (equivalent to 0.03 pH units) in a 60-year period (from 20 to 80 years of age) (Frassetto and Sebastian 1996). In bone, a decrease in blood flow leads to a progressive loss of medullary blood supply, which results in tissue hypoxia and acidosis (Bridgeman and Brookes 1996). In the first experimental chapter, I showed that normal human osteoclasts *in vitro* are extremely sensitive to pH changes, *i.e.* their resorptive activity increases significantly by lowering the extracellular pH. This decrease in pH levels leads to an increase in resorption by osteoclasts and over a period of time this could result in a measurable loss of bone mass, such as that seen in age-related osteoporosis. It is likely that *in vivo* acidosis strongly amplifies the resorption resulting from increased osteoclast formation by hypoxia (Arnett *et al.* 2003)

Another example of periods of low bone blood flow is at early stages of fracture healing. Following a bone fracture, local pH may fall to low levels owing to disruption of blood supply. pH measurements of samples taken from the femoral neck of rabbits during operation for impacted subcapital fracture has been shown to

be 6.87 (Richards and Brookes 1968). Following bone fracture, osteoclasts resorb away the damaged bone and participate in the remodelling of fracture callus. Therefore, the osteoclast's ability to increase resorption by low extracellular pH is a pathophysiological advantage in these situations.

Since the influential papers by Rodan and Martin (1981 and 1982), the prevailing view has been that activation of osteoclasts by PTH and other pro-resorptive factors are mediated indirectly by osteoblasts. In the mid-80s, Chambers and colleagues tested this hypothesis and showed that *in vitro* osteoblasts not only mediate the effect of PTH (McSheehy and Chambers 1986a; McSheehy and Chambers 1986b), but also that of IL-1 (Thomson *et al.* 1986), TNF- α and β (Thomson *et al.* 1987) and 1,25 (OH)₂ vitamin D₃ (McSheehy and Chambers 1987) on osteoclasts. My finding that human osteoclasts are activated by PTH in the absence of osteoblasts contradicts these observations, and are supported by data from our collaborators who showed the PTH receptor, PTH1R, is expressed by human osteoclasts derived from PBMCs (Dempster *et al.* 2005). Together, our data suggest that the dogma that PTH stimulation of osteoclasts requires osteoblast mediation is not correct. It may be reasonable to suppose that PTH exerts a dual regulatory action on bone resorption by acting directly on osteoclasts and also, indirectly, via osteoblasts.

A 'classical' action of growth factors and cytokines is to activate metabolism, resulting in increased proton efflux from cells. Investigations using osteoblast-like SaOS-2 cells, primary rat osteoblasts and whole bone organ cultures showed that IGF-1 or PTH application stimulates osteoblastic extracellular acidification (Barrett *et al.* 1997; Santhanagopal and Dixon 1999; Belinsky and Tashjian, Jr. 2000). This model system may resemble an *in vivo* situation, where acid production by osteogenic or stromal cells reduces local pH, through the action of bone-resorbing agents such as PTH, to a value optimal for osteoclastic bone resorption. These observations provide a plausible mechanism by which PTH, growth factors, cytokines, mitogens can influence osteoclast activity in an indirect manner.

The finding that PTH activates osteoclasts only when they have been acid-activated is consistent with the notion that acidification is the key initial requirement for osteoclasts to be able to excavate resorption pits. Following acid-activation, further stimulation with "conventional" pro-resorptive agents such as PTH,

1,25(OH)₂ vitamin D₃ (Murrills *et al.* 1998), nucleotides (Morrison *et al.* 1998; Hoebertz and Arnett 2003) or RANKL (Hoebertz and Arnett 2003) can then occur. Thus, extracellular H⁺ may be regarded as the long-sought 'osteoclast activation factor' (OAF). My results emphasise that extracellular pH is a critical influence on osteoclast activity which must be considered in the design, monitoring and interpretation of all bone resorption experiments.

