Molecular and Cellular Studies of Zoledronic Acid: A Potent Inhibitor of Multiple Myeloma-Induced Osteolysis

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DECLARATION

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BEIQING PAN

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ABSTRACT

Myelomatosis (MM) is an incurable B cell malignancy, characterised by the presence of osteolytic bone lesions, a major cause of morbidity and mortality. This study investigated the effect of zoledronic acid, a potent nitrogen-containing bisphosphonate (BP), on myeloma cells and osteoblasts-like cells to establish the molecular and cellular mechanism responsible for the clinical effectiveness of BPs in the treatment of patients with MM.

These studies indicate that zoledronic acid induces apoptotic and non-apoptotic cell death in myeloma cell lines by (1) inducing S phase arrest in a time and dose dependent manner, (2) inhibiting the mevalonate pathway, and (3) up regulating TRAIL expression and uncoupling the regulated expression of TRAIL death and decoy receptors.

Using an established model of osteoblast differentiation, zoledronic acid was found to increase the number of mature osteoblast/osteocyte-like cells, with a concomitant decrease in the number of stromal precursor cells. In accordance with these findings, zoledronic acid was found to enhance the osteoblast-like cells' ability to form a mineralised bone matrix, when used at concentrations in the range of 5μM and 25 μM. Therefore, we conclude that zoledronic acid mediates the differentiation of osteoblasts-like cells, which accounts for the enhanced bone formation. Zoledronic acid also upregulates the gene expression of IL-1β and TNF-α, which serve to increase the number of osteoprogenitors cells which in turn can respond to zoledronic acid. Furthermore, zoledronic acid was found to decrease the expression of membrane-associated RANKL by increasing TACE expression, a metalloproteinase disintegrin. In conclusion, the studies presented herein show that zoledronic acid enhances bone formation by directly acting on osteoblasts.

In conclusion, work reported in this thesis suggests that myeloma cell death, and proliferation and maturation of osteoblasts represent two mechanisms that zoledronic acid inhibits MM-induced ostolysis.

ABBREVIATIONS

A_{450}	Absorbance at 450 nm
α-MEM	Alpha Minimal Essential Medium
AP/ALP	Alkaline phosphatase
ASC-2P	L-ascorbic acid 2-phosphate
ATP	Adenosine triphosphate
bp	Base pair(s)
BP/BPs	Bisphosphonate/Bisphosphonates
BM	Bone marrow
BMMNC	Bone Marrow Mononuclear cells
BSP	Bone sialoprotein
BSA	Bovine Serum Albumin
CAM	Cell adhesion molecule
CAM-DR	CAM - mediated drug resistance
cAMP	Cyclic adenosine monophosphate
CBFA-1	Core binding facter alpha-1
CD	Cluster of Differentiation
cDNA	Complementary DNA
CFU-F	Colony Forming Unit-Fibroblast
cm	Centimetre
COLI	Collagen type I
CSF	Colony Stimulating Factor
d0, d1, d2…	Day 0, day 1, day 2…
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone sodium phosphate
DMEM	Dulbecco's Modified Eagles Medium

DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetra-acetic acid
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
FN	Fibronectin
FPP	Farnesylgeranylation
G-CSF	Granulocyte-CSF
GGO	Geranylgeraniol
GGPP	Geranylgeranyldiphosphate
GM-CSF	Granulocyte/Macrophage-CSF
HBSC	Human bone stromal cell
HBSS	Hank's balanced salt solution
HHF	HBSS supplement with 5% FCS
HLA	Human Leucocyte Antigen
h	Human
HGF	Hepatocyte growth factor
hr	Hour
i.u.	International Units
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IgG_1	Immunoglobulin gamma-1 isotype
IgG_{2a}	Immunoglobulin gamma-2a isotype
IgG_{2b}	Immunoglobulin gamma-2b isotype

IgM	Immunoglobulin M
IL-1, 3, 6	Interleukin-1, 3, 6
IL-1RA	Interleukin-1 receptor antagonist
IMEM	Iscove's Modified Dulbecco's Medium
IMVS	Institute of Medical and Veterinary Science
kDa	Kilodalton
LFA	Leukocyte Function-Associated Antigen
LRP	Lung resistance protein
M	Molar
mAb	Monoclonal antibody
M-CSF	Macrophage-colony stimulating factor
MDR	Multi-drug resistance
mg	Milli gram
mIgG,M	Mouse immunoglobulin G or M
min	Minute
ml	Milli litre
mm	Milli metre
mM	Milli Molar
MM	Myelomatosis or Multiple Myeloma
MMP	Metalloproteinase
MNC	Multinuclear cell
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
µg	Micro gram
µm	Micro metre
µM	Micro Molar

ng	Nano gram
nm	Nano metre
NHBC	Normal human bone cells
NOD	Normal osteoblast donor
NP-40	Nonidet P40
OAFs	Osteoclast-activating factors
OB	Osteoblast/Osteoblasts
OC	Osteoclast/Osteoclasts
OCN	Osteocalcin
ODF	Osteoclast differentiation factor
ON	Osteonectin
O/N	Overnight
OP	Osteopontin
OPG	Osteoprotegrin
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PO_{4i}	Inorganic phosphate
Ppi	Inorganic pyrophosphate
PTH	Parathyroid hormone
PTHrp	Parathyroid hormone related-protein
PTP	Protein tyrosine phosphotase
RANK	Receptor activator of nuclear factor-κB
RANKL	RANK ligand

RNA	Ribonucleic acid
r IgG	Rabbit Immunoglobulin G
RT	Room temperature
RT-PCR	Reverse transcription- polymerase chain reaction
SEM	Standard error of mean
7-AAD	7-aminoactinomycin
TNF-α	Tumour necrosis factor- α
TNF-β	Tumour necrosis factor- β or lymphotoxin
TACE	TNF-alpha converting enzyme
TPTX	Thyroparathyroidectomized
TRAIL/Apo2L	TNF-related apoptosis-inducing ligand
TRANCE	TNF-related activation-induced cytokine
TRAP	Tartrate-resistant acid phosphatase
2-ME	2-Mercaptoethanol
UV	Ultra violet
VCAM–1	Vascular Cell Adhesion Molecule-1
v/v	Volume per volume
VLA	Very late acting antigen
w/v	Weight per volume
Zole	Zoledronic acid

CHAPTER 1

INTRODUCTION

Myelomatosis (multiple myeloma, MM) is an incurable B cell malignancy, characterised by the presence of a monoclonal population of end-stage B cells (plasma cells), a monoclonal immunoglobulin (Ig) protein (M-, or para-protein) and "punched out" lytic bone lesions. Osteolytic bone destruction, a major cause of morbidity and mortality, is a striking clinical feature for approximately 80% of patients with MM. It is generally accepted that the osteolysis is related to the accumulation of the myeloma cell clone within the bone marrow (BM), in close vicinity to the bone trabeculae. Although current evidence suggests that myeloma cells cannot directly resorb bone, they produce a variety of osteoclast-activating factors (OAFs) which lead to the recruitment and activation of osteoclasts (OC) (Michigami *et al.*, 2000; Roodman, 1997). The normal equilibrium between bone formation by osteoblasts (OB), and bone resorption by multinucleated OC, is therefore disrupted leading to localised bone loss (Hjorth-Hansen *et al.*, 1999). It has been known for many years that cultures of human myeloma cells *in vitro* produce several OAFs, including tumour necrosis factor-α (TNF-α), TNF-β (lymphotoxin), interleukin-1β (IL-1β), and IL-6, macrophage-colony stimulating factor (M-CSF) and parathyroid hormone related protein (PTHrP) (Costes *et al.*, 1998; Filella *et al.*, 1996; Pfeilschifter *et al.*, 1989). However, most of the studies identifying factors produced by myeloma cells *in vivo* are inconclusive due to the presence of other contaminating cell types, including stromal cells and macrophages. In the last three years, a newly identified cytokine system has provided us with a greatly improved understanding of bone remodelling physiology. This system includes receptor activator of nuclear factor-κB ligand (RANKL), which is a member of the tumour necrosis factor (TNF) ligand family, its receptor, termed receptor activator of nuclear factor-κB (RANK), and its soluble decoy receptor, osteoprotegerin (OPG). Studies by our group (Farrugia *et al.*, 2002) and others (Croucher *et al.*, 2001; Giuliani *et al.*, 2001; Hofbauer *et al.*, 2001; Sezer *et al.*, 2002) have demonstrated that this system plays a very important role in myeloma-induced osteolysis.

Understanding the molecular mechanisms, by which MM cells induce osteolysis is essential for the development of new drugs to cure bone disease. To this end, the discovery of bisphosphonates (BPs), which are stable analogues of naturally occurring pyrophosphate-containing compounds, represents a significant milestone in the history of treatment of bone

disease. BPs inhibit resorption of bone predominantly through an effect on OC, and have provided us with an expectation of improved treatment for the skeletal complications of myeloma. Several double blind, placebo-controlled randomised trials have demonstrated that BPs reduce the skeletal complications of multiple myeloma, decrease the associated morbidity (Berenson *et al.*, 1996; McCloskey *et al.*, 1998) and improve the survival outcomes in some patients (Berenson *et al.*, 1998). The underlying mechanisms, by which these compounds act on the tumour cells and OB, require further elucidation.

In this project, we investigated the role of the most potent BP, zoledronic acid (formerly referred to as zoledronate), on myeloma cells and OB, to clarify the underlying molecular mechanism of clinical effectiveness.

1.1 MYELOMATOSIS

1.1.1 General Description

Myelomatosis is a differentiated B cell neoplasm characterised by the malignant clonal expansion of plasma cells that accumulate in the BM. It accounts for 2.9% of all malignancies in black people and 1.0% in white people. The median age of diagnosis is 69 and 71 years for males and females, respectively. The diagnosis of MM depends on the demonstration of increased numbers of marrow plasma cells (>10%) which present as atypical and immature forms, the presence of monoclonal paraprotein (M-protein, >3/dl) in serum, Bence-Jones protein in urine, and osteolytic skeletal lesions. Extensive bone disease is a hallmark of MM and contributes to most of the debilitating morbidity and mortality associated with this disease (Roodman, 1995). Multiple myeloma induced osteolytic bone lesions or osteoporosis cause intractable bone pain and other distressing symptoms such as pathological fractures after trivial injury, spinal cord compression, and disturbance in calcium homeostasis. At diagnosis, approximately 50% of patients have vertebral fractures, and approximately 30% have non-vertebral fractures (Lahtinen *et al.*, 1992). Valuable adjunct therapies for bony complications in addition to chemotherapy and BM transplantation, are therefore necessary for improving the quality of life for MM patients.

1.1.2 Myeloma and Drug Resistance

Despite initial responses to chemotherapy, myeloma patients ultimately develop drug resistance and become unresponsive to a wide spectrum of anti-cancer agents. This

phenomenon is termed multi-drug resistance (MDR). MDR presents a major obstacle to curing myeloma, and understanding factors that determine drug response and the development of drug resistance are essential in developing means of preventing or overcoming this problem (Dalton, 1997).

The development of intrinsic cellular resistance to front-line chemotherapeutic drugs, such as melphalan (an alkylating agent) and doxorubicin (an anthracycline), is a major factor responsible for treatment failure of MM. Classically, investigators of MDR have focused on the drug resistant single cells, selected by exposing cells to cytotoxic agents. In summary, these studies have revealed a number of potential mechanisms including: (1) Reduction of intracellular drug concentration due to the overexpression of membrane pump proteins such as P-glycoprotein, MRP, and LRP, (2) Altered drug metabolism or enhanced drug detoxification (changes in glutathione and glutathione-associated enzymes), (3) Alterations in the drug target that reduce drug efficacy (alterations in topoisomerase II), (4) Enhanced cellular repair of drug-induced damage (Dalton, 1997), and (5) Cell adhesion-mediated drug-resistance (for review see Dalton and Jove, 1999; Damiano and Dalton, 2000).

The integrin family of cellular adhesion molecules represents a major family of cell-surface receptors that mediate both cell-cell and cell-extracellular matrix (ECM) interactions. They are transmembrane heterodimers composed of noncovalently bound α- and β-subunits. To date, 16 different α subunits have been identified, which associate with as many as nine known β-subunits (for review see (Hynes, 1992; Hynes and Zhao, 2000)). The α4β1 (Very Late Activation Antigen 4, or VLA-4), α5β1 (VLA-5) and α4β7 heterodimers are the major fibronectin (FN) receptors of the integrin family (Clark and Brugge, 1995; Hynes, 1992). α4β1 is unique among the integrins as it is the only heterodimer that has conclusively been shown to mediate cell-ECM as well as cell-cell interaction (Pulido *et al.*, 1991). α4β1 is one of the main adhesion receptors involved in the homing and localization of myeloma cells to the BM microenvironment. A better understanding of its regulatory adhesive mechanisms might contribute to a greater knowledge of the pathology of MM (Sanz-Rodriguez *et al.*, 1999).

It is well recognised that cell-cell and/or cell-ECM adhesion may regulate apoptosis and cell survival in a wide variety of tumour types (Clark and Brugge, 1995). It was demonstrated

that myeloma cell lines and patient-derived myeloma cells express the FN receptors α4β1 and α5β1 (Damiano *et al.*, 1999), which facilitate the attachment and communication between the myeloma cells and the BM microenvironment (Barker *et al.*, 1992; Jensen *et al.*, 1993). Fibronectin, as well as VCAM-1, are expressed on the surface of MM stromal monolayers (Faid *et al.*, 1996; Lokhorst *et al.*, 1994), and antibodies to VCAM-1 partially inhibit myeloma cell adhesion to MM stroma (Kim *et al.*, 1994; Robledo *et al.*, 1998). In addition, anti-α4 and anti-β1 antibodies have been reported to inhibit the adhesion of myeloma cells to total FN and intact MM stroma, which indicates that α4β1 has an important role in this adhesion (Lokhorst *et al.*, 1994; Uchiyama *et al.*, 1992; Uchiyama *et al.*, 1993; van Riet *et al.*, 1994).

Damiano and colleagues (1999) recently presented data suggesting that integrin receptors on myeloma cells may be responsible for cell adhesion-mediated drug resistance (CAM-DR) following attachment to FN (Damiano *et al.*, 1999). These investigators showed that the drug-sensitive myeloma cell line RPMI 8226, known to express both α4β1 and α5β1 integrin FN receptors, were relatively resistant to the apoptotic effects of doxorubicin and melphalan, when cells were pre-adhered to FN, compared to cells exposed to the drug while in suspension culture. Consistent with previous studies, which demonstrated that α4β1 mediated adhesion decreased the proliferation of haemopoietic progenitor cells (Hurley *et al.*, 1995), Damiano and co-workers reported that chronic exposure of the myeloma cell line, RPMI 8226, to either doxorubicin or melphalan, prolonged cell doubling times by over-expressing α4 integrin (Damiano *et al.*, 1999). This suggested that integrin-mediated adhesion decreased the response of RPMI 8226 cells to chemotherapeutic drugs. Clinically, elevated FN receptor expression or function in myeloma cells within the BM may be an indicator of a more aggressive tumour cell that has a survival advantage against the cytotoxic effects of anti-cancer drugs. *In vivo* alterations in FN receptor expression or function may have a magnified effect on myeloma cell survival when they are in direct association with stromal cells and ECM component of the BM. The CAM-DR mediated by FN adhesion may be sufficient to allow the eventual emergence of drug resistance mechanism such as the up-regulation of P-glycoprotein, MRP, and topoisomerase II, which then become the predominant cytoprotective processes.

Blocking intercellular communications by blocking cell adhesion may represent a new approach for the treatment of myeloma. These findings provide evidence that antagonists of cellular adhesion, or signaling events related to adhesion, may serve as a means of inducing myeloma cell apoptosis or improving the efficacy of anti-cancer therapy for myeloma.

IL-6 is a potent growth factor for myeloma cells. It is secreted from both tumour and stromal cells in response to co-adhesion and $\alpha 4\beta 1$ ligation (Uchiyama *et al.*, 1993), where it is thought to play a role in drug resistance in MM patients. Autocrine IL-6 reflects a highly malignant phenotype of myeloma cells, and autocrine IL-6 production and deregulated apoptosis may induce expansion of IL-6^+ myeloma cells resistant to spontaneous and drug-induced cell death (Frassanito *et al.*, 2001).

1.2 BONE REMODELLING

1.2.1 Physiological Bone Remodelling

Bone is a highly organised structure composed of a calcified connective tissue matrix (hydroxyapatite) formed by OB, bone lining cells and osteocytes. Bone remodelling represents the predominant form of bone turnover in the adult human skeleton and serves to prevent the accumulation of micro-damage and allow the skeleton to respond appropriately to changes in mechanical loading. Although trabecular bone represents 20% of the skeletal mass, it accounts for up to 80% of the bone turnover. The process of bone remodelling, is mediated by the highly coordinated activities of two cell types, namely the bone resorbing OC and the bone forming OB (Parfitt, 1984). Osteoclastic bone resorption is preceded by a cascade of events that starts with the activation of haemopoietic stem cells and is followed by proliferation, differentiation, chemotaxis, attachment, and subsequent terminal differentiation and fusion of the OC precursors into functioning multinucleated OC. After activation, OC start to erode and form a cavity (lacuna) on the bone surface. This process occurs for approximately two weeks. After completion of resorption, the OC disappear and OB subsequently differentiate and form new bone at the same site. Newly formed sites then remain quiescent, normally for 2-3 years, until a new activation stimulus event occurs at this site.

1.2.2 Osteoblasts

Osteoblasts represent a heterogeneous population of cells, which are derived from the stromal or mesenchymal cell system. This family includes mature OB, the bone lining cells, which

cover bone surfaces, and osteocytes, which are buried within bone and communicate with each other *via* the canalicular system.

Mature OB line the bone surface, and have a single eccentric nucleus and a well-developed endoplasmic reticulum and Golgi apparatus (for review see Mundy, 1995). They are identified morphologically by their cuboidal appearance and their association with newly synthesized mineralized bone matrix (hydroxyapatite) at sites of active bone formation. This may be mediated in part by subcellular particles known as matrix vesicles, which are generated from the OB cytoplasm, are enriched in alkaline phosphatase. Osteoblasts also produce other bone matrix constituents that may be important in the mineralisation process such as phospholipids and proteoglycans. These committed osteogenic cells are characterised histologically by the synthesis of certain bone-associated matrix proteins, such as collagen type I (COLI), osteocalcin (OCN), bone sialoprotein (BSP), osteonectin (ON), and osteopontin (OP). Approximately 95% of the organic bone matrix is comprised of COLI, with the remainder consisting of various other molecules such as proteoglycans, other collagen types and non-collagenous proteins (Robey, 1996). Osteoblasts secrete the growth factors that are stored in the bone matrix, such as transforming growth factor β, bone morphogenic proteins, platelet-derived and the insulin-like growth factors (Hauschka *et al.*, 1986). Expression of the membrane-bound ectoenzyme alkaline phosphotase (ALP) is one of the earliest, and most widely accepted indicators that a cell has initiated a program of osteogenic differentiation. ALP probably plays an essential role in bone mineralisation and is frequently used by bone cell biologists as a marker of cells with an OB phenotype, and by clinicians as a serum marker of OB activity. The majority of OB die by apoptosis, and those remaining have two alternative fates: to become lining cells or osteocytes (Jilka *et al.*, 1998).

The fact that bone remodelling process continues throughout adult life, and both OB and OC have a limited functional lifespan, points to the existence of precursor cell populations with the potential for extensive proliferation and further differentiation. The osteogenic cell lineage belongs to the stromal fibroblastic system of the BM, which includes other stromal tissue, such as cartilage, smooth muscle, and fat (Beresford, 1989; Owen and Friedenstein, 1988). Although there is heterogeneity within the OB lineage with regard to their proliferative capacity and functional state, these cells have a common stromal cell precursor capable of differentiation into a number of mature lineages, including adipocytes, reticular cells, fibroblasts and chondrocytes (Liu *et al.*, 1997). Although the factors involved in

controlling the differentiation of precursors into cells of the OB lineage have not yet been fully determined, a number of growth regulatory factors such as transforming growth factor β (TGFβ), fibroblast growth factors, bone morphogenetic proteins (BMPs), and platelet-derived growth factor (PDGF), have been implicated (as reviewed by Mundy, 1995).

The exact location of BM stromal precursor cells in the marrow spaces is still a matter of conjecture. Histological evidence suggests that multipotential stromal progenitors may reside in the soft fibrous tissue of the marrow stroma. The osteogenic potential of the marrow stroma was attributed to the presence of a small population of highly adherent, clonogenic cells, termed colony-forming units fibroblastic (CFU-F) (Owen and Friedenstein, 1988). A monoclonal antibody (mAb) STRO-1 has been used to recognize a trypsin-resistant cell surface antigen present on a osteoprogenitor stem cell subpopulation of BM cells, including essentially all detectable CFU-F (Simmons and Torok-Storb, 1991). BM mononuclear cells sorted on the basis of STRO-1 expression are capable of establishing an adherent stromal layer *in vitro*, consisting of a number of phenotypically distinct stromal cell types, including fibroblasts, smooth muscle cells, adipocytes, and OB (Gronthos *et al.*, 1994). In addition, it has been demonstrated that the STRO-1^+ population of human BM cells is capable of osteogenic differentiation and that osteoprogenitors are present in the STRO-1^+ population (Gronthos *et al.*, 1994). The growth factors required for the expansion of the marrow CFU-F population have also been characterised (Gronthos and Simmons, 1995).

Using two independent cell surface markers, STRO-1 and ALP, cells of the OB lineage at different stages of maturation have also been identified (Gronthos *et al.*, 1999; Stewart *et al.*, 1999). The bone/liver/kidney isoform of ALP is broadly used as a general marker of committed OB, and STRO-1 is used as a marker representiative of a preosteoprogenitor phenotype. Gronthos *et al* (1999) showed that the majority of normal human bone cells (NHBCs), which express the STRO-1^-/ALP^+ and STRO-1^-/ALP^- phenotypes, appear to represent fully differentiated OB, while the cells that express the STRO-1^+/ALP^+ phenotype are considered as osteoprogenitors, and cells which express the STRO-1 antigen are stromal precursors (Gronthos *et al.*, 1999). This observation is based on the expression of bone-associated matrix proteins and the ability of each subpopulation to form a mineralised bone matrix *in vitro*.

Studies on human bone have shown that cells of the OB lineage express a range of integrin α and β subunits, both *in situ* and in culture (Clover *et al.*, 1992; Gronthos *et al.*, 1997; Grzesik and Robey, 1994; Saito *et al.*, 1994). β1 integrins appear to be the predominant adhesion receptor subfamily used by stromal precursor cells to adhere to matrix glycoproteins commonly found in the BM microenvironment and bone surfaces (Gronthos *et al.*, 2001). In addition, the ability of human BM stromal cells (BMSC) to initiate matrix mineralisation was found to be significantly diminished in the presence of a functional blocking monoclonal antibody to the β1 integrin subunit, demonstrating a role for this integrin subfamily during bone formation (Gronthos *et al.*, 2001). Collectively, these studies suggest that the resorption of bone by OC may be mediated through integrins such as αvβ3 and perhaps αvβ5, while OB development and bone formation may be more dependent on β1 integrin interactions.

1.2.3 Osteoclasts

Osteoclasts are derived from pluripotent precursors of the monocyte macrophage lineage in the BM (for review see Mundy, 1995). They are seen frequently at active sites of bone remodelling, such as the metaphyses of growing bones or adjacent to collections of tumour cells. Usually the large multinucleated cells have less than 10 nuclei, and have primary lysosomes, numerous and pleomorphic mitochondria, and a specific area of the cell membrane that forms adjacent to the bone surface, known as the ruffled border. This area of the cell membrane comprises folds and invaginations, which facilitates intimate contact with bone resorption sites and the formation of resorption pits (also known as the Howship's lacuna or resorption bay). The ruffled border is surrounded by a clear zone, which contains actin filaments, and appears to anchor the ruffled border area to the bone surface undergoing resorption. This structure may be critical to the polarisation of the OC, which occurs immediately when the cell is activated prior to bone resorption.

Osteoclasts are produced in the BM where they are in physical contact with stromal cells. Experimental data suggest that OB and their progeny (bone lining cells) may act as accessory cells for osteoclastic resorption. Interactions may occur by either direct cell-cell contact or by the production of soluble mediators that are critical for the proliferation and differentiation of haemopoietic OC precursors. Using the mononuclear fraction of peripheral blood of adults as a source of precursors, terminally differentiated OC capable of extensive resorption pit

formation can be cultured *in vitro* either in the presence of OB or essential mediators (Azuma *et al.*, 2000; Fujikawa *et al.*, 1996; Matayoshi *et al.*, 1996).

1.3 MECHANISM OF INCREASED OSTEOCLAST ACTIVITY IN MM

It is widely accepted that the underlying mechanism of osteolysis in MM patients is increased osteoclastic bone resorption. Myeloma cells are found in the close vicinity of bone trabeculae, suggesting a strong relationship between tumour cells and their environment (for review see Van Riet *et al.*, 1998). Recent studies suggest that there is considerable interdependence between malignant myeloma cells and BM stromal cells present within the bone environment, through the interaction of different adhesion receptors with their ligands. The BM microenviroment itself is a site of synthesis of numerous cytokines which may be important in the proliferation and/or survival of myeloma cells (Bataille *et al.*, 1996). The adhesion of myeloma cells to MM stroma triggers synthesis of cytokines, including IL-6, which augments myeloma cell growth and survival and confers drug resistance. IL-6, a potent growth factor for myeloma cells, is secreted from both tumour and stromal cells in response to co-adhesion and $\alpha 4\beta 1$ ligation (Uchiyama *et al.*, 1993). In addition, the important OAFs secreted by myeloma cells and OB, such as IL-6, IL-1β, TNF-α, TNF-β, and PTHrP, are also increased (Bataille *et al.*, 1992b; Caligaris-Cappio *et al.*, 1992) (figure 1.1). It has been showed that MM patients with advanced aggressive disease, secrete significantly higher levels of IL-6, TNF-α, and IL-1 than normal controls (Filella *et al.*, 1996; Lichtenstein *et al.*, 1989).

1.3.1 Interleukin-6

Interleukin-6 (IL-6), a pleiotropic cytokine produced by a variety of cells, is a critical growth factor for normal B-cell growth and development and represents a major tumour survival and proliferation factor for MM cells (Klein *et al.*, 1995; Nilsson *et al.*, 1990; Ohtake *et al.*, 1990). Although the origin of IL-6 is controversial, some primary plasma cells have been shown to produce, or can be induced to produce, IL-6. This is thought to mediate autocrine growth of human myeloma cells by modulating IL-6 signalling and IL-6 receptor expression in myeloma cells (Hirano *et al.*, 1990; Jernberg-Wiklund *et al.*, 1992; Kang *et al.*, 1996). Autocrine IL-6 production reflects a highly malignant phenotype of myeloma cells, and together with deregulated apoptosis, may induce expansion of selective IL-6^+ myeloma cells resistant to spontaneous and drug-induced cell death (Frassanito *et al.*, 2001).

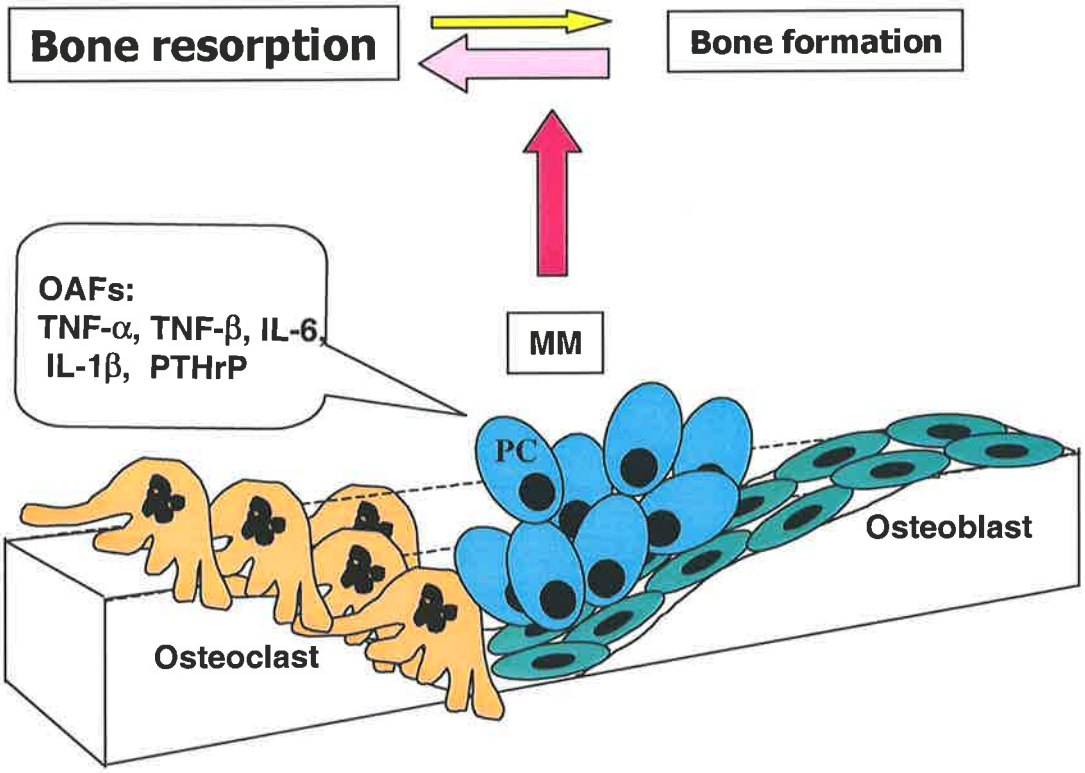


Figure 1.1. Increased osteoclastic bone resorption is an underlying mechanism of osteolysis in MM. A number of OAFs including IL-6, IL-1β, TNF-α, TNF-β, and PTHrP, have been implicated in the pathogenesis of the ostelytic bone destruction observed in patients with myeloma.

The development of MM is a multistep transformation process and Hallek *et al* supported that several oncogenic events result in the selection and malignant expansion of a single IL-6^+ clone (Hallek *et al.*, 1998). On the other hand, some studies show that IL-6 is produced by stromal components grown from myeloma BM, suggesting a paracrine regulation of tumour growth, which implies that the proliferation of myeloma cells is dependent upon close contact with stromal cells (Lichtenstein *et al.*, 1989; Lokhorst *et al.*, 1994). The importance of the IL-6/IL-6R interaction in MM was demonstrated by de Hon *et al.* (1994), who showed that the growth of MM cell line XG-1 could be inhibited by the addition of mutant form of IL-6, which displayed a 5-fold enhanced affinity for IL-6R, but was incapable of signalling through the IL-6R (de Hon *et al.*, 1994). Although IL-6 can stimulate osteoclast-like cell formation (Kurihara *et al.*, 1990), the role of IL-6 in osteoclastic bone resorption is complex and remains unclear.

1.3.2 Interleukin-1

Serum interleukin-1 (IL-1) level in myeloma BM cells was found to be significantly higher than that found in similarly processed BM cells from control individuals (Filella *et al.*, 1996; Lichtenstein *et al.*, 1989). IL-1 was shown to be mainly responsible for IL-6 production in the tumoral BM environment of patients with MM through a prostaglandin E2 (PGE2) loop (Costes *et al.*, 1998; Lu *et al.*, 1995). Inhibition of PGE2 synthesis using an IL-1 receptor antagonist (IL-1RA) and indomethacin, an inhibitor of cyclooxygenase which can block PGE2 synthesis, was found to inhibit myeloma cell proliferation by reducing IL-1-induced endogenous IL-6 production *in vitro* and *in vivo* (Costes *et al.*, 1998). In addition, IL-1β is also a principal bone resorption agent present in the supernatants of myeloma cell cultures that enhances destructive bone lesions in the patients with MM (Cozzolino *et al.*, 1989; Yamamoto *et al.*, 1989). This bone-resorbing activity can be neutralised by an antibody directed against IL-1β (Cozzolino *et al.*, 1989; Kawano *et al.*, 1989). Thus, IL-1 can promote the secretion of IL-6, and both IL-1 and IL-6 enhance the bone lysis in MM.

