OSTEOCLAST HETEROGENEITY: THE IMPORTANCE OF
CELL SIZE AND PHASE OF ACTIVITY

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Pharmacology
University of Toronto

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CELL SIZE AND PHASE OF ACTIVITY

Doctor of Philosophy, 2000

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ABSTRACT

Osteoclasts are multinucleated cells that resorb bone. Their activity is required for normal bone remodeling, but excessive bone resorption is also caused by osteoclastic activity. The ultimate goal is to be able to interfere in bone disease processes such as to maintain enough osteoclastic activity for normal bone remodeling while eliminating the excessive resorption. The present study was undertaken to identify the most active osteoclasts and to determine how they differ, in order to lay the groundwork for future targeted inhibition of the most active osteoclasts. Since macrophage colony stimulating factor (MCSF) is abundantly present in inflammatory diseases associated with increased bone loss, we wished to determine what effect this cytokine had on osteoclasts. MCSF stimulated osteoclastic bone resorption in osteoclast-containing cultures obtained from rabbit long bones and cultured on bone slices. This effect was paralleled by an increase in the number of larger osteoclasts (as determined by the number of nuclei per cell) without changing the total number of cells. In addition, populations of large osteoclasts resorbed more per nucleus than populations of small osteoclasts, suggesting large osteoclasts are more effective resorbers than small osteoclasts. Next, due to the importance of proton extrusion in resorption and in order to determine how resorptive efficiency differs in osteoclasts, we
investigated whether the activity of the bafilomycin A1-sensitive V-ATPase and amiloride-sensitive Na\(^+\)/H\(^+\) exchanger differed between large and small osteoclasts. The basal pH\(_i\) of large (≥10 nuclei) osteoclasts cultured on glass coverslips was much higher than that of small (2-5 nuclei) osteoclasts. After acid-loading, a subpopulation of large osteoclasts (40%) recovered by V-ATPase activity alone, while all small osteoclasts recovered by Na\(^+\)/H\(^+\) exchanger activity. Interestingly, in 60% of the large osteoclasts, pH\(_i\) recovery was mediated by both the Na\(^+\)/H\(^+\) exchanger and V-ATPase activity. We postulated that this heterogeneity in large osteoclasts may be a function of the state of activity of the cells (resorbing vs. non-resorbing) and showed this to be true by studying pH regulatory mechanisms in osteoclasts cultured on collagen/hydroxyapatite-coated coverslips. The resorbing osteoclasts represented one category while nonresorbing represented the other. In addition, all resorbing osteoclasts (small and large) had higher basal pH\(_i\) than their nonresorbing counterparts. We re-examined resorptive efficiency on a per cell basis and found that while resorption per nucleus did not differ between individual large and small osteoclasts, large osteoclasts did resorb more per cell and a much greater proportion of large cells were resorbing, thus resulting in greater overall resorption by populations of large osteoclasts. The fact that only 5% of small osteoclasts were actively resorbing versus 40% of large ones suggests different activation mechanisms of these cells, which could be essential in finding a means of selectively inhibiting the most active osteoclasts.
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Submitted for Publication:

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**Lees RL, Sabharwal VK, Grinstein S, Heersche JNM.** Large and small osteoclasts differ in resorptive activity. *Canadian Connective Tissue Conference* (May 27-29, 1999)
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Lees RL, Grinstein S, Heersche JNM. Differences in pH regulatory mechanisms in resorbing and non-resorbing osteoclasts. *Canadian Connective Tissue Conference* (June 5-6, 1998)
Departmental Meetings:

Lees RL, and Heersche JNM. The role of matrix metalloproteinase 9 in osteoclast migration and osteoclastic resorption of bone. *Visions in Pharmacology Research Symposium, University of Toronto* (May 18, 1995) p.26

Lees RL, and Heersche JNM. Macrophage colony stimulating factor induces osteoclast spreading and increases osteoclast motility and resorptive activity. *Visions in Pharmacology Research Symposium, University of Toronto* (May 16, 1996) p.21

Lees RL, Grinstein S, Heersche JNM. Large osteoclasts and small osteoclasts exhibit striking differences in intracellular pH and regulation of proton transport. *Visions in Pharmacology Research Symposium, University of Toronto* (May 23, 1997) p.21

Lees RL, Sabharwal VK, Grinstein S, Heersche JNM. Large and small osteoclasts differ in resorptive activity and pH regulatory mechanisms. *Visions in Pharmacology Research Symposium, University of Toronto* (June 4, 1999) p.16
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<tr>
<td>α-MEM</td>
<td>alpha minimum essential medium</td>
</tr>
<tr>
<td>APD</td>
<td>3-amino-1-hydroxypropylidene-1,1-bisphosphonate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BCECF</td>
<td>2',7'-biscarboxyethyl-5-(6)-carboxyfluorescein</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHA</td>
<td>collagen/hydroxyapatite</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4'-diisothiocyanostilbene-2,2'-disulfonate</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-(N-ethyl-N-isopropyl)amiloride</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-ethanesulfonic acid</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobin G</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LT</td>
<td>leukotrienes</td>
</tr>
<tr>
<td>MCSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MIBA</td>
<td>5-(N-methyl-N-isobutyl)amiloride</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MNGs</td>
<td>multinucleated giant cells</td>
</tr>
<tr>
<td>MNP</td>
<td>mononuclear phagocyte</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NMG</td>
<td>N-methyl-D-glucamine</td>
</tr>
<tr>
<td>NPPB</td>
<td>5-nitro-2-(3-phenylpropylamino)benzoic acid</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>NSE</td>
<td>non-specific esterase</td>
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<tr>
<td>ODF</td>
<td>osteoclast differentiation factor</td>
</tr>
<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
</tr>
<tr>
<td>OPGL</td>
<td>osteoprotegerin ligand</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandins</td>
</tr>
<tr>
<td>pH$_i$</td>
<td>intracellular pH</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SITS</td>
<td>4-acetoamido-4'-diisothiocyanostilbene-2,2’-disulphonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>vacuolar proton adenosine triphosphatase</td>
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CHAPTER 1: GENERAL INTRODUCTION

1.1 Bone and bone cells

Bone is a highly specialized form of connective tissue that, together with cartilage, makes up the skeletal system. Its three primary functions are: to provide mechanical support, to protect vital organs and bone marrow, and to serve as a metabolic reserve of ions, especially calcium and phosphate (Baron 1996). The bone matrix is composed of organic and inorganic components. The organic matrix primarily consists of type I collagen (95%), while the remaining 5% of total protein consists of proteoglycans and numerous noncollagenous proteins (Marks and Hermey 1996). The mineralized matrix is composed primarily of spindle- or plate-shaped crystals of hydroxyapatite \( [\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2] \) found within and covering the organic component of the bone matrix. It is this hydroxyapatite which serves as a potential source of calcium and phosphate (Blair 1998).

The extracellular matrix of bone is formed, remodeled and maintained by 3 different cell types: osteoblasts, osteocytes and osteoclasts. Osteoblasts are the fully differentiated cells responsible for the production of the extracellular bone matrix. The osteoblast is a mononuclear cell that originates from a local mesenchymal stem cell (Aubin and Liu 1996). It is a typical protein-producing cell with a prominent Golgi apparatus, a well-developed rough endoplasmic reticulum and a strongly basophilic cytoplasm. It secretes type I collagen and noncollagenous proteins of the bone matrix and regulates the mineralization of this matrix, although the mechanisms of this are not completely understood (Marks and Hermey 1996).

Osteocytes derive from osteoblasts which have become trapped within the bone matrix that they have produced. The morphology of osteocytes varies according to their age.
and functional activity; young osteocytes have most of the ultrastructural characteristics of osteoblasts, although a decreased cell volume, less rough endoplasmic reticulum and fewer Golgi while older osteocytes have greater decreases in these parameters. Each osteocyte occupies a space or lacunae within the matrix and these cells have numerous and long cell processes rich in microfilaments, which are in contact with cell processes from other osteocytes and osteoblasts via canaliculi in the matrix. These filopodial connections permit communication between neighboring cells, internal and external surfaces of bone and with blood vessels traversing the matrix which is of great importance since diffusion of nutrients and metabolites through the mineralized matrix is limited (Puzas 1996). These cells may behave as mechanosensor cells and as a result play a role in local activation of bone turnover in response to mechanical demands (Nijweide et al. 1996).

Lastly, osteoclasts are large, multinucleated, highly motile and specialized cells involved in bone resorption. They are usually found in contact with the bone surface and within a lacuna that is the result of their resorptive activity. When active, they are polarized cells with several distinct plasma membrane domains: an area of extensive membrane folding in apposition to the bone where bone resorption takes place (the ruffled border) (Holtrop and King 1977; Jones and Boyde 1977), a microfilament-rich, organelle-free area that surrounds the ruffled border and serves as the point of attachment of the osteoclast to the underlying bone matrix (clear zone) (Holtrop and King 1977; Teti et al. 1991), and a basolateral membrane opposite the ruffled border with a central part where the degraded bone matrix components are released extracellularly after being transcytosed through the cell (Nesbitt and Horton 1997; Salo et al. 1997). Characteristic ultrastructural features of this cell include varying numbers of nuclei that are centrally placed and usually contain 1-2 nucleoli, abundant Golgi complexes usually located around each nucleus, a high density of
mitochondria, numerous lysosomal vesicles that arise from the Golgi and cluster near the ruffled border and a high content of tartrate-resistant acid phosphatase (TRAP) (Baron et al. 1986; Mundy 1996).

1.2 Osteoclast origin and differentiation

Osteoclasts are terminally differentiated cells which do not undergo mitosis (Fischman and Hay 1962). They are derived from hemopoietic stem cells (Ash et al. 1980) and possess some characteristics similar to macrophages and other cells of the mononuclear phagocyte (MNP) system such as motility, undulating membranes and affinity for the vital dye neutral red. Although osteoclasts do not bear the Fc and C3 receptors or a number of other markers typical of macrophages and many MNP cells, Baron et al. (1986) showed that osteoclast precursors shared the marker enzyme non-specific esterase (NSE) with cells of the MNP system. They found that osteoclast precursors differentiated from a pool of mononuclear NSE-positive cells which progressively acquired TRAP$^+$ vesicles and later became predominantly positive for TRAP. In addition, osteoclast precursors were found to be members of the MNP lineage and that during the differentiation process there was a progressive accumulation of cytoplasmic vacuoles and synthesis of lysosomal enzymes. It was suggested that the activation of lysosomal enzyme synthesis is the first characteristic step in the differentiation of MNP lineage cells in the osteoclastic pathway.

Much information regarding the differentiation pathway of osteoclasts has been obtained by examining the formation of osteoclast-like multinucleated cells from bone marrow or spleen cells cultured in the presence of 1α,25-dihydroxyvitamin D$_3$ and stromal cells (Sasaki et al. 1989; Seed et al. 1988; Takahashi et al. 1988; Thavarajah et al. 1991). The postmitotic osteoclast precursor is committed to the osteoclast lineage and becomes a
differentiating osteoclast precursor, which in turn differentiates into a mononuclear (or pre-) osteoclast which then fuses into a mature, multinucleated osteoclast (Suda et al. 1996). Terminal differentiation in this lineage is characterized by acquisition of mature phenotypic markers, such as calcitonin receptors, TRAP activity, integrin α,β3 and the capacity to resorb bone. Marks and Seifert (1985) found that the proliferation, migration and differentiation of osteoclasts from precursors requires 1-2 weeks, based on their in vivo results of the replacement of one population of osteoclasts in mice by another of distinctive phenotype.

1.2.1 Formation of multinuclear osteoclasts

Since early studies on osteoclast origins showed that osteoclast nuclei do not synthesize DNA and osteoclasts do not undergo mitosis, it was concluded that osteoclasts do not become multinucleated as a result of division of nuclei within the cell (Fischman and Hay 1962; Tonna 1960). Further studies on osteoclast formation have conclusively shown that multinucleated osteoclasts form and become larger by fusion of mononuclear cells with other mononuclears, of mononuclears with multinuclear cells, or by fusion between multinuclear cells (Feldman et al. 1980; Marshall et al. 1986; Zambonin Zallone et al. 1984). Parathyroid hormone (PTH) has a marked effect on this fusion process (Addison 1980), as PTH stimulated progressive incorporation of postmitotic cells into preexisting osteoclasts and increased fusion of resident pre-osteoclasts in mouse calvaria in vitro (Feldman et al. 1980). When left in culture for several days, even control cultures showed a decrease in the total number of osteoclasts because of fusions between osteoclasts producing larger but fewer cells (Feldman et al. 1980). Conversely, cyclosporin A decreased the fusion and multinucleation of osteoclasts and macrophages, but had no effect on myoblasts, suggesting an antifusion effect specific for cells of the monocyte-macrophage lineage (Orcel et al. 1985).
The fusion process consists of at least two steps: first the cells must meet for cell to cell contact and then, during the actual fusion process, the membranes must join and rearrange as the two cells unite. Although the high motility of osteoclasts increases the probability of cell to cell contact, very little is known of the actual process of membrane fusion. Cell to cell contact alone is probably not sufficient for fusion to take place as Zambonin Zallone et al. (1984) showed, using osteoclasts isolated from medullary bones of laying hens co-cultured with monocytes of the same hens, that 3 monocytes out of 30 in the field fused with the cytoplasm of an osteoclast after 2-3 hours of making contact and detaching while another 6 monocytes moved away after making contact without cell fusion occurring. This suggests that other processes, such as alteration of the membrane structure, may be required to prepare the cell for fusion, although this has not been studied in detail in osteoclasts. Kurachi et al. (1994) found that D-mannose residues, the membrane carbohydrates involved in fusion of enveloped viruses with eukaryotic cells, were expressed on the outer membrane of osteoclast progenitors and that these were involved in the osteoclast fusion process. In addition, Mbalaviele et al. (1995) showed that mononuclear osteoclast precursors and mature osteoclasts expressed E-cadherin, a Ca^{2+}-dependent adhesion molecule, on their cell surfaces and that antibodies to E-cadherin markedly decreased the formation of TRAP+ multinucleated cells and inhibited pit formation. Recently, Abe et al. (1999) found that meltrin-α (also known as ADAM-12) mRNA was expressed on mononuclear and multinuclear TRAP-positive cells and multinuclear cell formation was inhibited in cultures transfected with the antisense oligonucleotide to this cell surface protein. Interestingly, and in agreement with our findings reported in Chapter 2, a report by Amano et al. (1998) suggested that macrophage colony stimulating factor (MCSF)
stimulates the fusion process in osteoclasts. They postulated that this may be due to an effect of MCSF on cell survival and/or differentiation of precursor cells. Whether MCSF has a direct effect on the actual fusion process remains unknown and as a result, more comprehensive studies on membrane changes and the factors that regulate cell fusion in osteoclasts are required.

1.2.2 Role of macrophage colony stimulating factor in osteoclast differentiation

MCSF (also known as colony stimulating factor-1) is one of the hemopoietic growth factors required for proliferation, differentiation, activation and survival of MNP cells (Stanley et al. 1983). In homozygous osteopetrotic op/op mice, the synthesis of MCSF is impaired due to a point mutation in the coding region of the gene (Felix et al. 1990b; Yoshida et al. 1990). The op phenotype is characterized by a low number of macrophages and an almost complete lack of osteoclasts (Marks and Lane 1976; Wiktor-Jedrzejczak et al. 1982). Daily injections of MCSF into op/op mice reversed the osteopetrotic phenotype, providing conclusive evidence that the development of osteoclasts depends on this cytokine (Felix et al. 1990a). In addition, it was shown that MCSF is normally synthesized and secreted by cells of the osteoblast lineage (Shiina-Ishimi et al. 1986; Weir et al. 1993) and that the MCSF receptor, the proto-oncogene c-fms, is expressed by both osteoclast precursor cells and mature osteoclasts (Hofstetter et al. 1992; Weir et al. 1993). Although MCSF has been shown to bind to its receptor on mature osteoclasts (Yang et al. 1996), and to stimulate motility of these cells in short term culture (Fuller et al. 1993; Owens and Chambers 1993), its effects on the resorptive activity of these cells is not clear. Increased resorption, decreased resorption and no change in resorption have all been reported, thus suggesting a need for further studies (see Chapter 2).
1.2.3 Other regulators of osteoclast formation and differentiation

Although osteoclasts are dependent on MCSF for their ontogenesis, this cytokine alone is not sufficient for osteoclastogenesis. Recently a second essential stimulatory factor has been identified and named osteoprotegerin ligand (OPGL), also known as osteoclast differentiation factor (ODF) (Kong et al. 1999b; Lacey et al. 1998; Matsuzaki et al. 1998). OPGL is a member of the tumour necrosis factor (TNF) family of cytokines and exists in transmembrane and soluble (cleaved) forms (Lacey et al. 1998). It is expressed by osteoblast/stromal cells, and along with MCSF induces osteoclast differentiation without the need for any additional co-factors (Filvaroff and Derynck 1998; Lacey et al. 1998; Matsuzaki et al. 1998; Quinn et al. 1998). The synergy of MCSF and OPGL may be explained by the ability of MCSF to induce OPGL receptor expression on osteoclast precursors. Interestingly, OPGL may also activate preexisting osteoclasts and stimulate bone resorption both in vitro and in vivo (Lacey et al. 1998). Although the number of osteoclasts in control and OPGL-treated animals was similar, the size and nucularity of the osteoclasts in the OPGL-treated group significantly exceeded the controls in a dose-dependent manner. The authors remarked that this effect on cell size was the most pronounced effect of OPGL as osteoclast area more than doubled in OPGL-treated animals.

Another important player in this system is osteoprotegerin (OPG), a secreted glycosylated protein that acts as a soluble receptor for OPGL and as a result binds and neutralizes OPGL, thus inhibiting osteoclastic differentiation and activity (Boyce et al. 1999; Filvaroff and Derynck 1998). It has been postulated that it is the balance between OPG and OPGL which determines the amount of bone resorption (Kong et al. 1999a).
1.3 Osteoclastic resorption of bone

Bone resorption is a multi-stage process that includes degradation of both the mineral matrix and the organic matrix. In order for osteoclasts to resorb bone it is essential for them to first attach to the bone surface and form an isolated extracellular resorptive microenvironment. This attachment process involves, at least in part, membrane-bound proteins called integrins (Sato et al. 1990). Integrins are heterodimeric transmembrane proteins with extracellular matrix binding domains and an intracellular component interacting with the cytoskeleton (Mundy 1999). The integrin α₁β₃ is an essential component of the resorptive process as antibodies to this integrin block the capacity of osteoclasts to attach to and degrade bone (Teitelbaum 1996). Upon attachment, osteoclasts degrade bone mineral and collagen in a specific order as the inorganic phase must be removed from collagen bundles prior to collagenolysis (Teitelbaum et al. 1997). Once degraded, matrix collagens and other bone resorption products are removed by transcytosis through the cell and released into the extracellular space via the basolateral membrane (Nesbitt and Horton 1997; Salo et al. 1997).

1.3.1 Degradation of mineral matrix

The demonstration many years ago of the accumulation of weak basic dyes at the sites of osteoclastic bone attachment suggested that an acid environment was present at the site of bone resorption (Baron et al. 1985). Silver et al. (1988) demonstrated with microelectrodes that osteoclasts form an isolated extracellular microenvironment acidified to a pH of 5-6. Since then many groups have shown that osteoclasts generate protons intracellularly from carbonic acid, a process facilitated by high expression of carbonic anhydrase II, and then pump massive amounts of these protons across the ruffled border
membrane via vacuolar H\textsuperscript+-ATPases (V-ATPases) (Blair et al. 1989; Laitala and Vaananen 1994). The ruffled border is the resorptive organelle of the osteoclast and is the product of plasma membrane fusion with intracellular vesicles which contain V-ATPases and other enzymes (Teitelbaum 1996). The V-ATPases extrude protons against a concentration gradient which is an energy-intensive process fuelled almost exclusively by glucose (Williams et al. 1997). Interestingly, osteoclast secretion of protons can be modulated by regulators of osteoclastic bone resorption, as stimulators of resorption (such as parathyroid hormone and prostaglandin E2) increase acid secretion, while the resorption inhibitor calcitonin decreases acid secretion (Roodman 1999). Thus, osteoclastic acid transport deposits acid onto the strongly basic hydroxyapatite mineral which results in dissolution of this mineral and liberation of calcium and phosphate ions (Blair 1998).