In Chapter 3, I showed that the resorption-associated factors TRACP, cathepsin K and TRAF6 are upregulated by acidosis. These results add to previous reports showing that the expression of other resorption-associated factors such as carbonic anhydrase II (CAII), calcitonin receptor (CTR) and V-ATPase are upregulated by low pH (Nordstrom *et al.* 1997; Biskobing and Fan 2000). These data provide further insight into the "downstream" responses of osteoclasts to extracellular protons and indicate the earlier suggestion that low extracellular pH may favour resorption by a simple reduction of the gradient against which osteoclast must pump H⁺ is unlikely to account for the activation effect of low pH observed in osteoclasts (Arnett and Dempster 1986).

The results presented in Chapter 4 show that bone nodule formation by rat osteoblasts may be more sensitive to small pH changes than was previously appreciated. The steep response of osteoblasts to acidosis is reciprocal to the acid-activation curve for resorption pit formation by cultured rat and human osteoclasts (Chapter 2; Arnett and Dempster 1986; Brandao-Burch and Arnett 2004). At the same pH that activates osteoclasts optimally (*i.e.* 6.90 - 7.00), mineralisation by osteoblasts is completely abolished. It is possible that during chronic metabolic acidosis, the bone cells may be exposed to low pH levels and the function of the bone tissue will be 1) to liberate alkaline bone mineral due to increased mineral solubility; reduce osteoblast alkaline phosphatase activity and increased matrix Gla protein expression and 2) to increase osteoclast resorptive activity. The powerful inhibition of alkaline phosphatase activity by small pH reductions may conceivably involve cell-mediated H⁺-sensing mechanisms, such as the OGR1 receptor that respond over similar pH ranges (Ludwig *et al.* 2003) and is upregulated by acidosis in primary rat osteoblast cultures (Chapter 5).

Although the pH of arterial blood is normally ~7.40, and that of venous blood ~7.36, the pH of the extracellular fluid bathing cells is likely to be below pH 7.36 and subject to complex, dynamic gradients, depending on the metabolic activity of the cells and their distance from the nearest capillary. There are relatively few reliable measurements for interstitial pH in intact tissues. It has been demonstrated by non-invasive measurements that the pH in normal skin is around ~7.2 and the interstitial pH decreases by ~ 0.32 units at a distance of 50µm from a blood vessel (Martin and Jain 1994). My own results suggest that for bone turnover to occur *in vivo*, extracellular pH in the immediate bone environment should be in the approximate range of 7.15-7.30. My data emphasise the importance of the vasculature for bone health and suggest a rationale for the histological observation that bone formation normally takes place in proximity to blood vessels.

In Chapter 5, I showed that capsaicin activates normal human osteoclasts in non-acidified conditions where most pro-resorptive factors such as PTH is inactive (Chapter 2). Furthermore, this activation response occurred at “hormonal” concentrations of capsaicin that are at least 2 orders of magnitude lower than those commonly used pharmacologically. These results suggest that the TRPV1 receptor may be involved in the osteoclast response to acidosis. This receptor is a non-selective plasma membrane cation channel that is activated by protons, heat and capsaicin (Caterina *et al.* 1997).

Ongoing experiments in our laboratory suggest that capsaicin may exert an inhibitory effect of bone nodule formation by normal osteoblasts. Further *in vitro* work is required to assess whether the effect of capsaicin on osteoblasts mimics that of acidosis. This could be achieved by using a number of methods described in detail in Chapter 4. Alkaline phosphatase activity and osteoblast proliferation measurement is needed to verify whether capsaicin has an effect on osteoblast differentiation. The cultures should also be assessed for apoptosis in order to rule out any cytotoxic effect of capsaicin on osteoblasts. Analysis of collagen deposition by TEM and quantification by ³H-proline incorporation would provide direct evidence of the effects of capsaicin on matrix deposition and subsequent mineralisation. Moreover, the expression pattern of proteins involved in osteoid production, matrix mineralisation and the nucleation process and osteoblast

differentiation such as alkaline phosphatase, nucleoside triphosphate pyrophosphohydrolase (NTPPase or PC-1), annexins II, V and VI, matrix Gla protein, osteopontin, type I collagen, osteocalcin and the transcription factor Runx2 could also be investigated.