1.3.3 Tumour Necrosis Factor

Tumour necrosis factor-α (TNF-α), is found at higher levels in supernatants from BM cultures of MM patients, and is capable of markedly stimulating osteoclast-like multinucleated cell formation in human marrow cultures (Filella *et al.*, 1996; Lichtenstein *et al.*, 1989; Pfeilschifter *et al.*, 1989). The role of TNF-β in myeloma bone disease has been

downplayed by more recent studies, which failed to find significant differences in the amount of this cytokine in supernatants derived from fresh BM plasma cultures from myeloma patients, compared to controls. In addition, antibodies to TNF-β do not reduce the bone resorbing activity of fresh BM plasma from myeloma patients. Interestingly, TNF-α has a more potent effect than IL-6 in stimulating the growth of primary myeloma cultures from the same patient, indicating that TNF-α in selected myeloma patients has a growth-promoting effect equal to that of IL-6 (Borset *et al.*, 1994).

1.3.4 Parathyroid Hormone Related Protein

Parathyroid hormone related protein (PTHrP) is a widely expressed hormone that is a major mediator of malignancy-associated hypercalcemia and shows 70% homology to parathyroid hormone (PTH). The binding of PTHrP to the PTH receptor activates cyclic adenosine monophosphate (cAMP) and enhances osteoclastic bone resorption (Kaji *et al.*, 1993). The elevated PTHrP can be measured in the plasma of patients with myeloma (Horiuchi *et al.*, 1997; Schneider *et al.*, 1998). PTHrP released by myeloma cells acts in a paracrine manner by binding to the PTH receptor expressed by OC to produce local bone resorption. When secreted in large amounts by malignant cells, PTHrP may act in a humoral manner to cause systemic hypercalcemia (Firkin *et al.*, 1996). Thus, PTHrP might be an important contributor to the skeletal complications in MM.

Although several OAFs have been implicated in the enhanced OC activity, the precise role of any of these factors in osteolytic bone disease in patients with myeloma has not been clearly demonstrated *in vivo*. Recently, a new cytokine system RANKL/RANK/OPG was identified and provides us with a novel paradigm as to the mechanisms of osteoclastogenesis and bone resorption in physiological and pathological bone remodelling.

1.3.5 RANKL/ ODF/TRANCE/OPGL: the Ligand

Receptor activator of nuclear factor-κB ligand (RANKL) is a member of the TNF ligand family, variously termed TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), or OC differentiation factor (ODF) (Anderson *et al.*, 1997; Lacey *et al.*, 1998). It is secreted by BM OB/stromal cells, chondrocytes, mesenchymal cells of the periosteum, OC, endothelial cells and T cells (Anderson *et al.*, 1997; Lacey *et al.*, 1998; Yasuda *et al.*, 1998b). RANKL is a type II membrane-anchored polypeptide, which is released from the plasma membrane by a metalloprotease-disintegrin TNF-alpha convertase

(TACE) (Lum *et al.*, 1999). Truncated RANKL is an important component of the function of transmembrane RANKL in bone and immune homeostasis (Lum *et al.*, 1999).

The development of OC comprises several stages: recruitment, proliferation, differentiation of the progenitors into mononuclear OC, and fusion of mononuclear OC to form activated multinucleated OC capable of resorbing bone. Numerous studies demonstrate that RANKL provides an essential signal to OC progenitors, enabling their differentiation into OC (Yasuda *et al.*, 1998b). RANKL-deficient mice show severe osteopetrosis, defects in tooth eruption, and completely lack OC as a result of the inability of OB to support osteoclastogenesis (Kong *et al.*, 1999a; Kong *et al.*, 1999b). Soluble RANKL (sRANKL) causes murine as well as human OC differentiation when co-stimulated with macrophage-colony stimulating factor (M-CSF) *in vitro* (Quinn *et al.*, 1998; Udagawa *et al.*, 1999). This suggests that the RANKL-mediated signals can be transduced not only by cell-to-cell contact between OB/stromal cells and OC progenitors, but also by the cleaved or soluble form of RANKL in a paracrine manner. The cell-to-cell interaction is essential for OC formation in the co-culture system, suggesting a pivotal role for the membrane-bound RANKL in osteoclastogenesis. The cleaved-form of RANKL may play a role in pathological bone resorption mediated by abnormal osteoclastogenesis. Taken together, these results demonstrate that RANKL is an essential OC differentiation factor *in vivo* and *in vitro*.

1.3.6 RANK: the Receptor

Receptor activator of nuclear factor-κB (RANK) is a member of the TNF receptor family and represents the membrane-associated counter receptor for RANKL (Yasuda *et al.*, 1999). RANK is expressed by several cell types, including dentritic cells, B cells, and mainly by OC precursor cells and is capable of initiating osteoclastogenic signal transduction after ligation with RANKL or anti-RANKL agonist antibodies (Anderson *et al.*, 1997; Hsu *et al.*, 1999). RANK deficient mice show a profound defect in bone resorption and bone remodelling, resulting from an apparent block in OC differentiation and an intrinsic defect in OC function (Dougall *et al.*, 1999; Li *et al.*, 2000). Severe osteopetrosis was observed in transgenic mice expressing a soluble RANK-Fc fusion protein (Hsu *et al.*, 1999). Recombinant RANK-Fc bound with high affinity to RANKL *in vitro* and blocked OC differentiation and activation *in vitro* and *in vivo* (Hsu *et al.*, 1999). These results all indicate that RANK is an OC receptor capable of mediating the biological activities of RANKL during normal bone homeostasis

and in disease. The RANKL signalling pathway is the ultimate common mediator of humoral signals that regulate bone resorption and calcium metabolism.

1.3.7 OPG/OCIF: the Decoy Receptor

Osteoprotegerin (OPG), also called osteoclastogenesis inhibitory factor (OCIF), is a heparin-binding, basic glycoprotein. It has been isolated as both a monomer and a disulfide-linked homodimer, with a molecular weight of 60 kD and 120 kD, respectively. OPG is a secreted member of the TNF receptor family, containing four cysteine-rich domains and two regions which exhibit homology to death domains (Simonet *et al.*, 1997; Yamaguchi *et al.*, 1998). It acts as a naturally occurring decoy receptor for RANKL by disrupting the interaction between RANKL and RANK (Yasuda *et al.*, 1999). OPG inhibits osteoclastogenesis by blocking cell-to-cell signalling between stromal cells and OC progenitors (Yasuda *et al.*, 1998a). A mouse co-culture system using calvarial OB and BM cells prepared from OPG-deficient mice suggested that OPG produced by OB/stromal cells is a physiologically important regulator in OC differentiation and function (Udagawa *et al.*, 2000).

1.3.8 The Relationship between RANKL, RANK and OPG in MM

As discussed previously, RANKL promotes the differentiation of OC precursor cells to OC through the RANK receptor expressed on the OC precursor cells. OPG acts as a decoy receptor that regulates the signalling between RANKL and RANK. The over-expression of OPG in transgenic mice results in a muted osteopetrotic phenotype (Simonet *et al.*, 1997). Conversely, targeted ablation of OPG in knockout mice leads to severe osteoporosis (Bucay *et al.*, 1998). These results demonstrate that OPG is a key factor acting as a negative regulator of osteoclastogenesis. It has been demonstrated that the bone resorbing agents 1 alpha, 25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$), PTH, and PGE2 can upregulate RANKL expression, and stimulate OB-induced pit-forming activity of OC (Udagawa *et al.*, 1999). Other investigators also reported that a number of osteotropic agents including 1,25-$(OH)_2D_3$, PTH or IL-11, could increase the ratio of RANKL:OPG (Horwood *et al.*, 1998). Thus, OC formation may be determined principally by the relative ratio of RANKL to OPG in the BM microenvironment. Alterations in this ratio may be a major cause of bone loss in many osteolytic neoplasms including MM. It has been demonstrated that myeloma cells affect the RANKL/OPG ratio in the BM environment by stimulating OB to overexpress RANKL (Giuliani *et al.*, 2001), and OPG could inhibit the development of osteolytic bone disease in myeloma-bearing mice (Croucher *et al.*, 2001). Furthermore, fresh myeloma cells (as defined

by their high expression of the CD38 cell surface molecule) isolated from patients with MM, express RANKL and can facilitate bone pit formation in co-culture system in the absence of OB (Farrugia *et al.*, 2002). These observations confirm that the RANKL/OPG system is involved in the pathogenesis of MM induced osteolysis.

1.3.9 The Relationship between RANKL/RANK/OPG and OAFs Secreted by MM.

As previously discussed, recent *in vitro* studies suggest that MM cell-derived OAFs may play a central role in the pathogenesis of MM induced osteolysis. In terms of IL-6, Dai *et al* reported that alcohol ingestion induced osteoclastogenesis and bone loss through an IL-6 mediated mechanism in mice, and that IL-6 achieved this effect by inducing RANKL to promote osteoclastogenesis (Dai *et al.*, 2000). However, the data from another group showed IL-1β and TNF-α, but not IL-6, could stimulate RANKL gene expression in human osteoblastic cells (Hofbauer *et al.*, 1999). These studies demonstrate that IL-6 induced stimulation of OC activity remains controversial. In addition, IL-6 also appears to have a complex interaction with IL-1, TNF and PTHrP in MM. Therefore, determining which of these molecules represent the key factor in this complicated cytokine network remains to be investigated.

1.3.10 Other Molecules That Affect RANKL/RANK/OPG System in MM

(a) TRAIL

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, also called Apo2L) is a member of a superfamily of cell death-inducing ligands that also includes TNF-α and Fas ligand (FasL or CD95L). TRAIL induces apoptosis of malignant cells by interacting with a complex system of cell surface receptors, including the two death-signalling receptors DR4 (also known as TRAIL-R1) and DR5 (TRAIL-R2) (Pan *et al.*, 1997; Sheridan *et al.*, 1997). These 2 receptors transmit a pro-apoptotic signal *via* interaction of their intracellular death domains with adaptor proteins, which are not well characterised. The death domains of these adaptors constitutively bind to caspases, initiating a cascade of proteolytic activation of downstream caspases and apoptosis (Schulze-Osthoff *et al.*, 1998). Additional antagonist decoy receptors have also been described and include DcR1/TRID/TRAIL-R3 and DcR2/TRAIL-4. These receptors have an extracellular TRAIL-binding domain and a transmembrane domain, but lack an intracellular death signalling domain (Pan *et al.*, 1997; Sheridan *et al.*, 1997). Thus, ectopic expression of DcR1 and DcR2 protects mammalian cells from TRAIL-induced apoptosis and inhibits TRAIL cytotoxicity (Degli-Esposti *et al.*, 1997;

Sheridan *et al.*, 1997). However, one report showed that the expression of DcR1 or DcR2 did not appear to be a significant factor in determining the resistance or sensitivity of some tumour target cells to the effects of TRAIL (Griffith *et al.*, 1999). Although non-toxic to most normal human cells *in vitro* (Pitti *et al.*, 1996; Wiley *et al.*, 1995), TRAIL was found to effectively induce extensive apoptosis in RPMI 8226 and ARP-1 myeloma cell lines in a time- and dose-dependent manner, and also induce substantial apoptosis in freshly isolated, flow-sorted myeloma cells obtained from different MM patients (Gazitt, 1999). Furthermore, TRAIL-induced apoptosis in MM cell lines was not blocked by bcl-2 (Gazitt, 1999; Gazitt *et al.*, 1999), and not abrogated by IL-6, a major growth and survival factor for MM cells (Mitsiades *et al.*, 2001). Preclinical studies also suggest that TRAIL can overcome conventional drug resistance and provides the basis for clinical trials of TRAIL-based treatment regimens to improve survival outcomes in patients with MM (Mitsiades *et al.*, 2001).

It was subsequently found that OPG, a secreted homologue of the TNF receptor, represented a fifth TRAIL receptor (Emery *et al.*, 1998). OPG-Fc bound TRAIL with an affinity of 3.0 nM, which was marginally weaker than the interaction of DcR1-Fc or DR5-Fc with TRAIL. It was reported that OPG could inhibit TRAIL-induced apoptosis of Jurkat cells, whilst in contrast, TRAIL blocked the anti-osteoclastogenic activity of OPG (Emery *et al.*, 1998). These data suggest that the mechanisms of potential cross-regulation may be mediated by OPG and TRAIL.

(b) Syndecan-1

Syndecan-1 (CD 138) is a heparin sulfate bearing integral membrane proteoglycan that is expressed and actively shed from the surface of most myeloma cells (Dhodapkar *et al.*, 1998; Saunders *et al.*, 1989). Syndecan acts as a matrix receptor on human myeloma cells by binding type I collagen *via* heparin sulfate chains, and it is thought to participate in regulating myeloma cell adhesion to the BM stromal cell matrix (Ridley *et al.*, 1993). Therefore, syndecan-1 may play a dual role in inhibiting the metastasis of tumour cells by promoting cell adhesion to the extracellular matrix and suppressing the proteolytic activity needed for invasion (Kaushal *et al.*, 1999; Liebersbach and Sanderson, 1994). It has been found that elevated level of syndecan-1 in serum is a strong and independent negative prognostic factor for patients with myeloma (Seidel *et al.*, 2000b). However, some studies show that syndecan-1 could induce apoptosis and inhibit the growth of myeloma tumour cells. In addition,

syndecan-1 increased OB development and inhibited OC formation in murine BM (Dhodapkar *et al.*, 1998). Since syndecan-1 can bind with heparin-binding proteins, like OPG or hepatocyte growth factor, it has been suggested that it may also regulate the biological activity of heparin-binding cytokines (Borset *et al.*, 2000). These data indicate that soluble syndecan-1 may participate in the pathology of myeloma induced osteolysis by modulating RANKL/RANK/OPG activity within the BM (refer to section 1.3.8).

(c) HGF

Hepatocyte growth factor (HGF) is a cytokine overproduced by myeloma cells and has implications for the prognosis of patients (Borset *et al.*, 1999). The biological significance of HGF overexpression in multiple myeloma is likely to include effects on bone turnover and angiogenesis (Borset *et al.*, 1999). HGF exists in a complex with soluble syndecan-1. Washing myeloma cells with purified soluble syndecan-1 was found to effectively displace HGF from the cell surface, suggesting that soluble syndecan-1 could act as a carrier for HGF *in vivo* (Seidel *et al.*, 2000a). HGF is only one of several heparin-binding cytokines associated with myeloma (Seidel *et al.*, 2000). The relationship between HGF and syndecan-1 and the effects of HGF on bone turnover requires further investigation.

In summary, it appears that RANKL and OPG will be a key agonist and antagonist cytokine system in regulating MM induced osteolysis (figure 1.2). Other molecules, such as IL-6, IL-1β, TNF-α, PTHrP and syndecan-1, secreted by MM cells may play a complex role in the RANKL/RANK/OPG system that regulates bone turnover in MM patients. The understanding of precise mechanisms responsible for myeloma-induced bone loss may enable us to understand how BPs may achieve clinical therapeutic effect in the patients with MM by affecting the molecules involved in the osteoclastic bone resorption.

1.4 BISPHOSPHONATES

1.4.1 General Properties

Bisphosphonates represent stable pyrophosphate analogues that differ from pyrophosphate by replacement of the oxygen bridge with a carbon atom to form a P-C-P structure which has various side chains (figure 1.3). These compounds were first synthesised in 1865 (for review see Rodan and Fleisch, 1996). In the early 1960s, Neuman and Fleisch discovered that inorganic pyrophosphate (PPi), a naturally occurring polyphosphate, was present in serum

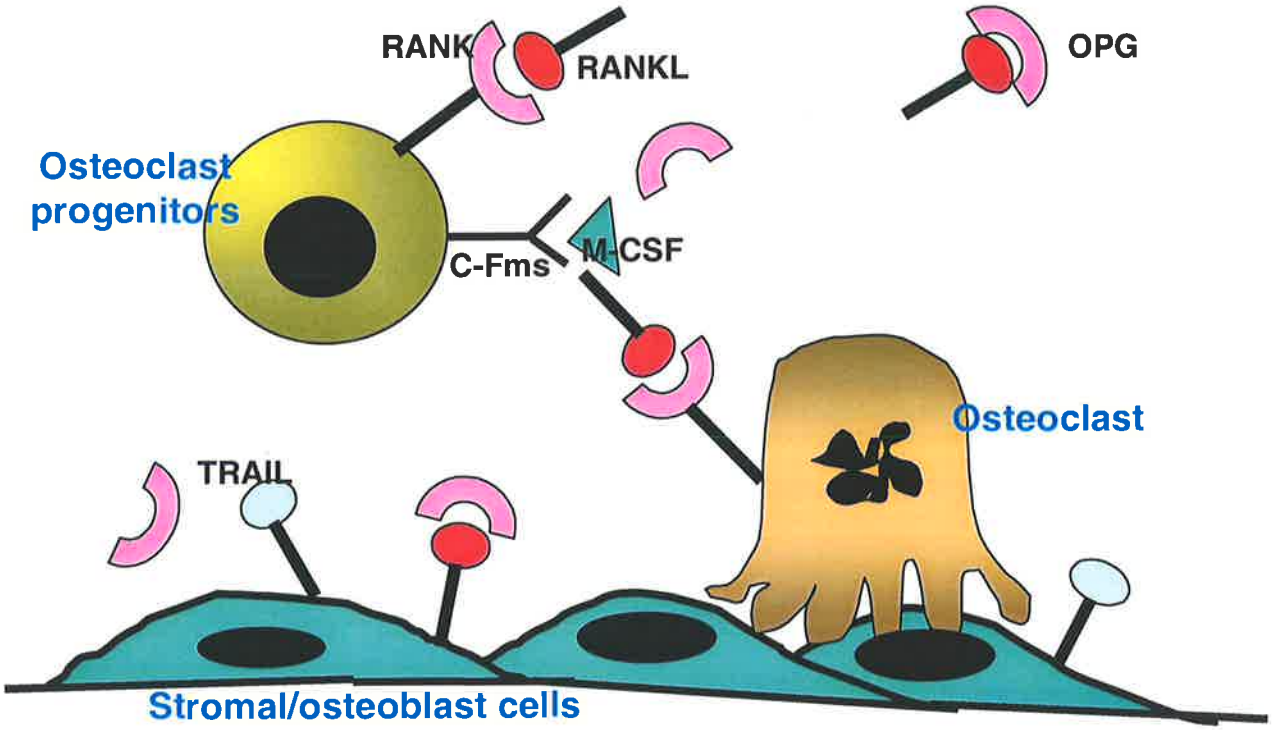


Figure 1.2. The role of the newly defined TNF-family members RANKL and OPG in osteoclastogenesis. RANKL promotes the differentiation of osteoclast precursor cells to osteoclasts via the RANK receptor expressed on osteoclast precursor cells. OPG acts as a decoy receptor that regulates the signalling between RANKL and RANK by binding to RANKL.

and urine, and could prevent calcification by binding to newly forming crystals of hydroxyapatite. BPs were found to be able to inhibit the dissolution of hydroxyapatite crystal and bone resorption both *in vitro* and *in vivo* (for review see Russell and Rogers, 1999). As a result, BPs have been developed and over the last three decades used in the treatment of a number of bone diseases, including Paget's disease, metastatic, osteolytic bone disease and hypercalcemia of malignancy.

BPs can be classified into at least two groups with different modes of action. BPs that closely resembles pyrophosphate (such as clodronate and etidronate) can be metabolically incorporated into nonhydrolysable analogues of adenosine triphosphate (ATP) that may inhibit ATP-dependent intracellular enzymes (figure 1.4). In contrast the more potent, nitrogen-containing BPs (such as pamidronate, alendronate, risedronate, ibandronate, tiludronate, incadronate and zoledronic acid) are not metabolized in this way, but serve to inhibit enzymes of the mevalonate pathway (figure 1.5), a biosynthetic pathway responsible for the production of cholesterol and isoprenoid lipids. Therefore nitrogen-containing BPs serve to prevent the biosynthesis of isoprenoid compounds that are essential for the posttranslational modification of small GTPases (refer to section 1.4.2).

The BPs, like pyrophosphate, bind to the bone mineral hydroxyapatite with high affinity, by virtue of the PCP structure, which accounts for their uptake in bone and their selective action on the skeleton. For example, it was demonstrated that the BP, alendronate, was rapidly eliminated from plasma and was not detectable 1 day after injection, whilst the concentration in bone was much higher than that in plasma. Approximately 30-40% of the dose was excreted in urine in 24 hours, whilst 60% of the dose was localised to the bone tissue; BPs that adsorbed to the bone remained as an unchanged compound for a long period of time (Lin *et al.*, 1991). Moreover, studies have demonstrated that BPs do not distribute uniformly in long bones (Azuma *et al.*, 1995; Sato *et al.*, 1991). The metaphysis of the tibia were found to possess a greater concentration than the diaphysis. Indeed, BPs are found to deposit where bone mineral is exposed to the surrounding fluids, especially where bone is formed and resorbed. It was reported that the distal femoral metaphyses of mice contained ~5% bone resorption surfaces, ~48% active bone formation surfaces, and the other surfaces were considered as resting and eroded surfaces (Sato *et al.*, 1991). Sato *et al.* reported that following injection of 0.4 mg/kg [^{3}H] alendronate to newborn rats, 72% of the osteoclastic

surfaces, 2% of the bone forming surfaces and ~13% of all other surfaces were densely labelled at day 1. In contrast, ~46% of the bone forming surfaces were found to be moderately labelled at day 1. It was assumed that the amount of alendronate in the densely labelled site was about five-fold higher than in the moderately labelled site. The lower level of [^{3}H] alendronate uptake on bone formation surfaces was thought to be due to the lower accessibility of alendronate to the hydroxyapatite embedded within the newly synthesised osteoid (Sato *et al.*, 1991). These observations suggest that bone is the specific binding organ for BPs, and bone resorption as well as bone formation surfaces are the major deposit sites for BPs. In addition, BPs bind to bone particles by strong pH dependence and 50 % of this drug would be released at pH 3.5 by virtue of the interaction of phosphate with hydroxyapatite (Sato *et al.*, 1991). Several studies showed that dense distribution of BPs was seen primarily under OC at day 1 following administration, but 5 days later, it was no longer observed under OC and was shifted from the metaphysis to the diaphysis (Azuma *et al.*, 1995; Sato *et al.*, 1991). These results imply that BP binding is strongly influenced by bone growth and, therefore, one may expect that BP treatment inhibits osteoclastic activity only at the level of the existing resorption sites. When new bone is laid down at sites of previous resorption, the BP remains buried and functionally excluded within the bone tissue. Therefore, as soon as the treatment is withdrawn, new remodelling sites will appear and hence a non inhibited bone turnover can be restored despite the extended physical presence of BPs in the skeleton. These observations may explain the need for repeated BP administration.

1.4.2 The Effect of Bisphosphonates on Osteoclasts

Since mature, multinucleated OC are formed by the fusion of mononuclear precursors of haemopoietic origin, BPs can inhibit bone resorption in the following ways: (1) Inhibition of OC recruitment to the bone surface (Hughes *et al.*, 1989; Schmidt *et al.*, 1996); (2) Inhibition of the excavation of resorption pits by mature OC directly (Murakami *et al.*, 1995; Sato and Grasser, 1990; van Beek *et al.*, 1997); (3) Shortening of the OC life span (Hughes *et al.*, 1995); (4) Secretion of an inhibitor of OC-mediated resorption by OB (Vitte *et al.*, 1996); and (5) Interfering with the attachment of OC to certain bone matrix proteins *via* cell-surface integrins (Colucci *et al.*, 1998).

It is likely that BPs are selectively internalised by OC because of their accumulation in bone and the phagocytic nature of OC. The uptake of bisposphonate by OC *in vivo* has been

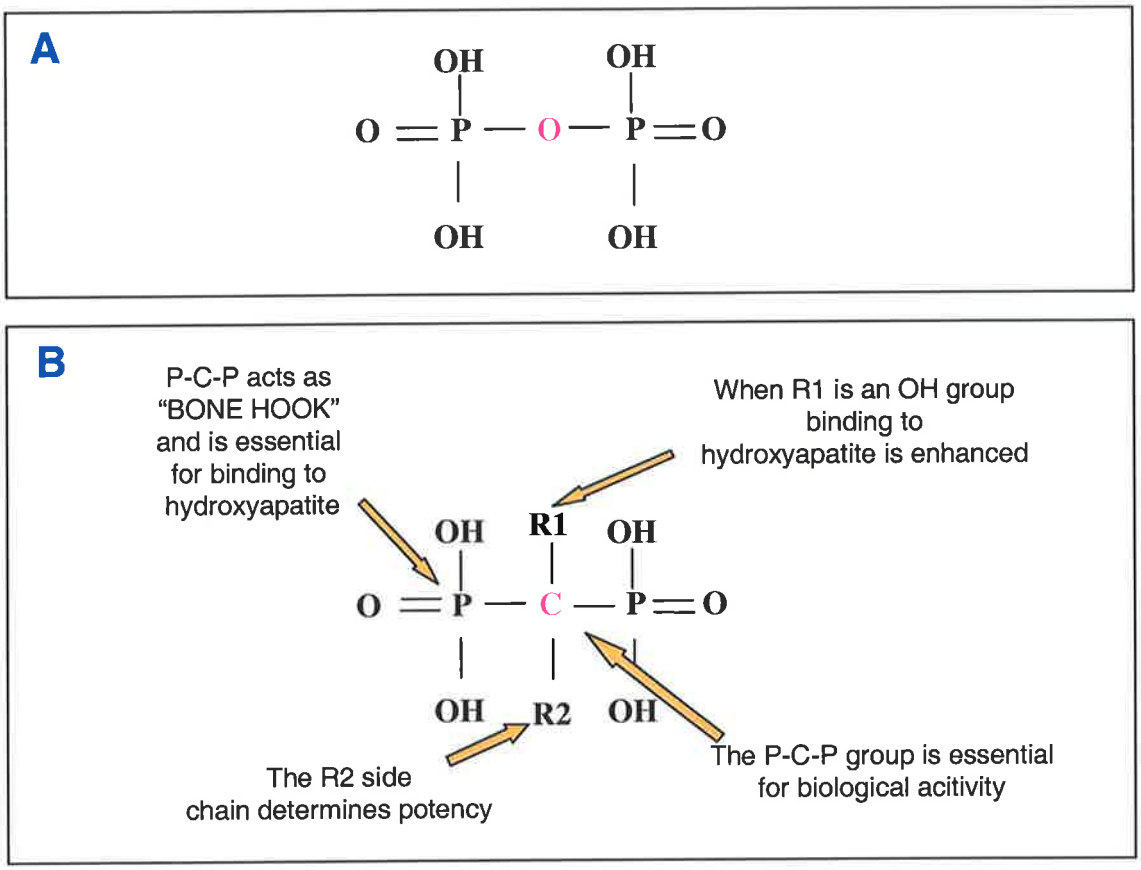


Figure 1.3. Chemical structure of a generic bisphosphonate. (A) Inorganic pyrophosphate (PPi). (B) Structure of a bone-active bisphosphonate, highlighting functional domains.

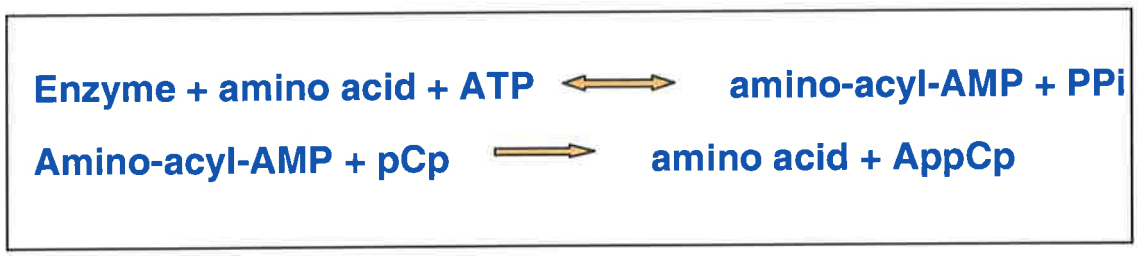


Figure 1.4. A BP that closely resembles pyrophosphate can be metabolically incorporated into nonhydrolysable analogues of ATP that may inhibit ATP-dependent intracellular enzymes.

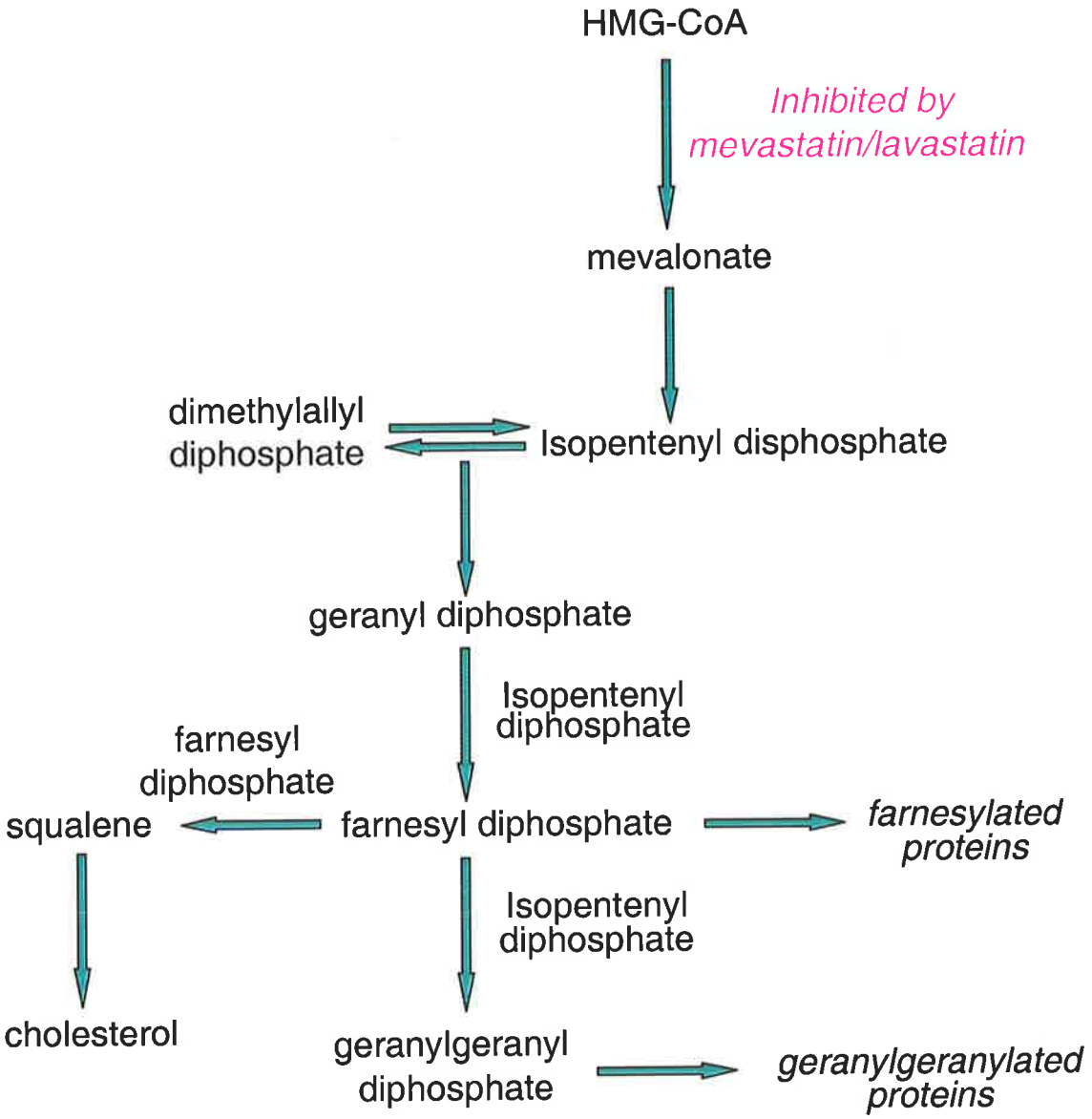


Figure 1.5. Schematic representation of the mevalonate pathway indicates sites of action of BPs.

confirmed using radiolabeled alendronate. These studies demonstrate that following internalisation, aledronate was localised to the intracellular vacuoles, and other subcellular compartments such as the cytoplasm, mitochondria, and nuclei (Masarachia *et al.*, 1996; Sato *et al.*, 1991). After cellular uptake, a characteristic morphological feature of BP treated OC is the lack of a ruffled border (Sato and Grasser, 1990). It was reported that the presence of alendronate on the bone surface *in vivo* and *in vitro* interfered with ruffled border formation in OC by causing an increase in the leakiness of ruffled border to ions that stopped bone resorption (Sato *et al.*, 1991). During the resorption process the low concentration of BPs in extracellular fluids can rise to a very much higher local concentration in the OC resorption lacuna. Sato *et al* (1991) demonstrated that acidification (Zimolo *et al.*, 1995) produced during the initiation of resorption caused the local release of alendronate with the concentration of 0.1-1.0 mM under the ruffled border of OC (Sato *et al.*, 1991). This is much higher than the concentrations of BPs required to affect OC morphology and cause OC apoptosis *in vitro* (Hughes *et al.*, 1995; Sato and Grasser, 1990). Breuil *et al* (1998) demonstrated that in a human model, inhibition of resorption by alendronate was mediated primarily by reduction of OC activity and OC number at very high concentrations (Breuil *et al.*, 1998).