1.3.2 Degradation of organic matrix

Although acidification is sufficient to dissolve bone mineral, organic matrix degradation requires proteolytic activity. Following demineralization, the organic phase of bone is degraded by lysosomal enzymes with a pH optimum approximating 4.5, reflecting that present in the resorptive microenvironment (Teitelbaum et al. 1997). These collagenolytic enzymes include the cysteine proteinases cathepsin B (Blair et al. 1993), cathepsin L (Goto et al. 1993), and the recently identified cathepsin K, which is the predominant proteinase in human osteoclasts (Bossard et al. 1996). They are delivered to the site of bone resorption by a specific carrier protein known as the mannose 6-phosphate receptor (Teitelbaum 1996). TRAP, an enzyme abundant in osteoclasts and used to identify these cells, is also believed to be somehow related to bone resorption although its precise role has not been elucidated (Minkin 1982).
A great deal of controversy has surrounded the function of matrix metalloproteinases (MMP) in osteoclastic degradation of bone. The MMPs are a family of zinc metalloenzymes divided into collagenases, gelatinases and stromelysins with optimal activity at neutral pH (Kleiner and Stetler-Stevenson 1993). Several groups have identified MMPs in osteoclasts, particularly the gelatinase MMP-9 (Okada et al. 1995; Reponen et al. 1994; Tezuka et al. 1994; Wucherpfennig et al. 1994). However, the acidic pH of the resorption lacuna, the lack of type IV collagen in bone (the preferred substrate of MMP-9) and the lack of effect of collagenase inhibitors on bone resorption (Delaisse et al. 1987) argue against a role for MMPs in bone resorption. Blavier and Delaisse (1995) suggested MMPs may be involved instead in the migration of pre-osteoclasts to bone surfaces. Others have suggested that initially bone type I collagen may be solubilized by cysteine proteinases and then degraded by MMP-9 into small peptides by its gelatinase activity once the osteoclast has moved on, or that gelatinases may be important as potentiators of collagenase activity (Everts et al. 1998; Lorenzo et al. 1992; Schepetkin 1997; Tezuka et al. 1994). To further complicate matters, Everts et al. (1992) did find that osteoclast MMPs were involved in bone degradation, but primarily in osteoclasts from calvarial bone and to a much lower degree in those from long bones (Everts et al. 1999).

1.3.3 Role of extracellular pH

It has long been known that bone degradation is influenced by systemic pH, as metabolic acidosis is associated with a negative calcium balance and increased bone resorption while metabolic alkalosis decreases calcium efflux by suppressing osteoclastic activity (Bushinsky 1996; Kraut et al. 1986). Several investigators have studied the effect of changing extracellular pH (by altering CO₂, bicarbonate or using buffered media) on various
types of *in vitro* osteoclast preparations (rat, rabbit, chicken). Invariably, they all have found that decreasing medium pH to 6.5-7.0 markedly increases bone resorption by these osteoclasts (Arnett *et al.* 1994; Arnett and Dempster 1986; Carano *et al.* 1993; Murrills *et al.* 1993; Shibutani and Heersche 1993). The mechanism by which low pH stimulates resorption by osteoclasts is not fully understood and initially it was suggested that low extracellular pH may simply aid in the creation of the acidic microenvironment associated with the resorption lacuna (Arnett and Dempster 1986). However, additional studies have shown that acidosis may stimulate osteoclast attachment to bone (Teti *et al.* 1989a), reduce responses to extracellular Ca$^{2+}$ concentrations (Grano *et al.* 1994), reduce the outwardly rectifying K$^+$ current (Arkett *et al.* 1994), stimulate clear zone formation (Murrills *et al.* 1993), and upregulate carbonic anhydrase II mRNA expression (Asotra *et al.* 1994). Whether all of these effects play a role in the stimulatory effect of low pH on osteoclast activity is not clear. It is clear, however, that larger pits are formed at low extracellular pH, suggesting an enhanced work output by each osteoclast. Analysis of resorption foci relative to the number of osteoclasts present also reveals that more osteoclasts are active at lower pH (Murrills *et al.* 1993). In addition, Arnett and Spowage (Arnett and Spowage 1996) found that the amount of resorption observed is closely related to time spent in low pH media, suggesting that extracellular proton concentration can modulate osteoclast activity in a continuous and reversible manner. The finding by Grano *et al.* (1994) and Nordstrom *et al.* (1997) that extracellular pH regulates human osteoclast-like cell basal intracellular pH, where low extracellular pH induces a rapid and sustained cell acidification, suggests that regulation of intracellular pH may be one of the determinants of osteoclastic activity.
1.4 Regulation of intracellular pH

The regulation and maintenance of intracellular pH (pHi) within a narrow physiological range of approximately 7.2 is imperative for survival and optimal activity of practically all mammalian cells (Fliegel and Dibrov 1996; Roos and Boron 1981). Cellular pH is under precise control during a large number of processes including cell proliferation and differentiation as small increases in pHi alter cell division and activate expression of specific genes (Busa 1986). As a result, cells have evolved several regulatory mechanisms to maintain this precise pH homeostasis including Na⁺/H⁺ exchange, Cl⁻/HCO₃⁻ exchange and proton pumping by V-ATPase, to name a few.

1.4.1 Na⁺/H⁺ exchanger

The Na⁺/H⁺ exchanger is an ubiquitous integral membrane protein present in low levels in the plasma membrane of many cells. It participates in a variety of cellular processes, including the control of pHi, maintenance of cell volume, transepithelial Na⁺ reabsorption, and facilitation of cell proliferation in response to growth factor stimulation (Orlowski and Shull 1996). Under physiological conditions, the exchanger catalyzes the net uptake of Na⁺ ions coupled to efflux of cytoplasmic H⁺ as the Na⁺ gradient drives alkaninization of the cell interior with an electroneutral stoichiometry of 1Na⁺:1H⁺. The exchanger is normally nearly quiescent when pHi is at the physiological level, however, activation occurs rapidly in the presence of hormones, chemotactic factors, neurotransmitters, cytokines and growth factors, the latter of which activate Na⁺/H⁺ exchange by increasing the exchanger’s affinity for protons (Shrode et al. 1996).

An important feature of the mammalian Na⁺/H⁺ exchanger is its sensitivity to the diuretic amiloride and related derivatives, all of which are competitive inhibitors of Na⁺
(Fliegel and Dibrov 1996). However, the Na\textsuperscript{+}/H\textsuperscript{+} exchanger has long been known to have different affinities for amiloride and its analogues depending on the tissue or cell type examined. This led to the discovery of multiple isoforms derived from multiple genes, which are expressed in isoform-specific patterns and at varying levels in different tissues and cells (Orlowski and Shull 1996).

The first known mammalian Na\textsuperscript{+}/H\textsuperscript{+} exchanger cDNA clone was found by Sardet et al. (1989) and later called the NHE1 isoform. The deduced protein contains 815 amino acids with a molecular weight of 110 kDa when glycosylated. Although little is known about the actual topology of the protein, the accepted model consists of an N-terminal hydrophobic domain with 10-12 putative transmembrane helices and a large hydrophilic C-terminal region with an intracellularly located cytoplasmic tail (Fliegel and Dibrov 1996). The first transmembrane region is suggested to form a signal sequence while the fourth transmembrane domain confers amiloride sensitivity. Wakabayashi et al. (1992) also firmly established that NHE1 may be activated by growth factors through direct phosphorylation of the C-terminal cytoplasmic domain.

Further screening using NHE1 cDNA fragments as probes under low stringency hybridization conditions resulted in the identification and isolation of additional Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoforms (termed NHE2, NHE3, NHE4 and NHE5). While NHE1 is the ubiquitous form present in nearly all mammalian tissues and functions mainly in pH\textsubscript{i} and volume regulation, NHE2 and NHE3 are mainly found in the gastrointestinal tract and kidney where they participate in transepithelial NaCl transport, while NHE4 occurs predominantly in the stomach and NHE5 resides primarily in non-epithelial tissue such as brain and spleen. The NHE1 and NHE2 isoforms are sensitive to amiloride with a K\textsubscript{i} of around 1 μM while NHE3 is the least sensitive to amiloride inhibition with a K\textsubscript{i} of 100 μM (Orlowski and Shull
Although the existence of additional isoforms has not been studied in osteoclasts, Gupta et al. (1996) did report the presence, distribution and regulation of the NHE1 isoform in avian osteoclasts. They found that in osteoclasts cultured on bone, small compacted cells had a peripheral staining pattern of NHE1 while larger, spread out cells had a similar pattern as well as some diffusely distributed intracellular staining. NHE1 was present at both the basolateral membrane and adhesion sites of the osteoclast, suggesting a role for Na\(^+\)/H\(^+\) exchange in migration or attachment as well as pH\(_{i}\) regulation.

An earlier study by Hall et al. (1992) found that Na\(^+\)/H\(^+\) exchange activity was essential during early activation of resorptive activity, perhaps in osteoclast attachment or spreading, but was not required for continual resorption, as addition of dimethylamiloride at the beginning of culture inhibited resorption but at later time points had no such effect. Similarly, Lehenkari et al. (1997) found that osteoclasts cultured on glass exhibited more Na\(^+\)/H\(^+\) exchange activity in regulating pH\(_{i}\) than their resorbing counterparts cultured on thin bone slices. They postulated that the different behaviour on glass could be due to altered cell motility or different mechanisms of spreading and attachment on this surface since the integrin-mediated attachment apparatus present on bone is missing. However, Ravesloot et al. (1995) found that although many rat osteoclasts cultured on glass regulated pH\(_{i}\) by Na\(^+\)/H\(^+\) exchange activity, 20% of their cells had no or very little activity of this antiporter. They were not able to classify these cells according to shape or any other morphological criteria and as a result could not explain these differences. Clearly heterogeneity in Na\(^+\)/H\(^+\) exchange activity exists between osteoclasts although the reason for this is still unknown.
1.4.2 Cl⁻/HCO₃⁻ exchanger

The Cl⁻/HCO₃⁻ anion exchanger is a large 95 kDa integral membrane protein that exchanges intracellular bicarbonate for extracellular chloride. It was first discovered in red blood cells where it is essential for the transport of CO₂ from tissues to lungs (Rothstein 1984). Three anion exchanger isoforms are known, which are all encoded by distinct genes and show differences in their tissue expression: AE1 is found in erythrocytes and kidney, AE2 in a number of epithelial and nonepithelial cells, and AE3 primarily in excitable tissues (Kopito 1990). The Cl⁻/HCO₃⁻ anion exchanger exists as a dimer with each subunit consisting of two distinct domains. The N-terminal 43 kDa is a water-soluble cytoplasmic domain that serves as an attachment site for the membrane skeleton. The remainder of the protein is hydrophobically associated with the membrane and functions as a catalyst for anion exchange (Jennings 1989; Kopito 1990). The current consensus is that this exchanger spans the erythrocyte membrane 14 times with both termini facing the cytosol (Casey and Reithmeier 1998; Fujinaga et al. 1999). Disulfonic stilbenes, such as 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS), inhibit the Cl⁻/HCO₃⁻ anion exchanger by binding to a distinct site that interacts with the Cl⁻ binding site (Casey and Reithmeier 1998). The binding of DIDS involves a rapid reversible interaction followed by a slower covalent reaction (Rothstein 1984) and it is inhibitory only when applied from the outside, suggesting its binding site is on the outer surface of the membrane.

This anion exchanger constitutes a very efficient mechanism for disposing of alkaline equivalents, particularly for cells that actively secrete large quantities of protons, such as intercalated cells of the renal tubule and osteoclasts (Bastani et al. 1996; Jennings 1989). In osteoclasts, Bastani et al. (1996) localized Cl⁻/HCO₃⁻ anion exchanger immunoreactivity to only the basolateral membrane of chicken osteoclasts and showed that its presence, intensity
of staining and distribution was not altered by a state of increased bone resorption. Earlier studies showed that osteoclasts have a Na⁺-independent Cl⁻/HCO₃⁻ exchanger important in pHᵢ regulation which can be inhibited by DIDS (Teti et al. 1989b) and that DIDS inhibits bone resorption, presumably by inhibition of anionic exchange which in turn could inhibit the release of protons (Hall and Chambers 1989; Klein-Nulend and Raisz 1989). Based on this, Hall and Chambers (1989) suggested that optimal bone resorption by isolated osteoclasts requires Cl⁻/HCO₃⁻ exchange, although more recent reports also show a DIDS-inhibitable chloride channel on the ruffled border of osteoclasts which may also play a key role in regulating bone resorption (Blair and Schlesinger 1990; Kelly et al. 1994; Schlesinger et al. 1997). Recently, Weinreb and Halperin (1998), using an antibody to an AE2-specific sequence found that both mature multinuclear and mononuclear osteoclast precursors expressed the Cl⁻/HCO₃⁻ exchanger, indicating that fusion of osteoclast precursors to multinucleated osteoclasts was not required for the induction of this molecule.

1.4.3 Vacuolar H⁺-ATPase

The V-ATPases are a family of ATP-driven proton pumps present in a variety of intracellular compartments and on plasma membranes of eukaryotic cells that allow the rotational energy released on ATP hydrolysis to drive the formation of a linear proton gradient across the membrane (Forgac 1998). They are related structurally and evolutionarily to the F-ATPases which are involved in ATP synthesis in mitochondria, chloroplasts and bacteria (Rastogi and Girvin 1999; Wilkens et al. 1999). However, V- and F-ATPases differ in many important respects, particularly in their function and regulation. V-ATPases are crucial in a variety of physiological processes such as renal acidification, maintenance of neutral cytoplasmic pH in macrophages and neutrophils, and degradation of
the bone matrix by osteoclasts (Blair 1998; Forgac 1998).

The V-ATPases are composed of two functional domains: the $V_1$ domain which is a 570 kDa peripheral complex responsible for hydrolysis of ATP and the $V_0$ domain which is a 260 kDa integral complex that is responsible for proton translocation across the membrane (Keeling et al. 1997). The $V_1$ domain is composed of eight different subunits (A-H), where both the A and B subunits participate in nucleotide binding but only the A subunit contains the catalytic site. The $V_0$ domain contains five different subunits ($a$, $c$, $c'$, $c''$, d), where the “$a$” subunit is believed to form water-filled channels that allow protons to gain access to the buried carboxyl group of subunit “$c$” and to exit this site to the opposite aqueous compartment. The “$a$” subunit (also known as the 100 kDa subunit in yeast or the 116 kDa subunit in mammalian cells) is a transmembrane glycoprotein possessing an N-terminal hydrophilic domain and a C-terminal hydrophobic domain containing multiple putative transmembrane helices. It has been suggested to be responsible for targeting the V-ATPase to different compartments in the cell (Forgac 1999).

One property that distinguishes the V- and F-ATPases is their sensitivity to bafilomycin A$_1$. This compound is a macrolide antibiotic produced by Streptomyces griseus and is a potent and highly specific inhibitor of the V-ATPases (Gagliardi et al. 1998). It is believed to bind to the 100 kDa subunit, however, since it is not selective for any particular subclass of V-ATPase, it is toxic when administered to animals due to a generalized inhibition of all essential V-ATPases.

Regulation of V-ATPase activity is essential for optimal functioning of cells and maintenance of pH. Several possible mechanisms have been suggested based on evidence found in different systems. These include rapidly reversible dissociation of the $V_1$ and $V_0$ domains which is independent of new protein synthesis, covalent modification of the active
site through disulfide bond formation, activator and inhibitor proteins, uncoupling of proton transport and ATPase activity by mild proteolysis or high ATP concentrations, control of pump density through intracellular targeting of V-ATPases, and finally, regulation of counterion conductance which in most cases in vivo appears to occur through the action of a parallel chloride channel (Forgac 1998). Because of the diversity of functions of V-ATPases, it is likely that a number of mechanisms are employed in controlling their activity.

A 100 kDa lysosomal membrane protein was first observed on the osteoclast ruffled border by Baron et al. (1985). Blair et al. (1989) and Vaananen et al. (1990) later showed this to be a subunit of the V-ATPase and along with additional studies it has become evident that V-ATPases are polarized to the ruffled border membrane in actively resorbing osteoclasts and are responsible for the acidification of the extracellular resorption zone necessary for bone resorption (Bastani et al. 1996; Laitala and Vaananen 1993; Laitala-Leinonen et al. 1996; Mattsson et al. 1994). As in kidney cells, V-ATPase activity on the osteoclast ruffled border is charge coupled to passive chloride permeability (Blair et al. 1991). Inhibitors of the V-ATPase, such as bafilomycin A₁ and antisense RNA and DNA molecules targeted against V-ATPase also inhibit bone resorption (Laitala and Vaananen 1994; Sundquist et al. 1990). Interestingly, the bisphosphonate tiludronate also inhibited osteoclast V-ATPase function although this may not have been the major mechanism of osteoclast inhibition as the dose required was very high and other bisphosphonates had no effect (David et al. 1996). Other evidence suggests that bisphosphonates inhibit osteoclastic activity by promoting apoptosis (Hughes et al. 1995). Chatterjee et al. (1992) also found that vanadate, a classic inhibitor of P-ATPases, inhibited the osteoclast proton pump at a very high concentration, suggesting a unique pharmacological profile for the osteoclast V-ATPase, but this observation has not been substantiated by any other group.
Although early studies indicated that V-ATPases were only localized on the ruffled border, or diffusely throughout the cytoplasm in non-resorbing osteoclasts, it has become apparent that V-ATPases are also located on the basolateral membrane (Gupta et al. 1997). This raises the question of the function of these non-ruffled border membrane proton pumps. Ravesloot et al. (1995) and Lehenkari et al. (1997) suggested that some osteoclasts regulated pH\textsubscript{i} by V-ATPase activity. However, their classification of osteoclasts by cell shape or resorptive activity, respectively, did not entirely correlate with pH\textsubscript{i} regulation by V-ATPase activity. In addition, Nordstrom et al. (1995; 1997) found that pH\textsubscript{i} regulation by V-ATPase activity was negligible in cells cultured at normal pH but became apparent in 40% of the cells following prolonged acidosis. Although the importance of the V-ATPase in bone resorption is clear and transcripts for V-ATPase subunits are abundantly expressed in osteoclasts (Sakai et al. 1995) the role of these proton pumps in pH\textsubscript{i} regulation is not clear.

1.5 Diseases of bone loss

1.5.1 Osteoporosis

A common disorder of bone loss is osteoporosis, a condition characterized by a decrease in bone mass and a deterioration in the bone microarchitecture. Osteoporosis occurs progressively in all aged individuals and may become evident as early as age 50 to 60, particularly in postmenopausal women. The clinical manifestations include bone fractures, which may occur spontaneously or after minimal trauma. With the increase of life expectancy as a result of advanced health care, osteoporosis is increasingly a major medical and socioeconomic problem (Lin 1996). Postmenopausal osteoporosis occurs as a result of increased bone turnover, where the number of bone remodeling units increases and the amount of bone laid down by osteoblasts in each unit is less than the amount removed by
osteoclasts. Estrogen deficiency is associated with postmenopausal osteoporosis, possibly because it leads to higher levels of the cytokines IL-6, IL-1 and TNF which in turn stimulate the generation and activity of osteoclasts (Boyce et al. 1999; Pacifici 1996). As a result, the most effective means of preventing and treating postmenopausal osteoporosis is estrogen supplementation, although due to other health risks associated with estrogen treatment, recent efforts have been directed toward developing nonestrogenic anti-osteoporotic agents (Teitelbaum 1996). The most promising of these are the bisphosphonates which are carbon-substituted pyrophosphate compounds with a high affinity for hydroxyapatite and which clearly inhibit osteoclastic bone resorption (Kanis et al. 1995; Lin 1996; Suda et al. 1997). Although their precise mechanism of action has been under investigation for some time and suggested to involve induction of osteoclast apoptosis (Hughes et al. 1995) and inhibition of protein-tyrosine phosphatase activity in osteoclasts (Schmidt et al. 1996), recent evidence has clearly shown that bisphosphonates act directly on osteoclasts to induce caspase cleavage of Mst1 kinase leading to the induction of apoptosis (Reszka et al. 1999). However, the long-term effects and usefulness of the bisphosphonates are not known yet.

1.5.2 Paget’s disease

Paget’s disease of bone was first described by Sir James Paget in 1876 and is a common disorder of unknown etiology characterized by increased bone remodeling and abnormal bone architecture (Tiegs 1997). Rates of bone remodeling may be enhanced up to 20-fold, and there is an increase in both osteoclastic bone resorption and new bone formation (Krane 1986). However, the coupling between bone formation and resorption is imperfect and the new bone that is formed is poorly organized and structurally unsound. The disease is more prevalent among men than women and prevalence increases progressively with age.
The pelvis, femur, spine, tibia, skull and humerus are most commonly involved and although most patients are asymptomatic, pain is the most common presenting symptom (Tiegs 1997). Increased resorption by osteoclasts is thought to be the primary abnormality in Paget’s disease as pagetic osteoclasts are abnormal morphologically and the effects on bone remodeling are reversed by anti-osteoclastic drugs (Mundy 1999). Interestingly, Pagetic osteoclasts are enormous in size, containing an average of 20 nuclei per cell as compared to 3-4 in normal osteoclasts, and they have increased levels of TRAP per cell, as measured by intensity of staining using spectrophotometry (Kukita et al. 1990; Rebel et al. 1976). These nuclei have increased numbers of nucleolar organizer regions (Chappard et al. 1998) and Pagetic nuclei can contain inclusion bodies which may be of viral origin, but whose exact function or effect is not known (Roodman 1996). The cytokine interleukin-6 (IL-6) appears to play an important role in the enhanced osteoclast formation and bone resorption in patients with Paget’s disease as IL-6 may act as an autocrine/paracrine factor produced by these osteoclasts and responsible for activity at sites distant from disease activity (Roodman 1999). Bisphosphonates have been used extensively in the treatment of Paget’s disease and cause a dose-dependent suppression of bone resorption although the long-term usefulness of these agents remains to be assessed (Kanis et al. 1995; Smith 1999). Although the therapeutic advances continue, the cause of Paget’s disease remains unknown and the mechanisms responsible for the local formation of very large osteoclasts remains unclear.