Capsaicin is the active ingredient of chilli peppers. Data are rather limited but some reports have shown that this alkaloid can be absorbed in the gastrointestinal system in its active form and is metabolised in the liver before reaching the general circulation. In rats, traces of capsaicin have been detected systemically in the blood and brain in its unchanged form a few hours after intragastrical administration (Donnerer *et al.* 1990). It has also been reported that following oral ingestion of 3 mg of capsaicin (an amount similar to that ingested by rural Thai people per meal) in experimental rats, 10% of the dose was detected in non-metabolised form in the faeces and a further 9% was excreted in the urine, indicating absorption of significant quantities into the circulation (Kawada and Iwai, 1985). In rats exposed nasally to 1, 5 and 50 mg/ml of capsacin, circulating concentrations of up to 90 ng/ml have recently been measured (Reilly *et al.* 2002). Therefore, bone cells could potentially be exposed *in vivo* to the capsaicin concentrations within the range shown to modulate osteoclast activity in my study.

It has been reported that systemic injection of capsaicinoids in rats results in a decrease of BMD in the metaphyses of the tibia and femur. Further histomorphometric analysis showed that the loss of BMD is probably due to an increase in osteoclast numbers and impaired osteoblast activity (Offley *et al.* 2005). The results obtained from Offley and colleagues (2005) need to be interpreted with caution. The systemic administration of capsaicin is unpleasant and results in profound depressions of core body temperature (Dib 1983; Szolcsanyi 2004). We have recently shown that temperature reductions cause significant increase in osteoclast formation *in vitro* (Utting *et al.* 2005).

In the *Capsicum* genus the content of capsaicin may be up to 1% by dry weight (*e.g.* in a habanero chilli) (Cordell and Araujo 1993). In some countries, such as Mexico, Thailand and India, consumption of hot chilli peppers is often high. It has been reported that in Mexico City, for example, the average consumption of chilli peppers is 20g / person per day, which is equivalent up to 200 mg / person per day of

capsaicinoids (Lopez-Carrillo *et al.* 1994). To date, there are no reports in the literature of the relationship between chilli pepper consumption and bone mass.

Anandamides, endogenous agonists of cannabinoid receptors (CB₁ and CB₂), have been shown to activate both native and recombinant TRPV1 receptors *in vitro* in a manner that is modulated by pH and temperature (Di, V *et al.* 2002). It has been reported recently that the endocannabinoid system plays an important role in bone regulation. *CBI*-knockout mice were shown to have increased bone mass and were protected from ovariectomy-induced bone loss. Additionally, pharmacological approaches showed that selective antagonists to these receptors (CB₁ and CB₂), expressed by bone cells, inhibit osteoclast activation both *in vivo* and *in vitro*, whereas the agonist anandamide stimulated osteoclast formation *in vitro* (Idris *et al.* 2005). In view of the potential 'cross-talk' between the vanilloid and cannabinoid system, investigation on whether the anandamide effect on bone cells mimics that of capsaicin, *i.e.* stimulation of bone resorption at physiological pH levels, would prove very useful for further understanding of osteoclast biology.

In chapter 5, I also showed that the G-protein coupled receptors OGR1 and TDAG8, which respond sensitively to pH changes in the range of 7.5 – 6.8 with increases in inositol phosphate and cyclic AMP respectively, are expressed by bone cells and that the TRPV1 receptor, a non-selective plasma membrane cation channel that is activated by protons, heat and capsaicin is upregulated by acidosis in human osteoclast cultures. My results provide evidence for a possible mechanism by which extracellular protons are sensed by osteoclasts by multiple receptors such as OGR1, TDAG8 or TRPV1; the signalling is then transmitted via a cascade that would eventually activate transcription factors to bind to promoters of TRACP, cathepsin K, CAII, CTR and V-ATPase genes, all known to be upregulated in acidified conditions (Nordstrom *et al.* 1997; Biskobing and Fan 2000; Brandao-Burch *et al.* 2003). Given the importance of H⁺ sensing receptors to bone cells, it should not be surprising that multiple pathways are activated upon exposure of bone cells to H⁺ as outlined in Chapter 5.