One possible molecular mechanism of BPs activity on OC is based on its ability to inhibit some of the enzymes involved in the pathway for cholesterol synthesis. Two groups of enzymes were shown to be inhibited by BPs: squalene synthetase, a lipid metabolism enzyme which has farnesyl pyrophosphate as its substrate (Ciosek *et al.*, 1993), and protein tyrosine phosphatases (PTP), which control tyrosine phosphorylation involved in signal transduction pathways (Schmidt *et al.*, 1996; Endo *et al.*, 1996).

Inhibition of geranylgeraniol PPi (GGPP) synthase has been implicated in the induction of apoptosis following BP treatment. GGPP is required for the prenylation and subsequent membrane localisation of proteins, particularly small GTP proteins of the Rho/Rac/Rab family. The Rho/Rac/Rab family of proteins are involved in a number of cellular processes including assembly of the actin cytoskeleton, vesicular trafficking, signal transduction and cell adhesion (Hall, 1998; Zerial and Stenmark, 1993). BPs inhibit the farnesylation and geranylgeranylation of proteins, by inhibiting enzymes required for synthesis of farnesyldiphosphate (FPP) and geranylgeranyldiphosphate (GGPP), and therby disrupt OC function. Several studies have demonstrated that alendronate and tibudronate disrupt the

formation of actin rings in polarised, resorbing OC (Murakami *et al.*, 1995; Sato *et al.*, 1991). The ability to inhibit GGPP syntheses appears to be restricted to the nitrogen-containing BPs, suggesting that different BPs may have distinct targets. Disruption of the cytoskeleton also could be brought about indirectly by the inhibition of protein kinases or phosphatases that regulate cytoskeletal structure. Indeed, several recent reports have shown that alendronate can inhibit several protein tyosine phosphatases (PTP), which determine the level of tyrosine phosphorylation and play an important role in the transduction of signals that control cell growth and differentiation (Endo *et al.*, 1996; Schmidt *et al.*, 1996). It is likely that BPs inhibit metabolic pathways that firstly affect OC function (eg. *via* disruption of the OC cytoskeleton and ruffled border) and therefore cause OC cell death as a later effect (figure 1.6). Differences probably also exist between the ability of different BPs to cause apoptosis, depending on their molecular mechanism of action.

Previous studies of direct actions of BPs on bone have been limited, mainly to their effects on bone-resorbing OC cells. Although several studies have demonstrated that the inhibition of osteoclastic bone resorption induced by BPs may be mediated indirectly through paracrine factors produced by the bone-forming OB cells, little is known about the direct actions of BPs on the important bone-forming OB.

1.4.3 The Effect of Bisphosphonates on Osteoblasts

It has been established that the osteoblastic cells are important in the formation of tartrate-resistant acid phosphatase-positive multinucleated cell ($TRAP^+$ MNC) induced by $1,25(OH)_2 D_3$ and PTH in mouse marrow cultures (Takahashi *et al.*, 1988) and in co-culture of spleen cells with osteoblastic cells (Takahashi *et al.*, 1988a). Therefore, it is postulated that direct cell-cell interaction is crucial in the process of osteoblastic cell-supported OC formation and maturation (Kurihara *et al.*, 1989). Thus, BPs may act *via* OB to inhibit bone resorption, by stimulating OB to produce osteoclast-inhibitory factors, or by differentiating OB progenitor cells into mature and functional OB.

It is now generally accepted that cells of osteoblastic lineage control the recruitment and activity of OC under physiological and pathological conditions. The inhibitory effect of BPs on osteoclastic bone resorption might be mediated, at least in part, through the regulation of the production of cytokines secreted by OB. The first experimental evidence to support this

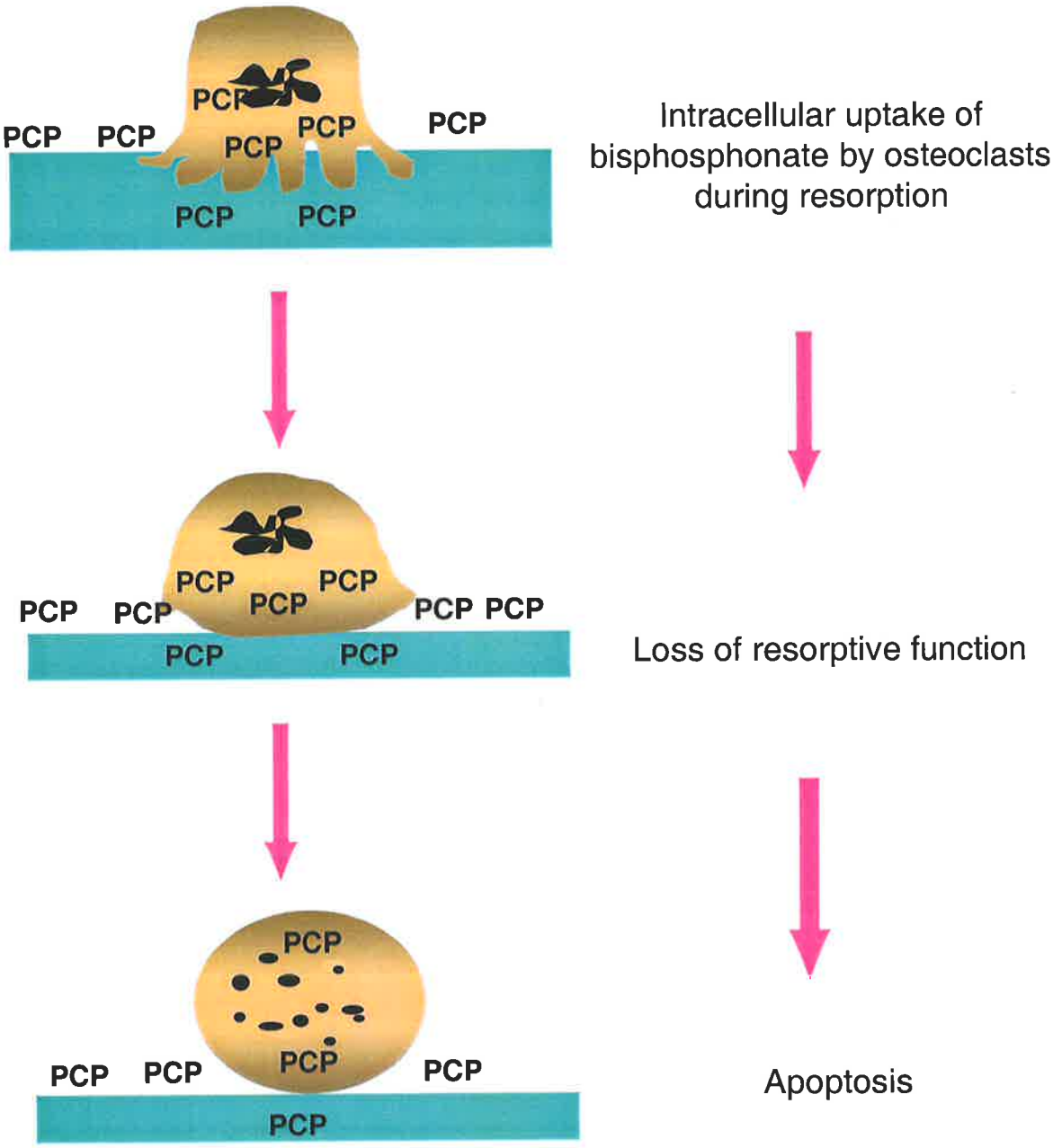


Figure 1.6. The mechanism of bisphosphonates action on bone-resorbing osteoclasts.

notion comes from studies which demonstrate that BPs can inhibit the synthesis of prostaglandin E_2 (Ohya *et al.*, 1985) and osteocalcin (Stronski *et al.*, 1988) in murine osteoblastic cells. It was also reported that BPs could inhibit IL-6 secretion by human OB-like cells in a dose-dependent manner (Giuliani *et al.*, 1998b). Recently, it has been shown that conditioned media taken from rat CRP 10/30 osteoblastic cells briefly (5min) (Sahni *et al.*, 1993) or continuously (Nishikawa *et al.*, 1996) exposed to low concentrations of potent BPs contains a factor(s) that reduces osteoclastic bone resorption in culture. The decrease in resorption was subsequently found to be due to the secretion from OB of a protein with a molecular weight between 1 and 10 kD, which was able to inhibit OC recruitment and survival (Nishikawa *et al.*, 1996; Vitte *et al.*, 1996). Another study has shown that the OB–like cell line UMR-106 significantly reduces its PTH-induced pit formation when pre-treated with BPs in co-culture with rat OC. Furthermore, conditioned media from UMR-106 cells pretreated with ibandronate was found to reduce bone resorption when added to cultures of rat OC. This effect however was abrogated by culturing ibandronate-pretreated UMR-106 cells in 10^{-8} M PTH before harvesting the conditioned media. It is apparent that osteoclastic bone resorption can be affected independently by the ibandronate-induced osteoclast-inhibiting factor and the PTH-induced osteoclast-stimulating factor, but the final level of bone resorption depends on the relative concentration of these two factors (Yu *et al.*, 1996). However, a contradictory finding showed that a low concentration of the nitrogen-containing BPs APD and Me_2APD act synergistically with PTH and increase osteoclastic resorption, possibly also through interference with factor(s) produced by osteogenic cells (van der Pluijm *et al.*, 1991a).

Further studies have demonstrated that BPs can inhibit the secretion of molecules such as prostaglandin E_2, osteocalcin, and IL-6 by OB-like cells (Giuliani *et al.*, 1998b; Ohya *et al.*, 1985; Stronski *et al.*, 1988). Pamidronate and zoledronic acid were found to inhibit the production of IL-6 by HBSCs at concentration of 1 μmol/L or less (Derenne *et al.*, 1999). A statistically significant increase of TNF-α and a mild increase of IL-6 in serum are observed in patients receiving BP treatment, with the greatest effects seen with the highest concentration of both pamidronate and zoledronic acid. No changes in IL-1 were observed with any agents. These increased cytokine production, which are demonstrated to be transiently produced by macrophages and monocytes, are considered as an acute-phase response associated with BPs (Thiebaud *et al.*, 1997).

Like pyrophosphate, BPs can affect mineral dissolution. Previous data indicate that BPs have a negative effect on the mineralisation of bone matrix at a very high dose, with osteomalacia and defective mineralisation reported (Adamson *et al.*, 1993). Impaired mineralisation associated with the first generation BPs, etidronate, has led to the development of more potent second and third generation compounds, in which anti-resorptive efficacy is dissociated from mineralisation effects. It has now been demonstrated that the low concentrations of BP, and especially the newer more potent ones necessary to produce a pharmacological effect, have no significant impact on mineral dissolution. It is notable that concentrations of BPs as low as 10^{-11} M have an effect on OB. Animal and clinical studies show that BP treatment markedly decreases trabecular bone turnover with normal mineralisation (Balena *et al.*, 1993; Chavassieux *et al.*, 1997; Storm *et al.*, 1993). Furthermore, other investigators have also described an enhancement of OB function with BPs (Giuliani *et al.*, 1998a; Tenenbaum *et al.*, 1992; Toolan *et al.*, 1992; Tsuchimoto *et al.*, 1994). In one study, the BP-induced formation of CFU-F did not show a clear dose dependency but rather an "on-off effect". There was also a biphasic effect on the formation of bone-like nodules, with a dose-dependent stimulation at lower concentrations and an inhibitory effect at higher concentrations (Giuliani *et al.*, 1998a). The differences in efficacy and in effects on mineralisation among different BPs require further investigation at a molecular, cellular and physiological level.

It is not yet clear how BPs access the OB *in vivo*. One possibility is that they come directly from the general circulation after their administration. A more likely explanation, consistent with their stability and long duration of action, is that they are released from bone into the microenvironment of OB, either by passive diffusion or during resorption. The actual concentration ranges of the BPs that OB and other cells in the body are exposed to under pharmacological conditions are unpredictable. In one study, at pharmacologically effective doses, ^{3}H-alendronate labelled eightfold more OC surface than OB surface. In contrast, ^{3}H-etidronate labelled approximately equal fractions of OC and OB surface. Therefore, alendronate, at pharmacological active doses, showed higher uptake on resorption versus formation surfaces than etidronate. However, the extent of bone formation on surfaces containing alendronate or etidronate was similar to that of controls (Masarachia *et al.*, 1996). Therefore, the differences in the distribution of these different drugs in the skeleton cannot explain the differences in efficacy and effects on mineralisation among different BPs.

Proliferation and differentiation of OB or preosteoblast are essential steps in bone formation. It has been demonstrated that pamidronate and zoledronic acid decreased OB proliferation but enhanced the differentiation and bone-forming activities of OB (Reinholz *et al.*, 2000). Furthermore, BPs also have been reported to inhibit apoptosis of the murine osteocyte cell line MLO-Y4 and primary murine OB (Plotkin *et al.*, 1999). The underlining mechanisms however remain unclear. It was known that agents such as prostaglandins, PDGF, FGF, TGFβ and bone morphogenetic proteins were shown to stimulate OB proliferation *in vitro* and *in vivo* (Canalis *et al.*, 1993). The receptors for these proteins are all protein kinases. Therefore, the finding that BPs inhibit the PTP activity of a novel receptor type protein tyrosine phosphatase sigma (PTPσ) and stimulate the proliferation of quiescent calvaria OB (1-10 μM) supports the notion that PTP activities play an important role in the regulation of cellular proliferation (Endo *et al.*, 1996). However, the concentrations of circulating BPs do not reach the concentrations required to inhibit PTPσ in OB. But it is possible that other PTPs that are not yet identified or tested are more sensitive to BPs. The increase in bone mass which is produced by BP treatment may not be fully explained by inhibition of bone resorption. Therefore, the finding that BPs inhibit the activity of PTP expressed in OB and stimulate the proliferation of these cells could provide additional clues regarding the action and molecular targets of BPs.

1.4.4 The Effect of Bisphosphonates on Myeloma Cells

In a randomised, double blind, phase III study, the second-generation BP, pamidronate, prevented bony complications in the patients with active Durie-Salmone stage III MM. Skeletal-related events, including the incidence of pathologic fracture, the use of radiotherapy to skeletal sties of disease, bone pain, episodes of hypercalcemia, and the need for narcotic analgesia, were all significantly reduced in pamidronate-treated patients (Berenson *et al.*, 1996). Similar results were found with clodronate in MM patient in another clinical trial (McCloskey *et al.*, 1998). There was also a significant prolongation of survival from 14 to 21 months in the patients who were on salvage chemotherapy receiving pamidronate (Berenson *et al.*, 1998). These survival advantages suggest that BPs not only decrease morbidity and mortality associated with bone events but also may induce an anti-tumor effect resulting in a longer survival rate. In support of these observations, several *in vitro* studies have shown that BPs such as pamidronate and incandronate can decrease cell proliferation and induce cytostasis and apoptosis in some human myeloma cells (Aparicio *et al.*, 1998; Shipman *et al.*, 1997). The third generation BP, YM529 at a concentration of 50 μM, also induced inhibition

of proliferation in all MM cell lines dose-dependently (Takahashi *et al.*, 2001). A synergistic induction of apoptosis was demonstrated when myeloma cell lines were exposed to a combination of zoledronic acid and Dex (Tassone *et al.*, 2000). In addition, it was reported that the BP, incadronate, caused apoptosis of myeloma cells *in vitro* by inhibiting the mevalonate pathway (Shipman *et al.*, 1998). Bisphosphonate-induced apoptosis has also been demonstrated in breast cancer cells. The osteolytic bone lesions induced by metastatic MDA-231 breast carcinoma cells, as well as tumour burden in a mouse model of breast cancer, could be reduced by risedronate (Sasaki *et al.*, 1995). These effects are considered to be the results of both decreased osteoclastic bone resorption and inhibited release of bone-derived tumour growth factors. However, although the potent BP ibandronate did not reduce tumor burden, it significantly reduced the occurrence of osteolytic bone lesions in myeloma-bearing mice (Cruz *et al.*, 2001; Dallas *et al.*, 1999; Shipman *et al.*, 2000). Despite these promising *in vitro* and animal model studies, there is no direct evidence to support the notion that that BPs induce apoptosis of myeloma cells in patients with MM.

Overall, these observations suggest that BPs might have some benefit not only on the improvement of bone disease but also on the prolongation for survival in some myeloma patients.

1.4.5 The Effect of Bisphosphonates on Cell Adhesion

There is also evolving evidence that BPs may act by inhibiting integrin-mediated adhesion. As described in section 1.1.3, molecules that mediate cell adhesion molecules, such as laminin and E-cadherin, play key roles in several important events involved in cancer cell invasion and metastasis. Cell adhesion molecules (CAMs) mediate not only cell-to-cell but also cell-to-substratum communications. The most abundant CAMs are integrins. Integrins have been shown to mediate attachment of cancer cells to vascular endothelial cells and to matrix proteins such as laminin and FN.

A large cohort of adhesion molecules modulating adhesion between myeloma cells and stromal cells have been defined (Tricot, 2000; Uchiyama *et al.*, 1993; Vidriales and Anderson, 1996). Preliminary data suggest that BPs modulate adhesion molecule profile and thereby overcome drug resistance (refer to Section 1.1.2). Adhesion assays using bone slices pretreated with alendronate, at the established active concentration, showed that, although the morphology of OC plated onto pretreated bone slices was not modified, the number of

adherent OC was reduced about 50% compared with controls. The inhibitory effect of alendronate on cell adhesion may be due to the interference with receptors specifically recognising bone matrix proteins, such as $\alpha_v\beta_3$ integrins (Colucci *et al.*, 1998). For instance, FN can be recognised not only by $\alpha_5\beta_3$, but also by $\alpha_v\beta_3$, and bone sialoprotein (BSP) acts as an adhesion molecule with which tumour cells establish contact with the bone matrix. Van der Pluijm *et al* (van der Pluijm *et al.*, 1996) showed that bone slices that were incubated with BPs were resistant to the adhesion molecules of tumour cells. Another study showed drastically reduced adhesion properties of breast and prostate carcinoma cell lines when the cells were pretreated with BPs (Boissier *et al.*, 2000).

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases with proteolytic activity for a large range of components of the extracellular matrix, are also known to play a critical role in bone remodelling and tumour invasion. Members of the MMP family are divided into three classes based on their substrate specificity: collagenases, gelatinases, and stromelysins. Interstitial collagenase (MMP-1) is the only protease beside neutrophil collagenase that is known to initiate the degradation of types I collagen at neutral pH. Collagen I is the major structural protein of bone and osteoid layer and the degradation of collagen I is critical for the initiation of bone resorption. After fragmentation by MMP-1, the denatured collagen I becomes a substrate for the gelatinases MMP-2 and MMP-9. It has been reported that myeloma cells express MMP-9 and induce BM stromal cells to express MMP-1 and MMP-2 (Barille *et al.*, 1997). An increase in MMP-1 levels reflects an enhanced capacity to degrade collagen I. The conversion of proMMP-2 into its active form will actively contribute to the final collagenolytic activity by achieving the degradation of collagen I initiated by MMP-1. The beneficial effects of the BPs on the metastatic process may be related to inhibition and downregulation of various genetically distinct MMPs (MMP-2, MP-9, MMP-13, and membrane type MMPs (MT-MMPs)) that are obligatory for successful tumor cell invasion, and bone destruction at a metastatic site (Teronen *et al.*, 2000).

In bone metastases, it is conceivable that BPs released from resorbed bone inhibit the proteolytic activity of MMPs secreted from tumor cells and stromal cells. It has been demonstrated that the inhibitory activity of BPs on tumor cell invasion requires the R_2 chain of the molecule, which is effective at low concentrations. Inhibition of MMP activity by BPs

occurs however through the so-called "bone hook" of the molecule, which is effective only at high concentrations (Boissier *et al.*, 2000).

The many different actions of BPs suggest that inhibition of bone resorption can be due to the overlap of several different actions ocurring together to exert their final effect.

More potent third-generation BPs are now available and have been shown to be 100-1000 times more potent than pamidronate, a commonly used BP in the treatment of MM. These new BPs may therefore represent a better treatment option for patients with myeloma. Bisphosphonates containing a hydroxyl group at the R1 side chain and a tertiary nitrogen within a ring structure in the R2 side chain appear to be the most potent anti-resorptive BPs discovered to date, including zoledronic acid, which contains an imidazole ring.

1.4.6 Zoledronic Acid

Zoledronic acid (formerly referred to zoledronate), CGP 42' 446 [2-(imidazol-1-yl)-1-hydroxyethylidene-1,1-bisphosphonate], is a heterocyclic imidazole, nitrogen-containing third-generation BP (figure 1.7). Among the clinically tested BP compounds, it is the most potent amino-substituted BP, which appeares to be 850 times more active than pamidronate in the thyroparathyroidectomized (TPTX) rats with hypercalcemia induced by 1, 25-$(OH)_2$ vitamin D3, and 40-100 times more potent than pamidronate in inhibiting calcium release induced by vitamin D_3, parathyroid hormone, parathyroid hormone-related protein, or recombinant human interleukin-1β from mouse calvaria *in vitro* (Green *et al.*, 1994). Unlike the first generation BPs, zoledronic acid produces potent and specific inhibition of bone resorption at low dose level without any significant detrimental effects on bone growth and mineralization. Although both pamidronate and zoledronic acid cause a dose-dependent suppression of cancellous bone turnover and resorption, and augment cancellous bone formation in a rat model, zoledronic acid is 100 times more potent than pamidronate *in vitro* and 850 times more active than pamidronate *in vivo* (Pataki *et al.*, 1997). Recent clinical trials show that very small doses of zoledronic acid can effectively normalise high calcium levels in patients with tumour-induced hypercalcemia (Body *et al.*, 1999). In addition, zoledronic acid has the largest therapeutic ratio of any BPs tested in terms of the desired inhibition of bone resorption versus unwanted renal toxicity and inhibition of bone mineralisation (Arden-Cordone *et al.*, 1997; Body, 1997; Buckler *et al.*, 1999). It was also demonstrated that a short duration, single intravenous bolus injection of zoledronic acid was

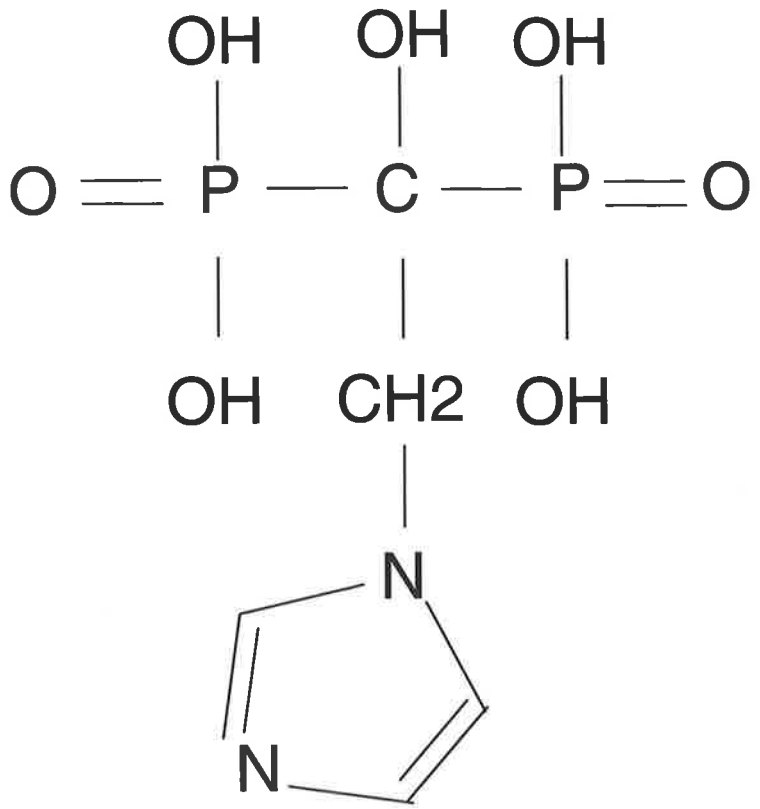


Figure 1.7. Chemical structure of zoledronic acid. Zoledronic acid is a nitrogen-containing heterocyclic imidazole bisphosphonate. It is 100 times more active than pamidronate *in vitro* and 850 times more active than pamidronate *in vivo*.

safe and well tolerated, and effectively suppressed bone resorption in cancer patients with metastatic bone disease, in a phase I clinical study (Berenson *et al.*, 2001a; Berenson *et al.*, 2001b). Furthermore, a phase II trial demonstrated that zoledronic acid (4mg and 8mg) was superior to pamidronate (90mg) in normalising serum calcium in cancer patients with moderate to severe hypercalcemia (Major *et al.*, 2001).

In addition to its highly potent effects on bone resorption, several preclinical studies suggest that zoledronic acid exhibits anti-tumor activity. *In vitro* studies demonstrate that zoledronic acid not only inhibits the proliferation of breast carcinoma cells (Fromigue *et al.*, 2000) and prostate cancer cells (Lee *et al.*, 2001), but also induces cytostasis and apoptosis of myeloma cells (Aparicio *et al.*, 1998). Zoledronic acid also may interfere with the process of metastasis at very low concentrations. It has been demonstrated that zoledronic acid at concentrations of 10^{-12}-10^{-6} M, inhibits the ability of human prostate and breast carcinoma cells to invade the extracellular matrix *in vitro*, without inducing apoptosis and inhibiting tumour cell migration. However, apoptosis is induced at a higher concentration (10^{-4} M) (Boissier *et al.*, 2000). These preliminary results suggest that zoledronic acid may prove to be a powerful drug in reducing tumour burden and increasing patient survival, and as such warrants further investigation.

1.5 HYPOTHESES

- BPs and TRAIL act synergistically to enhance myeloma cell death.

- BPs disrupt the integrin-mediated cell adhesion of myeloma cells to Stromal/OB cells leading to myeloma cell death.

- BPs act *via* OB to inhibit bone resorption, by stimulating or inhibiting OB to produce osteoclast-inhibitory or osteoclast-activating factors, respectively.

- BPs possess anabolic activity and are capable of differentiating OB progenitor cells into mature and functional OB.

1.6 AIMS AND EXPERIMENTAL RATIONALE OF THIS PROJECT

- To examine the mechanism(s) of BP- and TRAIL-induced myeloma cell death.

- To examine if BPs reduce bone resorption by acting on OB cells as well as OC cells.

- To examine if BPs can induce new bone formation.

1.7 SIGNIFICANCE OF PROJECT

The osteolytic lesions in MM represent one of the major clinical manifestations of the disease. The new RANKL/RANK/OPG cytokine system and OC activating factors play an important role in the normal bone remodelling and pathological bone resorption including MM induced osteolysis. Numerous studies have shown that BPs provide great benefits to MM patients including improved life quality and prolonged life expectation. Better understanding of the mechanism of actions of these compounds on osteolysis in cancer patients provides a new means by which the mechanism of tumour metastasis can be understood. Furthermore, it also enables us to obtain a greater understanding of normal bone remodeling biology as well as its precise mechanisms of action.

This project is centred on investigating two aspects of BP activity, and include (1) finding a new drug combination to overcome multiple drug resistance in MM patients, and (2) understanding the normal biology of OB and the underlying mechanism of BPs on osteoblastic bone formation.

We anticipate that this study will provide some novel and valuable insights into the molecular and cellular mechanisms responsible for the clinical effectiveness of BPs on the skeletal complications associated with MM.

CHAPTER 2
MATERIALS AND METHODS

2.1. SOLUTIONS, BUFFERS AND MEDIA

2.1.1 Sodium Pyruvate

Stock solutions of 100 mM sodium pyruvate (100×) were prepared by dissolving 1.1 g of sodium pyruvate powder (α-Ketopropionic Acid; 2-Oxopropanoic Acid, Sigma, USA, Cat. No. P-5280) in 100 ml Milli-Q water. The solution was filter sterilised and stored at 4°C.

2.1.2 2-Mercaptoethanol (2-ME)

A 1M stock solution of 2-ME was prepared by diluting 0.7 ml of 14.27 M β-mercaptoethanol (Sigma, St. Louis, MO, USA, Cat. No. M-3148) into 9.3 ml Hank's Balanced salt Solution (HBSS, Infectious Diseases Laboratories, IMVS) and was stored at -20°C. The 1 M stock solution was further diluted 1:10 (in HBSS) to prepare a 0.1 M working solution. This was filter sterilised and stored as 500 µl aliquots at -20°C.

2.1.3 PI/Triton X-100 Staining Solution with RNAse A

The solution was prepared freshly by adding 0.2 mg/ml of DNAse-free RNAse A (Roche Diagnostics, Castle Hill, NSW, Australia, Cat. No. C 109169), 20 µg /ml of propidium iodide (PI, Sigma Chemical Co. Ltd, St Louis, MO, USA), and 0.1% (v/v) Triton X-100 (ICN Biomedicals Inc, Ohio, USA, Cat. No. 807423) to PBS.

2.1.4 Fluorometric Caspase 3 Assay Buffer

The fluorometric caspase 3 assay buffer (pH 7.4) was prepared by adding 1.2% (w/v) HEPES, 10% (w/v) sucrose (BDH AnalaR®, Victoria, Australia), and 0.1% (w/v) CHAPS (3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (Pierce, Ukkinios, USA) to MilliQ-water.

2.1.5 Blocking Buffer for Flow Cytometric Analysis

HBSS was supplemented with 0.4% (w/v) bovine serum albumin, 4%(v/v) normal human serum (NHS; Red Cross, Adelaide, South Australia), 5%(v/v) foetal calf serum (FCS; CSL Limited, Victoria, Australia, Cat. No. 09702301) and penicillin (50 i.u./ml)/streptomycin

sulphate (50 μg/ml)(CSL Biosciences, Victoria, Australia, Cat. No. 05081901). The buffer was filter sterilised through a 0.22 μm bottle filter (Nalge Nunc International Corp, USA) and stored at 4°C.