1.5.3 Rheumatoid arthritis

Rheumatoid arthritis is a chronic multisystemic inflammatory disease with autoimmune features that is of unknown etiology and results in progressive damage to joint
tissues (Fujikawa et al. 1996; Odeh 1997). The pathogenesis of this serious disease seems to be multifactorial, where several cytokines, especially IL-1, TNF-α and MCSF are involved in the induction and perpetuation of the inflammatory process (Arend and Dayer 1990; DiGiovine et al. 1988; Firestein et al. 1988; Lipsky et al. 1989; Seitz et al. 1994). Although bone loss in rheumatoid arthritis is common and severe and has been widely recognized for decades, much attention has focused on the immunologic aspects of rheumatoid arthritis and to the pathophysiology of articular cartilage loss, while the state of the bone has received little attention (Bogoch and Moran 1998). Recently, this has changed as rheumatoid arthritis is now understood to be a condition also characterized by a major increase in the rate of bone remodeling where a net bone loss results from an increase in bone resorption by osteoclasts (Bellingham et al. 1995; Bogoch et al. 1988; Gravallese et al. 1998; Shimizu et al. 1985). The osteoclasts have been shown to increase both in number and size in rheumatoid joints (Aota et al. 1996; Takashima et al. 1989). In experimental inflammatory arthritis of the rabbit knee, an animal model of human rheumatoid arthritis developed by Bogoch and colleagues (1988), there is a reduction in fracture toughness associated with arthritis which the authors suggest is due to osteoclastic bone resorption that increases trabecular porosity (Bellingham et al. 1995). In the same study it was also found that treatment with a high dose of a bisphosphonate (3-amino-1-hydroxypropylidene-1,1-bisphosphonate, APD, 0.3 mg/kg/day) resulted in normal fracture toughness suggesting that bisphosphonates hold considerable promise for inhibiting bone loss associated with rheumatoid arthritis.

1.5.4 Periodontal disease

Diseases of the periodontium cause loss of more teeth in the adult than any other disease. In nearly every case the condition begins as a minor localized disturbance, usually
due to bacterial infections, which unless adequately treated, may gradually progress until the alveolar bone is resorbed and the tooth is exfoliated (Shafer et al. 1974; Socransky and Haffajee 1994). One of the major consequences of periodontitis and periodontal diseases is the loss of alveolar bone through osteoclastic bone resorption (Schroeder and Lindhe 1980). As in other inflammatory diseases such as arthritis and osteomyelitis, the factors responsible for increasing the osteoclastic resorption of bone in periodontitis are most likely cytokines such as MCSF, IL-1, and platelet activating factor (Schepetkin 1997). Shibutani et al. (1997) found in their model of experimentally induced periodontitis in dogs that inflammatory changes in the gingiva rapidly induced the appearance of multinucleated TRAP+ cells and from their figures it was apparent that the resorbing osteoclasts were quite large. When quantifying the relationship between osteoclast size (number of nuclei) and the extent of the resorbing surface in hamster periodontal disease, Makris and Saffar (Makris and Saffar 1982) found that the differences between control and experimental animals were highly significant with regard to the number of osteoclast nuclei per unit of bone surface and that this value varied closely with the extent of resorbing surface. They found that osteoclastic activity was due to an increase in the number as well as activity of osteoclasts. With the exception of postmenopausal osteoporosis, all other diseases of bone loss, including endstage renal disease and hyperparathyroidism (Kaye et al. 1985), are characterized by an increase in osteoclast size as determined by number of nuclei per cell.

1.6 Cell multinuclearity: functional implications

The functional significance of the multinucleated state of osteoclasts is not yet clear. Addison (1980) postulated that the fusion of mononucleated osteoclasts or precursor cells may be the result of membrane changes necessary for other phenomena, such as calcium
uptake and release, or the formation of the ruffled border membrane and as such multinuclearity may be incidental to these more important changes. Alternatively, he also speculated that the larger cells may have advantages over small cells, such as a more efficient ruffled border or a tighter attachment or sealing zone, even though the small cells could compensate by being more numerous.

In several types of multinucleated cells, the gain or loss of certain characteristics has been associated with cells becoming multinucleated. For multinucleated giant cells (MNGs), Vignery et al. (1991) reported that gene expression changed with multinuclearity: mononuclear rat macrophages expressed the α-1 isoform of the Na⁺/K⁺ ATPase only, while the α-3 isoform appeared when these cells fused to produce multinuclear cells. Enelow et al. (1992) found that, while microbicidal activity of human MNGs was enhanced in parallel with increased size and number of nuclei, the oxidative activity per unit cytoplasmic protein was significantly increased in the larger cells. As there was no difference in DNA content per unit cytoplasmic protein between MNGs and macrophages, larger MNGs appeared to have enhanced functional activity as compared to their smaller mononuclear counterparts. Papadimitriou and Van Bruggen (1986) reported that succinate dehydrogenase and NSE activities per nucleus were not different between rat MNGs and macrophages, but that acid phosphatase activity per nucleus was slightly higher in MNGs. The multinucleated cells also had significantly higher protein and RNA levels per nucleus as compared to the similarly cultured mononuclear macrophages, suggesting potential differences in gene expression. With respect to enzyme content, Elleder (1986) found that there was considerable heterogeneity in enzyme “equipment” not only between categories of human MNGs (inflammatory, foreign body, osteoclasts) but also between these and their mononuclear counterparts. The most striking differences observed were that multinucleated osteoclasts
had low NSE activity and high TRAP, aminopeptidase and dipeptidylpeptidase IV activities when compared to their mononuclear precursors. No distinction was made, however, between proliferative TRAP-negative osteoclast precursors, non-proliferative TRAP-negative osteoclast precursors, TRAP+ pre-osteoclasts or TRAP+ mononuclear osteoclasts (Scheven et al. 1986; Taylor et al. 1993). Also, with regard to osteoclast-like cells, Woods et al. (1995) reported that chick macrophages expressed high levels of mRNA for retinoic acid receptors alpha and gamma as well as the vitamin D receptor, while the osteoclast-like MNGs that formed in long-term cultures of these same cells had lower expression of these transcripts. Previous observations in our laboratory, using time-lapse video recordings of osteoclasts cultured on bone slices, indicated that mononuclear osteoclastic cells can actually demineralize the bone surface, but do not degrade the organic matrix as do multinucleated cells (Kanehisa and Heersche, unpublished observations). In summary, the above observations suggest that multinucleation is likely to confer different characteristics on the cell type involved when compared with its mononuclear counterpart.

Another interesting facet of osteoclast physiology which pertains to the question of multinuclearity is the great variability in the number of nuclei in mature, active osteoclasts. However, the significance of this variability is not known (Addison 1979). As stated earlier, the increase in size of osteoclasts in many diseases of excessive bone resorption suggests that larger osteoclasts (i.e. those with more nuclei) may be more efficient on a per nucleus basis at resorbing bone. However, initial studies by Piper et al. (1992) found that while a positive correlation was found between the size of the osteoclast and the volume of the resorption pit made, the volume resorbed per nucleus tended to decrease with increasing nuclear number. This suggested that resorptive efficiency (expressed as resorption per nucleus) was lower in the very large cells. Additional studies will be required to explain this discrepancy.
1.7 Osteoclast heterogeneity

It has been observed in virtually every assay system that a marked heterogeneity exists in the behaviour of osteoclasts and their responsiveness to stimulatory and inhibitory agents. One striking observation was made by Kanehisa (1989), who investigated calcitonin-induced inhibition of osteoclast function. He found that, while the majority of cultured rabbit osteoclasts exposed to calcitonin stopped migrating and started contracting, others showed no discernible changes in cytoplasmic motility or general morphology. In addition, while osteoclasts at 9 of 23 separate resorption sites regained resorptive activity due to spontaneous escape from calcitonin-induced contraction, the remaining 14 did not. Another example indicating osteoclast heterogeneity was reported by Owens and Chambers (1993), who found that, while MCSF increased the percentage of migrating rat osteoclasts from 10% to 60%, the remaining 40% of osteoclasts did not respond to this cytokine. We have made similar observations in rabbit osteoclast cultures (Lees and Heersche 1996). Equally striking were the observations of Hall et al. (1993), who found that, while 75% of rat osteoclasts required continued mRNA and protein synthesis to resorb bone when cultured in vitro, some cells still actively resorbed in the presence of the inhibitors actinomycin D and cycloheximide. Yu and Ferrier (1993) also found heterogeneity in osteoclast responses to IL-1α, as only 27% of osteoclasts in one-day cultures responded with an increase in intracellular Ca^{2+} concentration while 84% responded in three-day cultures. In some of our own laboratory’s previous investigations, we also observed marked heterogeneity in the responsiveness of osteoclasts in terms of increased V-ATPase activity in response to a decrease in extracellular pH (Nordstrom et al. 1997). No attempts were made in any of these investigations to correlate the size of the osteoclasts with the phenomena observed.

The evidence presented above led us to a series of studies to systematically
investigate differences between cells of different size. In the first of these, changes in cell motility and contraction in response to changes in pH of the culture medium were evaluated in view of the previously discussed finding that a decrease in extracellular pH increases osteoclast activity (see section 1.3.3). The differences in contractility found between large and small osteoclasts were clearly related to cell size (Goto and Heersche, unpublished observations). The rest of the size-related experiments form the basis of this thesis.

1.8 Research Objective

Diseases in which excessive bone loss leads to problems, such as renal osteodystrophy, rheumatoid arthritis, Paget's disease and periodontal disease are characterized by an increase in the number and/or size of osteoclasts, particularly in areas of increased resorption. However, the relationship between osteoclast size and resorptive activity and machinery is not clear. Also, significant heterogeneity has been observed in the behaviour of osteoclasts and it has been postulated that this heterogeneity may be due to differences in size or phase of activity of the osteoclasts.

The hypothesis to be evaluated in this research project is that osteoclast size (as defined by the number of nuclei per cell) is a major factor in determining the efficiency with which individual and/or populations of osteoclasts can resorb bone and that resorptive efficiency is related to the activity of the pathways in osteoclasts involved in proton transport.

1.8.1 Outline of Chapter 2

Since MCSF is abundantly present in inflammatory areas in diseases associated with increased bone loss, we wished to determine what effect this cytokine had on activity,
number and size of osteoclasts. Upon addition of exogenous MCSF to osteoclast-containing cultures obtained from rabbit long bones, a striking increase in osteoclastic resorption of bone was found in terms of area, number, size and depth of resorption pits. This increase was not accompanied by a change in osteoclast number, however, it was paralleled by the formation of larger osteoclasts. Additionally, the stimulation of resorption occurred only upon long-term addition of MCSF when there were many more large (>10 nuclei) osteoclasts formed. Calculations of resorption per nucleus on populations of large and small osteoclasts revealed that as a whole, populations of large osteoclasts resorbed considerably more per nucleus than populations of small osteoclasts, suggesting that large osteoclasts are more efficient at resorbing bone than small osteoclasts.

1.8.2 Outline of Chapter 3

Due to the importance of proton extrusion and intracellular pH regulation in the normal functioning and resorptive process of osteoclasts, we wished to determine whether the increased resorptive activity of large osteoclasts relative to that of small osteoclasts seen in Chapter 2 was reflected in the activity of their proton transport pathways. When cultured on glass coverslips large osteoclasts (≥ 10 nuclei) did have a higher basal pH than small osteoclasts (2-5 nuclei), which was maintained by greater V-ATPase activity in these large cells. Although differences in the activities of both the V-ATPase and Na⁺/H⁺ exchangers were found between large and small osteoclasts, not all differences could be explained by cell size. There were two populations of large osteoclasts, detected by studying recovery from an acid load: a Na⁺-dependent group which recovered largely by Na⁺/H⁺ exchange activity and a Na⁺-independent group which recovered largely by V-ATPase activity. This suggested that while differences in pH regulatory mechanisms did exist between small and
large osteoclasts that could underlie differences in resorptive efficiency, there were factors other than cell size alone important in determining whether V-ATPase or Na\(^+\)/H\(^+\) exchange were operating.

1.8.3 Outline of Chapter 4

This led to the investigation of whether the above proton transport mechanisms differed in actively resorbing versus non-resorbing osteoclasts \textit{in vitro} of both size categories and whether the two populations of large osteoclasts observed in Chapter 3 could represent cells in different states of activity (i.e. resorbing vs. non-resorbing). Rabbit osteoclasts were cultured on recently developed collagen/ hydroxyapatite (CHA)-coated coverslips and pH\(_i\) changes determined. All resorbing osteoclasts had a high initial pH\(_i\) and V-ATPase activity was much greater in these cells as compared to their non-resorbing counterparts. Large resorbing and non-resorbing osteoclasts did recover from an acid load in the same manner as the two populations of large osteoclasts observed in Chapter 3, suggesting that the different populations observed on glass represented the resorbing and non-resorbing phenotypes. However, since small and large resorbing osteoclasts had similar V-ATPase activities and these cells differed only slightly in that small resorbing osteoclasts also had Na\(^+\)/H\(^+\) exchange activity, we re-examined the question of differences in resorptive efficiency observed in chapter 2 for populations of small and large osteoclasts. When studied on a per cell basis on CHA-coated coverslips as opposed to on a population basis on bone slices, large and small osteoclasts did not differ in resorptive efficiency, although large osteoclasts did resorb considerably more per cell. The most striking observation was that a much larger percentage of large osteoclasts (40%) was resorbing than of small osteoclasts (5%), which explains why in the population study the resorption per nucleus of small osteoclasts was underestimated, as
very few of the nuclei counted were actually involved in resorption. Thus, although individual cell size is not a major factor in determining resorptive efficiency as we originally hypothesized, the considerable difference in the proportion of large and small osteoclasts involved in resorption suggests differences in activation mechanisms of these cells based on cell size, which may open up a new avenue of research for inhibiting the most active osteoclasts.
CHAPTER 2:

MACROPHAGE COLONY STIMULATING FACTOR INCREASES BONE RESORPTION IN DISPERSED OSTEOCLAST CULTURES BY INCREASING OSTEOCLAST SIZE

Rita L. Lees and Johan N.M. Heersche

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Rita Lees performed all of the experiments and wrote the manuscript.
2.1 INTRODUCTION

Several cytokines (MCSF, IL-1, IL-6, TNF-α, transforming growth factor β, leukemia inhibitory factor), leukotrienes (LTB₄) and prostaglandins (PGE₂) are known to be abundant in inflammatory areas in diseases such as rheumatoid arthritis, osteoarthritis and periodontal disease (Arend and Dayer 1990; DiGiovine et al. 1988; Firestein et al. 1988; Fontana et al. 1982; Lipsky et al. 1989; Moilanen 1994; Odeh 1997; Seitz et al. 1994; Shibutani et al. 1997; Smith et al. 1990; Smith et al. 1997). These diseases are also characterized by excessive bone loss and an increase in osteoclast size and number in the areas where bone loss is most prominent (Aota et al. 1996; Bogoch et al. 1988; Shibutani et al. 1997; Shimizu et al. 1985; Takashima et al. 1989). Among the factors inducing the increased bone resorption associated with inflammatory diseases, MCSF has been identified as the major factor regulating osteoclast formation and differentiation. However, the effects of MCSF on mature osteoclasts and their resorptive activity are not clear.

MCSF was first shown to be required for osteoclast formation in studies with the naturally occurring murine op/op mutants. These mice express an osteopetrotic phenotype due to an almost complete lack of osteoclasts (Felix et al. 1990a) as a result of a point mutation in the coding region of the MCSF gene (Yoshida et al. 1990). When daily injections of MCSF were administered to these mice, new and biologically active osteoclasts formed, resulting in the reversal of the osteopetrotic phenotype (Felix et al. 1990a; Kodama et al. 1991). Further studies convincingly demonstrated that MCSF is essential for osteoclast proliferation, differentiation and survival and that MCSF is produced by osteoblasts as well as other mesenchymal cells in the marrow environment (Rubin et al. 1996; Scheven et al. 1997; Weir et al. 1993).

Hofstetter et al. (1992), Weir et al. (1993) and Yang et al. (1996) showed that mature
osteoclasts also contain MCSF receptor transcripts (encoded by the proto-oncogene c-fms) and that they specifically bind MCSF, thus implying a role for this factor not only in osteoclast precursor proliferation but also in the regulation of osteoclast activity. Some investigators have found an inhibition of osteoclastic resorptive activity upon short-term exposure of rat or rabbit osteoclasts to MCSF (Fuller et al. 1993; Hattersley et al. 1988; Teti et al. 1998), while others (Edwards et al. 1998; Sarma and Flanagan 1996) reported an increase in both osteoclast number and bone resorption when human bone marrow cells or mature human osteoclasts were cultured in the presence of MCSF. These same results were found with MCSF addition to metatarsal explants in culture or injection into rats in vivo for several days (Antonioli-Corboz et al. 1992; Orcel et al. 1993) and the authors argued that the enhanced resorption was a result of increased osteoclast recruitment. Also, Weir et al. (1996) reported that MCSF inhibited resorptive activity in the absence of osteoclast recruitment, as anti-MCSF antibody added for 3 days to fetal rat long bones in culture significantly increased both basal and PTH-stimulated resorption. However, Yang et al. (1996) observed a clear stimulation of resorption after MCSF addition to murine calvarial explants without a change in osteoclast number. Recently Amano et al. (1998) reported that MCSF induces osteoclast fusion in cultured rat osteoclasts and suggested that tyrosine kinases are involved in this process. They also suggested that resorptive activity in MCSF-treated cultures was increased when compared to cells cultured under control conditions.

Because of the existing controversy regarding the effects of MCSF on osteoclast resorptive activity, we decided to investigate the role of MCSF on osteoclast formation and activity in more detail. We demonstrate here that MCSF clearly increases both osteoclast size and amount of bone resorbed in longer term rabbit bone marrow cultures containing stromal cells, osteoclast precursors and mature osteoclasts. Our results also suggest that the
MCSF-induced enhanced resorption is the result of an increase in the average size of the osteoclasts.

2.2 MATERIALS AND METHODS

2.2.1 Osteoclast Isolation

Osteoclasts were isolated from rabbit long bones as described previously (Asotra et al. 1994; Kanehisa and Heersche 1988). The femora, tibiae, humeri and radii of one-day-old New Zealand white rabbits were dissected out. After removal of adherent soft tissues, the shafts were placed in a sterile petri dish containing medium 199, cut longitudinally and the interior surfaces curetted to release the bone cells. The medium containing cells and bone fragments was agitated by pipetting to release additional cells attached to the bone fragments. These marrow cell suspensions containing osteoclasts were centrifuged at 200 g for 10 minutes and the pellet of cells obtained from the bones of one rabbit resuspended in 8 ml of α-MEM (α-minimum essential medium, pH 7.4) with 10% fetal calf serum (FCS) and antibiotics (100 μg/ml Penicillin G, 0.5 μg/ml Gentamycin, 0.3 μg/ml Fungizone).

2.2.2 Preparation of Bone Slices and Culture Methods

Bovine cortical bone was cleaned of soft tissue and devitalized by freeze-thawing in distilled water 3 times. After cutting the bone into blocks of 3.5x1.5x3.5 cm, cross sectional slices of 120-150 μm were cut using a Buehler Isomet low speed saw. Circular slices of approximately 5 mm in diameter were punched out from the slices, cleaned in a sonicating water bath and stored frozen in medium 199 containing 10x antibiotics. Prior to the addition of osteoclasts bone slices were placed in wells of 96-well plates and washed 2x with 150 μl α-MEM to remove the excess antibiotics. Following this, the bone slices were incubated for
24 hours in 150 μl α-MEM containing 10% FCS and antibiotics at 37°C in humidified air and 5% CO₂. Then 50 μl of the osteoclast-containing cell suspension was added to the 150 μl of α-MEM on each bone slice and the cells were allowed to attach for 18 hours.

2.2.3 Resorption Studies

After 18 hours, the cultures were washed with α-MEM and recombinant human MCSF (1.5x10⁵ U/μg, R&D Systems, Minnesota) was added at 4, 40 and 400 ng/ml for 12, 24 or 48 hours. Assuming similar unit activity and taking into account the low stromal cell density in our osteoclast preparations, we have calculated from the information published (Kanzaki et al. 1995; Rubin et al. 1997; Rubin et al. 1996; Sarma et al. 1998; Sarma and Flanagan 1996; Weir et al. 1993), that control media of cells cultured for 48 hours would probably contain no more than 0.03 ng MCSF per ml. At the end of the culture period, osteoclasts were identified by staining for TRAP, a marker for osteoclasts. The bones were fixed in 4% neutral buffered formalin for 10 min and washed with phosphate-buffered saline. A solution of Michaelis veronal acetate buffer (pH 5) containing naphthol AS-MX phosphate as substrate, hexazonium pararosanilin as coupler, and 20 mM L-(+)-tartaric acid was then added to the cells for 5 minutes at room temperature. TRAP-positive cells stained red. The number of multinuclear TRAP+ cells and the number of nuclei per cell were counted using light microscopy. Most of the cells were then removed by washing with 0.25 N ammonium hydroxide.