Clearly, it will now be important to study bone density, histology/histomorphometry, tooth eruption in H⁺ receptor knockout mice such as TRPV1 (Caterina *et al.* 1997) and G2A (a GPCR that belongs to the same family as

OGR1, GPR4 and TDAG8) (Le *et al.* 2001), together with functional analysis to assess osteoclast activation, osteoclastogenesis and bone nodule formation from cells obtained from these knockout mice. Knockout mice for OGR1, TDAG8 and GPR4 will be available to us shortly. Small interfering RNA technology will also enable complementary *in vitro* studies. Recently-published data indicate that this technology is effective in gene knockdown in primary osteoclast cultures (Yin 2005). A pharmacological approach could also be taken to study further the involvement of other pH-modulated receptors on osteoclast and osteoblast activity. Functional responses of bone cells to icilin and menthol, which activate the pH-modulated TRPM8 'cold' receptor, could be examined in detail (Andersson *et al.* 2004). Also of interest are TASK 1 and 3 receptors (TWIK-related acid-sensing K⁺ channel 1 and 3), members of the background K⁺ channel family. These channels have been reported to respond to extracellular pH in the pathophysiological range and hypoxia (Kemp *et al.* 2004).

The actions of extracellular pH on bone cells presented in this thesis suggest novel means of manipulating bone cell function, based either on adjustment of systemic acid base balance by diet or alkaline salt ingestion, or by pharmacological intervention at proton sensing receptors present on bone cells. Awareness of the potential risks of hormone replacement therapy in postmenopausal women has increased recently (Beral 2003). Moreover, long-term and short-term bisphosphonate administration has recently been linked with nephrotoxicity (Smetana *et al.* 2004). Therefore, there is a renewed need for research into alternative approaches to limiting bone loss in older individuals.

Evidence suggests that in post-menopausal women, administration of 60 to 120 mmol per day per 60 kg of potassium bicarbonate increases the blood pH from 7.39 to 7.41. This increase was accompanied by a significant increase in both calcium and phosphorus balance and a prompt decrease in net renal acid excretion, indicating that endogenous acid was almost completely neutralised (Sebastian *et al.* 1994). It has also been shown that thiazide diuretics, which are widely used as anti-hypertensive agents (Moser 1998), reduce bone loss in normal post menopausal women (Reid *et al.* 2000). The mechanisms by which thiazides influence bone metabolism are not fully elucidated, however it has been suggested that thiazide administration causes

metabolic alkalosis (Peh *et al.* 1993). Beta-blockers, taken alone or in combination with thiazides have also been shown to decrease the risk of fractures in both men and women, whether this is due to a decrease in blood acidity still remains to be elucidated (Schlienger *et al.* 2004). Many elderly patients that suffer from osteoporosis and hypertension may benefit from treatment with beta-blockers and thiazide diuretics. These drugs are of beneficial value as they are very inexpensive and do not have many side effects.

Dietary manipulation is currently receiving a lot of attention, a diet which is rich in fruit and vegetables may be beneficial to the skeleton although there are some controversies associated with this notion as detailed in Chapter 1.

The direct effect of systemic acidosis on osteoclast and osteoblast activity needs to be investigated further *in vivo*. One approach would be to examine changes in markers of bone turnover in response to vigorous exercise carried out by healthy volunteers. Excessive exercise is known to induce acute, severe systemic acidosis; after ten 10-second sprints in a cycle ergometer separated by 30-second passive recovery intervals, the blood pH has been shown to drop from ~7.4 to ~7.2 in men (Ratel *et al.* 2002). Serum and urine would have to be collected at regular intervals and markers for bone resorption such as serum cross-linked C telopeptide of type I collagen, urinary cross-linked N telopeptide levels, RANKL, OPG and serum tartrate resistant acid phosphatase 5b and for bone formation (*e.g.* serum type I terminal propeptide and bone specific alkaline phosphatase) measured, together with PTH and calcitonin levels. If a subsequent rise in bone resorption markers would be observed it would provide evidence that there is a net negative effect of acidosis on the skeleton.