2.1.6 Flow Cytometry Fixative (FACS Fix)

To 1 litre of PBS, 10 ml of formaldehyde (40% w/v, ACE Chemical Company, Adelaide, Australia), 20 g of D-glucose (BDH AnalaR®, $C_6H_{12}O_6$, Victoria, Australia), and 0.2 g of sodium azide (Sigma, USA, Cat.No. S-8032) were added.

2.1.7 Double-Strength Iscove's Modified Dulbecco's Medium (2×IMDM)

To prepare 500 ml of 2×IMDM (Cytosystems, Castle Hill, NSW, Australia, Cat. No. 50-016-PA), one sachet (17.67g) of IMDM powder was dissolved in Milli-Q water. To this, sterile stocks of penicillin, streptomycin, DEAE-Dextran (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden, Cat. No. 170350-01) and L-asparagine (Sigma, Cat. No. A-0884) were added at concentrations of 200 i.u./ml, 200 μg/ml, 5.2 mg/ml and 0.4 mg/ml, respectively. Filter sterilised medium was stored as 50 ml aliquots at -20°C or used immediately.

2.1.8 10%(w/v) Bovine Serum Albumin (BSA)

To prepare 10%(w/v) BSA, 20 g BSA (CSL Limited, Victoria, Australia, Cat. No. 06711701) was carefully overlayed on the surface of Milli-Q water (88.4 ml), and was allowed to sit at 4°C for 24 hours. Once dissolved, 3 g of AG® 501-X8 (D) Resin (Bio-Rad Laboratories, CA, USA, Cat. No. 142-6425) was used to deionise the BSA and to remove trace elements and contaminants. The beads were removed by filtration through Whatman No. 1 paper (Whatman Laboratory Division, England) when beads had changed colour from green to yellow. The procedure was repeated 3 times. An equal volume (100 ml) of 2× IMDM (or 2×PBS) was added to the BSA and sterilised by filtration through a 0.22 μm bottle filter, prior to storage at -20°C.

2.1.9 Dulbecco's Modified Eagles Medium-10 (DMEM-10)

To prepare 500 ml of DMEM-10, Dulbecco' Modified Eagles Medium (JRH Biosciences, USA, Cat. No. 51444-500M) was supplemented with penicillin (50 i.u./ml)/streptomycin sulphate (50 μg/ml), 10% (v/v) foetal calf serum (FCS, CSL Limited, Victoria, Australia, Cat. No. 09702301), 2 mM of glutamine (JRH Biosciences, USA, Cat. No. 59202-100M), 1 mM of sodium pyruvate, 15 mM of HEPES buffer (JRH Biosciences, USA, Cat. No. 59205-

100M / TRACE Scientific LTD, Melbourne, Australia, Cat. No. 21-115-0100V), and was buffered with 1 g of sodium bicarbonate (Infectious Diseases Laboratories, IMVS). The medium was subsequently filter sterilised and stored at 4°C. The medium was replenished with 2mM L-glutamine at weekly intervals.

2.1.10 Alpha Minimal Essential Medium-10 (α-MEM-10) for Osteoblast-like Cell Culture

To prepare 500 ml of single strength α-MEM-10, 410 ml of α-MEM (JRH Biosciences, USA, Cat. No. 51451-500M) was supplemented with penicillin (50 i.u./ml)/streptomycin sulphate (50 μg /ml), 10% (v/v) FCS, 2 mM of glutamine, 1 mM of sodium pyruvate, 15 mM of HEPES buffer, 100 μM L-ascorbate-2-phosphate (WAKO pure chemical industries, LTD, Japan, Cat. No. 013-12061), and buffered with 1 g of sodium bicarbonate. The medium was subsequently filter sterilised and stored at 4°C. The medium was replenished with 2 mM L-glutamine at weekly intervals.

2.1.11 Alpha Minimal Essential Medium-10 (α-MEM-10) for Myeloma Cell Culture

To prepare 500 ml of single strength α-MEM-10, 415 ml of α-MEM was supplemented with penicillin (50 i.u./ml)/streptomycin sulphate (50 μg /ml), 10% (v/v) FCS, 2 mM of glutamine, 1 mM of sodium pyruvate, 15 mM of HEPES buffer, 0.45% (w/v) D-glucose (BDH AnalaR®, $C_6H_{12}O_6$, Victoria, Australia) and buffered with 1 g of sodium bicarbonate. The medium was subsequently filter sterilised and stored at 4°C.

2.2 CELL CULTURE

2.2.1 Human Myeloma Cell Lines

Myeloma cell lines RPMI 8226, ARH-77, and U266 were purchased from the American Type Culture Collection. WL2 was kindly provided by Dr. Doug Joshua, Royal Prince Alfred Hospital, Sydney). All the myeloma cell lines were cultured in α–MEM-10 supplemented with glucose. The cells were cultured at a cell density of 2×10^5 cells/ml and the medium was changed every 2 to 3 days. Cells were cultured at 37°C in the presence of 5% CO_2.

2.2.2 Murine Cytokine-Dependent Myeloid Cell Line, FDC-P1

The murine cytokine-dependent myeloid cell line, FDC-P1, was cultured in DMEM-10 supplemented with 100 U/ml of murine granulocyte macrophage-colony stimulating factor

(GM-CSF; which was kindly provided by Dr. Thomas J. Gonda, Hanson Institute). Cells were cultured at 37°C in the presence of 5% CO_2.

The cells were washed with DMEM-10 thrice and cultured in DMEM-10 without GM-CSF. Following starvation for 24 hrs, the cells were harvested and prepared as a positive control for apoptosis and intranucleosomal fragmentation analysis (refer to section 2.3.5).

2.2.3 Normal Human Bone Cells (NHBC)

Trabecular bone specimens were obtained from osteoarthritis patients, during routine knee and hip replacements, from the Department of Orthopaedic Surgery and Trauma at the Royal Adelaide Hospital. Bone chips were cultured in T-75 tissue-culture flasks in α-MEM-10 supplemented with 100 μM L-ascorbate-2-phosphate. Medium was changed two times per week for 5-6 weeks, and cultures were incubated at 37° C in the presence of 5% CO_2 until confluent.

2.2.4 Normal Osteoblast Donor (NOD)

Normal OB-like cells (NOD's) were derived from young healthy donors as part of an institutional ethics approved Normal Bone Marrow Donor Program. Briefly, the donated bone marrow was strained using a cell strainer (Becton Dickinson, USA) and the small bone chips were washed through with HHF (HBSS supplement with 5% FCS) and cultured in α-MEM-10 supplemented with 100 μM L-ascorbate-2-phosphate. Medium was changed twice weekly for 5-6 weeks, and cultures were incubated at 37° C in the presence of 5% CO_2 until confluent.

2.2.5 Enzymatic Digestion of Osteoblast-like Cell Cultures

Single-cell suspensions were obtained from confluent primary osteoblast-like cell cultures by enzymatic digestion. The cells were washed twice in PBS, and then digested in a 2 ml mixture of collagenase (1 mg/ml, Worthington Biochemical Corporation, NJ, USA) and dispase (1 mg/ml, Boehringer Mannheim, Cat. No. 165859) for 45 mins at 37°C. The detached cells were transferred to a 50 ml Falcon tube, following a wash in 10 ml PBS. The remaining adherent cells were treated with Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4 Na, GIBCO Invitrogen Corporation, Canada, Cat. No. 15400-054) for 5 mins at 37° C. After washing with HHF, cells were mixed and pelleted by centrifugation at 1,400 rpm for 5 mins and used in experiments outlined below.

2.2.6 *Cryopreservation of Cells*

Cells were cryopreserved in FCS containing 10% (v/v) of the cryoprotectant, dimethyl sulphoxide (DMSO, BDH AnalaR® Merck Pty Limited, Victoria, Australia). Immediately prior to freezing, 0.5 ml of sterile freeze mix (20% (v/v) DMSO in FCS) was added dropwise with mixing, to 0.5 ml of FCS containing 5-10 × 10^6 cells. The cell mixture was kept on ice and immediately transferred to cryoampoules (Greiner Labortechnik) for liquid nitrogen storage (-196° C).

2.2.7 *Thawing Cryopreserved Samples*

Following removal from liquid nitrogen storage, cryoampoules were rapidly thawed at 37°C, and the cells were transferred to a 14 ml polypropylene round-bottom tube (Becton Dickinson, NJ, USA). Dropwise, 3 ml of appropriate medium was added, and then the sample was made up to a final volume of 10 ml. Cells were pelleted by centrifugation at 1,400 rpm for 5 minutes, and subsequently washed twice in medium to remove residual DMSO. Finally, cells were resuspended in appropriate growth medium and cultured in T75 flask (Greiner Bio-one, Germany) at 37°C in the presence of 5% CO_2.

2.3 *EFFECT OF ZOLEDRONIC ACID ON THE CELL GROWTH*

2.3.1 *Reagents Used in This Study*

Zoledronic acid was kindly provided by Dr Kevin Lynch (Novartis Pharmaceuticals). Recombinant soluble human TRAIL (Peprotech, Rocky Hill, NJ, USA) and TNFα were kindly provided by Drs. Andreas Evdokiou and Pu Xia, respectively (Hanson Institute).

2.3.2 *Cell Proliferation Assay*

2.3.2.1 *Dose Dependent Effects of Zoledronic Acid on Myeloma Cells*

Different myeloma cell lines were seeded in 96-well plates (Nalgene Nunc International, Denmark), at a density of 1×10^4 cells per well in 100 µl of α-MEM-10. Cells were cutured with zoledronate at different concentrations for 3 days. The relative number of viable cells in each well was determined on day 3 using the colorimetric assay reagent WST-1 (Roche Molecular Biochemicals, Cat. No. 1644807). Briefly, 10 µl of WST-1 was added to each well, including three wells containing only medium, to enable background subtraction. WST-1 is a tetrazolium salt that is cleaved to formazan by cellular enzymes. An expansion in the

number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenase in the sample that leads to an increase in the amount of formazan dye. After a 2.5-hour incubation at 37°C in the dark, the colour of the medium turned to orange, which was proportionate to the degree of cell viability and cell number. The absorbance of dye solution was measured directly with a plate reader (BIO-RAD Model 3550 microplate reader, CA, USA) using the test wavelength of 450 nm. Different treatment groups were performed in triplicate and the results were compared against controls using the Student's *t*-test.

2.3.2.2 Dose Dependent Assay of TRAIL on Myeloma Cells

Myeloma cell lines were seeded in 96-well plates as described in section 2.3.2.1. Alternatively, the cells were treated with recombinant soluble human TRAIL at different concentrations. On day 3, the cell number and viability were assessed using WST-1, as described above.

2.3.2.3 Cell Proliferation Assay of Osteoblast-like cells Treated with Zoledronic Acid

Osteoblast-like cells from three different donors (two NHBCs and one NOD) were seeded into 96-well plates using the ACDU facility of the FACStar cell sorter at a cell density of 4000 cells/well and incubated at 37°C for 24 hrs in α-MEM culture medium. The medium was then replaced with 100 µl/well fresh medium containing various concentrations of zoledronic acid. The cell number was assessed using WST-1 on day 3, as described before.

Alternatively, when exploring the effect of zoledronic acid on the growth of osteoblast-like cells, STRO-1^+ and STRO-1^- cells were sorted directly into the 96-well plates at a cell density of 4000 cells/well, and cell proliferation assay was performed as discribed above.

2.3.2.4 Cell Proliferation Assay Examing the Effects of Zoledronic Acid and TRAIL on Myeloma Cells

Myeloma cell lines were seeded into 96-well plates as described in section 2.3.2.1. The cells were exposed to both TRAIL and zoledronic acid. The RPMI 8226 cell line was treated with zoledronic acid at concentrations from 0.01 to 25 µM and TRAIL at constant concentration of 30 ng/ml. The concentration of zoledronic acid used for the ARH-77, WL2 and U266 cell lines was from 0.1 to 50 µM with 100 ng/ml of TRAIL. The cell number was assessed on day

3 and day 5 by using WST-1, as described previously. The experiment was performed twice with triplicate well samples.

2.3.3 Time Course Experiment of Zoledronic Acid on Myeloma Cells

Myeloma cells in exponentially grown phase were seeded into 48-well plates at an initial concentration of 5×10^4/ml. Zoledronic acid was added at the specified concentrations (10 μM for ARH-77, RPMI 8226 and Balm, 50 μM for WL2 and U266). At each time point after treatment, the cells were harvested and counted in triplicate using a haemocytometer. The viability was determined by trypan blue dye exclusion. Two separate counts were performed on each sample and the results were compared against controls using Student's *t*-test.

2.3.4 Analysis of Nuclear Morphology

To assess the effect of zoledronic acid on cell apoptosis, myeloma cells were seeded into 96-well plates at the cell density of 1×10^4 cells per well and treated with 0, 5 and 25 μM zoledronic acid for 48 hrs, respectively. The cells were cytospun onto slides using a Shandon cytospin 2 (500 rpm for 5 min), air dried over night and fixed in FACS fix for 10 mins at room temperature. The cells were stained with 1 μg/ml DAPI (4'-6-diamidino-2-phenyl-indole, Sigma, USA) in PBS for 1-2 mins and examined with the fluorescence microscope. Apoptotic cells were defined on the basis of the characteristic changes in nuclear morphology, including condensation of chromatin to produce crescent shapes around the periphery of the nucleus and apoptotic bodies.

Alternatively, osteoblast-like cells were cultured in 8-chamber slides (Nalge Nunc International, Denmark) and treated with 5 μM zoledronic acid. The cells were centrifuged before being fixed with FACS fix for 10 mins. The cells were stained with DAPI and assessed using fluorescence microscopy as described above.

2.3.5 Agarose Gel Analysis of Intranucleosomal Fragmentation

The osteoblast-like cells from different donors were detached by trypsin and washed once with cold PBS. The cell viability was determined using typan blue exclusion. The cell pellets were treated with lysis buffer (10 μl/10^6 cells with a minimum of 50 μl) for 10 seconds: 1% (v/v) NP 40 (Nonidet®P 40, Fluka, BioChemika), 20 mM EDTA, 50mM Tris-HCl (pH 7.5) in MilliQ-water. The nuclei were pelleted by centrifugation at 1,600 ×g for 5 mins. The

supernatant was collected and the nuclei were resuspended in 50µl of lysis buffer. The suspension was brought up to final concentration of 1% SDS (5µl of 10% SDS for 50 µl) and treated for 2 hrs with RNAse A (5µg/µl) at 56°C, and subsequently digested with Proteinase K (PK, 2.5 µg/µl, Sigma, Cat. No. P 2308) for 2 hrs at 37°C. This was followed by the addition of 0.5 volumes of 10 M ammonium acetate, and the DNA was precipitated with 2.5 volumes of absolute ethanol. The reaction was incubated on ice for 10 mins, and centrifuged at 12,000×g at 4°C for 15 mins. The DNA pellet was air dried and then dissolved in loading buffer. The DNA ladder was separated by electrophoresis on 1% (w/v) agarose gel and visualised with SYBR Gold (Quantum Scientific, Cat. No. S-11494) staining.

2.3.6 Cell Cycle Analysis

Untreated and zoledronate-treated myeloma cells (10 µM for RPMI 8226 and 25 µM for WL2) were harvested and washed twice with PBS, and then fixed in 70% (v/v) cold ethanol for at least 2 hrs at 4°C. After fixation, the cells were pelleted by centrifugation at 1100 rpm for 5 mins. The cells were washed once with PBS and stained with 1 ml of PI/Triton X-100 staining solution (containing RNAse) for 30 mins at room temperature (or at 37°C for 15 mins) in the dark. Cell cycle distribution was determined by analysing 10,000 events on a Excel flow cytometer (Beckman Coulter) using the red fluorescence of excited propidium iodide-stained nuclei as a measure of DNA content. Linear displays of fluorescence emissions were fitted to cell cycle distribution analysis by use of the MODFIT program for MAC V2.0. The degraded sub- G_0/G_1 DNA content was quantitated and considered characteristic of an apoptotic cell.

2.3.7 Caspase Activity Assay

Caspase-3 activity was measured by determining the degradation of the fluorometric substrate DEVD (Ac-Asp-Glu-Val-Asp-AFC), which contains the amino acid sequence of the caspase-3 cleavage site in poly (ADP-ribose) polymerase (PARP), conjugated with AFC. Cells were lysed in caspase lysis buffer (5 mM Tris-HCl [pH7.4], 5 mM EDTA [pH 7.6], 0.5% NP 40) by incubating on ice for 20 mins with shaking. Protein concentration was measured using Bicinchoninic Acid (Pierce, Illinios, USA). Lysates (15 µg protein) were incubated with 200 µl of caspase activity assay mixture (Fluorometric caspase 3 assay buffer [pH 7.4] supplemented with 10 mM DTT and 9 µM caspase-3/ CPP 32 substrate [Ac-DEVD-AFC, Kamiya biomedical company]) for 4 hours at room temperature in the dark. The

released fluorescent molecule AFC (7-amino-4-trifluoromethyl coumarin) was measured in a microplate fluorescence reader FL 500 (Bio Tek Instruments, Winooski, Vermont USA) with emission wavelength of 505 nm.

2.4 DETECTION OF GENE EXPRESSION IN CELLS

2.4.1 TRIzol™ Isolation of Total RNA

Total RNA was extracted from 5-10×10^6 cells by adding 1 ml of TRIzol™ (Life Technologies, Cat/ No. 15596-026). Washing cells before addition of TRIzol reagent was avoided as this increases the possibility of mRNA degradation. RNA was solubilised by passing the lysate through a pipette several times. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. To each tube, 0.2 ml of chloroform (APS Finechem, A division of Asia Pacific Specialty Chemicals Limited CAN, NSW, Australia) was added per 1 ml of TRIzol Reagent. The tubes were shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2 to 3 mins. The samples were centrifuged at 12,000×g for 15 minutes at 4° C to separate the mixture into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA that remained exclusively in the aqueous phase was carefully removed and transferred to a fresh 1.5 ml eppendorf tube (Scientific Specialties Incorporated Life Technologies Australasia, Cat. No. 1220-00). The total RNA was then precipitated by the addition of 0.5 ml of isopropyl alcohol (BDH Chemicals, Victoria, Australia, Cat. No. 10224) per 1 ml of TRIzol Reagent used for the initial homogenization. Following incubation for 10 mins at room temperature, the RNA was pelleted by centrifugation at 12,000 × g for 10 mins at 4°C. The RNA pellet was washed once with 75% (v/v) ethanol by adding at least 1 ml of 75% ethanol per 1 ml of TRIzol Reagent used for the initial homogenization. The samples were mixed by vortexing and were centrifuged at 7,500 × g for 5 mins at 4° C. At the end of the procedure, the RNA pellet was briefly air-dried at 37° C for 5-10 mins. RNA was resuspended in RNase free diethylpyrocarbonate (DEPC, Sigma Aldrich Ltd, Cat. No. D 5768)-treated Milli-Q water, and to facilitate RNA solubility, the samples were incubated for 10 minutes at 55 to 60° C. The concentration of RNA was determined by spectrophotometry, and the integrity of the RNA was determined by 1% (w/v) agarose gel electrophoresis. RNA was stored at −80°C or used immediately for cDNA synthesis.

2.4.2 *Determination of RNA Concentration*

The concentration of RNA in solution was determined by measuring the absorption at 260 nm on a Beckman UV spectrophotometer (Beckman Instruments, Mt. Waverley, Victoria, Australia), assuming that an A_{260} of 1.0 represents 40 μg/ml of RNA. Alternatively, the sample was electrophoresed in agarose gels and the intensity of the sample's ethidium bromide-stained bands was compared with the intensities of bands containing RNA standards of known concentration.

2.4.3 *Reverse Transcription (RT) Polymerase Chain Reaction (PCR) Amplification of DNA*

2.4.3.1 Synthesis of Complementary DNA (cDNA)

Total cellullar RNA from cell lines or tissues was prepared with TRIzol, as described in section 2.4.1. The following components were added to a nuclease-free microcentrifuge tube: 1 μl random hexamers (Geneworks, Cat. No. RP-6) at the concentration of 250 ng/μl, 1μg of total RNA and RNase free DEPC-treated Milli-Q water to 12 μl. The mixture was heated at 70°C for 10 mins and quickly chilled on ice. The contents of the tube was collected by brief centrifugation and was then added to a mixture containing 4 μl of 5× first strand buffer, 2 μl of 0.1M DTT, and 2 μl of 5 mM dNTP Mix (5 mM each dATP, dGTP, dCTP and dTTP at neutral pH). The contents of the tube were mixed gently and incubated at room temperature for 10 mins. One μl (200 units) of SUPERSCRIPT II (Gibco Life Tech, Cat. No. 18064-014) was added and mixed by pipetting gently up and down. The mixture was incubated at 42°C for 50 mins and the reaction was inactivated by heating at 70°C for 10 mins. The cDNA was then used as a template for amplification in PCR.

2.4.3.2 Polymerase Chain Reaction (PCR) Amplification of cDNA

cDNA was amplified by PCR to generate products corresponding to mRNA encoding the gene products. One μl of each cDNA synthesis reaction was ustilised as template DNA in each PCR reaction. Routinely, the cDNA mixture was added to a 200 μl microcentrifuge tube, to which 2 μl of 10 × PCR amplification buffer (10 × PCR buffer: 670 mM Tris HCl pH 8.8, 166 mM $(NH_4)_2SO_4$, 4.5% Triton-X 100, 2 mg/ml gelatin), 1.2 μl of 25 mM $MgCl_2$, 2 μl of 2 mM dNTP mixture, 100 ng each of the appropriate sense and antisense primers (table 1), 0.2 μl (1 Unit) of Amplitaq DNA Polymerase (Applied Biosystems, Roche, Switzerland),

and DEPC-treated water were added to a final volume of 20 µl. Amplitaq Gold is a "hot start" DNA polymerase that has an inactivating protein modification that only allows the enzyme to become active at high temperatures. Amplification was achieved by incubation in a Corbett Research thermal cycler. DNA was amplified under the following typical cycling conditions: denaturation at 94°C for 1 minute, annealing at the temperatures suitable for different primers for 1 minute, extension at 72°C for 1 minute for 20-40 cycles, with a final 10 minutes extension performed at 72°C, such that all products were assayed in the exponential phase of the amplification curve. Following amplification, the PCR products (10 µl of each reaction mixture) were separated on 2% (w/v) agarose gel according to molecular weight, and visualised by SYBR Gold staining under UV light at 570 nm.

2.4.4 Primers Used in This Study

All the primers used in these studies are displayed in table 1. The cycle numbers were determined by cycle course analysis, and annealing temperatures were determined empirically using AmpliTaq Gold in a gradient thermal cycler.

2.5 IMMUNOFLUORESCENCE STAINING AND FLOW CYTOMETRY

Flow cytometry was performed using an Epics®-XL-MCL analyser (Beckman Coulter). Cells to be analysed were immunostained with primary antibodies that recognise specific antigens of interest. Cell populations were analysed on the basis of their forward and side light scattering properties, which is indicater of cell size and cell density, respectively.

2.5.1 One-Colour Flow Cytometric Analysis

Cells were harvested from culture (2×10^5 cells per condition), and pelleted by centrifugation at 1,400 rpm for 5 mins at 4°C. Prior to immunolabelling, cells were incubated in blocking buffer at a concentration of 5×10^6 cells/ml, and were incubated on ice for 30 mins. For each condition, aliquots of 2×10^5 cells were incubated with 50 µl of antibody or isotype-matched control (used as culture supernatants or purified mAbs at a final concentration of 10 µg/ml). After a 45 minute incubation at 4°C, the cells were washed twice in chilled HHF to remove unbound antibody, and the pellets were resuspended in 50 µl HHF containing 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE) conjugated goat anti-mouse Ig (Southern Biotechnology Associates Inc. Birmingham, AL, USA). Following

Table 1: RT-PCR primers and conditions for the specific amplification of human mRNA.

Target Gene	Sense/ Antisense (5'-3') Primer Sequences	Annealing temperature (°C)	Cycle number	Product Size	Ref.
GAPDH	CACTGACACGTTGGCAGTGG/ CATGGAGAAGGCTGGGGCTC	60	20-22	417	1
M-CSF	CAGTTGTCAAGGACAGCAC/ GCTGGAGGATCCCTCGGACTG	58	35	670	2
IL-1β	AGGAAGATGCTGGTTCCCTCTC/ CAGTTCAGTGATCGTACAGGTGC	60	30	151	1
IL-6	ATGAACTCCTTCTCCACAAG/ GTGCCTGCAGCTTCGTCAGCA	62	30♣ 40♣♣	546	2
TNF-α	TCAGATCATCTTCTCGAACC/ CAGATAGATGGGCTCATACC	62	35	361	2
RANKL (4/5)	AATAGAATATCAGAAGATGGCACTC/ TAAGGAGGGGTTGGAGACCTCG	62	35♣ 38♣♣	668	2
RANKL (7/5)	AACAGGCCTTTCAAGGAGCTG/ TAAGGAGGGGTTGGAGACCTCG	62	35♣ 38♣♣	538	2
OPG	TGCTGTTCCTACAAAGTTTACTG/ CTTTGAGTGCTTTAGTGCGTG	62	30	435	2
CBFA-1	GTGGACGAGGCAAGAGTTTCA/ TGGCAGGTAGGTGTGGTAGTG	62	30	632	3
BSP	TCAGCATTTTGGGAATGGCC/ GAGGTTGTTGTCTTCGAGGT	62	30	657	3
OCN	ATGAGAGCCCTCACACTCCTC/ CGTAGAAGCGCCGATAGGC	62	30	289	3
Cox2	AACACAACAGAGTATGCGATGTG/ CAATGGAAGCCTGTGATACTTTC	62	30♣ 34♣♣	437	4
TRAIL	ACAGCAGTCAGACTCTGACAGG/ CATGGTCCATGTCTATCAAGTGC	62	35	838	5
DR4	TGCTGCAGCTCGTACCTAGCTC/ TTGCTGCTCAGAGACGAAAGTGG	66	34	646	5
DR5	CTGCAACTGTGACTCCTATG/ GTCTGCTCTGATCACCCAAC	66	34	424	5
DcR1	TCCTAGCTTACTCTGCCACCACT/ CACAATTAGAACTATGATCCCTAC	66	34	700	5
DcR2	TTCTCATGGGACTTTGGGGGACAA/ CTGTTACTCAGGGTCTCGTTGC	66	34	855	5
TACE	AAGAGTGTGATCCTGGCATC/ CATCCTGTACTCGTTTCTCAC	60	30	483	6

♣ Osteoblast-like cells ♣♣ Myeloma cell lines

1. Atkins *et al.*, 2000b
2. Atkins *et al.*, 2000a
3. Gronthos *et al.*, 1999
4. Genebank accession number NM_000963
5. Evdokiou *et al.*, 2002
6. Genebank accession number XM_015606

incubation with secondary antibody for a further 45 mins at 4°C, cells were washed as above and fixed in FACS fix. Typically, for each sample 10,000 events were analysed on the flow cytometer, and stored as list mode data for further analysis using WinMDI software (Windows Multiple Document Interface Flow Cytometry Application, 1993-1998 Joseph Trotter).

Alternatively, when cells were stained with rabbit anti human antibodies (OCN and RANKL), a 1:200 dilution of RDF (Sheep Anti-Rabbit Immunoglobulin F $(ab)_2$ Fraction Fluorescein conjugated, AMRAD Biotech, Victoria, Australia, Cat. No. 984141020) was used as a secondary antibody.

2.5.2 Intracellular Antigen Detection

Single cells were blocked in blocking buffer for 30 mins on ice and then washed twice in PBS. After fixing in 1% (w/v) paraformaldehyde (BDH Chemical Ltd, England) in PBS for 20 mins at room temperature, cells were washed twice by HHF containing 0.1% saponin (w/v, Sigma Cat. No. S-4521). The cells were resuspended with 50 µl of culture supernatant or purified mAbs at a final concentration of 10 µg/ml (diluted in HHF containing 0.1% saponin). Following a 60 min incubation at 4°C, the cells were washed twice in HHF (containing 0.1% saponin), and resuspended in wash medium containing a 1:50 dilution of goat anti-mouse IgG (γ-chain specific)-FITC and a goat anti-mouse IgM (µ-chain-specific)-PE. After a further incubation at 4°C for 45 mins, the cells were washed as described above and then fixed in FACS fix prior to analysis.

2.5.3 Two-Colour Flow Cytometric Analysis

The single cells were incubated in blocking buffer for 30 mins. For each condition, aliquots of 2×10^5 cells were incubated with 100 µl of STRO-1 mAb supernatant and 100 µl of B4-78 at the concentration of 10 µg/ml for 1 hour on ice. The isotype matched negative control antibodies, IgG_1 (1B5) and IgM (1A6.12) were used under identical conditions. The cells were then washed in HHF and incubated with a goat anti-mouse IgG–FITC (1:50) and a goat anti-mouse IgM-PE (1:50) for 45 mins on ice. The cells were then washed twice and resuspended in 300 µl of FACS fix prior to analysis.

2.5.4 *Analysis of Cell Cycling Status of STRO-1$^+$ Cells by Ki-67 Reactivity*

Single cell suspension of osteoblast-like cells was stained with STRO-1 using a goat anti-mouse IgM-PE conjugated second label as described in section 2.5.1. After staining, the cells were washed twice with ice cold PBS and then fixed with cold ethanol (70%) for 10 mins on ice. Following this, the cells were washed thrice with PBS and then incubated in blocking buffer for 15 mins. The monoclonal antibody FITC-conjugated Ki-67 (DAKOPATTS a/s, Denmark) was added directly to the cells (1/10 dilution in blocking buffer) for 45 mins on ice. The FITC conjugated mouse IgG_1 negative control antibody was used under the same conditions. After washing and fixation, the cells were analysed by dual-colour flow cytometric analysis.

2.5.5 *Three-Colour Flow Cytometric Analysis*

Single cell suspension of osteoblast-like cells were dual stained with ALP and STRO-1 using a goat anti-mouse IgG-FITC and IgM-PE conjugated second label as described in section 2.5.3. After staining, the cells were fixed in 0.5% (wv) paraformaldehyde in PBS (2×10^6 cells/ml) for 30 mins at 4°C. Then cells were washed once in TPBA (PBS supplement with 0.1% Trixton 100, 0.1% BSA and 0.1% sodium azide) and resuspended in 500 µl TPBA containing 5µg RNAse. The cells were incubated at 37° C for 20 mins and washed once by TPBA. The cells were resuspended in 500 µl PBA (PBS supplement with 0.1% BSA and 0.1% sodium azide) containing 5 µg/ml 7-aminoactinomycin D (7-AAD, Molecular Probes, Netherlands, Cat. No. A-1310) and stored in dark at 4°C until ready to analyse (at least 1 hr after staining).

2.5.6 *Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Labelling of Cells*

The cell-permeant fluorescein-based dye CFSE covalently attaches to cytoplasmic components of cells, resulting in uniform bright fluorescence. Upon cell division, the dye is distributed equally between daughter cells, allowing the resolution of up to eight cycles of cell division by flow cytometry. This technique was used to determine division-related phenotypic and functional change during differentiation of osteoblast-like cells.

The osteoblast-like cells were detached as described previously. The cells were washed once and resuspended in 1 ml of PBS/0.1% BSA, and 2 µl of 5 mM CFSE (final 10 µM) was added to the cells and incubated at 37°C for 10 mins. The staining was quenched by adding 5

volumes of ice cold culture media (α-MEM supplemented with 10% FCS), followed by incubation on ice for 5 mins. The cells were washed three times in the culture medium and *in vitro* cultures were established as described below.