The resorption lacunae were identified by immunohistochemical staining as described previously (Shibutani and Heersche 1993). The bone slices were pre-incubated with 3% sheep serum in PBS to minimize background staining and then incubated overnight with sheep IgG anti type I collagen (1:10, gift from Dr. Jaro Sodek, Faculty of Dentistry,
University of Toronto). After washing, biotinylated donkey anti sheep IgG (1:100, Sigma) was added for 1 hour, followed by treatment with avidin-biotin-peroxidase complex (ABC, Dimension Labs). Antibody binding was visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as substrate. The bone slices were then washed and dried and plan area resorbed quantified with a Zeiss interactive digital analysis system using light microscopy. Depth of lacunae was estimated by differential focusing of the edge and centre of the pits using a 40x objective. In order to avoid differences based on the variable size of pits, 90 lacunae of approximately the same shape and size (round, 60-80 μm diameter) were selected per treatment group for the depth measurements.

2.2.4 Statistics

Data were analyzed statistically by one way analysis of variance and with an unpaired, double-sided Student’s t-test with Bonferroni’s correction to allow for multiple comparisons. All results are expressed as the mean ± standard error of the mean (SEM).

2.3 RESULTS

When osteoclast-containing cell suspensions were cultured for 48 hours on bone slices in media to which 4, 40 or 400 ng/ml MCSF were added, MCSF caused an increase in total plan area resorbed per bone slice at all concentrations studied (figure 1A). For example, total area resorbed per osteoclast increased from 3000 μm² in control cultures to 6500 μm² in cultures treated with 40 ng/ml MCSF. Resorption expressed as area resorbed per multinuclear osteoclast was also increased and this increase was dose-dependent (figure 1B). In addition, MCSF increased the number of pits excavated per multinuclear osteoclast (figure 1C) and dose-dependently increased average pit size (figure 1D). This is illustrated by the
photographs shown in figure 2. The numbers of mononuclear and multinucleated TRAP+ cells (approximately 250 cells each) in the different treatment groups were the same at the end of the 48 hour culture period. We also measured the average depth of approximately circular resorption lacunae (60-80 μm) in control and MCSF (400 ng/ml)-treated cultures. Average depth in MCSF-treated cultures was 21.0 ± 0.6 μm, significantly higher than the average depth of similarly sized pits in the control group (16.4 ± 0.6 μm, P<0.001).

Since several reports indicated that MCSF decreased osteoclastic bone resorption or had no effect on resorption when rat osteoclasts were cultured for 6-24 hours in the presence of this factor (Fuller et al. 1993; Hattersley et al. 1988; Teti et al. 1998), we also investigated the effect of a short-term treatment of MCSF on our rabbit cell cultures. No effect was seen on any of the resorptive parameters after a 12 hour incubation with 40 ng/ml MCSF (table I).

Next we compared the effects of a 24 hour and 48 hour incubation with MCSF, and also explored in this same experiment whether cell density might be a major factor determining resorptive activity of individual osteoclasts. At the high plating density, no significant effects on resorbed area or area resorbed per multinuclear osteoclast were observed at the 24 hour time point (figure 3A), while culture in the presence of MCSF for 48 hours resulted in a 2-fold increase in total area resorbed (figure 3A) and in area resorbed per multinuclear osteoclast (figure 3B). The effect of cell density was determined by plating the same osteoclast preparation at high and low (1/5 of high) plating densities. Resorption per multinucleated cell was 8-fold less at the lower plating density than that at the higher plating density. However, the MCSF-induced fold increase in plan area resorbed and in area resorbed per osteoclast was not affected by cell density (approximately 2-fold increase, figures 3A and B).

We then investigated whether the increase in resorptive activity per osteoclast
induced by a 48 hour treatment with MCSF was related to an increase in size of the osteoclasts in MCSF-treated cultures (figure 4). At both high and low plating densities, a 48 hour treatment with MCSF increased the number of large osteoclasts by a factor of 2 (figures 4B, 4C). A 24 hour treatment with MCSF also increased the number of large osteoclasts (figure 4A). In all experiments the total number of multinucleated cells was the same in control and MCSF treated cultures.

To determine whether large osteoclasts, as a population, are more efficient resorbers than smaller osteoclasts, i.e. resorb more bone per nucleus, we calculated resorption per osteoclast nucleus. Resorptive activity per osteoclast nucleus, averaged over the whole osteoclast population, was not significantly different in control and MCSF-treated cultures (figure 5). However, when we calculated the resorption per nucleus for large osteoclasts and small osteoclasts separately, using the total number of nuclei per bone slice in osteoclasts of a given size (table II) and the total area resorbed (figure 3A), resorption per nucleus was 4 times greater in osteoclasts with >10 nuclei than for osteoclasts with ≤10 nuclei (figure 6). A similar calculation for the categories of osteoclasts with >5 nuclei and ≤5 nuclei showed a 20 times greater resorption per nucleus for osteoclasts with >5 nuclei than for smaller osteoclasts with ≤5 nuclei. The same calculation for the low plating density experiment (data from table II and figure 3A) resulted in similar relative resorptive activities, although as expected, values for resorption/nucleus were considerably lower (figure 6).
Figure 1: The effect on resorption of culturing osteoclasts on bone slices for 48 hours in the presence or absence of MCSF. The total number of mononuclear and multinuclear TRAP+ cells per bone slice was 260 and 250 cells, respectively. Plan area resorbed per bone slice was significantly higher in all three MCSF treatment groups as compared to control (A), as were the plan area resorbed per multinuclear osteoclast (B), the number of pits formed per multinuclear osteoclast (C), and the size of resorption pits (D). Error bars indicate SEM. [*P<0.001 vs control, aP<0.01 vs next highest dose, n = 9]
Figure 2: Osteoclasts cultured on bone slices for 48 hours in the presence of MCSF (400 ng/ml) formed large, compound resorption lacunae (A). Osteoclasts cultured under control conditions formed smaller lacunae (B). [Original magnification 500x]
TABLE I: The effect on resorption of culturing osteoclasts on bone slices in the presence or absence of MCSF (40 ng/ml) for 12 hours.

<table>
<thead>
<tr>
<th></th>
<th>Area resorbed/ bone slice (µm²)</th>
<th>Area resorbed/ Multinuclear osteoclast (µm²)</th>
<th>Number of pits/ Multinuclear osteoclast (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5660 ± 1100</td>
<td>145.4 ± 30</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>MCSF</td>
<td>6660 ± 2000</td>
<td>138.9 ± 39</td>
<td>0.15 ± 0.04</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM of 10 bone slices per group. The average number of mononuclear and multinuclear TRAP+ cells per bone slice in each group was 70 and 45, respectively.
Figure 3: Bone resorption by osteoclasts cultured on bone slices for 24 or 48 hours in the presence or absence of MCSF (40 ng/ml) at two different plating densities. The total number of mononuclear and multinuclear TRAP+ cells per bone slice in the high density groups averaged 110 and 100 cells respectively, while that in the low density groups averaged 25 and 30 cells. Plan area resorbed per bone slice (A) and plan area resorbed per multinuclear osteoclast (B) was significantly higher only in those groups exposed to MCSF for 48 hours. However, when starting with fewer cells the resorptive activity per cell was considerably reduced in both control and MCSF treated cultures. Error bars indicate SEM. [*P<0.001 vs control, n = 8]
Figure 4: The number of osteoclasts in cell populations cultured on bone slices for 24 or 48 hours in the presence or absence of MCSF (40 ng/ml). At 24 hours (A), there was a significant increase in the number of large osteoclasts with greater than 10 nuclei in the MCSF-treated group as compared to control, which became even greater at 48 hours (B) while the number of smaller cells decreased at this time point. When the original plating density was decreased to 1/5 the number of cells (C), very few large cells formed in either group although there was a significant increase after MCSF treatment in the number of cells with 6-10 nuclei as compared to control. Error bars indicate SEM.[*P<0.001 vs control, n=8]
Figure 5: Bone resorption expressed as amount of bone resorbed per osteoclast nucleus in osteoclast-containing cell populations cultured on bone slices for 24 or 48 hours in the presence or absence of MCSF (40 ng/ml) at two different plating densities. The total number of mononuclear and multinuclear TRAP+ cells per bone slice in the high density groups averaged 110 and 100 cells respectively, while that in the low density groups averaged 25 and 30 cells. Plan area resorbed per nucleus was 8-fold lower in the control group at the lower plating density, while in the MCSF treated group it was 5-fold lower. There was a slight but not significant increase in area resorbed per nucleus in the MCSF treated groups as compared to control at both plating densities. Error bars indicate SEM. [# P<0.001 vs high plating density, n=8]
Figure 6: Calculated values for bone resorption per osteoclast nucleus for osteoclast-containing cell populations cultured on bone slices for 48 hours in the presence or absence of MCSF (40 ng/ml) at high and low plating density. (A) High plating density: Bone resorption per nucleus in large osteoclasts (x) and in small osteoclasts (y) for the categories of osteoclasts with \( \leq 10 \) nuclei and \( > 10 \) nuclei was calculated using two equations. Equation one: \( 0.41 \text{mm}^2 = 347.63x + 530.75y \), where \( 0.41 \text{mm}^2 \) is the total area resorbed per bone slice in MCSF-treated cultures from figure 3A, 347.63 is the average total number of nuclei in osteoclasts with \( > 10 \) nuclei in these cultures and 530.75 is the average total number of nuclei in osteoclasts with \( \leq 10 \) nuclei in these cultures (table II). Equation two: \( 0.24 \text{mm}^2 = 134.25x + 541.88y \), where \( 0.24 \text{mm}^2 \) is the total area resorbed per bone slice in control cultures from figure 3A, 134.25 is the average total number of nuclei in osteoclasts with \( > 10 \) nuclei in these cultures and 541.88 is the average total number of nuclei in osteoclasts with \( \leq 10 \) nuclei in these cultures (table II). Combining these equations allows calculation of \( x \) and \( y \). Similar calculations were performed for the categories of osteoclasts with \( > 5 \) nuclei and \( \leq 5 \) nuclei.

(B) Low plating density conditions: the same calculations were performed as described above, but values were obtained from the low plating density experiment (figure 3A, table II). For all calculations we have assumed that resorptive activity of osteoclasts of the same size in control and MCSF treated cultures does not differ. This seems likely, based on our results indicating that a 12-24 hour culture period in the presence of MCSF has no effect on osteoclast resorptive activity (see table I). Published results of others (Antonioli-Corboz et al., 1992; Fuller et al., 1993; Hattersley et al., 1988; Teti et al., 1998) also support the fact that short term culture in MCSF-containing media has either no effect or is inhibitory in terms of resorptive activity.
**TABLE II:** The total number of nuclei per bone slice in osteoclasts of specific sizes after 48 hours of culture in control or MCSF (40ng/ml).

<table>
<thead>
<tr>
<th></th>
<th>Total number of nuclei in:</th>
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<tr>
<td></td>
<td>osteoclasts with ≤10 nuclei</td>
<td>osteoclasts with &gt;10 nuclei</td>
<td>osteoclasts with ≤5 nuclei</td>
<td>osteoclasts with &gt;5 nuclei</td>
</tr>
<tr>
<td>Control (high plating density)</td>
<td>541.88 ± 17</td>
<td>134.25 ± 17</td>
<td>318.38 ± 15</td>
<td>357.75 ± 20</td>
</tr>
<tr>
<td>MCSF (high plating density)</td>
<td>530.75 ± 34</td>
<td>347.63 ± 28</td>
<td>247.75 ± 13</td>
<td>630.63 ± 50</td>
</tr>
<tr>
<td>Control (low plating density)</td>
<td>78.6 ± 15</td>
<td>4.6 ± 3</td>
<td>66 ± 3</td>
<td>17.2 ± 8</td>
</tr>
<tr>
<td>MCSF (low plating density)</td>
<td>97.8 ± 15</td>
<td>22 ± 11</td>
<td>59.4 ± 7</td>
<td>60.4 ± 18</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM of 8 bone slices per group.
2.4 DISCUSSION

MCSF stimulates osteoclast progenitor proliferation and differentiation (Jimi et al. 1995; Takahashi et al. 1991; Tanaka et al. 1993), but the effects of MCSF on resorptive activity of mature osteoclasts are not clear: increased activity (Edwards et al. 1998; Orcel et al. 1993; Sarma and Flanagan 1996; Yang et al. 1996), decreased activity (Fuller et al. 1993; Hattersley et al. 1988; Sarma and Flanagan 1996; Teti et al. 1998; Weir et al. 1996) or no change in activity (Antonioli-Corboz et al. 1992; Fuller et al. 1993) have all been reported. Since the dispersed osteoclast preparations or the bone culture systems used in these experiments differ in many respects, for example cell density of the cultures, serum content of the culture medium, time in culture and presence of stromal cells, we initiated the current study to try to understand and explain these different outcomes with respect to how osteoclastic resorption is affected by MCSF.

We found that MCSF causes a time-dependent stimulation of bone resorption when added to osteoclast-containing rabbit bone marrow cultures that also contain stromal cells and osteoclast precursors. Concomitant with the stimulation of resorption, we also found that a 48 hour treatment with MCSF increased the number of large (>10 nuclei) osteoclasts and decreased the number of small (2-5 nuclei) osteoclasts in the cultures. Since the total number of nuclei per culture present in osteoclasts was increased, and the number of TRAP+ osteoclast precursors in control and MCSF-treated cultures was similar in all experiments, these observations suggest that MCSF also increased proliferation and differentiation of mononuclear TRAP+ osteoclast precursors. In addition, the total number of nuclei in osteoclasts may also have increased as a result of decreased osteoclast apoptosis or decreased cell death in MCSF-treated cultures. Fuller et al. (1993) reported previously that MCSF stimulated survival of rat osteoclasts cultured in serum-free medium. Decreased apoptosis
could contribute to the increase in the proportion of large osteoclasts if larger osteoclasts were selectively undergoing apoptosis in the absence of MCSF. An increase in size of osteoclasts after MCSF administration was also reported by Amano et al. (1998) for rat osteoclasts. Similar to our 24 hour results, they found that after 16 hours osteoclasts with 2-8 nuclei were present in similar numbers in the control and MCSF treated groups while the number of larger osteoclasts with >10 nuclei was increased by MCSF. In their cultures, the maximum size increase was seen at 16 hours, although the number of nuclei per osteoclast remained higher in MCSF-treated cultures for up to 72 hours. This differed from our results as we found a greater number of large cells after 48 hours treatment with MCSF when compared to a 24 hour treatment. The difference may be explained by the fact that Amano et al. (1998) cultured their cells in the presence of hydroxyurea to inhibit cell proliferation, thus limiting the supply of osteoclast precursors and the number of cells available for fusion and formation of larger cells at later time points.

Edwards et al. (1998) showed that MCSF increased osteoclast survival and thereby increased resorption in serum-free cultures of freshly isolated human osteoclasts cultured for 18 hours. We calculated from their data that resorptive activity per osteoclast in MCSF-treated cultures was not increased in their experiments. However, their culture conditions (i.e. no serum, low cell density, short culture time) were such that osteoclast fusion did not occur, as shown by the fact that MCSF had no effect on number of nuclei per cell in their experiments. Previous experiments of these authors with long term human bone marrow cultures containing 10% serum had shown that MCSF increased the number of 23c6+ cells (a marker for osteoclasts) under those culture conditions and also increased resorptive activity. The proportion of 23c6+ cells with 3 or more nuclei was 11-fold greater in the MCSF-treated cultures. Thus, the increase in the number of larger cells may have been responsible for the
increase in resorptive activity observed, which is entirely compatible with the conclusions reached from our own experiments. Yang et al. (1996) also observed that MCSF increased resorption in mouse calvaria cultured for 48 hours in the presence of MCSF without increasing osteoclast number, but they did not measure osteoclast size. Based on the observation that MCSF increased osteoclast size in our experiments and in those of Amano et al. (1998), both using culture conditions in which the diversity of cells present is in many respects similar to that present in bone tissue, we suggest that in their experiments increased osteoclast size may have been responsible for the increased resorptive activity.

That Weir et al. (1996) found an inhibition and Antonioli-Corboz et al. (1992) found no change in resorption upon MCSF administration may be a result of the fact that the explanted fetal rat radii used by them may not have allowed for the extensive osteoclast fusion that occurred in our cell culture system. Similarly, although the disaggregated rat osteoclast culture system used by Fuller et al. (1993) and by Hattersley et al. (1988) is comparable to our rabbit cell culture system, the low numbers of osteoclasts per bone slice reported in those experiments (less than 10 osteoclasts per bone slice), and in particular the short incubation times of 6-24 hours may not have allowed for sufficient formation of the larger osteoclasts. This may account for the discrepancy between their conclusion that MCSF inhibited or did not affect resorptive activity of osteoclasts and our demonstration of a clear increase in resorption after 48 hours of culture in the presence of MCSF.

The observation by us and others (Kaye et al. 1985; Makris and Saffar 1982; Piper et al. 1992) that large osteoclasts resorb more bone than smaller osteoclasts on a per cell basis raises the question of whether large osteoclasts are also more efficient resorbers using resorption per osteoclast nucleus as a parameter. Piper et al. (1992) found a trend for the volume resorbed per nucleus to decrease with increasing number of nuclei per osteoclast
when observing the resorptive activity of individual large and small avian osteoclasts. When we calculated resorptive activity per osteoclast nucleus by averaging the total number of nuclei in all osteoclasts in control and MCSF-treated cultures, the increase in resorptive activity per nucleus in MCSF-treated cultures was not significant (figure 5). However, when we calculated the resorption per nucleus for larger osteoclasts and small osteoclasts separately, the resorption per nucleus was greater in larger osteoclasts than small osteoclasts (figure 6). As already referred to in the legend for figure 6, this calculation assumes that MCSF does not increase resorptive activity of existing osteoclasts. Since short term incubation of osteoclasts with MCSF has no stimulatory effect on osteoclastic resorptive activity (Fuller et al. 1993; Hattersley et al. 1988); and our 12 and 24 hour experiments, this assumption seems justified. Our results indicating that the resorptive activity per nucleus of a population of large osteoclasts is greater than a population of smaller osteoclasts are not necessarily in conflict with the aforementioned observations of Piper et al. (1992): the explanation could be that the proportion of resorbing cells is greater in the population of large osteoclasts than in the population of smaller osteoclasts.

Our results also demonstrate that cell density is an important factor in regulating both osteoclast activity and osteoclast size. This is compatible with a previous study by Woods et al. (1995), who showed that cell density affected formation of osteoclast-like multinucleate giant cells which could resorb bone. It seems likely that part of the explanation is that the stromal and osteoblastic cells in our preparations produce factor(s) that stimulate osteoclast fusion and activity in addition to MCSF (i.e. OPGL, (Lacey et al. 1998; Yasuda et al. 1998)). The lower number of these cell types in the low density groups is likely one of the factors accounting for the smaller osteoclast size and decreased resorptive activity in our low density cultures.
In conclusion, we have demonstrated that MCSF increases resorptive activity in long term cultures of osteoclast-containing marrow cultures through an MCSF-induced increase in the proportion of larger osteoclasts in the population. Based on our calculations of resorptive activity per nucleus, we suggest that larger osteoclasts, as a population, are more effective resorbers than small osteoclasts, which raises the possibility that differences exist in the mechanisms or signaling pathways activating the resorptive process in large and small osteoclasts.

2.5 Statement of significance

In this chapter we showed clearly, and for the first time, that long-term culture of osteoclasts with MCSF stimulates osteoclastic resorption of bone by increasing osteoclast size. Short-term culture resulted in no change in cell size or area resorbed. This result revealed the reasons for the existing controversy regarding the effects of MCSF on osteoclast resorptive activity. The importance of cell size in determining resorptive activity was further emphasized by calculations of resorption per nucleus (resorptive efficiency) of populations of osteoclasts, which demonstrated that large osteoclasts as a population resorbed more per nucleus than small osteoclasts. This suggested some inherent differences between small and large osteoclasts in their resorptive capabilities.
CHAPTER 3:

DIFFERENCES IN REGULATION OF INTRACELLULAR pH IN LARGE (≥10 NUCLEI) AND SMALL (≤5 NUCLEI) OSTEOCLASTS

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Rita Lees performed all of the experiments and wrote the manuscript.
3.1 INTRODUCTION

Osteoclasts are multinucleated cells responsible for bone resorption. Osteoclastic bone resorption is a complex process starting with migration and attachment of osteoclasts to the bone surface. This is followed by the development of a sealing zone, the formation of a ruffled border and the subsequent extrusion of protons, chloride ions and proteolytic enzymes into the extracellular resorption zone between the ruffled border and the bone surface (Vaaninen 1996). Proton extrusion is accomplished by a vacuolar type H⁺-ATPase (V-ATPase), localized on the ruffled border (Blair et al. 1989; Vaananen et al. 1990). Maintenance of intracellular pH (pHᵢ) is believed to be controlled mainly by Na⁺/H⁺ exchangers and Cl⁻/HCO₃⁻ exchangers on the basolateral membrane (Hall and Chambers 1990; Nordstrom et al. 1995; Teti et al. 1989b).

Osteoclasts from humans, rats and rabbits can under normal circumstances contain anywhere from 2-30 nuclei, with an average of 3-10 nuclei. However, in diseases characterized by increased bone resorption such as Paget’s disease, end stage renal disease, periodontal disease and rheumatoid arthritis, osteoclast size is generally increased (Aota et al. 1996; Kaye et al. 1985; Makris and Saffar 1982; Singer and Roodman 1996). In Paget’s disease osteoclasts have been reported to reach a diameter of 100 μm and to contain up to 100 nuclei (Singer and Roodman 1996).