In conclusion, this study shows that acidosis exerts an important “double negative” action on bone cells and provides insights into the underlying mechanisms, summarised in **Figure 6.1**. This is in accordance with the physiological role of bone as a reserve of base that is available as a “fail-safe” mechanism to buffer protons if the kidneys and lungs are unable to maintain acid-base balance within narrow physiological limits (Barzel 1995; Frassetto *et al.* 1996). The mechanism by which acidosis regulates bone cell function may have evolved in vertebrates as a last line of defence to protect the body against the effects of systemic acidosis.

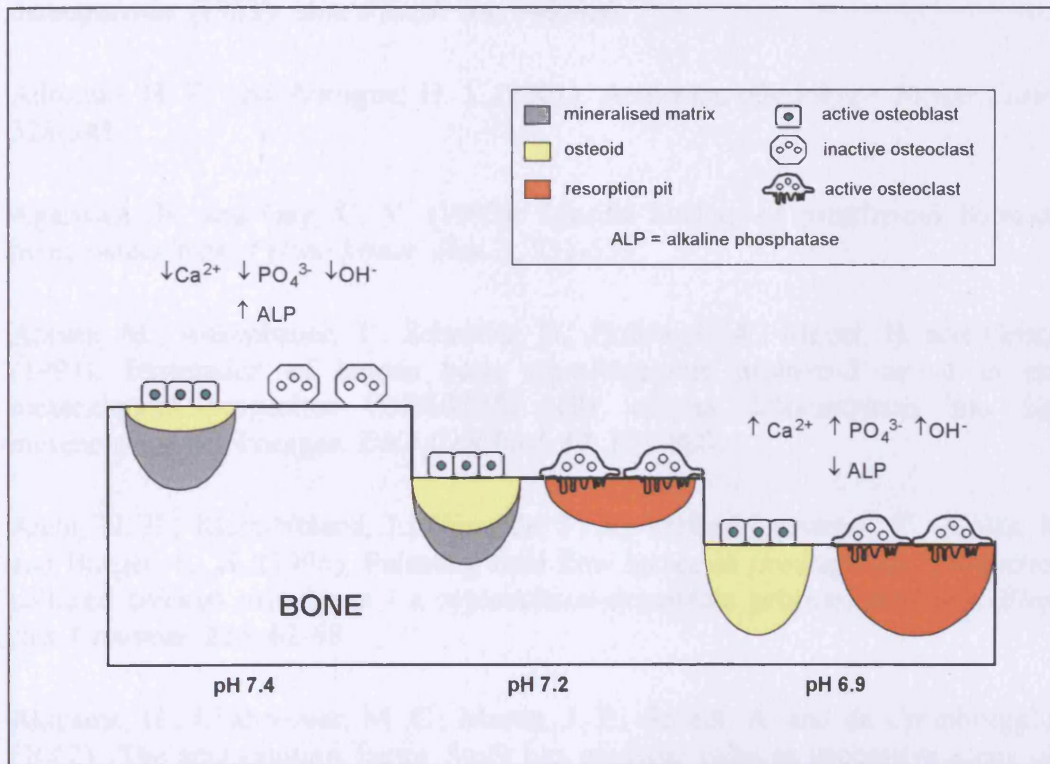


Figure 6.1. Schematic of extracellular pH actions on bone cell function.