At appropriate time points, the cells were detached by Trypsin-EDTA and were stained with STRO-1 or ALP mAbs coupled with PE as described above. Dual-colour flow cytometric analysis was performed to analyse samples.

2.5.7 Fluorescence-Activated Cell Sorting (FACS)

After detachment, approximately $1x10^7$ osteoblast-like cells were pelleted in 14 ml polypropylene tubes and resuspended in 2 ml (100 µl/ 5×10^5 cells) of a saturating concentration of STRO-1 supernatant for 60 minutes on ice. The monoclonal mouse isotype control Ig M (1A6.12) was used under identical conditions. The cells were then washed in HHF and incubated with a goat anti-mouse IgM–PE (1/50) in a final volume of 1 ml (50 µl/ 5×10^5 cells) for 45 minutes on ice. The cells were resuspended to approximately 10^6 cells/ml and passed through a cell strainer to obtain a single cell suspension prior to analysis using FACStar^{PLUS} flow cytometer (Becton Dickinson, Mountain View, CA, USA). Positive fluorescence for STRO-1 staining was defined as the level of fluorescence greater than 99% of the corresponding isotype-matched control antibody.

STRO-1 positive and negative cells were collected and cultured in the presence/absence of different treatments. Single cell deposition was performed using the ACDU facility of the FACStar^{PLUS} cell sorter and 4000 single cells were sorted directly into the appropriate medium in 96-well plates for cell proliferation assays.

2.5.8 Antibodies Used in This Study

All the antibodies used in this study are listed in the table 2.

2.6 CELL ADHESION ASSAYS

2.6.1 Calciene Labelling of Non-Adherent Cells.

Untreated and zoledronate (5 µM) treated myeloma cells were washed twice and resuspended in 500 µl cell adhesion buffer (IMDM-0.2%BSA). Ten µM calciene-AM (stock 1 mM in

Table 2: List of Antibodies Used in This Study

Antibody Name	Specificity	Isotype	Source
B4-78	anti-human bone/liver/kidney isoform of ALP	mIgG$_1$	DSHB[1]
STRO-1	Erythroid, Stromal cells, CFU-F, Endothelial cells	mIgM	DSHB
LF32	Osteoclacin (OCN), Bone-associated matrix protein	rIg G	Dr. L. Fisher[2]
Ki-67	Cell cycle antigen (G_1, S, M, &G_2)	mIg G$_1$	DAKO[3]
TRANCE	TNF-related activation induced cytokine	mIgG$_{2b}$	R&D[4]
RANKL	Receptor activator of nuclear factor-κB ligand	mIg G	Santa Cruz[5]
6G10	vascular cell adhesion molecule-1 (VCAM-1), CD106	mIg G$_1$	Dr. B. Masinovski[6]
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand	mIg G$_1$	R&D
DR4	h TRAIL-R1, Death-signalling receptor of TRAIL	mIgG$_{2a}$	R&D
DR5	h TRAIL-R2, Death-signalling receptor of TRAIL	mIg G$_1$	R&D
DcR1	h TRAIL-R3, Antagonist decoy receptors of TRAIL	mIg G$_1$	R&D
DcR2	h TRAIL-R4, Antagonist decoy receptors of TRAIL	mIg G$_1$	R&D
1B4.B12	β_2 microglobulin (MHC class I α subunit)	mIg G$_1$	Dr. L. Ashman[7]
HLA Class I	Membrane-bound glycoproteins expressed on the surface of nearly all nucleated cells	mIgG$_{2a}$	DAKO
β_2 microglobulin	Subunit of HLA Class I	mIg M	PharMingen[8]
61.2C4 (β1)	CD29, Integrin β1subunit	mIg G$_1$	Dr. J. Gamble[9]
8A2 (β1)	CD29, Integrin β1subunit(mAb shown to activate β1 integrin function)	mIg G$_1$	Drs. J. Harlan and N. Kovach[10]
1998 (α2β1)	CD49b/CD29, very later antigen (VLA-2α), integrin α2	mIg G$_1$	Chemicon[11]
1992 (α3β1)	CD49c/CD29, VLA-3α, integrin α3	mIg G$_1$	Chemicon

Table 2: List of Antibodies Used in This Study, Continued.

Antibody Name	Specificity	Isotype	Source
P4C2 (α4)	CD49d/CD29, VLA-4α, integrin α 4	mIg G_1	Dr. L. Ashman
1999 (α5β1)	CD49e/CD29, VLA-5α, integrin α 5	mIgG_{2a}	Chemicon
PHM2 (α5β1)	CD49e/CD29, VLA-5α, integrin α 5	mIg G_1	Prof. R. A. Atkins[12]
4F10 (α6β1)	CD49f/CD29, VLA-6α, integrin α 6	mIgG_{2b}	Serotec[13]
1997 (αvβ3)	Integrin αvβ3	mIg G_2	Chemicon
23 C6	Integrin αvβ3	mIg G_1	Dr. L. Ashman
CD11a	Leucocyte Function Associated-1 antigen (LFA-1), alpha chains	mIg G_1	Immunotech[14]
H9H11	Phagocytic glycoprotein-1 (Pgp-1), CD44	mIg G_1	Dr. P. Simmons[15]
CD45	Leucocyte Common Antigen (LCA)	mIg G_1	Becton Dickinson[16]
1H4	Intracellular Cell Adhesion Molecule-1 (ICAM-1), CD54	mIgG_{2b}	Dr. L. Ashman
CD56	Natural killer cell, N-CAM, NKH1	mIgG_{2b}	Immunotech
CD58	LFA-3	mIgG_{2a}	Dr. L. Ashman
CD138	Syndecan-1, a heparin sulfate bearing integral membrane proteoglycan	mIg G_1	Immunotech
1B5	Isotype-matched negative control/ α-Giardia	mIg G_1	Dr. G. Mayrhofer[17]
1D4.5	Isotype-matched negative control/α-Salmonella	mIgG_{2a}	Dr. L. Ashman
1A6.11	Isotype-matched negative control/α-Salmonella	mIgG_{2b}	Dr. L. Ashman
α-MAP	Multiple antigenic peptide(MAP, HBV 21-47 pre S_1)	rIg G	Dr. G. Atkins[18]
1A6.12	Isotype-matched negative control/α-Salmonella	mIg M	Dr. L. Ashman

DSHB[1]: Developmental Studies Hybridoma Bank, University of Iowa, IA, USA

Dr. L. Fisher[2]: Craniofacial and Skeletal Disease Branch, National Institute of Dental and Craniofacial Research, National Institute of Health, bethesda, maryland, USA

DAKO[3]: DAKAOPATTS A/S, Glostrup, Denmark

R&D[4]: R&D Systems Inc. Minneapolis, MN, USA

Sants Cruz[5]: Santa Cruz Biotechnology, Inc. Santa Cruz, California, 95060, USA

Dr. B. Masinovski[6]: FCOS Corp., Seattle, WA, USA

Dr. L. Ashman[7]: School of Biomedical Science, The University of Newcastle, NSW, Australia

PharMingen[8]: BD Biosciences, San Diego, California, USA

Dr. J. Gamble[9]: Dept. Human Immunology, Hanson Institute, I.M.V.S, Adelaide, South Australia

Drs. J. Harlan and N. Kovach[10]: University of Washington, Seattle, USA

Chemicon,CA[11]: ChemIcon Inc. Pittsburgh, PA, USA

Prof. R. A. Atkins[12]: Monash Medical Centre, Melbourne, Victoria, Australia

Serotec[13]: Serotec, Oxford, England

Immunotech[14]: Bio Online, Inc. Berkeley, CA, USA

Dr. P. Simmons[15]: Stem Cell Biology Laboratory, Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia

Becton Dickinson[16]: Becton, Dickinson and Company, NJ, USA

Dr. G. Mayrhofer[17]: Molecular Lifescience, University of Adelaide, South Australia, Australia

Dr. G. Atkins[18]: Department of Orthopaedics and Trauma, University of Adelaide, South Australia

DMSO) were added to the cells and incubated at 37°C for 60 mins. Cells were washed twice in adhesion buffer and resuspended at an appropriate density (1-2× 10^4 cells/ well). The assay was performed in triplicate.

2.6.2 Cell Adhesion Assays of Myeloma Cells on Fibronectin or VCAM-1

After labelling with calciene, myeloma cells were washed twice with adhesion assay buffer, resuspended in 25 µl of adhesion buffer at a concentration of 8× 10^5 cells/ml, and chilled on ice for 10 minutes prior to assay. The same volume of different test antibodies was added to the cells and 50 µl of the labelled cell suspension was placed, in triplicate into 96 wells previously coated with Fibronectin or VCAM-1 (10 µg/ml, 50 µl/well). The entire procedure was carried out on ice. Plates were centrifuged at 1,000 rpm for 5 mins at 4°C to sediment cells into direct, uniform contact with treated surfaces. Plates were incubated in a humidified (100%) incubator at 37°C for the indicated periods (usually 90 mins). Assay medium was removed by aspiration and wells were washed three times by adding 150 µl of the adhesion assay medium and flicking it off. After the last wash, cell adhesion was examined using an inverted-contrast microscope before adding 150 µl of 1% (w/v) sodium dodecyl sulphate (SDS) in H_2O. Plates were scanned using the Fluorimager^{TM} 595 (Sunnyvale, CA, USA) using the 530 nm filter.

2.7 IN VITRO MINERALISATION AND MEASUREMENT OF CALCIUM PHOSPHATE PRODUCTION

The conditions for the induction of human bone marrow stromal cells to develop mineralised bone matrix *in vitro* have been reported by Gronthos et al (Gronthos *et al.*, 1994). Briefly, primary BM stromal cells were seeded into 96-well plates (8×10^3 cells/well), in α-MEM supplemented with 10% FCS, β-mercaptoethanol (5×10^{-5} M), dexamethasone sodium phosphate (10^{-8} M), and KH_2PO_4 (1.8 mM) at 37°C in the presence of 5% CO_2. The medium was supplemented with zoledronic acid at the different concentrations. Culture medium (200µl) was changed at weekly intervals for a period of 5 weeks. Alternatively, cells were only treated with zoledronic acid at day 0 (d0). Calcium levels were determined as described below at weekly intervals. The cultures were washed three times with Ca^+ and Mg^+ free PBS and then solublized in 0.6 N HCl (100 µl per well) overnight. Samples from each well were then reacted with o-cresol-phthalein-complexone, and the colorimetric reaction was read at 570 nm. The absolute calcium concentration was determined by a standard curve for calcium

according to the manufacturer's recommendations. The method used here is based on the cresolphthalein complexone (CPC) method of Moorehead and Briggs (Moorehead and Biggs, 1974). CPC reacts with calcium and magnesium in alkaline solution to form a deeply coloured complex. The intensity of the purple colour formed is proportional to the calcium concentration and can be measured photometrically between 540 and 600 nm with maximum absorbance at 575 nm. Alternatively, the calcium levels were also detected using AAnalyst 300 Atomic Absorption Spectrometer (Perkin Elmer intruments). Statistical significance ($p \leq 0.05$) in the level of calcium detected between the zoledronic acid-treated BM stromal cell cultures and the untreated cells was determined using the paired *t*-test.

2.8 STATISTICAL ANALYSIS

Results are expressed as the mean ±the standard error of the mean (SEM). Student's *t*-test was used to determine the statistical significance of differences between the means of several experiments for normal distribution data. Wilcoxon test was used for non-parametric data. Spearman's test was used for correlation analysis. A probability value ≤ 0.05 was considered to be statistically significant. Each experiment was repeated at least two times for these analyses.

CHAPTER 3

MYELOMA CELL LINES EXHISIT INHIBITION OF CELL PROLIFERATION AND ENHANCED CELL DEATH IN RESPONSE TO ZOLEDRONIC ACID

3.1 INTRODUCTION

It has been demonstrated that BPs prevent bony complication and prolong the survival of myeloma patients in clinical trials (Berenson *et al.*, 1998; Berenson *et al.*, 1996; McCloskey *et al.*, 1998). This survival advantage has been attributed to anti-tumour effects mediated by the BP family of drugs. *In vitro* studies have demonstrated that BP such as pamidronate and incandronate, can decrease cell proliferation and induce cytostasis and apoptosis in some human myeloma cells (Aparicio *et al.*, 1998; Shipman *et al.*, 1997; Takahashi *et al.*, 2001). A synergistic induction of apoptosis was also observed when myeloma cell lines were exposed to a combination of zoledronic acid and dexamethasone (Tassone *et al.*, 2000).

Simultaneously, it was found that TRAIL can effectively induce extensive apoptosis in some myeloma cell lines in a time- and dose-dependent manner, and also induce substantial apoptosis in myeloma cells freshly isolated from MM patients (Gazitt, 1999). Importantly, it is not toxic to most normal human cells *in vitro* (Pitti *et al.*, 1996; Wiley *et al.*, 1995). Furthermore, TRAIL-induced apoptosis in MM cell lines is not abrogated by IL-6, a major growth and survival factor for MM cells (Mitsiades *et al.*, 2001). Preclinical studies also suggest that TRAIL can overcome conventional drug resistance and provides the basis for clinical trials of TRAIL-based treatment regimens to improve outcome in patients with MM (Mitsiades *et al.*, 2001).

It is generally accepted that cultures of human myeloma cells, *in vitro*, produce several OAFs, including TNFα, IL-1β, IL-6, M-CSF (Costes *et al.*, 1998; Filella *et al.*, 1996; Pfeilschifter *et al.*, 1989). It has been recently demonstrated that myeloma cells, also express RANKL, an important molecule involved in the pathogenesis of MM induced osteolysis (Farrugia *et al.*, 2002). In addition, the anti-resorptive effects of BPs are considered, at least partially, to be the result of inhibited release of some OAFs from OB (Derenne *et al.*, 1999; Giuliani *et al.*, 1998b; Ohya *et al.*, 1985; Stronski *et al.*, 1988). However, to date, it is still unknown whether or not BPs inhibit the secretion of OAFs and RANKL from myeloma cells.

In this study, we have investigated the effect of zoledronic acid on cultures of human myeloma cell lines. In addition, we have investigated if zoledronic acid in combination with

TRAIL can mediate a synergistic anti-tumour effect. Finally, we have investigated whether zoledronic acid treatment of myeloma cells, leads to an inhibition of OAF synthesis.

3.2 RESULTS

3.2.1 Multiple Myeloma Cell Lines Display Differential Sensitivity to Zoledronic Acid

Initial experiments monitored possible cytotoxic effects of zoledronic acid on the myeloma cell lines using the colorimetric reagent WST-1. The WST-1 assay is a measure of the combined effects of cytostasis (inhibition of proliferation) and cytolysis (induction of target cell death). As shown in Figure 3.1, a significant reduction in cell number was observed when the RPMI 8226 cell line was exposed to zoledronic acid for 72 hours. At a concentration of 1μM, zoledronic acid induced a significant reduction in cell number ($p<0.005$). A classical dose-dependent sigmoid response curve was evident and a plateau was achieved at concentrations greater than 5 μM. In contrast, this drug had little or no effect on the myeloma cell lines WL2 and U266. The ARH-77 cell line was less responsive to zoledronic acid treatment than RPMI 8226, and a significant reduction in cell number was only observed when more than 10μM of zoledronic acid was used ($p<0.05$).

To investigate whether zoledronic acid was mediating its effect by inducing cytostasis or cell death, a time course study was performed by counting viable cells using trypan blue exclusion. In agreement with the above findings, a significant reduction in cell number was achieved at day 1 ($p<0.005$) in RPMI 8226 cells treated with zoledronic acid (figure 3.2). Zoledronic acid induced a significant reduction in cell number over the period of 5 days in RPMI 8226 cells ($p<0.005$). ARH-77 cell proliferation was also inhibited in the presence of zoledronic acid (B) ($p=0.025$), however, no effects were observed with the WL2 and U266 ($p>0.05$) cell lines. Interestingly, the proliferation rate of RPMI 8226 cells in the normal culture media was significantly greater than that of the other three myeloma cell lines.

3.2.2 Zoledronic Acid Induces Cell Death by Inhibiting Cell Cycle Progression in RPMI 8226

To identify the mechanisms of the cytoreductive effect of zoledronic acid on the myeloma cell line RPMI 8226, flow cytometric cell cycle analysis was performed (figure 3.3). There was a 2-fold increase in the number of cells, which accumulated in S-phase of the cell cycle after exposure to zoledronic acid for 24 hrs. After 48 hrs culture in zoledronic acid, there was an increase in the proportion of hypo-diploid cells in the sub-G_0/G_1 peak consistent with the onset of apoptosis. This increase in the sub-G_0/G_1 peak was time dependent. In contrast, no

Figure 3.1. Multiple myeloma cell lines display differential sensitivity to zoledronic acid. Four myeloma cell lines, ARH77, RPMI-8226, WL2 and U266 were seeded in 96-well plates at a cell density of 10,000 cells/ well. The cells were cultured in the presence or absence of zoledronic acid and the cell number and viability was quantitated at d3 using WST-1, as described in the methods (refer to section 2.3.2.1). Zoledronic acid induced a significant decrease ($p<0.005$, *t*-test) in cell number at a concentration of 1 μM in RPMI 8226 cell lines. A classical dose-dependent sigmoid response curve was evident and a plateau was achieved at concentrations greater than 5 μM (A). ARH-77 was less responsive to zoledronic acid treatment and the decrease in cell number was observed when more than 10μM of zoledronic acid was used (B, $p<0.05$, t-test). In contrast, myeloma cell lines WL2 (C) and U266 (D) were resistant to the effects of zoledronic acid treatment. Values represent means ±SEM of triplicate cultures of each concentration. The results displayed are a representative example of 2 independent experiments.

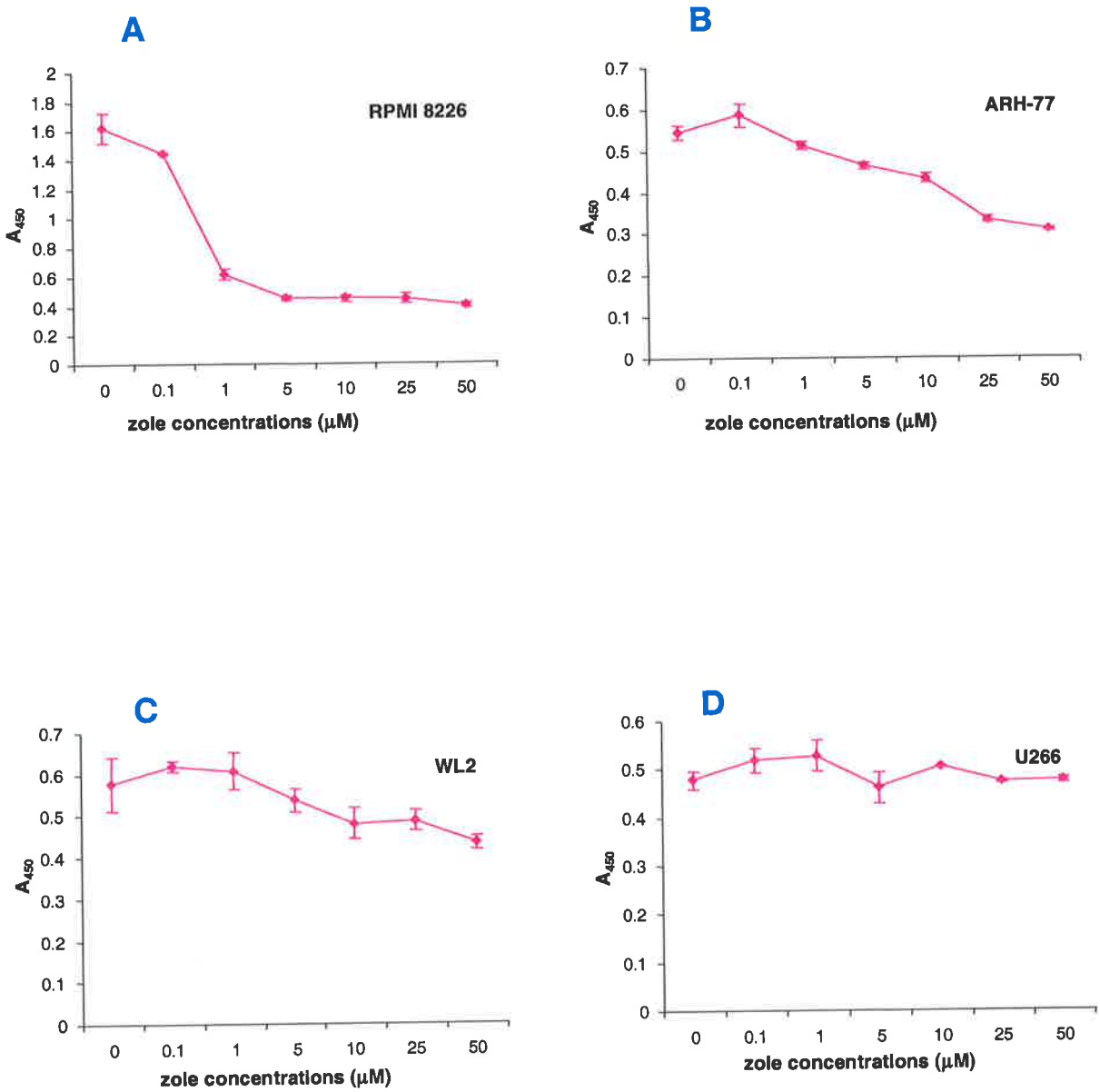

A
RPMI 8226
A_{450}
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 0.1 1 5 10 25 50
zole concentrations (μM)
B
ARH-77
A_{450}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 0.1 1 5 10 25 50
zole concentrations (μM)
C
WL2
A_{450}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 0.1 1 5 10 25 50
zole concentrations (μM)
D
U266
A_{450}
0.6
0.5
0.4
0.3
0.2
0.1
0
0 0.1 1 5 10 25 50
zole concentrations (μM)

Figure 3.2. Effects of zoledronic acid on the proliferation of myeloma cell lines. The myeloma cell lines were cultured in 48-well plates at a density of 5×10^4 cells/well. ARH-77 and RPMI 8226 cell lines were cultured in the presence of 10 μM zoledronic acid, whilst U266 and WL2 cell lines were treated with 50 μM zoledronic acid. Using trypan blue exclusion, viable cells were counted each day for the period of 5 days. The results showed that the proliferation rate of RPMI 8226 (A) was greater than the other three cell lines. The significant reduction in cell number was achieved at day 1 ($p<0.005$, *t*-test) in RPMI 8226 treated with zoledronic acid. Zoledronic acid induced a significant reduction in cell number over the period of 5 days in RPMI 8226 cells ($p<0.005$, Wilcoxon test). ARH-77 cell proliferation was also inhibited in the presence of zoledronic acid (B) ($p=0.025$, Wilcoxon test), however, no effects were observed in WL2 (C) and U266 (D) ($p>0.05$, Wilcoxon test) cell lines.

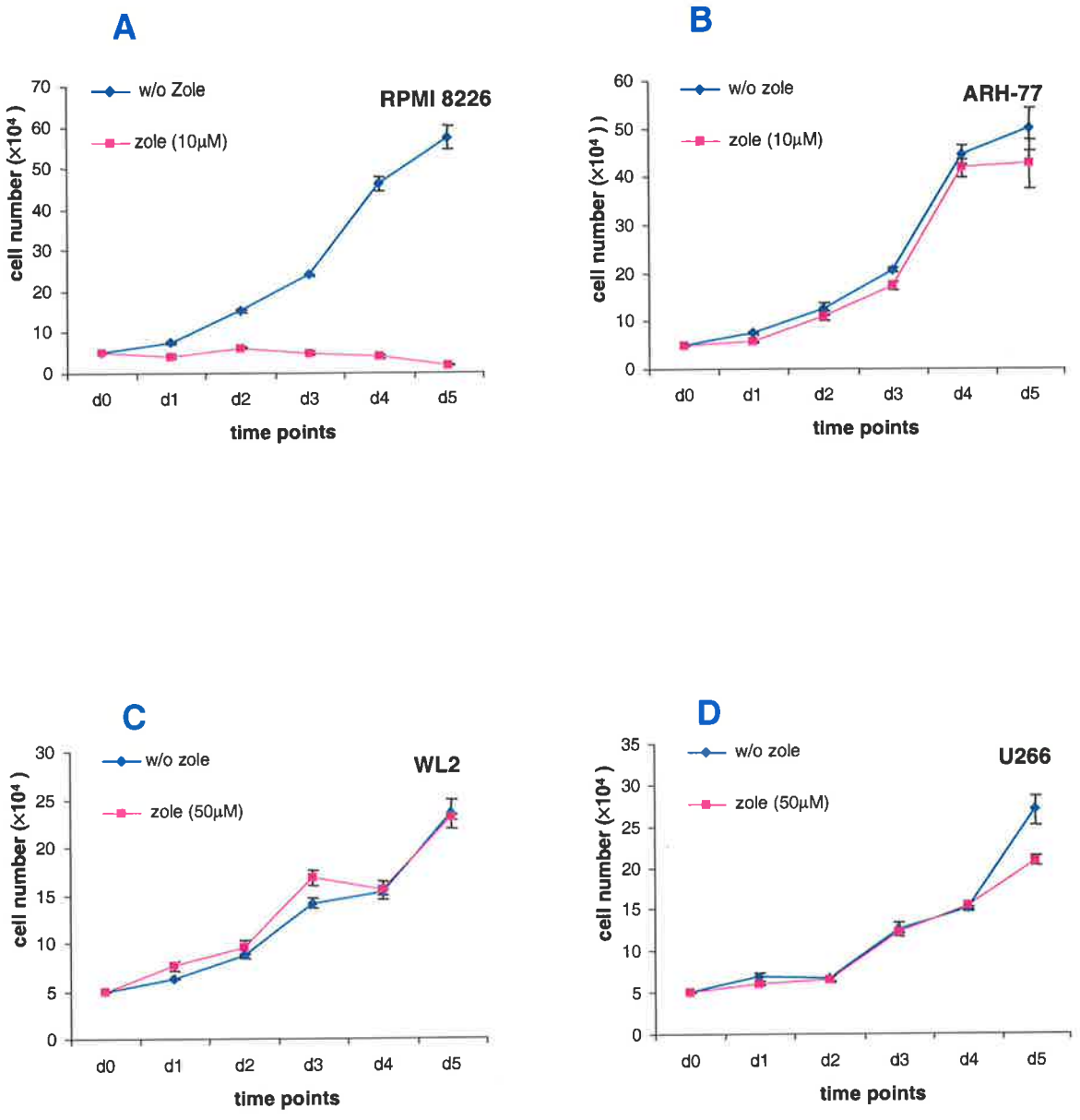

A
w/o Zole
zole (10μM)
RPMI 8226
cell number (×10⁴)
70
60
50
40
30
20
10
0
d0
d1
d2
d3
d4
d5
time points
B
w/o zole
zole (10μM)
ARH-77
cell number (×10⁴)
60
50
40
30
20
10
0
d0
d1
d2
d3
d4
d5
time points
C
w/o zole
zole (50μM)
WL2
cell number (×10⁴)
30
25
20
15
10
5
0
d0
d1
d2
d3
d4
d5
time points
D
w/o zole
zole (50μM)
U266
cell number (×10⁴)
35
30
25
20
15
10
5
0
d0
d1
d2
d3
d4
d5
time points

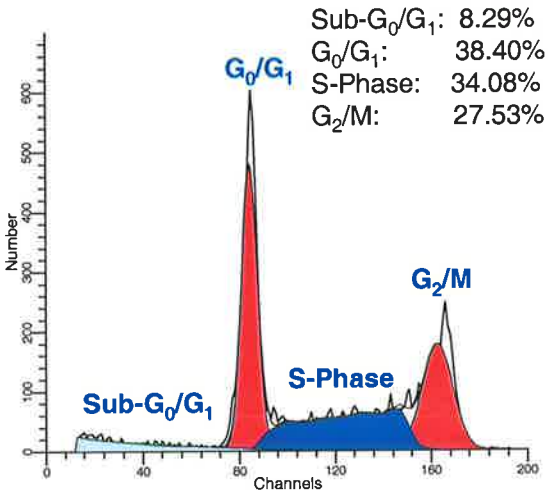
Figure 3.3. Zoledronic acid induces cell death by inhibiting cell cycle progression in RPMI 8226 cells. The myeloma cell line RPMI 8226 was treated with zoledronic acid at concentrations of 10 μM. Cells were harvested at different time points over a period of 5 days and stained with the DNA fluorochrome, propidium iodide (PI), as described in the methods. The samples were subsequently analysed by flow cytometry and ModFit LT for win 32 (version 2.0). There was a 2-fold increase in the number of cells, which accumulated in S-phase of the cell cycle after exposure to zoledronic acid for 24 hrs. After 48-hr culture there is an increase in the proportion of hypo-diploid cells in the sub-G_0/G_1 peak, consistent with the onset of apoptosis. This increase in the sub-G_0/G_1 peak was time dependent.