Osteoclasts become multinucleated as a result of cell fusion, either with other multinuclear osteoclasts or with mononuclear osteoclast precursors (Fischman and Hay 1962; Jaworski et al. 1981) and certain enzymes and receptors are up or down regulated upon formation of multinuclear osteoclasts from their mononuclear precursors (Elleder 1986; Woods et al. 1995). However, it is not known whether increased multinuclearity in osteoclasts is associated with changes in gene expression as has been reported for other
multinucleated cells (Enelow et al. 1992; Fais et al. 1994; Nishii et al. 1991; Papadimitriou and Van Bruggen 1986; Vignery et al. 1991). Piper et al. (1992) studied the relationship between osteoclast size (as determined by the number of nuclei) and resorptive ability of these cells and found a positive correlation between the size of an osteoclast and the volume of the resorption pit made. They also found that the volume resorbed per nucleus tended to decrease with increasing nuclear number. However, their observations included only osteoclasts associated with a resorption pit and did not take into account the possibility that osteoclasts can form several pits within a given period of time (Kanehisa and Heersche 1988). Thus they may have underestimated the activity of their osteoclasts. We found recently that in cultures with significantly greater numbers of large osteoclasts both pit size and pit number were increased, indicating that large osteoclasts were not only resorbing a larger surface area per pit but also formed more pits than smaller osteoclasts (Lees and Heersche 1999).

One striking characteristic of osteoclasts is the marked heterogeneity observed in their responsiveness to inhibitory and stimulatory agents, such as calcitonin (Kanehisa 1989) and MCSF (Owens and Chambers 1993). Osteoclast responses to actinomycin D and cycloheximide were not uniform either, as these inhibitors of mRNA transcription and protein translation, respectively, did not inhibit bone resorption by all osteoclasts in the studies reported by Hall et al. (1993). Heterogeneity was also observed with regard to cytoplasmic pH regulation: under conditions of chronic extracellular acidosis and induced acid load, 40% of osteoclasts recovered by V-ATPase activity while the remaining 60% did not (Nordstrom et al. 1997; Ravesloot et al. 1995). While some investigators have associated the variable responses with osteoclast size or shape (Arkett et al. 1992; Ravesloot et al. 1995), the reasons for the variable responses have not been elucidated.
Proton extrusion and cytoplasmic pH regulation have a pivotal role in the resorptive process. Interestingly, heterogeneity of osteoclasts with regard to activity of proton pumps has also been noted (Nordstrom et al. 1997; Ravesloot et al. 1995). Therefore, we sought to determine in this study whether small (2-5 nuclei) and large (≥10 nuclei) osteoclasts differed with regard to V-ATPase and Na⁺/H⁺ exchanger activities. We found that basal pHᵢ in large and small osteoclasts differed significantly as did the responses to Na⁺ removal and to the addition of the V-ATPase inhibitor bafilomycin A₁. Upon acid loading, small osteoclasts recovered by a Na⁺-dependent and amiloride-sensitive mechanism, while large osteoclasts consisted of two groups: a Na⁺-dependent group which was maximally inhibited by the addition of both amiloride and bafilomycin A₁ and a Na⁺-independent group which was sensitive only to bafilomycin A₁. These results clearly show that large and small osteoclasts differ with regard to the activity of proton extrusion mechanisms.

3.2 MATERIALS AND METHODS

3.2.1 Osteoclast isolation and identification

Osteoclasts were isolated from rabbit long bones as described previously in section 2.2.1. The osteoclast-containing cell suspension was plated on glass coverslips in 6-well tissue culture dishes (100 μl per coverslip) and allowed to attach overnight in α-MEM containing 10% FCS and antibiotics in humidified air, 37°C and 5% CO₂. The cells were then cultured for 3-8 hours in 25 mM N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES)-buffered, bicarbonate-free RPMI with 10% serum at pH 7.4 and 37°C in an air incubator before measurement of pHᵢ. In some cases, the cells were cultured for 24 hours under these conditions, but the results were pooled as no differences were found between short and long term culture in HEPES-buffered bicarbonate-free medium.
Osteoclasts were identified as cells containing 2 or more nuclei (as seen by phase contrast microscopy). A few of the cells in the initial experiments were also fixed and stained for TRAP after completion of the experiments, as described in section 2.2.3. Since all of these cells were TRAP-positive, TRAP staining was omitted in subsequent experiments.

3.2.2 Intracellular pH measurements

The intracellular pH was determined using the microfluorometric technique described previously for osteoclasts (Nordstrom et al. 1995). Coverslips with cells were placed into a Leiden coverslip dish and maintained at 37°C for the remainder of the experiment. The cells were loaded with the H⁺-sensitive fluorescent dye BCECF by incubation with 1 μM of the parent acetoxymethyl ester for 10 min, then washed with RPMI and incubated in Na⁺-containing medium (140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 20 mM HEPES, pH 7.4). For incubation in sodium-free conditions, Na⁺ was replaced with either K⁺ or NMG⁺. Test solutions were applied to the cells by pipetting 3 ml of the desired medium (with or without inhibitors) into the chamber. Previously used culture medium was removed by aspiration. In some experiments treatments were applied sequentially to the same cells (figures 2 and 3). Since the effects of amiloride are reversible, it was added first, then washed out with Na⁺-containing medium alone, after which bafilomycin A₁ was added. In the experiments shown in figures 5 and 6, treatments with transport blockers were all independent observations on different cells, however, a few cells in figure 6 (19 out of a total of 131) received sequential treatment as in the experiments shown in figures 2 and 3. No statistical difference was seen between the results of sequential and independent addition of inhibitors in the experiments shown in figure 6, thus the results were pooled. The rate of pHᵢ recovery shown in figures 5 and 6 is the initial maximal rate of change.
Fluorescence intensity in single cells was measured using a Nikon TMD-Diaphot microscope attached to a dual wavelength illumination system (490 nm and 440 nm) from Photon Technologies Inc. Illumination was on for 2 sec and off for 8 sec (to minimize photo-bleaching), and the photometric data were recorded at a rate of 5 points/sec. The excitation light was directed to the cells via a 510-nm dichroic mirror. Fluorescence emission collected by a Nikon Fluor 40x oil-immersion objective traversed a 542 ± 64 nm band-pass filter.

3.2.3 Acid loading of osteoclasts

For acid loading we used the NH$_4^+$ ‘prepulse’ method (Swallow et al. 1990). The adherent cells were first exposed to 40 mM NH$_4$Cl for 5-8 minutes, during which time there was a rapid rise in pH$_i$ as NH$_3$ entered the cell and combined with H$^+$ to form NH$_4^+$, followed by a slow decline in pH$_i$ due to influx of NH$_4^+$ which dissociated to form NH$_3$ and H$^+$. Osteoclasts were then transferred to NH$_4^+$-free medium resulting in a rapid loss of NH$_3$, subsequent dissociation of internal NH$_4^+$ into NH$_3$ and H$^+$ and a decrease in pH$_i$ to a level much lower than the starting pH$_i$. Recovery of osteoclast pH$_i$ was then monitored in the cells in the presence of different media and inhibitors of pH$_i$ regulatory mechanisms.

3.2.4 pH$_i$ calibration

Calibration of the fluorescence ratio versus pH of each individual osteoclast was performed using the K$^+/H^+$ ionophore nigericin. Upon completion of the pH experiments, the cells were equilibrated in K$^+$ medium (140 mM) of varying pH in the presence of 5 µM nigericin and calibration curves were constructed by plotting the extracellular pH (which is assumed to be identical to the internal pH; (Thomas et al. 1979)) against the corresponding fluorescence ratio. Additionally, some cells were calibrated by the null-point method as
originally described by Eisner et al. (1989) to verify the values found with the nigericin technique. The principle of this method is to find the mixture of permeant acid (A) and base (B) which upon entering the cell produces no change in pH. The pH can then be calculated from the composition of this mixture, according to the following equation: $pH = pH_o - 0.5\log\{[A]/[B]\}$. Thus with $pH_o$ of 7.4 we prepared Na$^+$-containing solutions (designated as $pH_{null}$) of pH 6.8-7.8 using butyric acid as the weak acid and trimethylamine as the weak base.

3.2.5 Statistics

See section 2.2.4 for statistical analysis of data.

3.3 RESULTS

3.3.1 Size distribution of osteoclasts

The osteoclast-containing cell cultures obtained from newborn rabbit long bones were comprised of a variety of stromal cell types as well as osteoclasts. Osteoclasts were defined as cells having 2 or more nuclei under phase-contrast microscopy and staining positively for TRAP after fixation. We have reported previously that cells so identified bind calcitonin and resorb bone when plated on bone slices (Shibutani and Heersche 1993; Turksen et al. 1988). The size and nuclear number of the osteoclasts ranged from cells with a diameter of 30 μm and 2 nuclei to cells with a diameter of 90 μm and 23 nuclei. We studied 2 groups of osteoclasts: small osteoclasts which contained 2-5 nuclei (60% of the total number of osteoclasts) and large osteoclasts that contained 10 or more nuclei (10% of the osteoclasts), and investigated the $pH_i$ changes in these cells. This choice was arbitrary, and made to decrease the likelihood of overlap for parameters which might increase or decrease in value.
3.3.2 Determination of cytoplasmic pH in osteoclasts by two independent methods of calibration

BCECF was uniformly distributed in the cytoplasmic compartment. Small osteoclasts maintained a steady resting pH$_i$ of 7.34±0.02 (n=94) when cultured on glass coverslips for 2-6 hours in bicarbonate-free HEPES-buffered RPMI with 10% FCS, as determined by the nigericin calibration technique. In contrast, large osteoclasts had a significantly higher pH$_i$ of 7.70±0.02 (n=156) under the same conditions (P<0.001). This difference in steady-state pH$_i$ between large and small osteoclasts was confirmed when another calibration technique (the null-point method) was used (n=12, figure 1A). pH$_i$ tracings for a single small osteoclast and a single large osteoclast, calibrated by both the null point method (horizontal bars) and then the nigericin method (y-axis) are shown in figures 1B and C. The pH$_i$ of the representative small osteoclast in figure 1B decreased when a null solution of pH 7.05 was added (bar) but increased after addition of a null solution of pH 7.45, indicating that this osteoclast had a resting pH$_i$ between these values, i.e. about 7.2. A similar procedure applied to the large osteoclast shown in figure 1C indicates a resting pH$_i$ of approximately 7.7. Due to the excellent agreement between the two calibration methods, and the greater ease of performing nigericin calibration, all future calibrations were performed with the nigericin technique.

3.3.3 Effect of amiloride and bafilomycin A$_1$ on osteoclast resting pH$_i$

Upon addition of amiloride (an inhibitor of the Na$^+$/H$^+$ exchanger) to Na$^+$-containing medium, the pH$_i$ of both small and large osteoclasts did not change significantly (figure 2A).
Upon the removal of amiloride by washing and the addition of bafilomycin A₁ (the specific and widely-used inhibitor of the V-ATPase) in Na⁺-containing medium to these same cells, the pHᵢ of the large osteoclasts decreased significantly from 7.59 ± 0.04 to 7.37 ± 0.05 at a rapid initial rate of -0.16 ± 0.01 pH units/min while the pHᵢ of small osteoclasts did not change. When these same cells were treated with both amiloride and bafilomycin A₁ there was a significant drop in pHᵢ of small osteoclasts from 7.34 ± 0.03 to 7.19 ± 0.05 while that of large osteoclasts remained at about the same level as seen with bafilomycin A₁ alone. Representative traces of a small (figure 2B) and a large (figure 2C) osteoclast treated with amiloride and then with bafilomycin A₁ are shown.

3.3.4 Chloride channels and V-ATPase activity in small and large osteoclasts

Since V-ATPase-mediated proton transport is often charge-coupled to passive chloride permeability, we wished to ascertain whether the observed differences in V-ATPase activity between small and large osteoclasts were due to differences in Cl⁻ channels in these cells. To evaluate this, the Cl⁻ channel inhibitor diisothiocyanato-stilbene disulfonic acid (DIDS) was added to Na⁺-containing medium in addition to bafilomycin A₁. Once again, the pHᵢ of small osteoclasts did not change significantly as compared to control Na⁺-containing medium (figure 3A). Under the same conditions, the pHᵢ of large osteoclasts decreased significantly from 7.55±0.05 to 7.35±0.06. Interestingly, however, DIDS alone raised the pHᵢ of both large and small osteoclasts by 0.2 pH units. Representative traces of a small and a large osteoclast are shown in figures 3B and 3C, respectively.

3.3.5 The importance of extracellular Na⁺ in maintaining high pH in large osteoclasts

When Na⁺-containing medium was replaced by K⁺- or NMG⁺-containing medium, the
pH$_i$ of small osteoclasts was not affected (figure 4A,B). Similar treatment of large osteoclasts resulted in a decrease in pH$_i$ by 0.40 pH units. This drop in pH$_i$ of large osteoclasts occurred rapidly (within 20 sec) and was maintained until Na$^+$ was added back to the external medium (figure 4C).

3.3.6 Effect of amiloride and bafilomycin A$_1$ on osteoclast pH$_i$ recovery

In order to determine which pH regulatory mechanisms were involved in the pH recovery of large osteoclasts after re-addition of Na$^+$, the effects of various inhibitors were evaluated independently in different cells (figure 5A). Under Na$^+$-free conditions, recovery was negligible, averaging 0.01 pH units/min. The addition of Na$^+$ alone resulted in immediate and rapid recovery at a rate of 0.20 pH units/min. Addition of amiloride resulted in a 25% inhibition in the initial maximal rate of Na$^+$-induced pH$_i$ recovery. Addition of bafilomycin A$_1$ resulted in a 70% reduction in the rate of pH$_i$ recovery of large osteoclasts in the presence of Na$^+$. Complete inhibition of pH$_i$ recovery was seen with addition of both amiloride and bafilomycin A$_1$, suggesting that both the Na$^+$/$H^+$ exchanger and the V-ATPase contribute to pH$_i$ recovery in the presence of Na$^+$. Representative traces of three different large osteoclasts and their recovery in the presence of amiloride, bafilomycin A$_1$, or amiloride and bafilomycin A$_1$ are shown in figures 5B-D, respectively.

3.3.7 Effect of amiloride and bafilomycin A$_1$ on osteoclast pH$_i$ recovery after acid load

Since the results shown above strongly suggested that both the Na$^+$/$H^+$ exchanger and V-ATPase activity operated to different degrees in large and small osteoclasts at physiologic pH$_i$, we decided to investigate whether under more acidic conditions similar differences would be detectable. We used the ammonium pre-pulse technique to decrease pH$_i$. 
Small osteoclasts did not recover in the absence of Na\(^+\), but did recover in the presence of Na\(^+\) (figure 6, 7A). Recovery of pH\(_i\) in small osteoclasts in the presence of Na\(^+\) was completely inhibited by amiloride, indicating that the recovery was mediated by amiloride-sensitive Na\(^+\)/H\(^+\) exchange.

Interestingly, 60% of the 80 large cells studied behaved like small osteoclasts in that they did not recover from an acid load in the absence of Na\(^+\), although in contrast to small osteoclasts, the Na\(^+\)-dependent recovery of these large cells was not completely inhibited by amiloride (figure 6, 7B). These cells were categorized as large, Na\(^+\)-dependent osteoclasts. However, in the remaining 40% of large osteoclasts, pH\(_i\) increased at the same rate in the presence or absence of Na\(^+\) and was not inhibited by amiloride, suggesting that recovery here was not mediated by the Na\(^+\)/H\(^+\) exchanger (figure 6, 7C). These were categorized as large Na\(^+\)-independent osteoclasts.

We next evaluated the role of V-ATPase in the recovery from acid load in Na\(^+\)-containing medium for the three different categories of cells. In small cells bafilomycin A\(_1\) had no effect, suggesting that the proton pump was not responsible for the recovery in pH\(_i\) (figure 6). In the large, Na\(^+\)-dependent osteoclasts, addition of bafilomycin A\(_1\) alone reduced the recovery rate by approximately 25%, but this decrease was not significant with the number of osteoclasts measured (P<0.17, n=23). However, in these large cells, the addition of both amiloride and bafilomycin A\(_1\) resulted in complete inhibition of pH\(_i\) recovery. The degree of inhibition was significantly different from that by amiloride alone, suggesting that both the Na\(^+\)/H\(^+\) exchanger and the proton pump were active in these cells. In the large, Na\(^+\)-independent cells, pH\(_i\) recovery was inhibited by bafilomycin A\(_1\) and addition of amiloride did not enhance this effect, suggesting that the proton pump was solely responsible for the pH\(_i\) recovery in these cells.
Figure 1: Basal pH$_i$ of small (2-5 nuclei) and large (≥10 nuclei) osteoclasts. Osteoclasts were cultured on glass coverslips in Na$^+$-containing HEPES-buffered medium and pH$_i$ recorded. Two independent methods of pH$_i$ calibration, the nigericin technique and the null-point calibration method, gave almost identical results (A): large osteoclasts have a resting pH$_i$ of 0.4 pH units above that of small osteoclasts. Results represent mean ± SEM of 12 observations. (B) Representative trace of a small osteoclast and (C) a large osteoclast calibrated first with the null-point technique and then the nigericin technique (calibration not shown). The ordinate axis represents the results of the nigericin method and the horizontal bars represent the addition of the indicated null-point solutions.
Figure 2: Effect of amiloride and bafilomycin A₁ addition on osteoclast pHᵢ. (A) Amiloride (1 mM, an inhibitor of the Na⁺/H⁺ exchanger) had no significant effect on pHᵢ of small osteoclasts (n=11) or large osteoclasts (n=8) cultured in Na⁺ medium. There was no significant effect of bafilomycin A₁ (200 nM, an inhibitor of the V-ATPase) on pHᵢ of small osteoclasts cultured in Na⁺ medium (n=11), while the pHᵢ of large osteoclasts decreased significantly (n=9). The addition of both amiloride and bafilomycin A₁ together resulted in a significant decrease in pHᵢ of both small (n=11) and large (n=7) osteoclasts. Results represent mean ± SEM, *p<0.01, p<0.05. (B) Representative trace of a small osteoclast and (C) of a large osteoclast treated first with amiloride and then with bafilomycin.
Figure 3: Effect of DIDS on V-ATPase activity in small and large osteoclasts. (A) DIDS (10-100 μM, an inhibitor of Cl⁻ channels) significantly alkalinized both small (n=6) and large (n=8) osteoclasts to the same extent. Simultaneous addition of DIDS and bafilomycin A₁ (200 nM) resulted in a significant acidification of large osteoclasts (n=10), but no effect on the pH of small osteoclasts (n=8). Results represent mean ± SEM, *P<0.05 and **P<0.01 as compared to Na⁺ medium. (B) Representative trace of a small and (C) a large osteoclast treated first with DIDS and then with both DIDS and bafilomycin A₁.
Figure 4: Effect of removal of extracellular Na$^+$ on osteoclast pH$_i$. Osteoclasts cultured on glass coverslips in Na$^+$-containing medium were transferred to Na$^+$-free medium. (A) Small osteoclasts (n=20) had a very small drop in pH$_i$ while large osteoclasts (n=45) had a significant decrease in pH$_i$. Results represent mean ± SEM, * P<0.001. (B) Representative trace of pH$_i$ changes in a small osteoclast and (C) a large osteoclast after removal and re-addition of Na$^+$ medium. The pH$_i$ of large osteoclasts decreased immediately upon removal of Na$^+$ and remained low until Na$^+$ was re-applied, at which time recovery was very fast to basal levels.
Figure 5: Rate of pH\textsubscript{i} recovery of large osteoclasts after Na\textsuperscript{+} removal in the presence of various inhibitors of pH\textsubscript{i} regulatory mechanisms. (A) Under Na\textsuperscript{+}-free conditions, recovery was negligible (n=45). Addition of Na\textsuperscript{+} resulted in rapid recovery (n=27). The addition of 1 mM amiloride to Na\textsuperscript{+} medium had a slight inhibitory effect on pH\textsubscript{i} recovery (n=9) as compared to Na\textsuperscript{+} alone. Bafilomycin A\textsubscript{1} (200 nM) significantly decreased the recovery in Na\textsuperscript{+} medium (n=7) while the combination of bafilomycin A\textsubscript{1} and amiloride completely inhibited pH\textsubscript{i} recovery (n=7). Results represent mean ± SEM, "P<0.05 and *P<0.001 as compared to recovery in the presence of Na\textsuperscript{+}, "P<0.05 as compared to bafilomycin only. Representative traces of 3 different large osteoclasts recovering in the presence of (B) amiloride, (C) bafilomycin, or (D) amiloride and bafilomycin.
Figure 6: Rate of pH<sub>i</sub> recovery of large and small osteoclasts after acid loading. Small osteoclasts recovered very slowly in the absence of Na<sup>+</sup> (n=35) and very rapidly in the presence of Na<sup>+</sup>. Recovery in the presence of Na<sup>+</sup> was completely and reversibly inhibited by 1 mM amiloride (n=27), while 200 nM bafilomycin A<sub>1</sub> had no effect (n=11). Large osteoclasts were divided into Na<sup>+</sup>-dependent cells, where pH<sub>i</sub> recovery occurred only in the presence of Na<sup>+</sup> (n=49), and Na<sup>+</sup>-independent cells, where pH<sub>i</sub> recovery occurred regardless of the presence of Na<sup>+</sup> (n=31). The pH<sub>i</sub> recovery of Na<sup>+</sup>-dependent large osteoclasts was only partially sensitive to amiloride (n=20) or bafilomycin A<sub>1</sub> (n=23), but inhibited to a much greater extent in the presence of both inhibitors (n=13). The pH<sub>i</sub> recovery of Na<sup>+</sup>-independent cells was significantly inhibited by bafilomycin A<sub>1</sub> (n=15), but not by amiloride (n=17). The addition of both bafilomycin A<sub>1</sub> and amiloride did not enhance the inhibitory effect of bafilomycin A<sub>1</sub> alone (n=5). Results represent mean ± SEM, *P<0.01 and *P<0.001 as compared to recovery in the presence of Na<sup>+</sup>, #P<0.05 as compared to amiloride only.
Figure 7: Representative $pHi$ tracings of a small osteoclast, a Na$^+$-dependent large osteoclast and a Na$^+$-independent large osteoclast upon acid loading. NH$_4$Cl was added for 5-8 minutes to osteoclasts cultured on glass coverslips. The isosmotic replacement of NH$_4^+$ with NMG$^+$ or K$^+$ resulted in a rapid and immediate drop in pH$_i$ in all osteoclasts. (A) The pH$_i$ recovery of the small osteoclast was completely inhibited by 1 mM amiloride. (B) The pH$_i$ recovery of the Na$^+$-dependent large osteoclast was only partially inhibited by amiloride while that of the Na$^+$-independent large osteoclast (C) was not affected by the addition of this inhibitor.
3.4 DISCUSSION