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Appendix I: Abbreviations

ABBREVIATION	FULL NAME
1,25-(OH) ₂ D ₃	1,25-dihydroxyvitamin D ₃
AP-1	Activator protein-1
ASIC	Acid sensing ion channel
ATP	Adenosine 5'-triphosphate
BGJb	Biggers, Gwatkin and Heyner medium
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
C-	Carboxy-
Ca ²⁺	Calcium
CA II	Carbonic anhydrase II
CAMP	Cyclic adenosine 5'-monophosphate
Cbfa1	Core binding factor 1
Cl ⁻	Chloride
COX	Cyclooxygenase
CO ₂	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylen-diamine tetraacetic acid
ER	Oestrogen receptor
F-actin	Filamentous actin
FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF	Fibroblast growth factor
Gla	γ-carboxylated glutamate
GPCR	G-protein-coupled receptor
GPR4	G-protein-coupled receptor 4
GTP	Guanosine 5'-triphosphate
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid

HCO ₃ ⁻	Bicarbonate
H ₂ CO ₃	Carbonic acid
IFN-γ	Interferon-γ
IGF	Insulin like growth factor
Ihh	Indian hedgehog
IL-1	Interleukin-1
IP ₃	Inositol 1,4,5-triphosphate
K ⁺	Potassium
Li	Lithium
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-stimulating growth factor
MMP	Matrix metalloproteinase
N-	Amino-
NFAT	Nuclear factor of activated T-cell
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa B
OGR1	Ovarian cancer G-protein coupled receptor
OPG	Osteoprotegerin
PBMCs	Peripheral blood mononuclear cells
P _i	Phosphate (inorganic)
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PGE ₂	Prostaglandin E ₂
PI-3	Phosphatidylinositol-3 kinase
PKC	Protein kinase C
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein/peptide
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
RER	Rough endoplasmic reticulum
RGD	Arg-Gly-Asn
TDAG8	T-cell death associated gene 8
TGF	Transforming growth factor

TNF	Tumour necrosis-related factor
TRAF	TNF receptor-associated factors
TRANCE	TNF-related activation-induced cytokine
TRACP	Tartrate-resistant acid phosphatase
TRPV	Transient receptor potential cation channel subfamily V
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
V-ATPase	Vacuolar-type ATPase

Appendix II : Publications

Brandao-Burch, A., Utting, J., Orriss, I. R. and Arnett, T. R. (2005). Acidosis inhibits bone formation by osteoblasts *in vitro* by preventing mineralisation. *Calcif Tissue Int.* (in press).

Dempster, D. W., Hughes-Begos, C. E., Plavetic-Chee, K., Brandao-Burch, A., Cosman, F., Nieves, J., Neubort, S., Lu, S. S., Iida-Klein, A., Arnett, T. and Lindsay, R. (2005). Normal human osteoclasts formed from peripheral blood monocytes express PTH type 1 receptors and are stimulated by PTH in the absence of osteoblasts. *J Cell Biochem.* **95**, 139-148.

Brandao-Burch, A. and Arnett, T. R. (2004) Activation of normal human osteoclasts by acidosis. *Osteoporos Int.* **15**, S133. (Abstract accepted for poster presentation at IOF conference, Brazil, 2004).

Brandao-Burch, A., Utting, J. C., Orriss, I. R. and Arnett, T. R. (2004) Osteoblast function is inhibited cooperatively by acidosis and hypoxia. *J Bone Miner Res.* **19**, 1031. (Abstract accepted for oral presentation at BATS conference, Oxford, 2004).

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Brandao-Burch, A., Utting, J. C., Orriss, I. R. and Arnett, T. R. (2004). Cooperative inhibition of osteoblast function by acidosis and hypoxia. *Calcif Tissue Int.* **74**, S55. (Abstract accepted for poster presentation at ECTS conference, Nice, 2004).

Brandao-Burch, A., Meghji, S. and Arnett, T. R. Acidosis strongly upregulates mRNA for cathepsin K, TRAP and TRAF-6 in bone. *Calcif Tissue Int.* **72**, S64. (Abstract accepted for poster presentation at ECTS conference, Rome, 2003).

Normal Human Osteoclasts Formed From Peripheral Blood Monocytes Express PTH Type 1 Receptors and Are Stimulated by PTH in the Absence of Osteoblasts

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