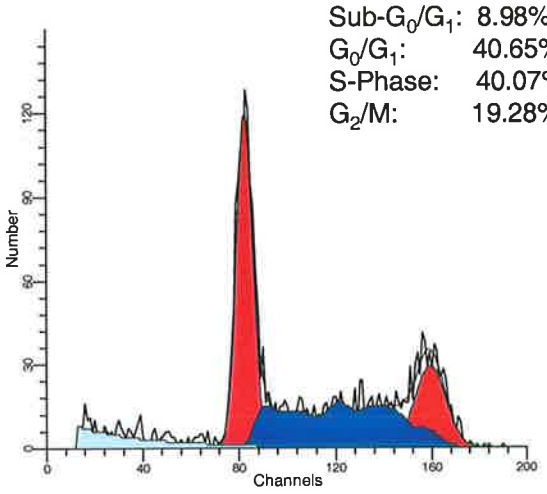
A

w/o zole

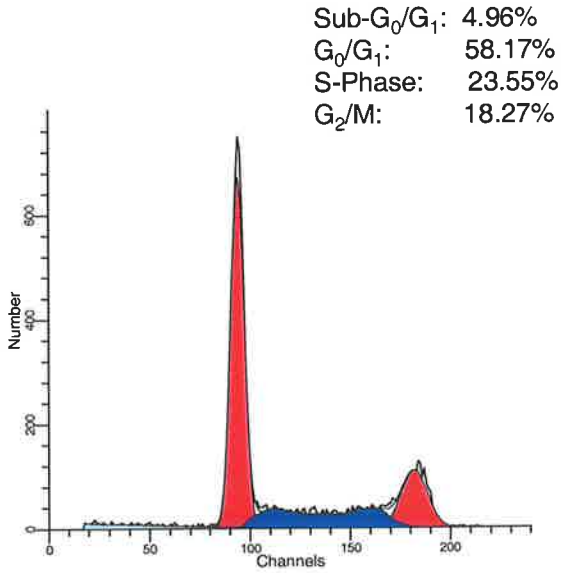
zole (10 µM)

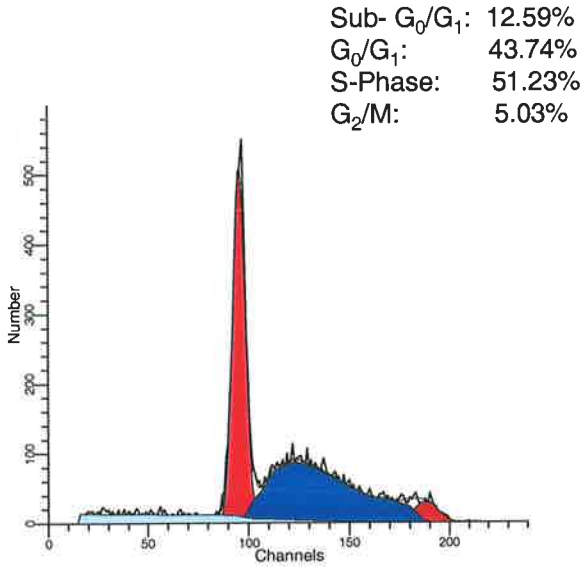
5h





d1





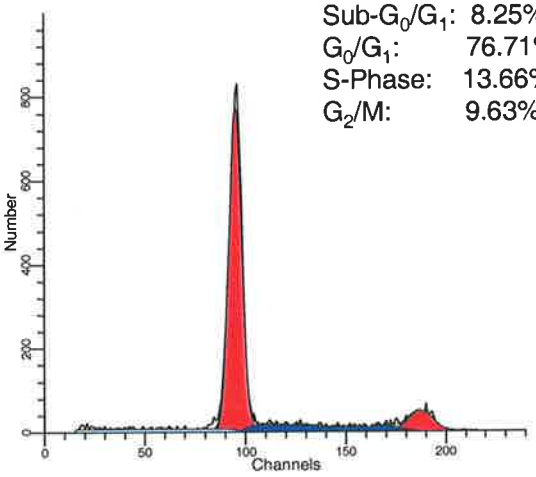
B

w/o zole

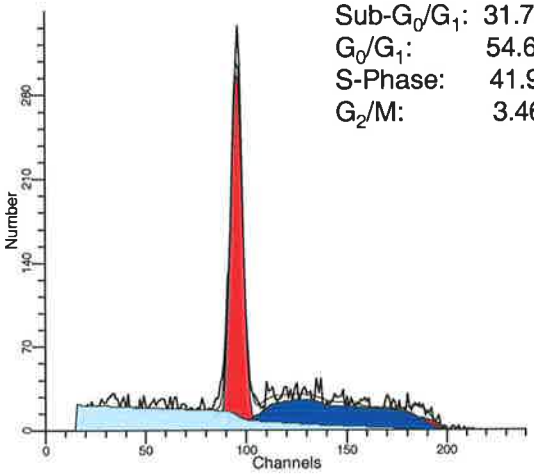
zole (10 µM)

d2

Sub-G_0/G_1: 8.25%
G_0/G_1: 76.71%
S-Phase: 13.66%
G_2/M: 9.63%

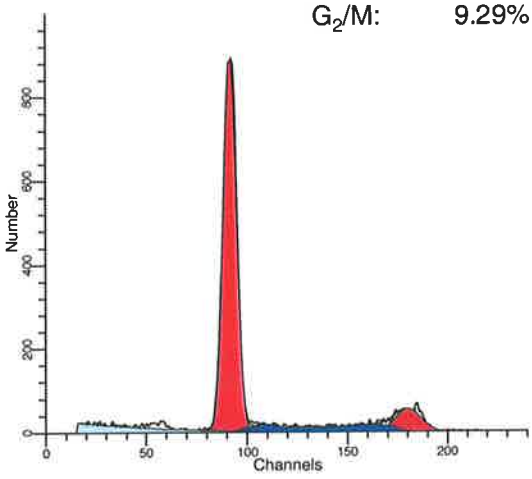


Sub-G_0/G_1: 31.72%
G_0/G_1: 54.62%
S-Phase: 41.92%
G_2/M: 3.46 %

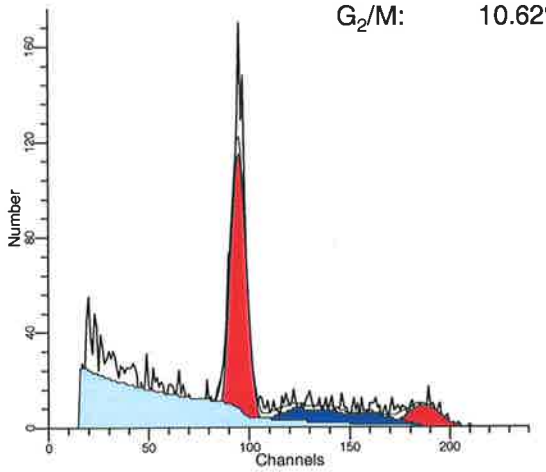


d3

Sub-G_0/G_1: 8.67%
G_0/G_1: 78.69%
S-Phase: 12.03%
G_2/M: 9.29%



Sub-G_0/G_1: 45.71%
G_0/G_1: 65.46%
S-Phase: 23.92%
G_2/M: 10.62%

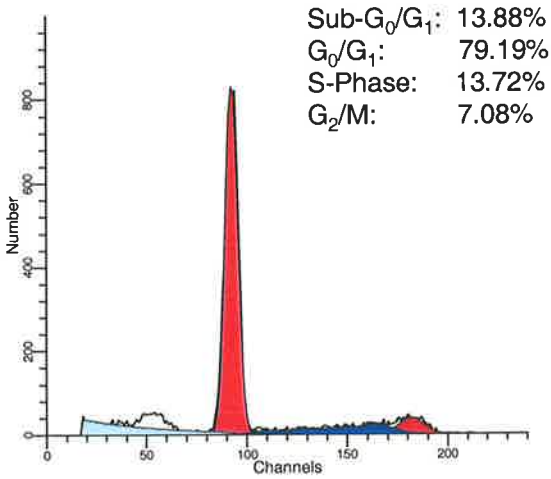


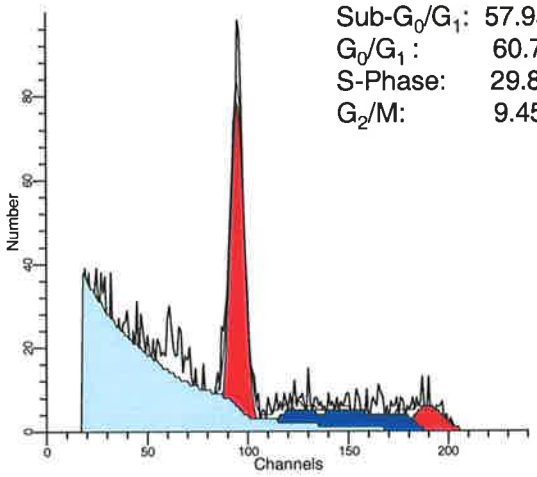
C

w/o zole

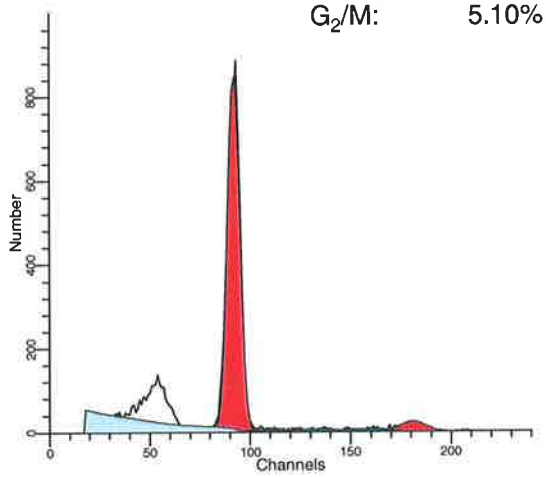
zole (10 μM)

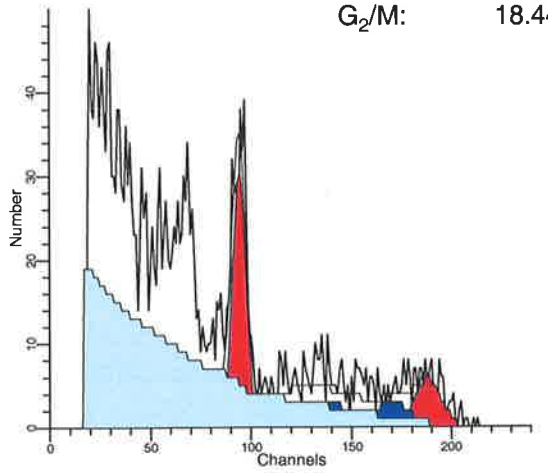
d4





d5





alteration of cell cycle was observed in the zoledronic acid-resistant cell line WL2 (data not shown).

The changes in nuclear morphology of myeloma cell lines induced by zoledronic acid were also examined by fluorescence microscopy. As shown in figure 3.4, morphological changes characteristic of apoptosis (Wyllie *et al.*, 1980) including condensation and the formation of dense, rounded apoptotic bodies are clearly evident in a small percentage of all four myeloma cell lines when compared with untreated cells. Consistent with the cell proliferation results (fig 3.1/3.2), fewer apoptotic nuclei were noted in ARH-77, WL2 and U266 compared with RPMI 8226. However, the proportion of cells with apoptotic nuclei was not proportional to the reduction in cell numbers. Therefore, the most profound effect mediated by zoledronic acid is the induction of cytostasis.

3.2.3 Zoledronic Acid Does not Increase Caspase Activity

It is generally acknowledged that apoptotic cell death can be triggered by caspase activation. Therefore, the caspase activity of myeloma cell lines RPMI 8226 and WL2 was investigated (figure 3.5). Consistent with previous observations, zoledronic acid significantly inhibited cell proliferation of RPMI 8226 ($p<0.001$) and ARH-77 cells ($p<0.05$) at the indicated concentrations. However, caspase inhibitor I did not prevent the zoledronic acid-mediated reduction in cell numbers. Interestingly, untreated myeloma cells displayed a relatively high caspase activity, and zoledronic acid did not enhance caspase activity in both of these myeloma cell lines. The caspase inhibitor I, z-VAD-FMK (50 μM) successfully inhibited the basal caspase activity.

The nitrogen-containing BPs inhibit the mevalonate pathway by inhibiting geranylgeranylation of small GTP-binding proteins such as Ras, Rho, Rac and Rab (Murakami *et al.*, 1995; Sato *et al.*, 1991), which is important for many cellular processes, including proliferation, cytoskeletal organisation and intracellular signalling (Hall, 1998; Zerial and Stenmark, 1993). To examine the involvement of the mevalonate pathway on the zoledronic acid-mediated apoptosis of myeloma cells, we assessed the ability of geranylgeraniol (GGO), an intermediate product in mevalonate pathway, to protect myeloma cell lines from zoledronic acid-induced apoptosis. Consistent with the findings of others (Miquel *et al.*, 1996; Ohizumi *et al.*, 1995; Ohizumi *et al.*, 1997), this study (figure 3.5/3.6) also showed that GGO alone induced cytostasis and cell death in RPMI 8226 cells in a dose-

Figure 3.4. Zoledronic acid induces apoptosis in myeloma cell lines. Myeloma cell lines were treated with 10 μM zoledronate for 72 hrs, stained with DAPI, and examined by fluorescence microscopy as described in the methods (refer to section 2.3.4). Morphological changes characteristic of apoptosis including condensation and the formation of dense, rounded apoptotic bodies are clearly evident in a small percentage of all four myeloma cell lines (B, D, F & H) when compared with untreated cells (A, C, E & G). Consistent with the cell proliferation results (figure 3.1/3.2), fewer apoptotic nuclei were noted in ARH-77 (D), WL2 (F) and U266 (H) compared with RPMI 8226 (B). The arrows display the apoptotic cells.

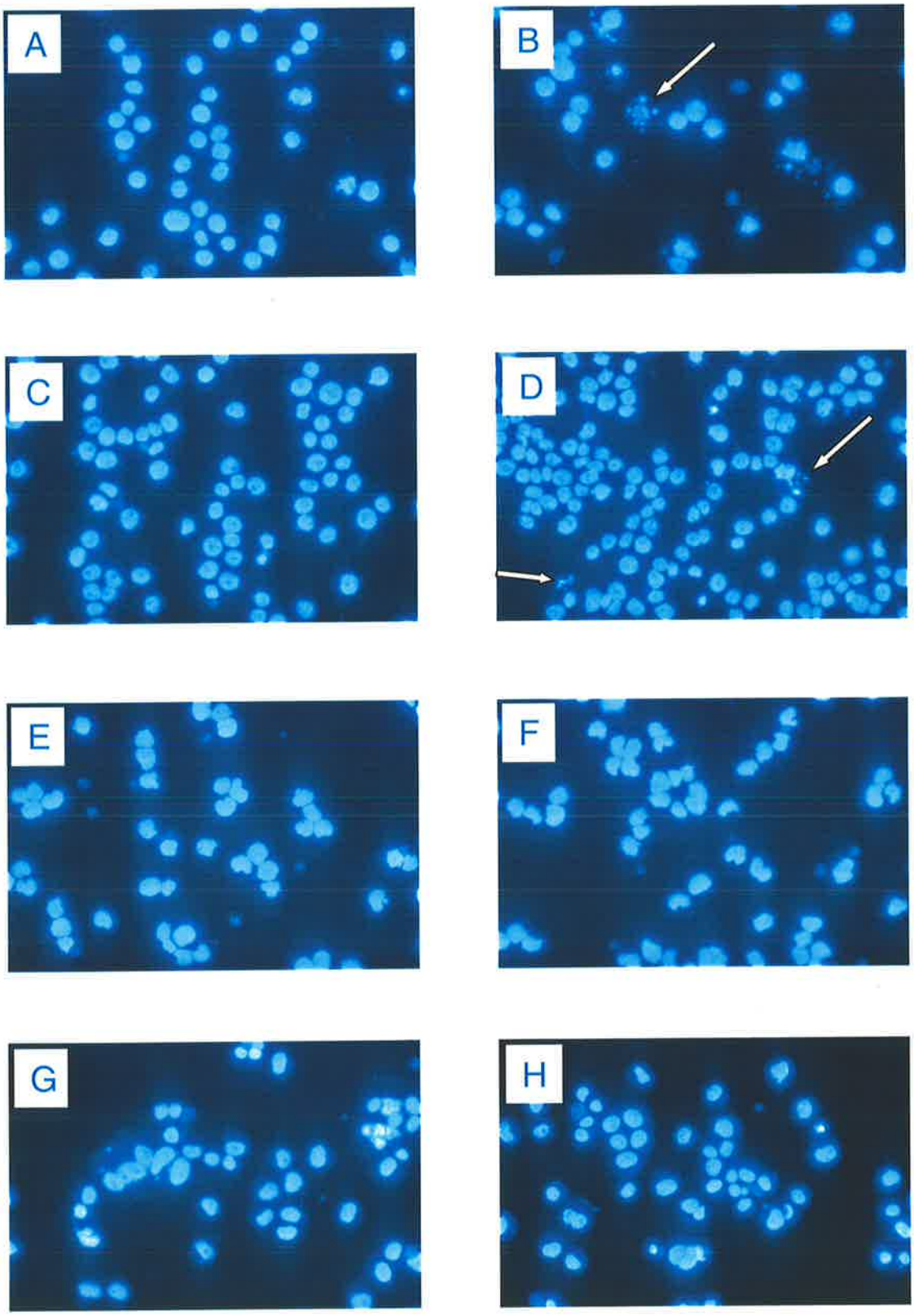
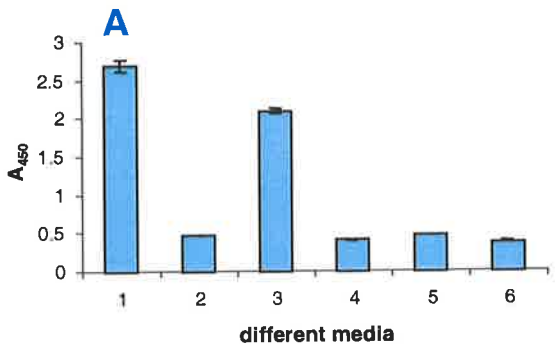
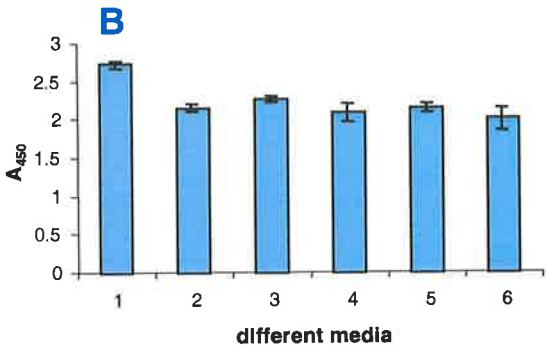

A
B
C
D
E
F
G
H

Figure 3.5. Zoledronic acid does not enhance caspase activity in myeloma cell lines. Myeloma cell lines RPMI 8226 and ARH-77 were seeded in 96-well plates under the different treatment conditions and the cell proliferation was assessed at day 3 using WST-1. At the same time, cells were also cultured in 24-well plates under the same treatment condition. Caspase-3 activity was measured at day 3 by measuring the caspase-3 dependent degradation of the fluorogenic substrate DEVAFC, as described in the methods. Consistent with the previous observations, zoledronic acid significantly inhibited cell proliferation of RPMI 8226 (A, $p<0.001$, *t*-test) and ARH-77 (B, $p<0.05$, *t*-test) cells at the indicated concentrations. Geranylgeraniol (GGO) alone, also decreased cell number in both cell lines (A&B, condition 3, $p<0.05$, *t*-test). However, caspase inhibitor I did not prevent the cells from a zoledronic acid-mediated reduction in cell number (A&B, 4^{th}, 6^{th} condition). Similarly, GGO at a concentration of 50 μM did not have a protective effect on the myeloma cell lines treated with zoledronic acid (A&B, condition 5&6). Interestingly, untreated myeloma cells displayed a relatively high caspase activity, and zoledronic acid and GGO did not enhance caspase activity in both of myeloma cell lines (C&D, condition 2&3). The caspase inhibitor I, z-VAD-FMK (50 μM) successfully inhibited the basal caspase activity (C&D, condition 4&6). The data represent means ± SEM of triplicate cultures of each condition.

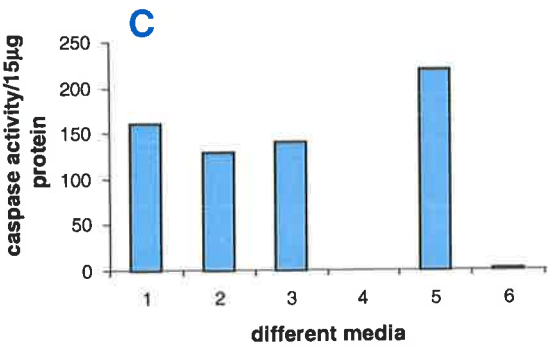
WST-1 assay of RPMI 8226 treated with different media (72h)



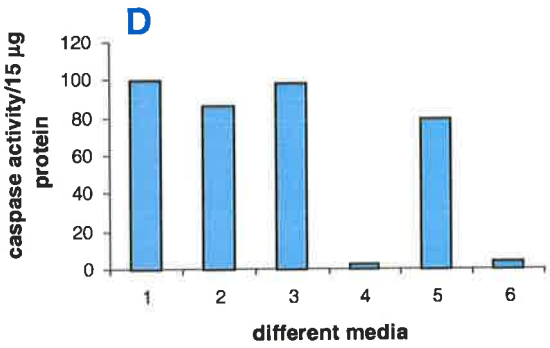
WST-1 assay of ARH-77 treated with different media (72h)



caspase activity in RPMI 8226 treated with different media (72h)



caspase activity in ARH-77 treated with different media (72h)



1. Control
2. Zole for 8226 (10 µM) ARH (25 µM)
3. Geranylgeraniol (GGO, 50 µM)
4. Zole + caspase inhibitor I (50 µM)
5. Zole + GGO
6. Zole + GGO + caspase inhibitor

dependent manner ($p<0.05$) at both d3 and d5. However, GGO did not enhance caspase activity in either myeloma cell lines ARH-77 or RPMI 8226 (figure 3.5). The results also show that GGO protects myeloma cells against the effects of zoledronic acid, in a dose-dependent manner at high concentrations (figure 3.6). Notably, this protection was observed when GGO was used at concentrations greater than 300 μM ($p<0.01$). The protective effect of GGO suggests that zoledronic acid, in part, mediates its effects by inhibiting the mevalonate pathway.

3.2.4 Gene Expression of Myeloma Cells Treated with Zoledronic Acid

The effect of zoledronic acid on the gene expression of the myeloma cell lines RPMI 8226 and WL2 was examined using semi-quantitative RT-PCR, as described in the methods. As seen in figure 3.7, TNF α, IL-1β and COX-2 gene expression was upregulated in RPMI 8226 cells following zoledronic acid treatment ($p<0.005$). In contrast, the WL2 cell line, which is resistant to zoledronic acid, showed no changes in gene expression.

3.2.5 Zoledronic Acid Upregulates the Cell Surface Molecule Expression on the Myeloma Cell Lines

Cell-cell and/or cell-ECM adhesion plays an important role in regulating apoptosis and cell survival in tumour cells (Clark and Brugge, 1995). Previous studies demonstrate that myeloma cells overexpress a variety of cell surface molecules (Leo *et al.*, 1992; Damiano, 1999 #59; Van Driel *et al.*, 2002), which may be responsible for cell adhesion-mediated drug resistance (Barker *et al.*, 1992; Damiano *et al.*, 1999; Jensen *et al.*, 1993). Therefore, the expression of various cell surface molecules by myeloma cells was examined using single colour flow cytometric analysis (figure 3.8). One interesting finding is that RPMI 8226 cells express high levels of HLA class I, β_2 microglobulin and CD138. In contrast, WL2, which is resistant to zoledronic acid, did not express any of these antigens. However, zoledronic acid had no effect on the expression of these molecules ($p>0.05$) at d1 or d3. Consistent with other studies (Clark and Brugge, 1995; Damiano *et al.*, 1999), both cell lines expressed $\alpha_4\beta_1$ integrin (61.2C4, P4C2), whilst the expression by the zoledronic acid-resistant cell line WL2 was greater when compared to the zoledronic acid-sensitive cell line RPMI 8226. Zoledronic acid marginally increased the expression of $\alpha_4\beta_1$ integrin in both cell lines ($p<0.05$). Moreover, zoledronic acid was also found to marginally increase the expression of CD44, CD45, CD54 and CD56 in both cell lines ($p<0.01$).

Figure 3.6. GGO partially protects against zoledronic acid-mediated cell death in a dose-dependent manner. RPMI 8226 cells were seeded in 96-well plates and cultured for 3 and 5 days in the presence of GGO alone or GGO in combination with zoledronic acid, at the indicated concentrations. The cell number and viability were quantitated using WST-1. The results show that GGO alone induced cytostasis and cell death of RPMI 8226 in a dose-dependent manner ($p<0.05$, *t*-test) at both d3 (A) and d5 (B). GGO was found to protect zoledronic acid-induced cytostasis and cell death at a concentration of 300 μM ($p<0.01$, *t*-test). The data represent means ± SEM of triplicate cultures of each condition. The cell morphology was assessed at d5 and representative examples are shown in C (negative control), D (zole, 10 μM), E (zole, 10 μM and GGO 25 μM), F (zole, 10 μM and GGO 100 μM), G (zole, 10 μM and GGO 300 μM), and H (zole, 10 μM and GGO 500 μM).

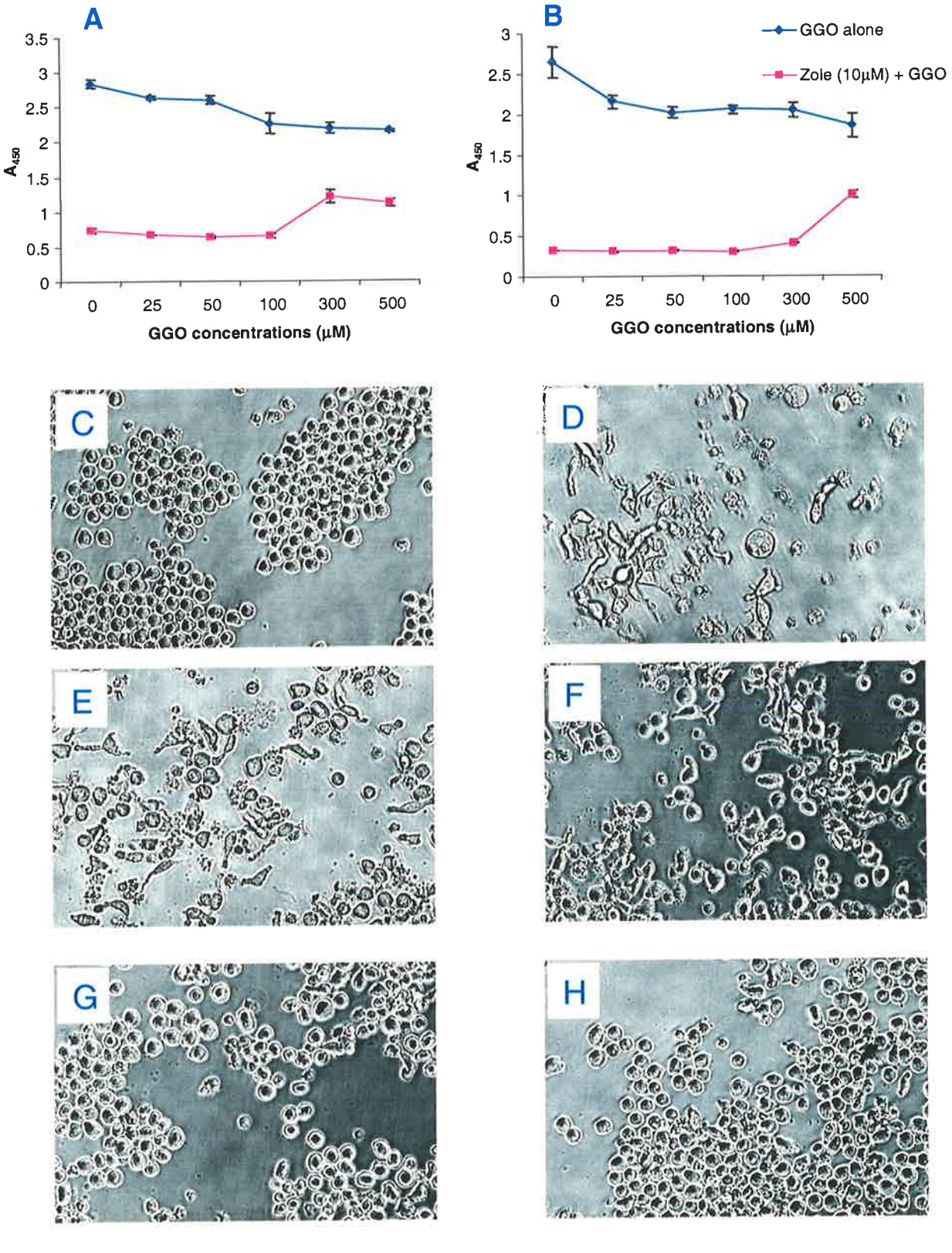
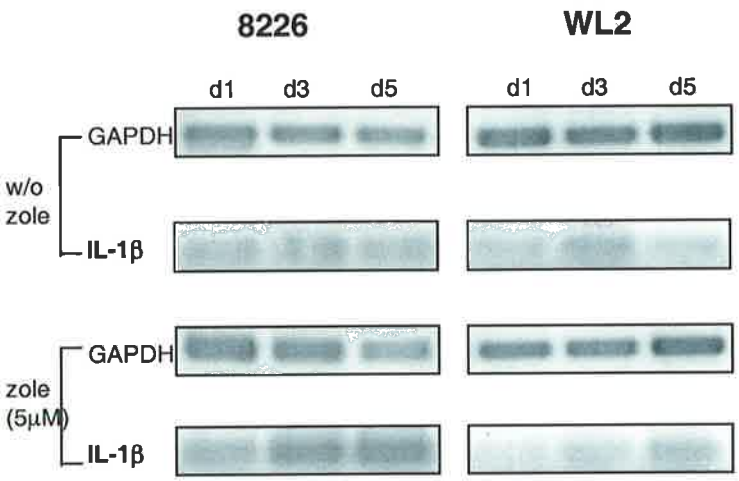
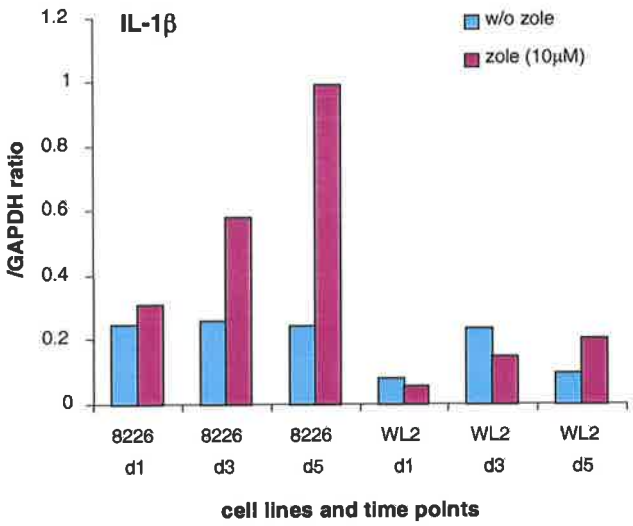

A
3.5
3
2.5
2
1.5
1
0.5
0
A_{450}
0
25
50
100
300
500
GGO concentrations (μM)
B
GGO alone
Zole (10μM) + GGO
3
2.5
2
1.5
1
0.5
0
A_{450}
0
25
50
100
300
500
GGO concentrations (μM)
C
D
E
F
G
H

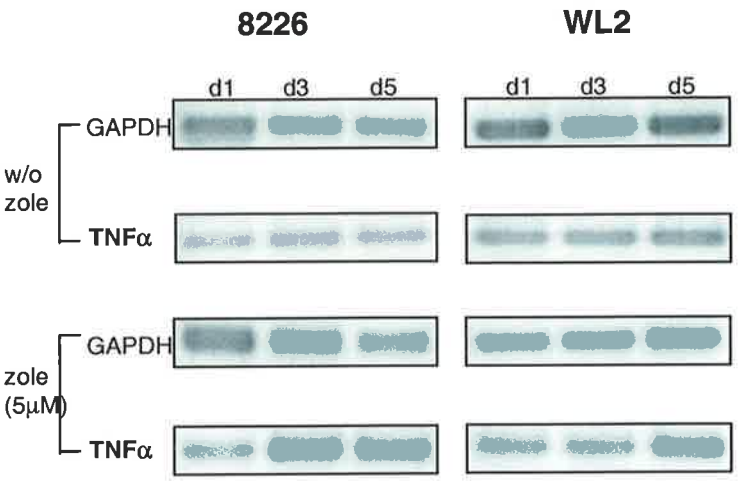
Figure 3.7. The effect of zoledronic acid on the gene expression of myeloma cell lines. Myeloma cell lines RPMI 8226 and WL2 were treated with zoledronic acid at a concentration of 10 μM and the cells were harvested at three different time points over a period of 5 days. Total RNA was isolated and semi-quantitative RT-PCR was performed as described in the methods. The house-keeping gene, GAPDH was used as an internal control for mRNA integrity and enabled relative gene expression to be determined. PCR products were subjected to electrophoresis on 1% agarose gel, stained with SYBR gold and visualised using a Fluorimager. The product bands were semi-quantitated and plotted as a histogram of the ratio of specific gene expression relative to the expression of GAPDH. The experiments were performed twice and the Wilcoxon test was employed for statistical analysis. The data shows that zoledronic acid upregulates IL-1β (A), TNF-α (B) and COX-2 (C) gene expression in RPMI 8226 cells ($p<0.005$, Wilcoxon test), which is sensitive to zoledronic acid treatment. However, no alteration was noted in the WL2 cell line.