The production and extrusion of protons and the maintenance of intracellular pH are essential processes in the function and activity of osteoclasts. It is widely accepted that V-ATPases found in the ruffled border of osteoclasts pump protons into the extracellular resorption zone, thus acidifying the bone surface and dissolving the mineral component of bone (Blair et al. 1989; Sundquist et al. 1990; Vaananen et al. 1990). Proton extrusion into the extracellular resorption zone is accompanied by passive Cl⁻ flux through a chloride channel. The driving force behind Cl⁻ movement is the potential difference that arises from electrogenic proton transport across the ruffled membrane during bone resorption (Blair and Schlesinger 1990; Schlesinger et al. 1997; Sims et al. 1991). With regard to maintenance of an appropriate pHᵢ, Teti et al. (Teti et al. 1989b) demonstrated that during bone resorption one of the mechanisms of alkaline export was a Na⁺-independent Cl⁻/HCO₃⁻ exchanger. Other reports indicate the presence and function of an amiloride-sensitive Na⁺/H⁺ exchanger (Gupta et al. 1996; Hall and Chambers 1990; Lehenkari et al. 1997; Nordstrom et al. 1995) and a proton conductance (Nordstrom et al. 1995). However, in many cases, differences in pHᵢ regulatory responses between osteoclasts of the same preparation have been reported, in particular with regard to Na⁺/H⁺ exchange and V-ATPase activities for which the investigators have had no explanation, or attributed them to differences in substrate, stage of resorption cycle or cell shape (Gupta et al. 1996; Lehenkari et al. 1997; Nordstrom et al. 1995; Ravesloot et al. 1995; Yu and Ferrier 1995; Zimolo et al. 1995).

The first striking finding in the present investigation was the existence of a significant difference (0.4 pH units) in steady-state pHᵢ of small and large osteoclasts maintained in HEPES-buffered Na⁺-containing medium at pH 7.4. The value obtained for small osteoclasts was in good agreement with that observed in a number of other cell types and with other
reports on osteoclasts cultured on glass in nominally bicarbonate-free, HEPES-buffered medium at pH 7.4 (Nordstrom et al. 1995; Nordstrom et al. 1997; Ravesloot et al. 1995; Teti et al. 1989b; Zimolo et al. 1995). However, large osteoclasts had an unusually high pH\(_i\) of 7.70. Since Boyarsky and colleagues (1996) recently reported that the nigericin technique to calibrate BCECF in vascular smooth muscle cells could lead to errors of up to 0.2 pH units in estimating steady-state pH, we verified our results using the null-point method. This method has been reported to be more reliable than the nigericin method in some instances because it is independent of K\(^+\) concentration, thus eliminating the error involved in estimating intracellular K\(^+\) (Babcock 1983; Boyarsky et al. 1996; Buckler et al. 1991; Eisner et al. 1989; Yamashiro and Maxfield 1987). The results of the two methods of calibration were in excellent agreement, indicating that the difference in pH\(_i\) between large and small osteoclasts is not an artifact of the recording system. Interestingly, Ravesloot et al. (1995) in their experiments with rat osteoclasts also found that a small percentage of the cells tested had high initial pH\(_i\) values, but did not attempt to associate this with a specific population of osteoclasts. Other reports on osteoclast pH\(_i\) regulation do not mention the size of osteoclasts studied, although Nordstrom et al. (1995) did specifically state that the majority of their studies were performed on compact osteoclasts containing 3-5 nuclei. The great abundance of small osteoclasts as compared to large ones (60% vs 10%) in rabbit, rat and chick osteoclast preparations cultured on glass coverslips (our results and (Asotra et al. 1994; Piper et al. 1992)) as well as the greater ease of performing single-cell experiments with cells of a smaller diameter would suggest that other investigators may also have concentrated mainly on smaller osteoclasts.

The cause of the high basal pH\(_i\) in large osteoclasts was further investigated. Firstly, we excluded the possibility that the high pH\(_i\) in large osteoclasts was a result of maintaining
the cells in HEPES-buffered medium. Buckler et al. (1991) had shown that in type I carotid body cells of neonatal rats the remarkably alkaline pH$_i$ (7.8) was due to the transfer of these cells from CO$_2$-containing to HEPES-buffered (CO$_2$-free) medium. They observed that, when measuring pH$_i$ within one hour of transfer, the pH$_i$ was 7.8, but if the cells were cultured in HEPES-buffered medium for 24 hours, the pH$_i$ was 7.3. However, this was not the case for osteoclasts in our study: large osteoclasts cultured for 24 hours in HEPES-containing medium had the same alkaline pH$_i$ as those cultured for 2 hours, while small osteoclasts had the same low pH$_i$ (7.3) at the two time-points. It is also relevant in this regard that Teti et al. (1989b) found that avian osteoclasts attached to bone and transferred to bicarbonate-free HEPES-containing medium became slightly acidified, not alkaline, within 2 minutes of transfer and then recovered to resting pH$_i$ within 20 minutes.

We next discovered that, while Na$^+$ was required for large osteoclasts to maintain their alkaline pH$_i$, the Na$^+$/H$^+$ exchanger was not the primary mechanism involved in this process as addition of amiloride did not lower the resting pH$_i$ of large cells. However, amiloride added after Na$^+$ removal slightly inhibited the recovery of these cells (25% inhibition) to their starting pH$_i$ upon re-addition of Na$^+$ to the extracellular medium, suggesting a small role for the Na$^+$/H$^+$ exchanger. Interestingly, the specific V-ATPase inhibitor bafilomycin A$_1$ inhibited the Na$^+$-dependent alkalinization by 70%, while the combination of bafilomycin A$_1$ and amiloride completely inhibited the recovery. The fact that V-ATPase activity was Na$^+$-dependent in these cells was surprising since V-ATPase activity is generally not linked to the presence of Na$^+$ in the extracellular medium. However, Mernissi et al. (1991) did report Na$^+$-dependent ATPase activity in the rat nephron which was clearly not a Na$^+$/K$^+$-ATPase, K$^+$-ATPase or Ca$^{2+}$-ATPase, but they did not look at pH regulation or bafilomycin-sensitivity to determine if it was a V-ATPase.
Another possible explanation for the apparent Na\(^+\)-dependence of the V-ATPase could involve Na\(^+\) loading of the cells. Normally Na\(^+\) is maintained at very low concentrations within the cytosol by the Na\(^+\)/K\(^+\)-ATPase, as K\(^+\) is pumped into the cell and Na\(^+\) is pumped out. However, the lack of K\(^+\) in the extracellular medium could have resulted in Na\(^+\) loading of the cells. The subsequent removal of Na\(^+\) from the extracellular bathing solution would have caused a reversal of the Na\(^+\)/H\(^+\) exchanger resulting in the outward movement of Na\(^+\) coupled to inward movement of protons (Moolenaar et al. 1983). This would then acidify the cells. If V-ATPase is also active in these cells then inhibition of V-ATPase by bafilomycin A\(_1\) upon re-addition of Na\(^+\) would maintain the low pH\(_i\) as seen in figure 5C. In order to test this possibility, K\(^+\) could also be added to the basal medium to ensure normal Na\(^+\) concentrations within the cell and V-ATPase activity could be re-examined.

Taken together, our findings suggest that both small and large osteoclasts cultured on glass contain active proton pumps, but that its role in pH\(_i\) regulation is much more important in large osteoclasts than in small osteoclasts. This was further supported by our observation that the addition of bafilomycin A\(_1\) resulted in a significant drop in basal pH\(_i\) in large but not small osteoclasts.

One possible explanation for the difference in V-ATPase activities between large and small osteoclasts could be the variation in number or activities of chloride channels in these cells. Since V-ATPase activity is often charge coupled to passive chloride permeability, a lack of anion conductance due to insufficient chloride channels may inhibit further V-ATPase activity due to the electrogenic nature of this pump (Blair et al. 1991; Sims et al. 1991). Thus lower V-ATPase activity in small osteoclasts could be due to fewer chloride channels in these cells as compared to large osteoclasts, and not directly as a result of fewer
or less active V-ATPases. Various reports have indeed demonstrated the presence of chloride channels in osteoclasts (Blair and Schlesinger 1990; Blair et al. 1991; Kelly et al. 1994; Schlesinger et al. 1997; Sims et al. 1991). Interestingly, Sims et al. (1991) and Kelly et al. (1994) reported that only 30-50% of their osteoclasts had a basal chloride current, however, they used very high concentrations of the chloride channel inhibitors DIDS and SITS (0.5-1 mM). Such high concentrations may have inhibited other ion currents as well, including V-ATPases (Blair et al. 1991). In order to study Cl− channel activity in our cells, we added 10-100 μM DIDS (shown by Blair et al. (1991) to reversibly inhibit Cl− channels in osteoclasts without affecting other proton transport mechanisms) in addition to 200 nM bafilomycin A₁. The pHᵢ of small osteoclasts did not change significantly while that of large osteoclasts did decrease, thus indicating that the differences in small and large osteoclast responses to bafilomycin A₁ were not caused by differences in Cl− channel activity. Surprisingly, DIDS addition alone resulted in a rise in pHᵢ in both large and small osteoclasts, likely due to inhibition of Cl−/HCO₃⁻ exchange activity in these cells. That this transporter continues to be active in our cells is possible since significant accumulation of HCO₃⁻ by carbonic anhydrase activity may continue in osteoclasts maintained in nominally bicarbonate-free medium as a result of CO₂ production in association with glycolysis and ATP generation in the mitochondria (Blair 1998). The fact that both large and small osteoclasts exhibited a similar increase is further evidence that anion translocation is not different in these cells, only V-ATPase activity.

To further study the characteristics of the pHᵢ regulatory mechanisms in large and small osteoclasts, we next examined the recovery of these cells from an acid load in the presence of various inhibitors. In agreement with previously reported data for osteoclasts (Nordstrom et al. 1995; Zimolo et al. 1995), the recovery of small osteoclasts from very low
pH; was Na\(^+\)-dependent, amiloride-sensitive and bafilomycin A\(_1\)-resistant. All of these data suggest a classical Na\(^+\)/H\(^+\) exchanger as the primary mechanism of re-alkalinization in these cells. The fact that this antiporter was very active in recovery from an acid load in small osteoclasts but was not very active at physiologic pH in these cells is consistent with other reports that Na\(^+\)/H\(^+\) exchangers only become active below a set-point pH of about 6.8 (Bidani \textit{et al.} 1994; Grinstein and Furuya 1986; Hays and Alpern 1990; Wakabayashi \textit{et al.} 1992).

The recovery from an acid load by large osteoclasts, however, was more complex. Surprisingly, 40\% of the large osteoclasts recovered at a very fast rate from an acid load in the absence of Na\(^+\) (Na\(^+\)-independent) while 60\% of the large osteoclasts recovered from an acid load in a Na\(^+\)-dependent manner (Na\(^+\)-dependent). The Na\(^+\)-independent cells did not respond to amiloride but were sensitive to inhibition by bafilomycin A\(_1\), indicating that V-ATPase was exclusively responsible for the re-alkalinization. Ravesloot \textit{et al.} (1995) also described a subpopulation of cells with active proton pumps in rat osteoclasts cultured on glass. Similar to our results, they found that these cells recovered from an acid load in the absence of Na\(^+\) and also had a higher mean initial pH\(_i\). They attempted to correlate presence of high initial pH\(_i\) and recovery from an acid load in the absence of Na\(^+\) with cell shape (round and well spread vs. polygonal with cytoplasmic extensions) but concluded that osteoclast shape was not sufficient to explain the differences. They did not report on the number of nuclei seen in their cells. We also examined the shape of our rabbit osteoclasts, and found that the great majority of our cells were round and did not display irregularly shaped cytoplasmic extensions as described by Ravesloot \textit{et al.} (1995). This may be due to the fact that rat osteoclasts take on a stellar appearance when transferred to serum-free medium (Fuller \textit{et al.} 1993), while similarly treated rabbit osteoclasts do not (Lees and Heersche, unpublished observations). Ingber \textit{et al.} (1990) and Schwartz \textit{et al.} (1991)
reported that spreading of bovine capillary endothelial cells and normal fibroblasts leads to rapid activation of the Na\(^+\)/H\(^+\) antiporter and elevation of pH\(_i\). They also found that fully spread cells had a higher pH\(_i\) than round cells and that local clustering of integrins led to activation of the Na\(^+\)/H\(^+\) antiporter and, hence, the rise in pH\(_i\). The evidence available for osteoclasts suggests that this explanation for the difference in pH\(_i\) of our cells is not likely as pH\(_i\) did not seem to correlate with the degree of spreading but rather with nuclear number.

In the Na\(^+\)-dependent category of large osteoclasts pH\(_i\) recovery was only partially inhibited by amiloride and bafilomycin A\(_1\), but when amiloride and bafilomycin A\(_1\) were added together, pH\(_i\) recovery was completely inhibited. The presence and activity of both a V-ATPase and Na\(^+\)/H\(^+\)-exchanger in the same cell has also been reported in other cell types i.e. alveolar macrophages, rabbit outer medullary collecting duct cells, rat papillary collecting duct cells and guinea pig pancreatic duct cells (Bidani et al. 1994; De Ondarza and Hootman 1997; Hays and Alpern 1990; Takeda et al. 1989). The findings of De Ondarza and Hootman (1997) in guinea pig pancreatic duct cells were similar to ours in that neither amiloride nor bafilomycin A\(_1\) alone significantly inhibited pH\(_i\) recovery from an acid load, while both inhibitors added together completely abolished this recovery.

Osteoclasts cultured on bone slices cycle between an actively resorbing phase and a migratory phase (Kanehisa and Heersche 1988). Lehenkari et al. (1997) reported that active, bone-resorbing osteoclasts regulate their pH\(_i\) differently from osteoclasts cultured on glass or non-resorbing cells cultured on bone. The active osteoclasts on bone had greater V-ATPase activity than Na\(^+\)/H\(^+\) exchange activity and had a slightly higher pH\(_i\) than inactive cells. It is tempting to hypothesize that in our experiments the category of large Na\(^+\)-independent cells that rely on V-ATPase activity exclusively for regulating their pH\(_i\) represent the actively resorbing population. If large resorbing osteoclasts remain activated when transferred into
our culture system, then this could account for our finding of two categories of large osteoclasts. This could imply that small osteoclasts and large Na\(^{+}\)-dependent osteoclasts are in an inactive or non-resorbing state. In the next chapter we use a system in which we can distinguish between actively resorbing and non-resorbing osteoclasts to test this hypothesis.

3.5 Statement of significance

This was the first demonstration of a clear difference in pH\(_i\) and its regulatory mechanisms between small and large osteoclasts. Large osteoclasts cultured on glass had a higher pH\(_i\) than their smaller counterparts and they regulated pH\(_i\) primarily by V-ATPase activity while small osteoclasts used primarily Na\(^{+}/H^{+}\) exchange activity. However, under acid loaded conditions, 60% of large osteoclasts exhibited Na\(^{+}\)-dependence and some Na\(^{+}/H^{+}\) exchange activity as well, thus suggesting that cell size alone was not sufficient to explain all the differences in pH regulatory mechanisms.
CHAPTER 4:

LARGE AND SMALL OSTEOCLASTS DIFFER IN RESORPTIVE ACTIVITY AND pH REGULATORY MECHANISMS

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Rita Lees wrote the manuscript and performed all of the experiments, except the quantification of area resorbed by individual osteoclasts and percentages of cells resorbing, which was done by Vageesh Sabharwal under the direct supervision of Rita Lees.
4.1 INTRODUCTION

Osteoclasts are multinucleated cells that resorb bone. They resorb bone by first attaching to the bone surface, then forming a sealing zone and ruffled border and then dissolving the bone matrix by extruding protons and secreting proteolytic enzymes into the extracellular resorption zone under the ruffled border of the cell (Mundy 1996; Vaananen 1996). Acidification of the extracellular resorption zone is accomplished by a vacuolar type V-ATPase localized on the ruffled border (Blair et al. 1989; Vaananen et al. 1990). Maintenance of pH_i, on the other hand, is believed to be controlled mainly by Na^+/H^+ exchangers and Cl^-/HCO_3^- exchangers on the basolateral membrane (Hall and Chambers 1990; Nordstrom et al. 1995; Teti et al. 1989b).

Under normal circumstances osteoclasts generally contain an average of 2-10 nuclei, but in diseases characterized by increased bone resorption, such as end stage renal disease, periodontal disease and rheumatoid arthritis, osteoclast size is generally increased, particularly in areas of increased bone resorption (Aota et al. 1996; Kaye et al. 1985; Makris and Saffar 1982; Shibutani et al. 1997). In diseases associated with excessive bone resorption such as Paget's disease osteoclasts have been reported to contain up to 100 nuclei (Singer and Roodman 1996).

Osteoclasts acquire more nuclei and become larger by fusing with other multinuclear osteoclasts or with mononuclear osteoclast precursors (Fischman and Hay 1962). Although multinuclearity of osteoclasts appears to enhance resorptive activity of these cells, the advantage of having a few larger osteoclasts over several smaller ones is not clear. Piper et al. (1992) studied the resorptive ability of individual osteoclasts and found a positive correlation between the size of an osteoclast and the volume of the resorption pit made. However, they also found that the volume resorbed per nucleus tended to decrease with
increasing nuclear number, thus suggesting that smaller osteoclasts are more efficient at resorbing bone than their larger counterparts. In Chapter 2, however, we found that when looking at populations of osteoclasts, large osteoclasts resorbed more bone per nucleus than small ones (Lees and Heersche 1999).

In Chapter 3 we demonstrated that osteoclasts of different size (as determined by the number of nuclei per cell) control their pH\textsubscript{i} using different pH\textsubscript{i} regulatory mechanisms (Lees and Heersche 2000): large osteoclasts (≥ 10 nuclei) had a significantly higher basal pH\textsubscript{i} than small osteoclasts (2-5 nuclei) and this high pH\textsubscript{i} was maintained by considerable V-ATPase activity in these large cells. Recovery from an acid load also proved different in these two types of cells as the pH\textsubscript{i} recovery of small osteoclasts was completely dependent on Na\textsuperscript{+}/H\textsuperscript{+} exchange activity, while in 40% of the large osteoclasts the pH\textsubscript{i} recovery was solely dependent on V-ATPase activity. In the remaining 60% of the large osteoclasts, pH\textsubscript{i} recovery was mainly dependent on Na\textsuperscript{+}/H\textsuperscript{+} exchange activity, although V-ATPase activity also played a minor role. Since we did not know the resorptive activity of these cells, we postulated that the Na\textsuperscript{+}-independent cells represented the resorptive population while the Na\textsuperscript{+}-dependent cells represented the non-resorptive population.

To determine whether osteoclasts in different phases of activity (i.e. resorbing and non-resorbing) regulate pH\textsubscript{i} differently, we examined in this study the proton translocating pathways in actively resorbing and non-resorbing osteoclasts as well as the resorptive efficiency of large (≥10 nuclei) and small (2-5 nuclei) osteoclasts. This approach became possible through the recent development of apatite collagen complexes by Doi \textit{et al.} (1996) and the modification of this process for covering glass coverslips with a thin layer of transparent collagen/hydroxyapatite (CHA) complex by Shibutani \textit{et al.} (2000). Osteoclasts cultured on this substratum resorb the CHA complex and can thus be identified as resorbing.
or non-resorbing. We found that actively resorbing osteoclasts, both small and large, have a significantly higher basal pHi than their non-resorbing counterparts and that these resorbing osteoclasts have greater V-ATPase activity than non-resorbing osteoclasts. The recovery from an acid load in large non-resorbing osteoclasts was Na⁺-dependent and regulated almost entirely by Na⁺/H⁺ exchangers while that of large resorbing osteoclasts was largely Na⁺-independent and regulated by V-ATPase activity. We could also confirm the observations of Piper et al. (1992) that large osteoclasts resorbed significantly more per cell than small osteoclasts, but that their resorptive activity as expressed per nucleus was similar. Interestingly, however, we discovered that a much greater percentage of large osteoclasts were actively resorbing as compared to small osteoclasts, thus explaining our previous observation in Chapter 2 (Lees and Heersche 1999) that, as a population, larger osteoclasts are more effective resorbers than small ones.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of collagen/hydroxyapatite-coated coverslips

Glass coverslips (round, 25 mm in diameter) were commercially coated with type I collagen (Matsunami Glass Company, Japan). A hydroxyapatite-type coating was precipitated on these coverslips by a modification of the method used by Doi et al. (1996) for the development of new bone substitutes (Shibutani et al. 2000). Briefly, the coverslips were incubated for 1 week at 37°C in 200 mM Tris(hydroxymethyl)aminomethane (TRIS) buffer (pH 8.5) containing 0.04% alkaline phosphatase, 0.04% phosvitin and 0.3% of the cross-linking reagent dimethyl suberimidate dihydrochloride. The coverslips were then gently washed in water, dried and placed in fresh 0.04% alkaline phosphatase and phosvitin solutions for 3 hours at room temperature. The coverslips were again washed and dried and
then placed in a 6 mM solution of calcium β-glycerophosphate for 20 hours at 37°C. The 3- and 20 hour incubations were repeated daily for 7-10 days.

4.2.2 Osteoclast isolation and cell culture

Osteoclasts were isolated from rabbit long bones as described previously in section 2.2.1. The osteoclast-containing cell suspension (100 μl per coverslip) was plated on the CHA-coated coverslips which were placed in 6-well tissue culture dishes. The cells were allowed to attach overnight in α-MEM containing 10% FCS and antibiotics in humidified air, 37°C and 5% CO₂. The cells were then cultured for 48 hours under the same conditions for determination of resorbed area or for 3-8 hours in 25 mM HEPES-buffered, bicarbonate-free RPMI with 10% serum at pH 7.4 and 37°C in an air incubator for measurement of pHᵢ.

4.2.3 Identification of resorbing and non-resorbing osteoclasts

Osteoclasts were identified as cells containing 2 or more nuclei (as seen by phase contrast microscopy) and classified either as small osteoclasts (those containing 2-5 nuclei) or large osteoclasts (those containing ≥10 nuclei). Those osteoclasts located on a resorption pit (as seen by phase contrast microscopy) were identified as resorbing osteoclasts while osteoclasts located on intact mineral and not associated with a pit were classified as non-resorbing osteoclasts. After completion of the experiments, cells were also stained for TRAP, a marker for osteoclasts, as described in section 2.2.3.

4.2.4 Intracellular pH measurements

Intracellular pH was determined using the microfluorometric technique described previously in section 3.2.2. Test solutions were applied in the same manner, except all
treatments were applied sequentially to the same cells in the experiments shown in figures 5 and 6.

4.2.5 Acid loading of osteoclasts

Osteoclasts were loaded with acid by the NH$_4^+$ ‘prepulse’ method as described previously in section 3.2.3.

4.2.6 pH; calibration

Calibration of the fluorescence ratio versus pH of each individual osteoclast was performed using the K$^+$/H$^+$ ionophore nigericin described previously in section 3.2.4.

4.2.7 Quantification of area resorbed by individual osteoclasts

The number of small and large, resorbing and non-resorbing osteoclasts were counted after TRAP staining. The plan area of each resorption pit associated with an osteoclast of known size was determined by tracing the area of the pit using the Zidas image analysis system from Zeiss. Results were pooled from a total of 10 coverslips.

4.2.8 Statistics

See section 2.2.4 for statistical analysis of data.

4.3 RESULTS

4.3.1 Osteoclast culture

Osteoclast-containing rabbit bone marrow suspensions were plated on CHA-coated coverslips and cultured for periods up to 48 hours. The population of cells cultured consisted
of osteoclasts of varying sizes (2-25 nuclei), stromal cells and monocyte-macrophage-like cells. Figure 1 shows a representative culture after staining for TRAP. Large and small resorbing and non-resorbing osteoclasts can be distinguished. Of the total osteoclast population, approximately 70% of the cells contain from 2-5 nuclei. These were classified as small osteoclasts for the purpose of this study. Of the population of cells with more than 5 nuclei, the category of cells classified as large osteoclasts (≥ 10 nuclei), comprised approximately 6% of the population.

4.3.2 Cytoplasmic pH

Osteoclasts were cultured for 24-48 hours in α-MEM containing 10% FCS on CHA-coated coverslips and then transferred to bicarbonate-free HEPES-buffered RPMI with 10% FCS. pHï measurements were conducted on cells maintained for 2-6 hours in HEPES-buffered media. Cells were loaded with BCECF for 10 min, at which time BCECF was uniformly distributed in the cytoplasmic compartment. Small non-resorbing osteoclasts maintained a steady state resting pHï of 7.31 ± 0.03, while small resorbing osteoclasts cultured under the same conditions had a significantly higher steady state resting pHï of 7.59 ± 0.02 (figure 2). Similarly, large resorbing osteoclasts had a significantly higher pHï of 7.60 ± 0.02 as compared to their non-resorbing counterparts (pHï of 7.46 ± 0.04).

4.3.3 Effect of bafilomycin A₁ on osteoclast resting pHï

Addition of bafilomycin A₁, a specific and widely-used inhibitor of V-ATPase activity, to osteoclasts maintained in control medium (Na⁺-containing), had no effect on the pHï of small non-resorbing osteoclasts (7.26 ± 0.04 vs 7.19 ± 0.06), while pHï of small resorbing osteoclasts decreased from 7.54 ± 0.05 to 7.29 ± 0.02 (figure 3). The effects of
bafilomycin A₁ addition were similar for large osteoclasts: the pHᵢ of large non-resorbing osteoclasts decreased slightly, but not significantly, from 7.46 ± 0.04 to 7.33 ± 0.06 while that of large resorbing osteoclasts dropped from 7.62 ± 0.04 to 7.35 ± 0.04.

4.3.4 The role of extracellular Na⁺ in maintaining pHᵢ

When Na⁺-containing medium was replaced by NMG⁺-containing medium, the pHᵢ of small non-resorbing osteoclasts did not change significantly (7.35 ± 0.04 vs 7.27 ± 0.04), while that of small resorbing osteoclasts decreased from 7.60 ± 0.04 to 7.33 ± 0.04 (figure 4). For both large non-resorbing and large resorbing osteoclasts, pHᵢ decreased by approximately 0.25 pH units upon removal of extracellular Na⁺. This drop in pHᵢ occurred rapidly (within 20 s) and was maintained until Na⁺ was added back to the external medium.

4.3.5 Effect of amiloride and bafilomycin A₁ on osteoclast pHᵢ recovery upon re-addition of Na⁺

Re-addition of Na⁺ after incubation in NMG⁺-containing Na⁺-free medium resulted in recovery of pHᵢ at a rate of 0.15-0.17 pH units/min in all small resorbing and large resorbing and non-resorbing osteoclasts (figure 5). Under Na⁺-free conditions, recovery was negligible in all cell categories, averaging less than 0.02 pH units/min. For small resorbing osteoclasts, addition of amiloride, an inhibitor of the Na⁺/H⁺ exchanger, resulted in a 30% inhibition of the Na⁺-induced pHᵢ recovery, while addition of the V-ATPase inhibitor, bafilomycin A₁, resulted in complete inhibition in the pHᵢ recovery. For large non-resorbing osteoclasts, addition of amiloride or bafilomycin A₁ alone did not result in significant inhibition of pHᵢ recovery with the number of osteoclasts evaluated, while addition of both inhibitors together completely inhibited recovery, suggesting that both the Na⁺/H⁺ exchanger and the V-ATPase
contribute to pH$_i$ recovery upon re-addition of Na$^+$. In large resorbìng osteoclasts, amiloride alone had no effect, while bafilomycin $A_1$ significantly inhibited the pH$_i$ recovery (by 53%). However, when both amiloride and bafilomycin $A_1$ were added together, pH$_i$ recovery was completely abolished, thus suggesting again that both the Na$^+/H^+$ exchanger and the V-ATPase play a role in regulating pH$_i$ recovery upon re-addition of Na$^+$. 

4.3.6 Effect of amiloride and bafilomycin $A_1$ on osteoclast pH$_i$ recovery after acid load

Since the results shown above strongly suggested that the Na$^+/H^+$ exchanger and V-ATPase activities operated to different extents in large and small resorbìng and non-resorbìng osteoclasts at physiologic pH$_i$, we decided to investigate whether under more acidic conditions similar differences would be detectable. We used the ammonium pre-pulse technique to decrease pH$_i$.

Non-resorbìng osteoclasts (both small and large) did not recover in the absence of Na$^+$, but did recover in the presence of Na$^+$ (figure 6, 7A). Recovery of pH$_i$ (in the presence of Na$^+$) was completely inhibited by amiloride, indicating that the recovery was mediated by amiloride-sensitive Na$^+/H^+$ exchange. In both cell types, bafilomycin $A_1$ inhibition was not significant. In small resorbìng osteoclasts, recovery was completely inhibited by both amiloride and bafilomycin $A_1$, indicating that both V-ATPase and Na$^+/H^+$ exchange involved in recovery from an acid load in these cells (figure 6). Compared to all other cells, large resorbìng cells in the absence of Na$^+$, recovered more rapidly from an acid load. Recovery after re-addition of Na$^+$ was completely inhibited by bafilomycin $A_1$ (figure 6, 7B). Na$^+/H^+$ exchange, however, also appears to play a role in these cells, although amiloride did not significantly inhibit recovery after re-addition of Na$^+$. 

Representative pH$_i$ tracings for a large non-resorbìng osteoclast and a large resorbìng
osteoclast are shown in figures 7A and B, respectively. Upon addition of NH$_4^+$-containing medium, the pH$_i$ rises immediately as a result of fast entry of NH$_3$ and then decreases progressively during the following 5-8 minutes as a result of slow NH$_4^+$ influx. When the cells are transferred to NH$_4^+$-free K$^+$- or NMG$^+$-containing medium, they acidify virtually instantaneously as a result of rapid efflux of NH$_3$.

4.3.7 Proportion of osteoclasts resorbing

Of the 700 small osteoclasts studied, only 39 (or 5.6%) were actively resorbing while of the 64 large osteoclasts studied, 25 (or 39.1%) were resorbing (figure 8A). Large osteoclasts resorbed a significantly greater total area per cell of 25.1 ± 3.5 x 10$^3$ μm$^2$ as compared to 10.3 ± 1.7 x 10$^3$ μm$^2$ resorbed per small osteoclast (figure 8B). There was no significant difference in the area resorbed per nucleus between large and small osteoclasts, as small osteoclasts resorbed a total of 2.4 ± 0.3 x 10$^3$ μm$^2$/nucleus, whereas large osteoclasts resorbed 2.0 ± 0.3 x 10$^3$ μm$^2$/nucleus.
Figure 1: Identification of resorbing and non-resorbing osteoclasts. Osteoclasts were cultured on CHA-coated glass coverslips, stained for TRAP and identified as resorbing (arrowhead) or non-resorbing (arrow).
Figure 2: Basal pH\textsubscript{i} of small (2-5 nuclei) and large (≥10 nuclei), resorbing and non-resorbing osteoclasts. Osteoclasts were cultured on CHA-coated glass coverslips in Na\textsuperscript{+}-containing HEPES-buffered medium and pH\textsubscript{i} recorded. Both small (n=23) and large (n=28) resorbing osteoclasts had significantly higher basal pH\textsubscript{i} values than their non-resorbing counterparts (n=23 and n=22, for small and large respectively). Results represent mean ± SEM, *P<0.01, *P<0.001.
Figure 3: Effect of bafilomycin A₁ addition on osteoclast pHᵢ. Bafilomycin A₁ (200 nM) had no significant effect on the pHᵢ of small (n=12) or large (n=8) non-resorbing osteoclasts cultured in Na⁺ medium. However, the pHᵢ of both small (n=8) and large (n=6) resorbing osteoclasts was significantly decreased in the presence of this V-ATPase inhibitor. Results represent mean ± SEM, *P<0.001.
Figure 4: Effect of removal of extracellular Na⁺ on osteoclast pHᵢ. Osteoclasts cultured on CHA-coated glass coverslips in Na⁺-containing medium were transferred to Na⁺-free medium and pHᵢ recorded. Small non-resorbing osteoclasts (n=12) had a very small drop in pHᵢ while their resorbing counterparts (n=10) had a significant decrease in pHᵢ. Both large non-resorbing (n=9) and large resorbing (n=13) osteoclasts had a significant decrease in pHᵢ upon Na⁺-removal. Results represent mean ± SEM, *P<0.05, **P<0.001.
Figure 5: Rate of pH\textsubscript{i} recovery of small resorbing and large resorbing and non-resorbing osteoclasts after Na\textsuperscript{+} removal in the presence of various inhibitors of pH\textsubscript{i} regulatory mechanisms. Under Na\textsuperscript{+}-free conditions, recovery was negligible in all cell categories (n=10, 9 and 13, respectively) while addition of Na\textsuperscript{+} resulted in rapid recovery in these same cells (n=8, 6 and 12, respectively). The addition of 1 mM amiloride (an inhibitor of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger) to Na\textsuperscript{+} medium had a significant although not complete inhibitory effect on pH\textsubscript{i} recovery in small resorbing osteoclasts (n=8) while the addition of bafilomycin A\textsubscript{1} (an inhibitor of V-ATPase, 200 nM) completely inhibited the recovery in these same cells (n=8). Although neither inhibitor alone significantly affected the pH\textsubscript{i} recovery of large non-resorbing osteoclasts (n=6 amiloride, n=7 bafilomycin A\textsubscript{1}), the addition of both inhibitors together completely inhibited the recovery in these cells (n=5). The recovery of large resorbing osteoclasts was not affected by amiloride addition (n=11), although bafilomycin A\textsubscript{1} addition resulted in significant inhibition (n=9) and a combination of amiloride and bafilomycin A\textsubscript{1} (n=7) resulted in complete inhibition of recovery in these cells. Results represent mean ± SEM, "P<0.05, *P<0.01, **P<0.001.
Figure 6: Rate of pH recovery of small and large resorbing and non-resorbing osteoclasts after acid loading. Small non-resorbing osteoclasts (n=11) recovered very slowly in the absence of Na\textsuperscript{+} and very rapidly in the presence of Na\textsuperscript{+}. Recovery in the presence of Na\textsuperscript{+} was completely and reversibly inhibited by 1 mM amiloride, while 200 nM bafilomycin A\textsubscript{1} had no significant effect. The recovery of small resorbing osteoclasts (n=9) was similar to that of their non-resorbing counterparts, except their recovery was also inhibited by bafilomycin A\textsubscript{1}. Large non-resorbing osteoclasts (n=7) recovered from an acid load in the same manner as small non-resorbing osteoclasts while the recovery of large resorbing (n=12) osteoclasts was completely inhibited by bafilomycin A\textsubscript{1}, but not by amiloride. Results represent mean ± SEM, *P<0.05, #P<0.01, *P<0.001.
Figure 7: Representative pH$_i$ tracings of a large non-resorbing and a large resorbing osteoclast upon acid loading. NH$_4$Cl was added for 5-8 minutes to osteoclasts cultured on CHA-coated glass coverslips. The isosmotic replacement of NH$_4^+$ with NMG$^+$ or K$^+$ resulted in a rapid and immediate drop in pH$_i$ in both the large non-resorbing and resorbing osteoclasts. While the pH$_i$ recovery of the large non-resorbing osteoclast was completely (and reversibly) inhibited by 1 mM amiloride (A), that of the large resorbing osteoclast was not affected by the addition of this Na$^+/H^+$ exchange inhibitor (B). Bafilomycin A$_1$ (200 nM), in contrast, had no effect on the pH$_i$ recovery of the large non-resorbing osteoclast but did completely inhibit recovery of the large resorbing osteoclast.
Figure 8: Resorptive capabilities of small and large osteoclasts. Osteoclasts were cultured on CHA-coated glass coverslips in α-MEM, 10% FBS and antibiotics for 48 hours and proportion of resorbing cells as well as plan area of resorption pit associated with each individual osteoclast of known nuclear number determined. The percentage of large osteoclasts resorbing was 8-fold greater than that of small osteoclasts (A). The resorption per cell of large osteoclasts (n=25) was significantly greater than that of small osteoclasts (n=39) while resorption per nucleus did not differ between the two size categories of osteoclasts (B). Results represent mean ± SEM, *P<0.001
4.4 DISCUSSION

Direct measurement of the activity of proton transporters in actively resorbing osteoclasts in vitro using fluorometric techniques has been very difficult due to the limited light-permeability of the bone substratum (bone slices) on which the osteoclasts have to be cultured for such assays. Direct measurements of these parameters in vivo are obviously not possible. However, several substrata mimicking bony substrata that can be resorbed by osteoclasts have been developed recently, mostly for use in surgical repair of osseous lesions. The most promising in this regard appear to be composites of hydroxyapatite and collagen (Iwano et al. 1988), in particular a recently developed apatite collagen complex (Doi et al. 1996). Using the same methodology as described by Doi et al. (1996), Shibutani and colleagues have recently succeeded in manufacturing glass microscope coverslips covered with a thin layer of transparent CHA complex (Shibutani et al. 2000) and demonstrated that isolated rabbit osteoclasts resorb such complexes in a pattern very similar to that observed for osteoclasts on bone slices. This development created the possibility of recording osteoclasts while resorbing or migrating over these surfaces using phase-contrast microscopy, and subsequently recording the activity of the proton transport systems in these same osteoclasts. Of particular advantage is the fact that this CHA coating is transparent enough to allow simultaneous determination of nuclear number and thus the size of the osteoclast.

Using this substrate and distinguishing resorbing from non-resorbing osteoclasts we found that all resorbing osteoclasts (both small and large) had significantly higher basal $pH_i$ than their non-resorbing counterparts. The high $pH_i$ of resorbing osteoclasts decreased upon addition of the V-ATPase inhibitor bafilomycin A1, while addition of bafilomycin A1 had no significant effect on the $pH_i$ of non-resorbing osteoclasts. This suggests that the high basal $pH_i$ of resorbing osteoclasts is due, at least in part, to V-ATPase activity. Lehenkari et al.
observed that bafilomycin A₁ addition to osteoclast populations cultured on very thin bone slices resulted in a drop in pHᵢ in most, but not all osteoclasts. Our results suggest that their non-responding cells may have been non-resorbing osteoclasts.

When we removed extracellular Na⁺ from the medium, the pHᵢ of all resorbing osteoclasts (small and large) and of large non-resorbing osteoclasts decreased significantly indicating a dependence on Na⁺ for the maintenance of pHᵢ. Only the small non-resorbing osteoclasts did not respond to Na⁺ removal with a significant decrease in pHᵢ, possibly related to the fact that their pHᵢ was already at a lower level. Re-addition of Na⁺ resulted in a rapid recovery of pHᵢ in all three responding cell types (0.15-0.17 pH units/min). Interestingly, in all resorbing osteoclasts (small and large) V-ATPase activity was responsible for most of the recovery. In small resorbing osteoclasts there was also significant Na⁺/H⁺ exchange activity. Interestingly, in both categories of large osteoclasts neither amiloride nor bafilomycin A₁ alone completely abolished pHᵢ recovery, while addition of both inhibitors resulted in complete inhibition of pHᵢ recovery. This synergistic inhibitory effect has been shown previously in rat papillary collecting duct cells (Takeda et al. 1989) and in guinea pig pancreatic duct epithelial cells (De Ondarza and Hootman 1997). De Ondarza and Hootman (1997) suggested that one pHᵢ regulatory mechanism may augment the activity of the other in the face of a large H⁺ gradient. We interpret our results as indicating that the extent of V-ATPase and Na⁺/H⁺ exchange participation differs between the cell types: in small and large resorbing osteoclasts, V-ATPase is primarily responsible for pHᵢ regulation, while in large non-resorbing osteoclasts, both V-ATPase and the Na⁺/H⁺ exchanger contribute equally.

In order to further study differences in pHᵢ regulatory mechanisms in the various categories of osteoclasts after more extreme changes in pHᵢ, we acid loaded the cells and
observed the recovery of pH\(_i\) in the presence of V-ATPase and Na\(^+\)/H\(^+\) exchange inhibitors. Under these conditions, Na\(^+\)/H\(^+\) exchange again predominated in non-resorbing osteoclasts. In contrast, in large resorbing osteoclasts V-ATPase activity was greater than Na\(^+\)/H\(^+\) exchange activity, while in small resorbing osteoclasts equivalent V-ATPase and Na\(^+\)/H\(^+\) exchange activities were observed. Thus small resorbing osteoclasts, while exhibiting the strong bafilomycin A\(_1\) sensitivity of large resorbing osteoclasts, also maintain the amiloride sensitivity of their non-resorbing counterparts. These results may explain why Lehenkari et al. (1997) found, using a mixture of small and large resorbing and non-resorbing osteoclasts that osteoclast recovery from an acid load was most sensitive to bafilomycin A\(_1\) inhibition, but also inhibited by amiloride. Zimolo et al. (1995), on the other hand, specifically looked at the largest osteoclasts they could find which were mainly on pits and found Na\(^+\)-independent recovery from an acid load (similar to our large resorbing osteoclasts). However, although a bafilomycin A\(_1\) sensitive V-ATPase was present in their cells, it did not appear to mediate most of the Na\(^+\)-independent acid extrusion, suggesting the existence of an additional and as of yet uncharacterized H\(^+\) extruder.