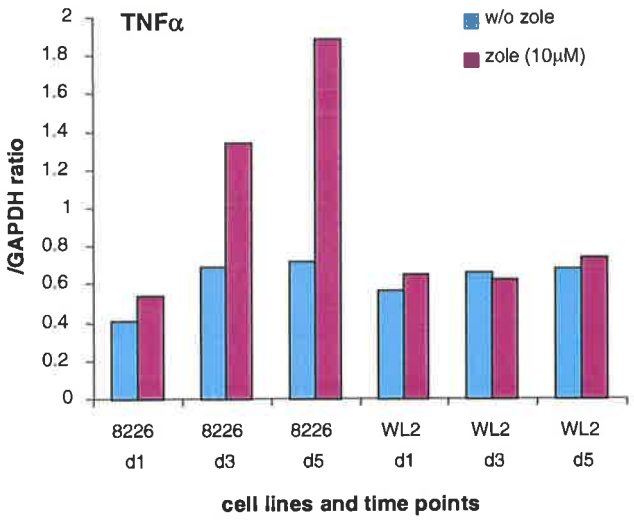
A


8226
WL2
d1
d3
d5
d1
d3
d5
GAPDH
w/o zole
IL-1β
GAPDH
zole (5μM)
IL-1β

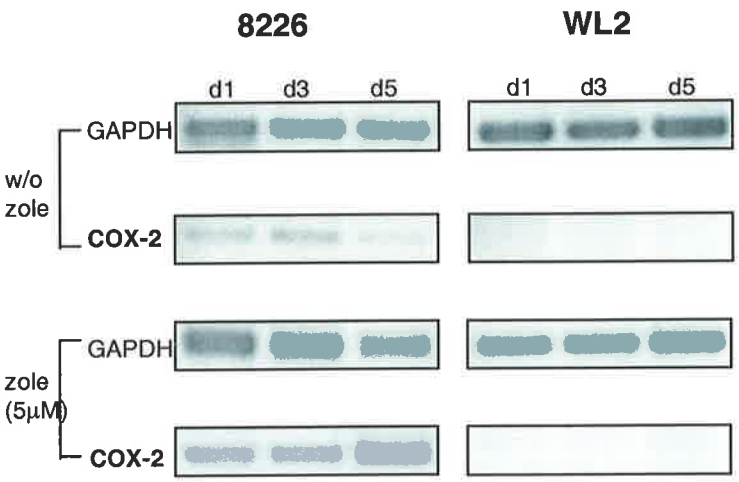

IL-1β
w/o zole
zole (10μM)
/GAPDH ratio
1.2
1
0.8
0.6
0.4
0.2
0
8226 d1
8226 d3
8226 d5
WL2 d1
WL2 d3
WL2 d5
cell lines and time points

B


8226
WL2
d1
d3
d5
d1
d3
d5
GAPDH
w/o zole
TNFα
GAPDH
zole (5μM)
TNFα


TNFα
w/o zole
zole (10μM)
/GAPDH ratio
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
8226 d1
8226 d3
8226 d5
WL2 d1
WL2 d3
WL2 d5
cell lines and time points

C


8226
WL2
d1
d3
d5
d1
d3
d5
GAPDH
w/o zole
COX-2
GAPDH
zole (5μM)
COX-2

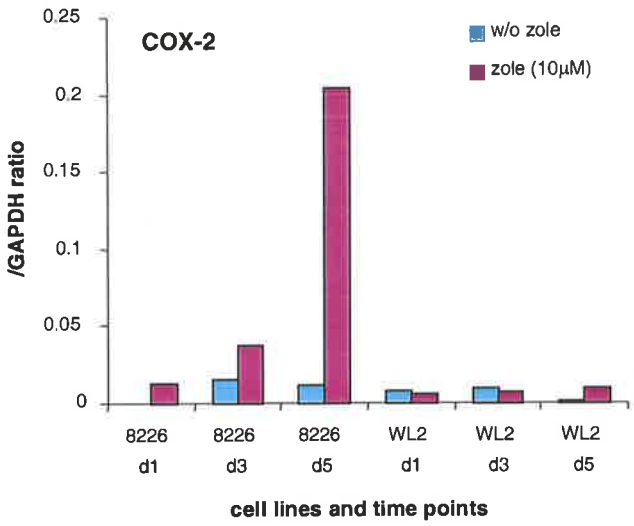
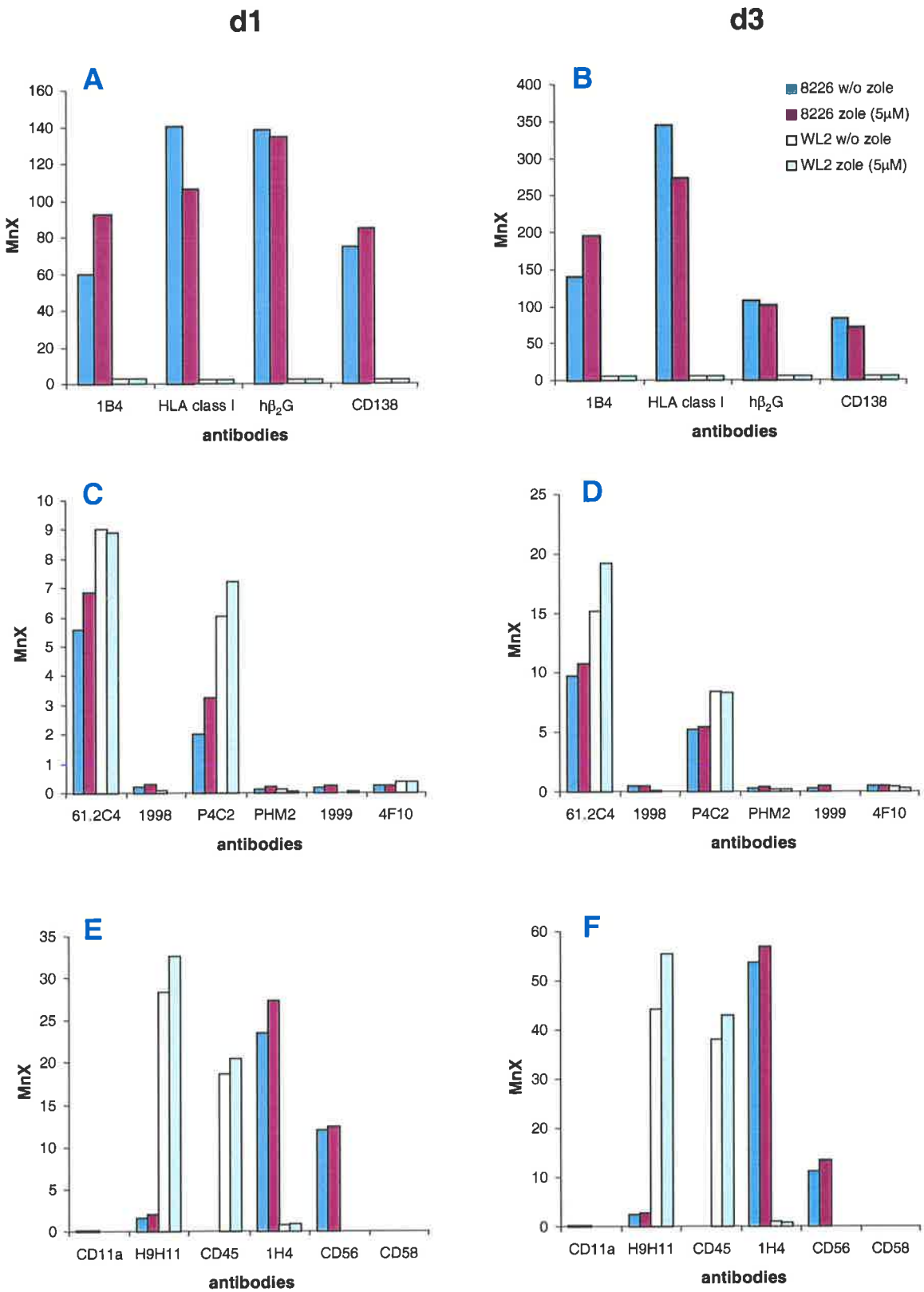

COX-2
w/o zole
zole (10μM)
/GAPDH ratio
0.25
0.2
0.15
0.1
0.05
0
8226 d1
8226 d3
8226 d5
WL2 d1
WL2 d3
WL2 d5
cell lines and time points

Figure 3.8. Zoledronic acid upregulates the expression of cell surface molecules on the myeloma cells. Myeloma cell lines RPMI 8226 and WL2 were treated with zoledronic acid and harvested at d1 and d3 for single-colour immunofluorescence staining of each of the different cell surface molecules, as described in the methods. Data are displayed as the mean fluorescence of each protein, from which the mean fluorescence value of the isotype-matched negative control has been subtracted. RPMI 8226 cells were found to express high levels of HLA class I, β_2 microglobulin and CD138 (A&B). However, zoledronic acid had no effect on the expression of these molecules ($p>0.05$, Wilcoxon test) at d1 or d3. In contrast, WL2 did not express any of these antigens (A&B). Consistent with other studies (Clark and Brugge, 1995; Damiano *et al.*, 1999), both cell lines expressed $\alpha_4\beta_1$ integrin (61.2C4, P4C2), whilst the expression by the zoledronic acid-resistant cell line WL2 was greater contrast to zoledronic acid-sensitive cell line 8226 (C&D). Zoledronic acid marginally increased the expression of $\alpha_4\beta_1$ integrin in both cell lines ($p<0.05$, Wilcoxon test). Furthermore, it was also found to marginally increase the expression of CD44, CD45, CD54 and CD56 expression in the both cell lines (E&F) ($p<0.01$, Wilcoxon test).



$h\beta_2G$: human β_2 microglobulin 61.2C4: β1 1998: α2β1 P4C2: α4 PHM2: α5β1 1999: α5β1 4F10: α6β1
H9H11:CD44 1H4: CD54

To investigate if zoledronic acid altered the functional status of the adhesion molecules expressed by myeloma cell lines, adhesion to FN and VCAM-1 was examined using a quantitative adhesion assay. The results show that myeloma cells adhere preferentially to VCAM-1 and not FN. RPMI 8226 cells use $\alpha_4\beta_1$ to adhere to VCAM-1 and CD44 to adhere to FN. WL2 cells use both $\alpha_4\beta_1$ and CD44 to adhere to FN, and use $\alpha_4\beta_1$ adhere to VCAM-1. This data also shows that zoledronic acid does not augment adhesion of myeloma cells to two important ligands (figure 3.9).

3.2.6 Differential Sensitivity of Myeloma Cell Lines to TRAIL–Mediated Cell Death

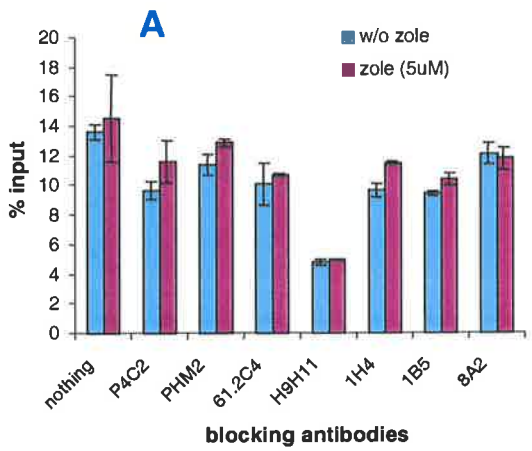
As shown in figure 3.10, myeloma cells are differentially sensitive to the cytotoxic effects of TRAIL. Interestingly, the response amongst the different cell lines was similar to the response seen following zoledronic acid treatment. In brief, TRAIL mediated a significant reduction in RPMI 8226 cell number in a dose-dependent manner. A significant decrease in cell number was found when the cells were treated with TRAIL at concentrations greater than 100 ng/ml ($p<0.05$). In contrast, TRAIL had no effect on the other cell lines, ARH-77, WL2 and U266.

3.2.7 TRAIL Enhanced the Effect of Zoledronic Acid When Used in Combination on the Myeloma Cells

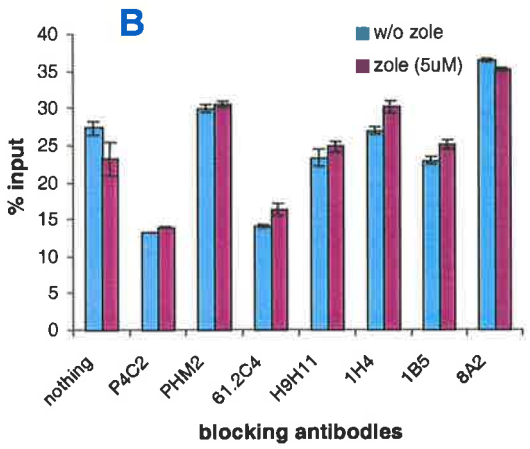
The culture of myeloma cell lines with zoledronic acid in combination with TRAIL resulted in a decrease in the cell number and viability that was consistently greater than that observed in cultures treated with either drug alone in the four cell lines tested (figure 3.11). Of note, there is no significant difference between d3 and d5, indicating that prolonged exposure of drugs did not enhance the effect. TRAIL rendered the cells more sensitive to zoledronic acid ($p<0.05$) at concentrations previously shown to have no significant effect on cell proliferation. When the concentration of TRAIL was increased to 100 ng/ml in RPMI 8226, zoledronic acid was only effective when used at a concentration greater than 5 μM ($p<0.05$). These results indicated that zoledronic acid might affect the cells through the TRAIL and TRAIL receptor pathway. The expression of TRAIL and TRAIL receptors in response to zoledronic acid treatment was therefore investigated using semi-quantitative RT-PCR and flow cytometry.

Figure 3.9. Zoledronic acid does not regulate integrin-mediated adhesion of myeloma cells to FN and VCAM-1. Calciene labelled suspensions of myeloma cell lines RPMI-8226 and WL2 were incubated with function-blocking antibodies directed against the various adhesion molecules. The cells were seeded into plates, previously coated with either FN or VCAM-1 as described in the methods. The data present the mean values ± SEM of triplicate wells, expressed as a percentage of the input population (2×10^4 cells). The level of significance for the percentage of attached cell in all conditions was compared between with zoledronic acid treated and untreated groups. The results show that myeloma cells adhere preferentially to VCAM-1 and not FN. RPMI 8226 cells use $\alpha_4\beta_1$ to adhere to VCAM-1 and CD44 to adhere to FN. WL2 cells use both $\alpha_4\beta_1$ and CD44 to adhere to FN, and use $\alpha_4\beta_1$ to adhere to VCAM-1. Futhermore, these data indicate that zoledronic acid does not augement adhesion.

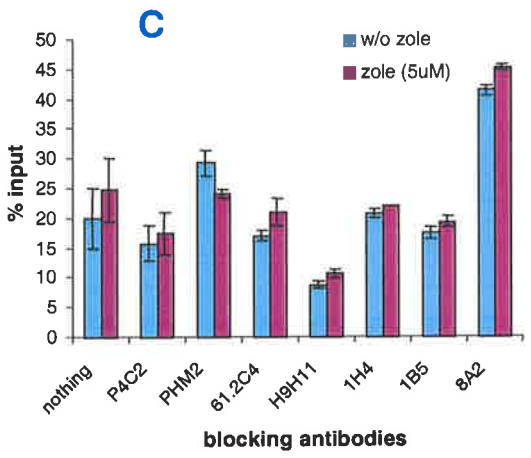
Adhesion assay of RPMI 8226 (FN)



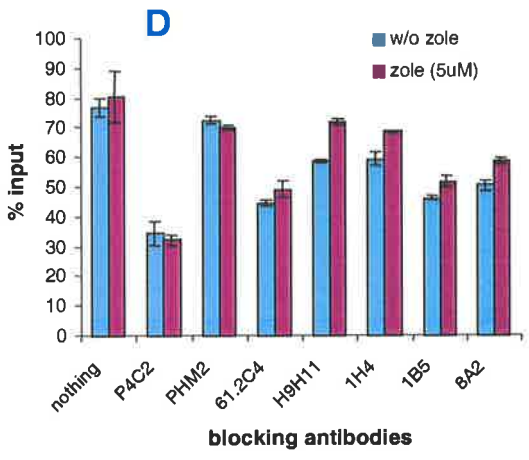
Adhesion assay of RPMI 8226 (VCAM- 1)



Adhesion assay of WL2 (FN)



Adhesion assay of WL2 (VCAM-1)



61.2C4: β1 1998: α2β1 P4C2: α4 PHM2: α5β1 1999: α5β1 4F10: α6β1 H9H11:CD44 1H4: CD54 8A2: β1

Figure 3.10. Differential sensitivity of myeloma cell lines to TRAIL-mediated cell death. The myeloma cell lines were seeded in 96-well plates at a cell density of 10,000 cells/well. The cell number and viability were assessed by WST-1 at d3. The results showed that TRAIL mediated a significant reduction in RPMI 8226 cell number (A) in a dose-dependent manner. A significant decrease in cell number was found when the cells were treated with TRAIL at concentrations more than 100 ng/ml ($p<0.05$). In contrast, TRAIL had no effect on the other cell lines, ARH-77 (B), WL2 (C) and U266 (D). Values represent means ±SEM of triplicate cultures of each concentration. The results displayed are a representative example of 2 independent experiments.

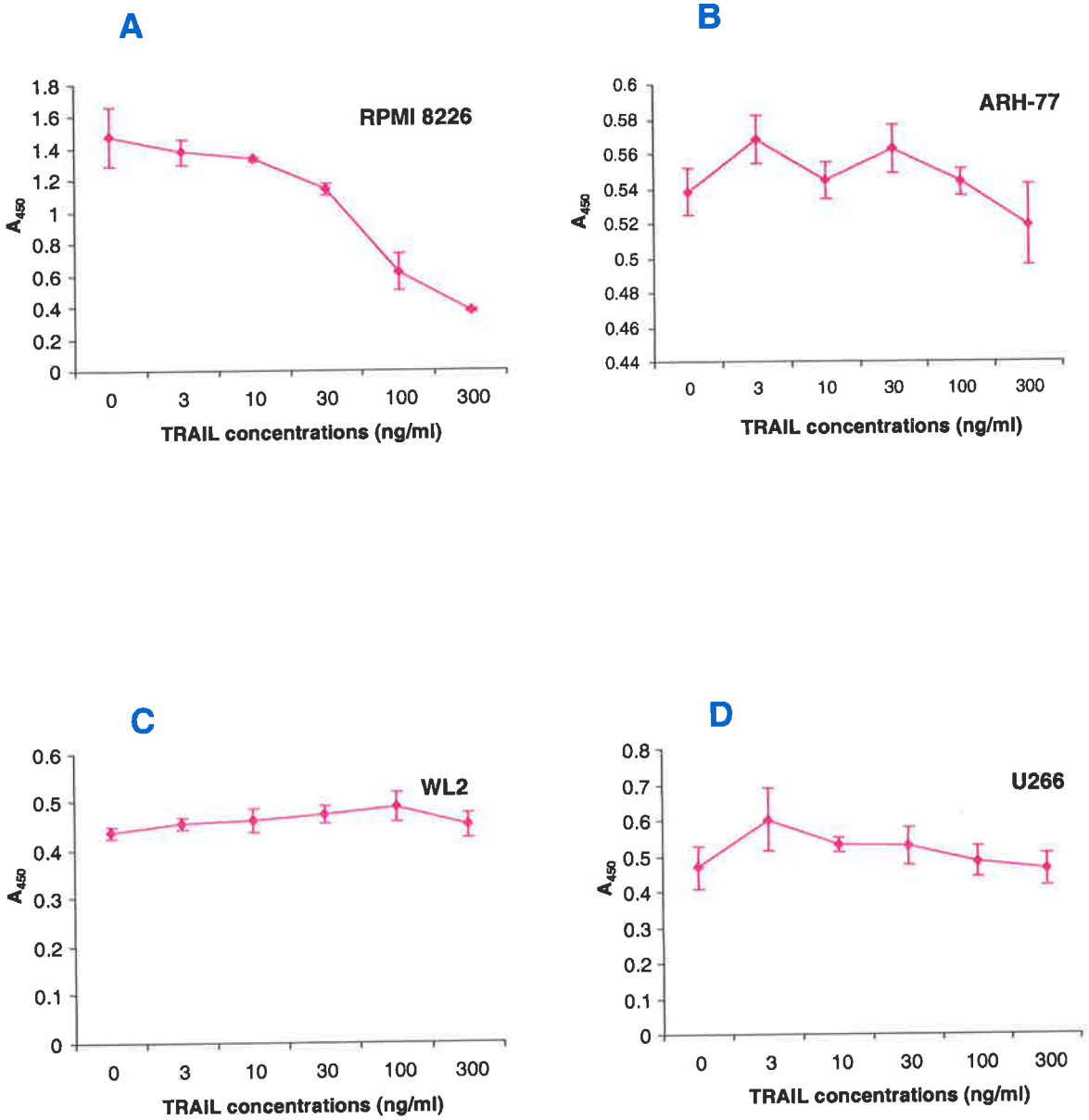
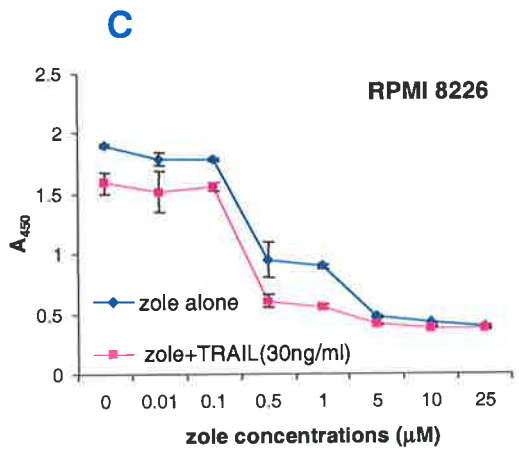
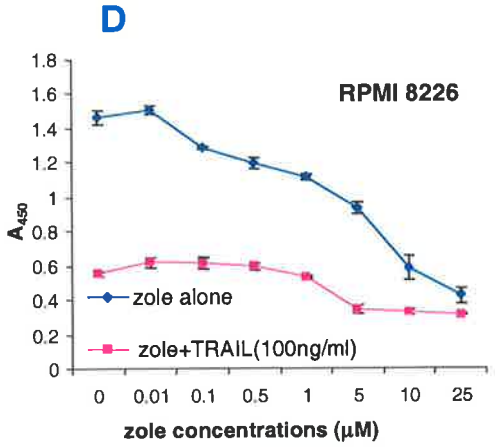
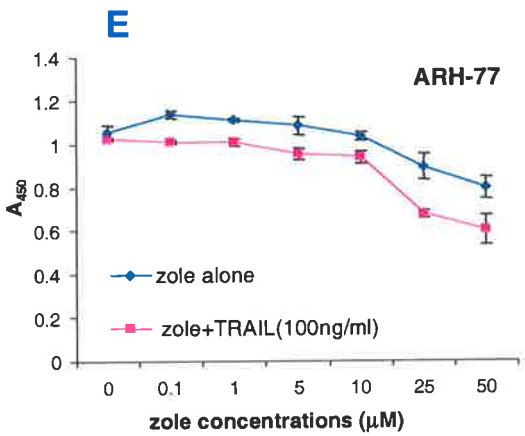

A
RPMI 8226
A_{450}
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
3
10
30
100
300
TRAIL concentrations (ng/ml)
B
ARH-77
A_{450}
0.6
0.58
0.56
0.54
0.52
0.5
0.48
0.46
0.44
0
3
10
30
100
300
TRAIL concentrations (ng/ml)
C
WL2
A_{450}
0.6
0.5
0.4
0.3
0.2
0.1
0
0
3
10
30
100
300
TRAIL concentrations (ng/ml)
D
U266
A_{450}
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
3
10
30
100
300
TRAIL concentrations (ng/ml)

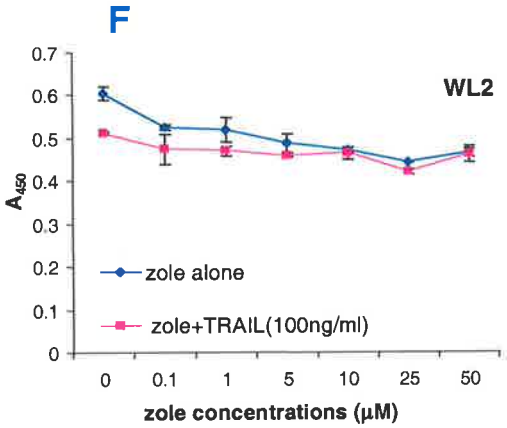
Figure 3.11. TRAIL enhanced the effect of zoledronic acid when used in combination on the myeloma cells. The myeloma cell lines were seeded in 96-well plates and treated with either zoledronic acid alone, or zoledronic acid and TRAIL at the concentrations as indicated. The cell number and viability were assessed at d3 and d5 using WST-1. Values represent means ±SEM of triplicate cultures of each concentration. The results displayed are a representative example of 2 independent experiments. Of note, there is no difference between d3 (A) and d5 (B) data, indicating that prolonged exposure of drugs did not enhance the effect. TRAIL rendered the cells more sensitive to zoledronic acid ($p<0.05$, Wilcoxon test) at concentrations previously shown to have no significant effects on cell proliferation [30 ng/ml for RPMI 8226 (C of d3 &C' of d5) and 100 ng/ml for other cell lines (E, F, G of d3 & E', F', G' of d5)]. When the concentration of TRAIL was increased to 100 ng/ml in RPMI 8226 (D&D'), zoledronic acid was only effective when used at concentrations greater 5 μM ($p<0.05$, *t*-test).

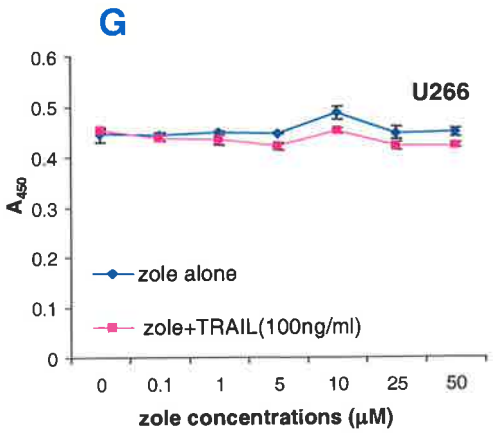
A


C
RPMI 8226
A450
zole alone
zole+TRAIL(30ng/ml)
0 0.01 0.1 0.5 1 5 10 25
zole concentrations (μM)


D
RPMI 8226
A450
zole alone
zole+TRAIL(100ng/ml)
0 0.01 0.1 0.5 1 5 10 25
zole concentrations (μM)

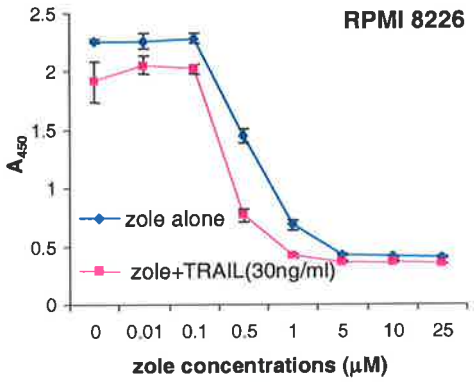

E
ARH-77
A450
zole alone
zole+TRAIL(100ng/ml)
0 0.1 1 5 10 25 50
zole concentrations (μM)


F
WL2
A450
zole alone
zole+TRAIL(100ng/ml)
0 0.1 1 5 10 25 50
zole concentrations (μM)

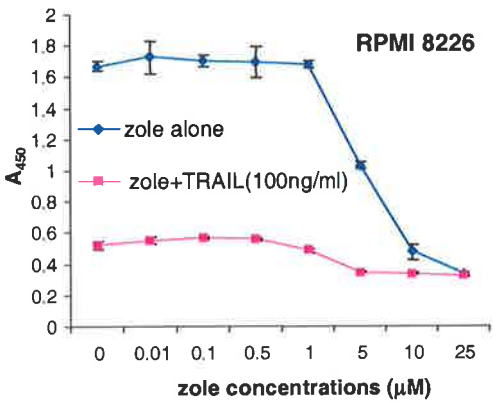

G
U266
A450
zole alone
zole+TRAIL(100ng/ml)
0 0.1 1 5 10 25 50
zole concentrations (μM)

B

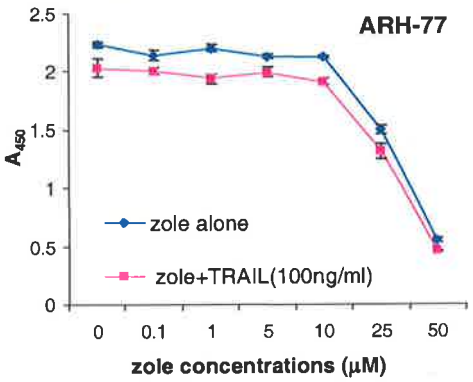
C'


RPMI 8226
A450
zole alone
zole+TRAIL(30ng/ml)
0 0.01 0.1 0.5 1 5 10 25
zole concentrations (μM)

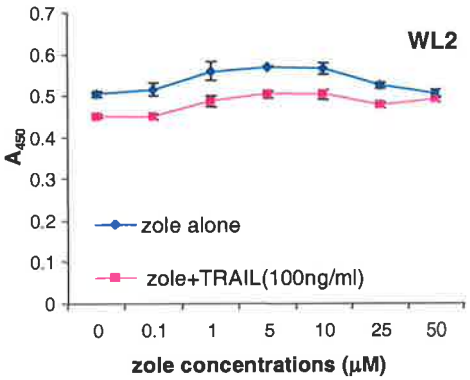
D'


RPMI 8226
A450
zole alone
zole+TRAIL(100ng/ml)
0 0.01 0.1 0.5 1 5 10 25
zole concentrations (μM)

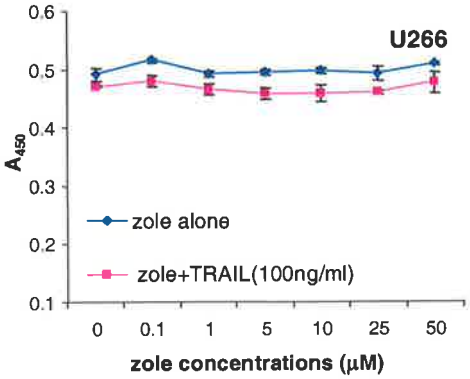
E'


ARH-77
A450
zole alone
zole+TRAIL(100ng/ml)
0 0.1 1 5 10 25 50
zole concentrations (μM)

F'


WL2
A450
zole alone
zole+TRAIL(100ng/ml)
0 0.1 1 5 10 25 50
zole concentrations (μM)

G'

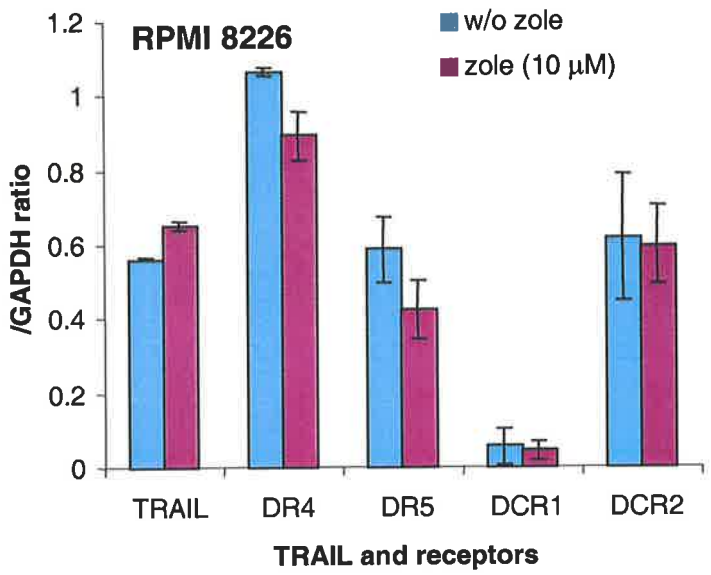

U266
A450
zole alone
zole+TRAIL(100ng/ml)
0 0.1 1 5 10 25 50
zole concentrations (μM)

3.2.8 *The Effect of Zoledronic Acid on the Expression of TRAIL and TRAIL Receptors on Myeloma Cell Lines*

TRAIL and its receptors were investigated at both the transcriptional and protein levels, using semi-quantitative RT-PCR and flow cytometry. Gene expression analysis showed that the TRAIL transcript was increased in response to zoledronic acid treatment in both cell lines (figure 3.12). The protein expression study indicated that the zoledronic acid sensitive cell line RPMI 8226 had increased TRAIL receptors DR4 and DR5 with a decreased or unchanged level of expression of decoy receptors (DcR1 and DcR2) in permeablised staining (figure 3.13). However, an increased DR4 and DR5 expression, coupled with increased DcR1 and DcR2 expression respectively, were noted in the resistant cell line WL2 when the cells were exposed to the zoledronic acid.