Comparing the results presented here with those obtained with osteoclasts cultured on glass in Chapter 3 (Lees and Heersche 2000), it became apparent that recovery from an acid load in large resorbing and non-resorbing osteoclasts was clearly comparable to recovery of large Na\(^+\)-independent and Na\(^+\)-dependent osteoclasts, respectively, cultured on glass coverslips. This strongly supports our hypothesis that the Na\(^+\)-independent cells represented cells in the resorbing state while the Na\(^+\)-dependent cells represented non-resorbing cells. Moreover, the observation that 40% of all large osteoclasts are resorbing agrees with the observation in our previous investigation that 40% of the cells were Na\(^+\)-dependent. This, however, also raises the question of why in cultures on glass we only observed small
osteoclasts sensitive to amiloride inhibition and unaffected by bafilomycin A₁ (i.e. the non-resorbing phenotype in this study), and not those affected by both amiloride and bafilomycin (i.e. the resorbing phenotype in this study). We think the explanation is related to the fact that 95% of small osteoclasts were not resorbing, while only 5% were resorbing. Thus only one in twenty of the small osteoclasts cultured on glass would likely be in the "resorbing phenotype" category. Thus, it is next to impossible to detect this phenotype in culture on glass.

Although large osteoclasts resorbed considerably more per cell than small osteoclasts, resorptive efficiency (expressed as resorption per nucleus) of individual small and large osteoclasts was the same. Both cell types, when resorbing, also had similar high pH₁ and V-ATPase activity. Small resorbing osteoclasts only differed from the large resorbing cells by their greater Na⁺/H⁺ exchange activity, a process not believed to be as important in osteoclastic resorption of bone as in initial spreading and attachment to bone (Hall et al. 1992). Calculations in Chapter 2 indicated that resorptive efficiency of large osteoclasts was greater than that of small osteoclasts when calculated over the total populations of large and small cells present. This calculation underestimates the resorption per nucleus of resorbing small cells due to the large proportion of the small cells that are not actively resorbing (only 5% of small cells are resorbing). Our present results confirm that, as a population, large osteoclasts resorb more actively than small osteoclasts. The fact that a greater percentage of large osteoclasts than of small osteoclasts is actively resorbing is likely an important contributing factor to the increased resorption seen in diseases where osteoclast size is greatly increased. To discover why the percentage of resorbing large cells is greater than that of small cells under the same culture conditions is a challenge for future investigation.

In summary, our results show that pH₁ regulation is associated with enhanced proton
pump activity in actively resorbing osteoclasts, while Na\(^+/H^+\) exchange activity is the primary mechanism of pH\(_i\) regulation in non-resorbing osteoclasts. We also found that 40% of large osteoclasts are resorbing, while only 5% of the small osteoclasts are active in resorption. That different proportions of large and small osteoclasts are resorbing, despite being exposed to the same culture environment may indicate that differences exist between mechanisms responsible for activation of these cells. This indicates that selective pharmacological inhibition of the most active osteoclasts is a potentially useful approach to inhibit pathological bone resorption.

4.5 **Statement of significance**

The significance of this study is that it explains the heterogeneity of large osteoclasts found in Chapter 3 to be a function of state of activity. In addition it was apparent that while some differences in pH regulatory mechanisms still existed between small and large osteoclasts, V-ATPase activity was similar in all resorbing osteoclasts, suggesting a need to re-examine resorptive efficiency on a per cell basis. While there was no difference in resorption per nucleus of small and large osteoclasts when examined individually, we showed for the first time that a much larger percentage of large osteoclasts were resorbing compared to small osteoclasts, explaining the different activities of populations of large and small osteoclasts found in Chapter 2. Also the fact that only 5% of small osteoclasts were actively resorbing versus 40% of the large ones suggests different activation mechanisms in small and large osteoclasts. This could be of crucial importance in selectively inhibiting the most active osteoclasts.
CHAPTER 5: SUMMARY AND GENERAL DISCUSSION

Clearly in some disease states the total number of osteoclasts increases and as a result there is more overall resorption. However, increased bone resorption in several bone-related diseases, particularly those associated with local increases in resorption such as arthritis, Paget’s disease and periodontal disease, is also frequently associated with an increase in the size of osteoclasts. This suggests that an increase in osteoclast size (number of nuclei per cell) may be related to increased resorptive activity. Because inflammatory processes are often associated with excessive bone loss, we chose to study the effects of MCSF on osteoclast activity, as it is an inflammatory cytokine present in large quantities in inflammatory diseases with associated bone loss. Interestingly, we found that addition of MCSF only stimulated resorption when there was a concomitant increase in size of the osteoclasts, with no change in osteoclast number (Chapter 2). Short term administration of MCSF resulted in no change in resorptive activity or size of osteoclasts while long term culture with MCSF increased both size of osteoclasts and amount resorbed. Calculation of resorptive efficiency of populations of large and small osteoclasts also indicated that populations of large osteoclasts resorbed more per nucleus than did populations of small osteoclasts.

Since resorptive efficiency differed between populations of large and small osteoclasts, we hypothesized that this might be a result of some inherent differences in the “resorptive machinery” of these cells (Chapter 3). We chose to study pH regulatory mechanisms, in particular those involving proton extrusion (V-ATPase and Na+/H+ exchanger), because of the pivotal role of proton extrusion and cytoplasmic pH regulation in the resorptive process. When cultured on glass, large osteoclasts had a significantly higher basal pH than small osteoclasts and this high pH was maintained by V-ATPase activity in
these cells. The differences between large and small osteoclast pH$_i$ regulation were confirmed in acid-loaded cells, where all small osteoclasts recovered their pH$_i$ almost entirely by Na$^+$/H$^+$ exchange activity while in 40% of large osteoclasts only V-ATPase activity was responsible for pH$_i$ regulation. However, in the remaining 60% of large osteoclasts both V-ATPase and Na$^+$/H$^+$ exchange activities were involved in pH$_i$ regulation, suggesting that size alone could not explain differences between pH$_i$ regulatory mechanisms in these cells. As a result, we hypothesized that large osteoclasts with V-ATPase activity only may be those expressing the resorptive phenotype, even though they were cultured on glass.

In order to determine whether resorbing osteoclasts regulate pH$_i$ mainly by V-ATPase activity and whether there are differences in resorptive machinery between small and large osteoclasts associated with differences in their resorptive efficiencies, we examined pH$_i$ regulatory mechanisms in small and large resorbing and non-resorbing osteoclasts (Chapter 4). This study was made possible by the availability of collagen and hydroxyapatite coated coverslips which were sufficiently light-permeable for pH studies and upon which resorbing and non-resorbing osteoclasts could be distinguished. All resorbing osteoclasts had a high basal pH$_i$ which was maintained by high V-ATPase activity. The two populations of large osteoclasts which recovered from an acid load differently on glass were very similar in their pH$_i$ regulatory mechanisms to large resorbing and non-resorbing osteoclasts, respectively, suggesting that phase of activity correlated with the differences observed in these cells. Interestingly, all non-resorbing osteoclasts (both small and large) regulated pH$_i$ by Na$^+$/H$^+$ exchange activity while all resorbing osteoclasts utilized V-ATPase (except that small resorbing osteoclasts also had Na$^+$/H$^+$ exchange activity), suggesting no inherent differences in resorptive efficiency of small and large resorbing osteoclasts. As a result we re-examined the issue of resorptive efficiency on an individual cell basis and found that while large
osteoclasts do resorb considerably more per cell than small osteoclasts, there was no
difference in resorption per nucleus of these two groups of cells. However, the percentage of
large osteoclasts that were resorbing was 8-fold higher than the percentage of small resorbing
osteoclasts, suggesting that activation mechanisms of these cells are very different. As a
result, the calculation of resorptive efficiency in Chapter 2 underestimated resorptive activity
per cell of small osteoclasts as many of the cells were not in a resorptive state.

The central hypothesis of this research project was that osteoclast size (as defined by
the number of nuclei per cell) is a major factor in determining the efficiency with which
osteoclasts can resorb bone and that resorptive efficiency is related to the activity of the
pathways in osteoclasts involved in proton transport. With regard to the first part of the
hypothesis, we have shown that actively resorbing large osteoclasts do not resorb more per
nucleus on an individual cell basis as compared to actively resorbing small osteoclasts.
However, as a population, large osteoclasts clearly are more effective resorbers than small
osteoclasts because the proportion of large osteoclasts that are resorbing is much greater than
the proportion of small osteoclasts that are resorbing. Thus, many small osteoclasts are not
as effective as a smaller number of large osteoclasts with the same total number of nuclei as
present in small osteoclasts. This explains why in disease states where there are more large
osteoclasts, there is also much more bone resorbed. This finding demonstrates the need to
look at more than just one parameter when considering resorptive efficiency of osteoclasts.

Piper et al. (1992) investigated only resorption per nucleus of individual osteoclasts and as a
result came to the conclusion that the larger the cell, the less they resorb, which was contrary
to the observed situation in certain pathological conditions of bone loss. Here we show that
while individual osteoclasts may have similar resorptive efficiencies (on a per nucleus basis),
it is the population as a whole which is important in determining total resorption.
With regard to the second part of the hypothesis, further studies into the mechanisms of proton transport and pH$_i$ regulation as related to resorptive efficiency showed that cells with similar resorptive efficiencies also regulated pH$_i$ using essentially the same mechanisms. In particular, we demonstrated that all resorbing osteoclasts used V-ATPase to regulate pH$_i$ while non-resorbing osteoclasts used the Na$^+$/H$^+$ exchanger almost exclusively, thus providing an additional indicator of state of activity (i.e. resorbing vs. non-resorbing) of osteoclasts. However, since a population of large osteoclasts resorbs more overall (on a per nucleus basis) than a population of small osteoclasts, but both populations use the same proton transport pathway, additional factors must differ between these cells.

We suggest that one of these differences may be differential activation of large and small osteoclasts. Although exposed to the same culture environment, a greater percentage of large osteoclasts were resorbing as compared to small osteoclasts, suggesting that large osteoclasts resorb more as a population because a greater proportion of them are "turned on" to resorb. Whether this is due to differences in receptor numbers for certain activating ligands, such as MCSF or OPGL remains to be determined. Preliminary results in our laboratory show that resorptive activity of large osteoclasts (as measured by the percentage of cells resorbing) may be more sensitive to inhibition by calcitonin than that of small osteoclasts. No difference was seen in a preliminary experiment with MCSF addition, although this may have been due to an unusually high percentage of cells resorbing in the control in this particular experiment. Future studies will focus on quantifying differences in receptor levels of activating ligands in small and large osteoclasts by in situ hybridization and immunofluorescence studies to determine whether this could account for the differential activation of large and small osteoclasts.

A limitation of this study involves the use of only one inhibitor for each of the pH$_i$
regulatory mechanisms studied. Although bafilomycin A₁ and amiloride are specific and classical inhibitors of V-ATPase and Na⁺/H⁺ exchange, respectively, and have been used exclusively in the literature in pH studies to identify these pH regulatory mechanisms, other inhibitors could also be used to verify these results. Extensive modifications of the structure of bafilomycin A₁ did not identify any new analogue more potent or significantly more selective for the osteoclast V-ATPase (Gagliardi et al. 1998). However, the structurally related macrolide antibiotic concanamycin A was more stable and showed greater potency and specificity for the V-ATPase as compared to bafilomycin A₁ when tested in bacteria (Drose et al. 1993). Amiloride analogues such as 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and 5-(N-methyl-N-isobutyl)amiloride (MIBA) were more potent than amiloride (Vigne et al. 1984) and gave a more complete suppression of Na⁺/H⁺ exchange activity than amiloride (Maidorn et al. 1993), however, a recent report demonstrated that these analogues were not specific for the Na⁺/H⁺ exchanger as they also inhibited the Na⁺/Ca²⁺ exchanger and the Ca²⁺ pump with the same potency as that for the Na⁺/H⁺ exchanger in cardiac myocytes (Murata et al. 1995). In addition, electrophysiological studies involving patch clamping would also ensure that other ion currents are not involved. Although Cl⁻ channels were ruled out by our experiments using DIDS, a more potent Cl⁻ channel inhibitor such as 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) could also be used to verify these results, however, as with all Cl⁻ channel inhibitors discovered to date, NPPB does not act on these channels alone as it also has non-specific effects on energy metabolism and functions as an effective protonophore (Keeling et al. 1991; Lukacs et al. 1991). The presence of protein and mRNA for V-ATPase and Na⁺/H⁺ exchanger in the various cells could also be studied by immunohistochemistry and single cell RT-PCR, respectively, to determine whether the different activities of these pH regulatory mechanisms correlate with mRNA and protein
levels. However, since V-ATPase activity is primarily a function of assembly and/or targeting to the membrane rather than mRNA and protein levels (Brown et al. 1992; Kane et al. 1992), the information obtained by evaluating these parameters is of limited importance (Bastani et al. 1991).

Another important issue is the question of the origin and history of the individual nuclei in small and large osteoclasts. Osteoclast lifespan may be longer than that of its nuclei as 1 nucleus can reside within an osteoclast for about 11.5 days (nuclear turnover averages 8% per day) (Jaworski et al. 1981), while estimates of osteoclast lifespan range from 1-4 weeks (Anderson et al. 1979; Gungor et al. 1982; Marks and Seifert 1985). As a result, it should be noted that size may not necessarily relate to age or stage of differentiation of the osteoclast, as we do not know the history of the individual cell or its nuclei. Time-lapse microcinematography studies have shown that in vitro, multinuclear osteoclasts fuse with each other as well as with mononuclear cells to form larger osteoclasts (Marshall et al. 1986; Zambonin Zallone et al. 1984; Kaneshisa and Heersche, unpublished). This process is observed frequently. Conversely, the splitting up of large osteoclasts in culture to form two smaller osteoclasts has also been observed, although much less frequently (Addison 1979; Kaneshisa and Heersche, unpublished).

It is also possible that differences in pH regulatory mechanisms between Na\(^{+}\)-dependent and Na\(^{+}\)-independent large osteoclasts seen in Chapter 3 are due to differences in whether they were formed from fusions of several mononuclear or fewer multinuclear cells. Similarly, large non-resorbing osteoclasts in Chapter 4 could be those generated from fusions of small osteoclasts while the large resorbing osteoclasts could be those resulting from fusion of larger cells. In addition, small resorbing osteoclasts could be cells which have split from larger osteoclasts and as such are capable of resorption, whereas the non-resorbing ones
could be cells that are at an early stage of differentiation and have just formed by fusion of mononuclear precursors.

To study these possibilities, the fate of individual nuclei and the cells which contain them must be determined. One possible approach to do this would be to use a technique similar to that used by Solari et al. (1995), who studied mononuclear cell generation from multinucleated giant cells. One osteoclast per CHA-coated coverslip would be microinjected with an impermeable fluorescent probe (such as Lucifer Yellow) which does not freely cross cell membranes. Its localization in only one cell can be determined immediately after injection and then the fate of this cell observed at various time intervals over a period of at least 24 hours. If the cell divides or joins with another cell, distinct and separate fluorescent areas will be observed which can be correlated with individual cells as seen under phase-contrast microscopy. Alternatively, the fate of individual nuclei could be determined by injecting a viable chromatin dye such as Hoechst 333342 into a cell and following those nuclei over time. Once the histories of the cells and/or nuclei are known, then amount resorbed and pH measurements can also be performed on these cells to determine whether cell fusion or splitting has any effect on these parameters.

In addition to the potential importance of the history of individual osteoclasts and their nuclei is the question of age and differentiation of these cells. If a differentiation pathway exists once osteoclasts are formed, then their characteristics may change along this pathway and as a result younger osteoclasts may differ from older ones. Although no such pathway has been shown to exist and osteoclasts are categorized as either mononuclear pre-osteoclasts or fully differentiated multinuclear osteoclasts (Suda et al. 1996), the question of age could be addressed by artificially inhibiting cell proliferation to stop the formation of new precursors and then examine the remaining osteoclasts over time without fear of
contamination by younger cells. This could be done by adding 1 mM hydroxyurea to the cultures (Lorenzo et al. 1983) or 3 μg/ml cytosine D-arabinofuranoside (Teti et al. 1988; Woods et al. 1995) and then examining resorption and measuring pH over several days. Tritiated thymidine or 5-bromodeoxyuridine can be added to detect any residual cells undergoing DNA synthesis to ensure complete block of proliferation (Gratzner 1982; Marshall and Davie 1991; Woods et al. 1995). Pitfalls of this proposed study include potential inhibition of RNA and protein synthesis by hydroxyurea (Yarbro 1968) and other unknown effects these compounds may have on osteoclast activity. In addition, although the recruitment of new cells is inhibited, the implied assumption with this approach is that all the remaining osteoclasts are of relatively similar age and level of differentiation, which may not be entirely true.

Another possible explanation for the differences seen between certain osteoclasts with regard to pH regulatory mechanisms and resorptive activities, which is not dependent on the size or phase of activity of osteoclasts, could be due to dedifferentiation of some of these cells. Dedifferentiation refers to the loss of differentiated characteristics which results in loss of morphologic or functional properties of the cell (Reischl et al. 1999). This leads to the cell adopting a less mature phenotype and often results in the cell re-acquiring proliferative capability which is once again followed by a new round of differentiation (Kitamura et al. 1986; Bar-Shavit et al. 1986). Thus dedifferentiation of small osteoclasts and/or Na⁺-dependent large osteoclasts could result in a loss of V-ATPase activity and resorptive potential in these cells which is completely independent of size of the cells. Several investigators have shown that dedifferentiation does occur in many different cell types, including rat adrenal glomerulosa cells (Engeland and Levay-Young 1999), mouse mast cells (Kitamura et al. 1986), rabbit cortical collecting tubule cells (Jamous et al. 1993), and bovine
oviduct epithelial cells (Reischl et al. 1999). Common to all of these was the resultant proliferation of the dedifferentiated cells to produce new cells and/or to regenerate the organs. Of particular interest was the study by Kintner and Brockes (1984) who found that multinucleated myofibrils dedifferentiate to mononucleated blastemal cells in newt limb regeneration. However, when Fischman and Hay (1962) and Washabaugh and Tsonis (1994) investigated the possible role of osteoclasts in the regeneration of newt limbs in vivo and in vitro, respectively, they clearly showed that osteoclasts do not dedifferentiate from stump tissues but rather form by the fusion of hemopoietic cells. In addition, Bar-Shavit et al. (1986) showed that mononuclear human leukemia cell line HL-60 cells dedifferentiate to a less mature phenotype upon removal of 1,25-dihydroxyvitamin D$_3$ from culture (as shown by a loss of 63D3 antigen, increased c-myc mRNA, increased cell volume and resumption of proliferation) whereas the osteoclast-like multinucleated giant cells also formed in these cultures did not dedifferentiate, as they remained viable, remained 63D3 antigen positive and did not proliferate. They hypothesized that the multinucleated cells stabilize their phenotype by achieving developmental commitment even before cell fusion. Thus, since all of the osteoclasts we studied were already multinucleated and osteoclasts clearly do not proliferate, dedifferentiation of some of the cells is not a likely reason for the differences in osteoclast activity we observed.

Although the reasons for differences between the resorptive capacity and activation of small and large osteoclasts remain unknown, it is clear from these studies that differences do exist. As a result large osteoclasts degrade more bone than small osteoclasts and the presence of greater numbers of large osteoclasts in certain diseases is responsible for the greater amount of bone loss.
CHAPTER 6: CONCLUSIONS

Osteoclast resorption of bone is required for normal bone remodeling and the total inhibition of all osteoclastic activity, while treating diseases of bone loss, would prevent bone renewal, and would thus be detrimental for bone quality. As a result, selective inhibition of the most active osteoclasts would be a valuable method of treatment, although not yet possible. The goals of this research project were to explain why increased resorptive activity appears to be associated with the presence of many large osteoclasts, to find the basis for heterogeneity among osteoclasts and to identify the most active osteoclasts such that in future it may be possible to target inhibitory drugs to these osteoclasts alone. We have obtained, for the first time, a clear indication that multinuclearity affects osteoclast function and regulation. Osteoclast size, as determined by number of nuclei per cell, is important in determining how much bone is resorbed. Large osteoclasts are more effective bone resorbers than small osteoclasts, although not on a per nucleus basis as we had originally anticipated, but rather as a whole population because a much greater proportion of large osteoclasts are resorbing as compared to small osteoclasts. We suggest that these different proportions reflect differences in activation mechanisms, rather than pH regulatory mechanisms (V-ATPase activity, Na+/H+ exchange activity), of these cells and hope that the results obtained here will form the groundwork that will make the selective pharmacological inhibition of the most active osteoclasts feasible.
CHAPTER 7: REFERENCES


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