Figure 3.12. The effect of zoledronic acid on the TRAIL and TRAIL receptor gene expression in myeloma cell lines. Myeloma cell lines RPMI 8226 and WL2 were cultured in 6-well plates in the presence or absence of zoledronic acid at a concentration of 10 μM. The cells were collected at day 3, total RNA was isolated and semi-quantitative RT-PCR were performed as described in the methods. The results revealed increased TRAIL gene expression in response to zoledronic acid treatment in both cell lines (A&B). Values represent means ±SEM of the ratio of TRAIL gene expression relative to the expression of GAPDH.

A


RPMI 8226
w/o zole
zole (10 μM)
/GAPDH ratio
1.2
1
0.8
0.6
0.4
0.2
0
TRAIL
DR4
DR5
DCR1
DCR2
TRAIL and receptors

B

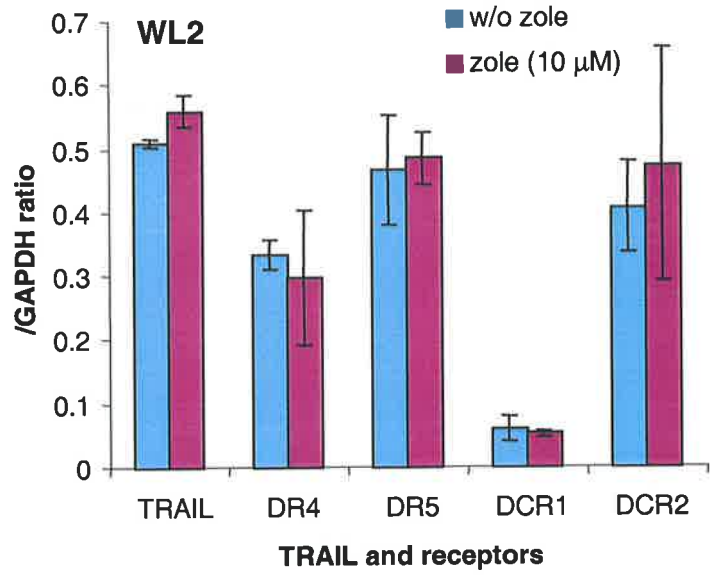
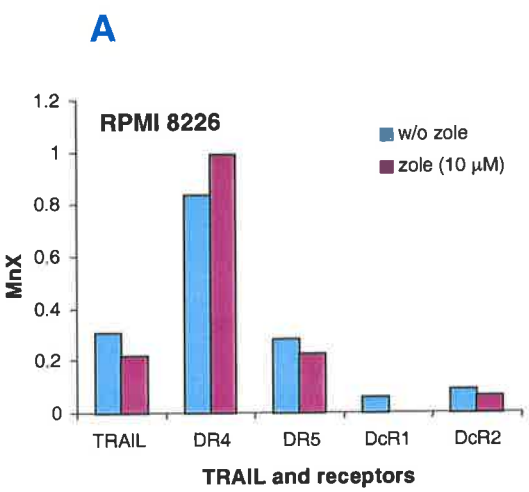
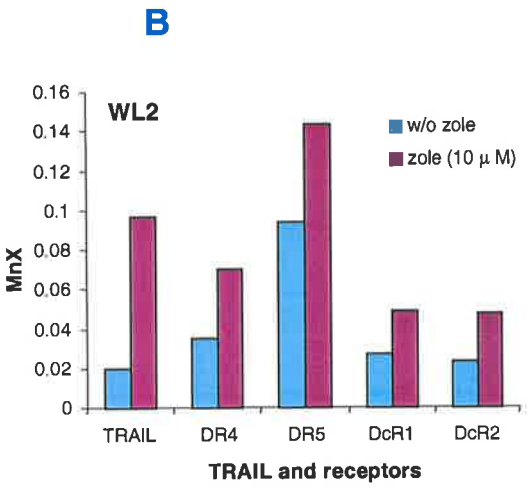

WL2
w/o zole
zole (10 μM)
/GAPDH ratio
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
TRAIL
DR4
DR5
DCR1
DCR2
TRAIL and receptors

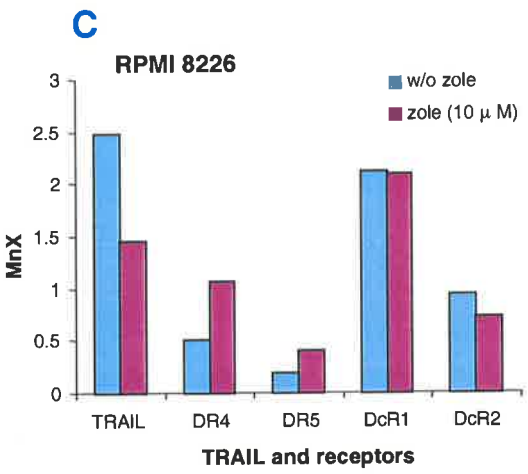
Figure 3.13. The effect of zoledronic acid on the protein expression of TRAIL and TRAIL receptors. The myeloma cell lines RPMI 8226 and WL2 were treated with zoledronic acid and both membrane and cytoplasmic protein expression of TRAIL and its receptors were detected as described in the methods. Data of d3 are displayed as the mean fluorescence of each protein from which the mean fluorescence value of the isotype-matched negative control has been subtracted. The results indicated that the zoledronic acid-sensitive cell line RPMI 8226 had increased TRAIL receptors DR4 and DR5 with the decreased or unchanged level of decoy receptor DcR1 and DcR2 when cytoplasmic protein expression was examined (C). However, the increased DR4 and DR5 expression, coupled with increased DcR1 and DcR2 expression respectively, were noted in zoledronic acid-resistance cell line WL2 (B&D).

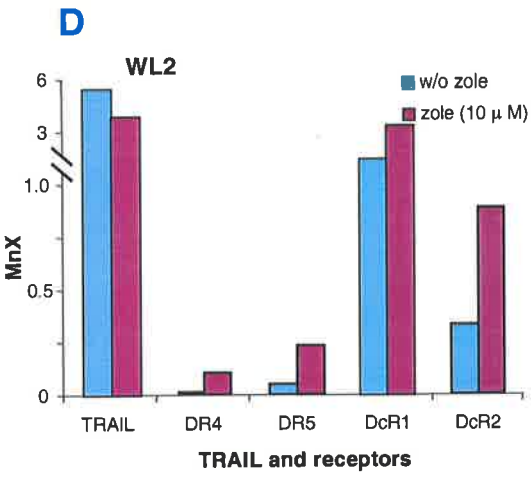
Membrane protein expression





Cytoplasmic protein expression





3.3 DISCUSSION

The prolonged survival benefit seen in the patients with MM treated with BPs (Berenson *et al.*, 1998; Berenson *et al.*, 1996; McCloskey *et al.*, 1998) has initiated numerous *in vitro* studies in an attempt to determine the mechanisms of the anti-tumour effect of BPs. The anti-tumour effects of BPs have been demonstrated in different cell lines, including human myeloma cells (Aparicio *et al.*, 1998; Shipman *et al.*, 1997; Takahashi *et al.*, 2001), breast carcinoma cells (Fromigue *et al.*, 2000; Sasaki *et al.*, 1995) as well as prostate cancer cells (Lee *et al.*, 2001). The studies reported herein, also demonstrate that zoledronic acid had a dose- and time-dependent cytotoxic effects on myeloma cell lines RPMI 8226 and ARH 77. Both dye exclusion (refer to figure 3.2) and WST-1 assays (refer to figure 3.1) revealed that zoledronic acid had a profound cytoreductive effect in the sensitive cell line RPMI 8226. In contrast, the myeloma cell lines, WL2 and U266 (which is an IL-6-autocrine MM cell line), were completely unaffected by zoledronic acid. Given the apparent difference in sensitivity between cell lines, it will be of interest to examine the underlying mechanism, for the differential sensitivity seen in myeloma cells.

As described in the results, zoledronic acid at concentrations greater than 5 μM induced a classical dose-dependent sigmoid response curve of cell number reduction. Therefore, in these *in vitro* studies, the concentrations of zoledronic acid used were generally over 5 μM. These doses are high relative to the peak serum concentrations achieved in the patients undergoing treatment with bisphosphonates. Thus, it remains to be determined whether sufficiently high levels of zoledronic acid could readily be attained in the patients for this anti-tumour activity. The peak serum concentrations of ibandroante achieved in the murine model were approximately 5 μM. This concentration did not affect the growth or viability of the mouse myeloma cell line 5TGM1 *in vitro*, and cytotoxic effects were seen only at doses of 50 μM and higher (Dallas *et al.*, 1999). Therefore, it is possible that BPs with different chemical structures may have different effects on tumour cell growth and apoptosis. Thus, the cytotoxic effects of BPs on myeloma cells may be limited to particular BPs with specific structural features. The concentration required to inhibit the growth of myeloma cells in the present study was far higher than the peak serum concentrations *in vivo*. However, owing to the singular skeletal distribution of administered BPs, marrow concentration may be sufficient to inhibit growth of myeloma cells. It was demonstrated that the pharmacological doses of BP required to inhibit bone resorption could lead to even higher concentrations, up

to 1000 μM, in the space beneath the ruffled border of osteoclasts *in vivo*, due to osteoclast-induced acidification (Sato *et al.*, 1991). Since the areas of active bone resorption have been described as specific homing sites of tumour cells in the patients with MM (Bataille *et al.*, 1995; Khan *et al.*, 1997; Mundy *et al.*, 1974), it is possible that the tumour cells may be exposed to relatively high concentrations of BPs, which are sufficient to induce cell death. However, the actual concentration of BPs encountered by cells other than osteoclasts remains unknown. Moreover, it is unclear as to the period of time that high concentrations in these areas are maintained.

The studies described here show that the zoledronic acid mediates its effect through a combination of cytostasis and cytolysis. Flow cytometric analysis of DNA content (refer to figure 3.3) demonstrated that following zoledronic acid treatment, there was an accumulation of cells in S-phase of the cell cycle. This may be due to slowing of progression through S phase or a block between S and G_2M phase, and hence inhibition of cell proliferation. These observations are consistent with reports that nitrogen-containing BPs can inhibit cell proliferation in other cell types (Fromigue *et al.*, 2000; Lee *et al.*, 2001; Sasaki *et al.*, 1995). The accumulation of RPMI 8226 cells in S phase was observed following treatment with zoledronic acid for 5 hours. After 48 hrs, there was a signifcant increase in the number of cells in the sub-G_0/G_1 phase of the cell cycle. The sub-G_0/G_1 hypodiploid population, is a characteristic of cells undergoing apoptosis, which have lost DNA owing to nuclear fragmentation. Apoptosis was also confirmed by morphologic analysis. Although only few apoptotic nuclei were observed, at least part of the cytoreductive effect induced by zoledronic acid could be attributed to the induction of apoptotic death. In contrast, the WL2 myeloma cell line was resistant to the effects of zoledronic acid. Of interest, RPMI 8226 was found to possess a greater proliferative rate than WL2 (refer to figure 3.2), which may account for the different sensitivity. Myeloma cells typically show a very low rate of proliferation in patients with MM. Therefore, the studies using myeloma cell lines are subject to limitations and myeloma cells isolated from the patient with MM should be used in the future studies. However, it should be emphasised that in our study, treatment of tumour cells with zoledronic acid was performed in the presence of serum, which is a survival factor for these cells. Therefore, it is conceivable that serum counteracts some pro-apoptotic effect of zoledronic acid.

Interestingly, zoledronic acid did not enhance the caspase activity in either RPMI 8226 or ARH-77 (refer to figure 3.5) although it induced apoptosis in a proportion of cells, which was confirmed by morphologic observation (refer to figure 3.4). Furthermore, caspase inhibitor I, which inhibits caspase activity, could not protect against cell death induced by zoledronic acid. This indicated that zoledronic acid may induce cell death in RPMI 8226 in a caspase independent pathway (via non-apoptotic programmed cell death with necrotic-like appearance, or via the mitochondrial pathway), which has also been reported (Mochizuki *et al.*, 2002; Daugas *et al.*, 2000; Kroemer, 1999)

We next examined whether zoledronic acid induces myeloma cell death *via* inhibition of the mevalonate pathway (refer to figure 3.6), a biosynthetic pathway responsible for the production of cholesterol and isoprenoid lipids. Consistent with previous reports (Shipman *et al.*, 1998; for review see Russell and Rogers, 1999), we have shown that the effects of zoledronic acid on myeloma cells is mediated, in part, through the inhibition of the mevalonate pathway. The cell death induced by zoledronic acid was reversed in the presence of GGO, an intermediate of the mevalonate pathway, at a concentration greater than 300 μM. However, the cell number was still lower than that in negative controls, suggesting that GGO could not fully overcome the inhibitive effect of zoledronic acid on myeloma cell proliferation. These results suggest that zoledronic acid induces its cytoreductive effect on drug sensitive myeloma cell lines partially through the mevalonate pathway. Consistent with the findings of others (Miquel *et al.*, 1996; Ohizumi *et al.*, 1995; Ohizumi *et al.*, 1997), our study also demonstrated that GGO caused a degree of cell death in RPMI 8226, in a dose-dependent manner, without activating caspases.

Human myeloma cells were reported to produce several OAFs, including TNFα, IL-1β, IL-6, M-CSF and RANKL *et al* (Costes *et al.*, 1998; Filella *et al.*, 1996; Pfeilschifter *et al.*, 1989), which play an important role in the pathogenesis of MM induced osteolysis. In addition, the anti-resorptive effects of BPs, at least partially, are considered to be the result of inhibited release of some OAFs from OB (Derenne *et al.*, 1999; Giuliani *et al.*, 1998b; Ohya *et al.*, 1985; Stronski *et al.*, 1988). To investigate whether BPs inhibit the secretion of OAFs from myeloma cells, gene expression was examined using semi-quantitative RT-PCR.

Surprisingly, zoledronic acid significantly upregulated gene expression of TNFα in the sensitive myeloma cell line RPMI 8226 (refer to figure 3.7). TNF was initially recognised and named for its ability to cause necrosis of tumour masses. It has been shown to also have dramatic systemic effects. At the cellular level, TNF-α has widespread pleiotropic actions and has been shown to modulate the fundamental processes associated with cell proliferation, differentiation, and apoptosis or necrotic cell death in a number of different cell types. TNF-α is a survival and proliferation factor for human myeloma cells (Borset *et al.*, 1994; Jourdan *et al.*, 1999), and a high level of TNF-α was found in supernatant from bone marrow cultures of MM patients, which was capable of markedly stimulating formation of OC-like multinucleated cells in human marrow cultures (Filella *et al.*, 1996; Lichtenstein *et al.*, 1989; Pfeilschifter *et al.*, 1989). In our study, TNF-α was upregulated in the zoledronic acid sensitive myeloma cell lines, whilst it remained unchanged in drug resistant cell line WL2, suggesting it may represent a rescue mechanism in response to zoledronic acid treatment. However, TNF-α was found to mediate apoptotic cell death in a mitotically active human erythroleukemic cell line, and to induce cell proliferation in mitotically quiescent cells (Baxter *et al.*, 1999). As we have shown in the results that the RPMI 8226 cell line had greater proliferative potential than WL2 cells (refer to figure 3.2), it is also possible that zoledronic acid induces cell death by up regulating TNF-α gene expression. To differentiate these two possibilities, blocking antibodies to TNF-α could be used to examine whether they can protect from zoledronic acid-mediated cell death in the myeloma cell line RPMI 8226.

Zoledronic acid also significantly upregulated gene expression of IL-1β and COX-2 in the sensitive myeloma cell line RPMI 8226 (refer to figure 3.7). IL-1β is a principal bone resorption agent present in the supernatant of myeloma cell cultures that enhances destructive bone lesions in the patients with MM (Cozzolino *et al.*, 1989; Yamamoto *et al.*, 1989). TNF-α and IL-1β produced by MM or accessory cells are also able to stimulate IL-6 production by OB, through a prostaglandin E2 (PGE2) loop and show additive effects (Costes *et al.*, 1998; Lu *et al.*, 1995; Thomas *et al.*, 1998), while IL-6 is a growth factor for MM. In addition, TNF-α and IL-1β are also able to increase the adhesion of MM cells to stromal cells (Thomas *et al.*, 1998). Thus, IL-1β can promote the secretion of IL-6, and both IL-1β and IL-6 enhance the bone lytic lesions in MM. Furthermore, both TNF-α and IL-1β can induce COX-2 expression. Cyclooxygenase (COX) is a family of enzymes that catalyse the conversion of arachidonic acid to prostaglandins (for review see (Hinz and Brune, 2002)). Two COX

isoforms have been identified: COX-1 and COX-2. COX-1 is constitutively expressed as a "housekeeping" enzyme and is ubiquitous in its distribution. COX-2 is inducible and it can be upregulated by various pro-inflammatory agents, including lipopolysaccharide, cytokines, and growth factors. The induction of COX-2 has been shown to promote cell growth, inhibit apoptosis, and enhance cell motility and adhesion in a number of cell and animal models. Expression of COX-2 appears to be important in tumour promotion, growth, metastasis and angiogenesis (Cianchi *et al.*, 2001; Williams *et al.*, 1999), which is a key step in carcinogenesis. Therefore, the upregulation of COX-2 may be in response to the increased expression of IL-1 β and TNF-α, or induced directly by zoledronic acid. It is our contention, however, that the upregulation of transcripts represent a rescue reaction of cell lines to the zoledronic acid treatment since COX-2, IL-1 β, and TNF-α are able to promote cell proliferation.

Cells have the capability of responding to a multitude of signals that they encounter in the extracellular environment. Therefore, an additional anti-tumour effect of BPs might occur secondary to effects on MM cell growth factors, which can be secreted by both myeloma cells and the bone marrow environment. In contrast, our study demonstrates that zoledronic acid does not inhibit the secretion of TNF-α, IL-1β and COX-2, at least at the transcriptional level. Although BPs were reported to potentially inhibit the production/release of IL-6 and MMP-1 from myeloma cells and myeloma bone marrow stoma cells (Derenne *et al.*, 1999), we did not detect a significant change in the IL-6 gene expression when the cells were exposed to zoledronic acid. It is well accepted that IL-6 is a potent myeloma growth factor which acts *via* an autocrine and paracrine mechanism (Kawano *et al.*, 1988; Klein *et al.*, 1989), and can be upregulated by TNF-α and IL-1β (Costes *et al.*, 1998; Lu *et al.*, 1995; Thomas *et al.*, 1998). In addition, zoledronic acid increased the expression of TNF-α and IL-1β in RPMI 8226, however, no upregulation of IL-6 gene expression was observed. The detection of protein levels of IL-6 should be considered in future studies. In contrast to breast cancer cells, myeloma cells are derived from haemopoietic cell lineages, which normally reside in the bone marrow. Thus, the marrow microenvironment may already be favourable for growth of the myeloma cells independent of local rates of bone resorption, or of the production of cytokines secreted by OB such as IL-6. This may explain why myeloma cells appear to be able to grow in the marrow cavity and completely replace the normal marrow cells even when osteoclastic activity is inhibited by BPs. Thus myeloma cells may be less

dependent than breast cancer cells on factors released by resorbing osteoclasts, for their growth in the bone marrow cavity.

Cell-cell and/or cell-ECM adhesion not only play important roles in regulating apoptosis and cell survival in tumour cells (Clark and Brugge, 1995), but are also involved in the migration of the MM cells into the extravascular compartment of the bone marrow, a process referred to as "homing". Therefore, an additional anti-myeloma mechanism mediated by zoledronic acid was considered to be the inhibition of the adhesiveness to bony matrix of circulation myeloma cells, preventing further myeloma tumour deposits within the skeleton, thereby overcoming the adhesion-mediated drug resistance (Boissier *et al.*, 2000; van der Pluijm *et al.*, 1996). The cell surface molecules of interest which are expressed by myeloma cell lines include β_2-microglobulin, integrin $\alpha_4\beta_1$, CD44, CD45, CD54, CD56. The expression of these molecules was examined using single-colour flow cytometric analysis in our study (refer to figure 3.8).

One interesting finding is that RPMI 8226 cells express high levels of HLA class I, its subunit β_2 microglobulin, and CD138. In contrast, WL2, which is resistant to zoledronic acid, did not express any of these antigens. It is generally accepted that the serum level of β_2-microglobulin is a reliable prognostic marker in the patient with MM (Bataille *et al.*, 1992; Bataille *et al.*, 1984; Durie *et al.*, 1990; Greipp *et al.*, 1988; Greipp *et al.*, 1993). A low β_2-microglobulin level is associated with a significantly better complete response rate among the patients undergoing transplantation for myeloma (Rajkumar *et al.*, 1999). The relationship between the serum level of β_2-microglobulin and cell surface expression on myeloma cells is unknown. It is possible that zoledronic acid-resistant cells secrete more soluble β_2-microglobulin, or this molecule is cleaved from the cell surface into serum. It would be interesting to investigate the relationship between drug resistance and β_2-microglobulin expression on myeloma cells derived from the patients, in future studies.

Consistent with other studies (Clark and Brugge, 1995; Damiano *et al.*, 1999), both cell lines were found to express $\alpha_4\beta_1$ integrin (61.2C4, P4C2), which allows attachment and communication between the myeloma cells and the bone marrow microenvironment (Barker *et al.*, 1992; Jensen *et al.*, 1993). Our study found that the expression of $\alpha_4\beta_1$ integrin by the zoledronic acid resistant cell line WL2 was greater in contrast to zoledronic acid sensitive

cell line RPMI 8226, suggesting that differential sensitivity to zoledronic acid between the two cell lines may be partially attributed to the extent of $\alpha_4\beta_1$ integrin expression.

Our results also showed that the zoledronic acid-resistant cell line WL2 strongly expressed CD44 and CD45. CD44 is a transmembrane glycoprotein expressed by wide variety of cell types. It functions in cell-cell adhesion and binds to components of the extracellular matrix, including fibronectin, collagen, hyaluronan (HA), and osteopontin. The many isoforms of these cell surface glycoproteins are encoded by a single gene that consists of standard exons (1s to 10s) and variant exons (1v to 10v). The standard exons encode the common part of the CD44 family members (CD44s, which is widely distributed), and the variant exons are alternatively spliced, giving rise to the different members of the family (Gunthert, 1993). It was demonstrated that CD44v10 mediates murine MM cells binding to bone marrow endothelium (Asosingh *et al.*, 2001). CD44v9 was involved in binding to bone marrow stromal cells which resulted in a significant induction of IL-6 secretion by bone marrow stromal cells, which may account for correlation between CD44v9 expression and adverse prognosis in MM (Stauder *et al.*, 1996; van Driel *et al.*, 1998; Van Driel *et al.*, 2002). CD45 antigen was initially characterised as leucocyte common antigen expressed on all haemopoietic cells except for mature erythrocytes and platelets. It has been reported to identify subpopulations of myeloma cells with higher proliferative capacity (Joshua *et al.*, 1996; Kawano *et al.*, 1993). The $CD45^+$ subpopulation of myeloma cells proliferate in response to IL-6 (Fujii *et al.*, 1999) and CD45 expression also can be induced by IL-6 stimulation (Mahmoud *et al.*, 1998). The activities of src family protein tyrosine kinases (PTKs) associated with the CD45 protein tyrosine phosphatase (PTP), seem to be a prerequisite for the myeloma cell proliferation. The activation of signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK1/2) by IL-6 were further required (Ishikawa *et al.*, 2002). Therefore, CD44 and CD45 are two other key molecules regulating myeloma cell adhesion, which may contribute to the resistance of WL2 to zoledronic acid treatment.

In contrast, the zoledronic acid sensitive myeloma cell line RPMI 8226 was found to strongly express CD54, and CD56. CD54, an intercellular adhesion molecule-1 (ICAM-1), was also expressed by myeloma cells (Leo *et al.*, 1992; Van Riet *et al.*, 1991) and anti-CD54 antibody had anti-tumour activity without inhibiting adhesion and cell proliferation (Huang *et al.*, 1995). This suggests that CD54 may not be a key molecule in the adhesion of myeloma cells.

CD56 antigen, a 140 kD isoform of the neural cell adhesion molecule (N-CAM), is a membrane glycoprotein and belongs to the immunoglobulin superfamily. It is also known as a differentiation antigen of natural killer (NK) cells and has been described as a prognostic marker in MM (Kaiser *et al.*, 1996). Down regulation of CD56 and upregulation of CD44 were found in extramedullary myeloma (Dahl *et al.*, 2002). The lack or weak expression of CD56 is a characteristic feature of plasma cell leukemia (PCL) (Pellat-Deceunynck *et al.*, 1998). It is difficult to compare myeloma cell lines and the myeloma cells isolated freshly from patients with MM since the cell lines were immortalised and cultured *in vitro* for a long time.

Our *in vitro* studies also showed that zoledronic acid marginally increased the expression of $\alpha_4\beta_1$ integrin, CD44, CD45, CD54 and CD56 expression in both cell lines, which could be considered as a rescue response of myeloma cells to drug treatment. The up regulation of CD54 may be a response to the upregulated IL-1β and TNF-α expression, since it was found that IL-1β and TNF-α can upregulate CD54 expression (Thomas *et al.*, 1998). It has been reported that during the course of initial or chronic drug exposure, myeloma cells overexpressing $\alpha_4\beta_1$ may have a selection advantage over cells expressing low levels of this protein. Selection pressure is required for $\alpha_4\beta_1$ upregulation in drug-resistant cells (Damiano *et al.*, 1999) and cells under selection pressure may then use soluble or cell-bound integrin ligands as a protective mechanism. Furthermore, myeloma cells in patients are surrounded by other cell types in the bone marrow microenvironment and they are exposed to the numerous cytokines secreted by both myeloma cells and OB, and as such, myeloma cells may react to zoledronic acid differently in an *in vivo* setting.

Our functional assays examining the adhesion of myeloma cell lines showed that both myeloma cells adhere preferentially to VCAM-1 and not FN. Both RPMI 8226 and WL2 cells use α4β1 to adhere to VCAM-1. RPMI 8226 use CD44, whilst WL2 cells use both α4β1and CD44 to adhere to FN. Surprisingly, zoledronic acid does not change adhesion of myeloma cells (refer to figure 3.9). This may be due to the relatively low concentration or short exposure time of the drug. Breast cancer cells were found to adhere rapidly to extracellular bone matrix, which can be inhibited by pre-treating of cortical or trabecular bone with BPs (van der Pluijm *et al.*, 1996). Moreover, the anti-adhesive effect of nitrogen-containing BPs was bone specific since pre-treatment of non-mineralised tissues with BPs did

not affect adhesion of the breast cancer cells (van der Pluijm *et al.*, 1996). The one underlying mechanism is that BPs, like pyrophosphate, specifically bind strongly to the bone mineral, hydroxyapatite, thereby non-mineralised tissue cannot retain BPs to affect breast cell adhesion. In addition, human bone cells express high levels of integrins (Hughes *et al.*, 1993) and BPs might affect the integrin expression on the bone instead of cancer cells. Thus, we postulate that zoledronic acid may inhibit the adhesiveness of circulating myeloma cell to bony matrix, and prevent further myeloma tumour deposits within the skeleton by inhibiting the integrin expression on the OB. As we have known, FN and VCAM-1 are expressed on the surface of MM stromal monolayers (Faid *et al.*, 1996; Lokhorst *et al.*, 1994), and antibodies to VCAM-1 partially inhibit myeloma cell adhesion to MM stroma (Kim *et al.*, 1994; Robledo *et al.*, 1998). Our preliminary experiments showed that zoledronic acid decreased VCAM-1 protein expression on the OB-like cells (data not shown), further confirming that zoledronic acid might affect the adhesion of cells by acting on OB.

TRAIL is a TNF superfamily member that is capable of inducing apoptosis. It interacts with five distinct receptors: DR4, DR5 (Pan *et al.*, 1997; Sheridan *et al.*, 1997), DcR1, DcR2 (Degli-Esposti *et al.*, 1997; Sheridan *et al.*, 1997) and OPG (Emery *et al.*, 1998). DR4 and DR5 are necessary and sufficient to mediate cell death, while DcR1, DcR2 and OPG may be protective receptors by acting either as membrane-bound or soluble antagonistic receptors (Degli-Esposti *et al.*, 1997; Emery *et al.*, 1998; Pan *et al.*, 1997; Sheridan *et al.*, 1997). Consistent with the findings of others (Gazitt, 1999), we found that TRAIL induced apoptosis in a dose-dependent manner in one of four myeloma cell lines we used in this study (refer to figure 3.10). Of note, our experiments showed that TRAIL and zoledronic acid had a similar pattern of effect on the myeloma cell lines. RPMI 8226 cells are sensitive to both TRAIL and zoledronic acid in a dose-dependent manner. TRAIL had no effect on the other myeloma cell lines, WL2 and U266. Although TRAIL had no significant effect on ARH-77, it may produce an effect if the dose was increased, since ARH-77 was only sensitive to zoledronic acid at high doses. Zoledronic acid-induced reduction in cell number was significantly enhanced by the addition of TRAIL in all four myeloma cell lines (refer to figure 3.11). However, when the concentration of TRAIL was increased to 100 ng/ml in RPMI 8226 cells, which alone can induce a significant reduction in cell number, zoledronic acid had limited effects on cells, suggesting zoledronic acid may partially act on the myeloma cells through the upregulation of TRAIL expression.

At the mRNA level, both RPMI 8226 and WL2 cells expressed TRAIL, DR4 and DR5 (refer to figure 3.12). At the protein level, although zoledronic acid-resistant cell line, WL2, hardly expressed cytoplasmic DR4 and DR5 receptors, both cell lines expressed membrane death receptors (refer to figure 3.13). Therefore, the level of surface expression of either DR4 or DR5 correlates with TRAIL-sensitivity in our panel of MM cell lines. It has been reported that some TRAIL-resistant cells have very strong expression of both receptors, in comparison to some TRAIL-sensitive cells (Mitsiades *et al.*, 2001). DcR1 and DcR2 expression has been suggested as another potential mechanism for protection of cells against TRAIL. DcR2 was uniformly expressed by both TRAIL sensitive and resistant cells by either RT-PCR or flow cytometric analysis. In contrast, DcR1 was hardly expressed at the mRNA level and membrane protein levels on both MM cell lines, but cytoplasmic DcR1 was strongly expressed on both cell lines. Therefore, TRAIL receptor status alone cannot serve as a reliable predictor of TRAIL sensitivity in MM cells, with the exception of the lack of both DR4 and DR5 expression. This lack of correlation of TRAIL decoy receptor expression with TRAIL sensitivity in MM cells observed in this study is consistent with another report (Mitsiades *et al.*, 2001). It should be emphasised that it is not known how the different TRAIL receptors interact with one another on the cell surface. Neither is it known how much DcR1 and DcR2 must be present on the surface of normal cells to prevent the formation of DR4 or DR5 signalling complexes to provide protection from the cytotoxic effects of TRAIL.

After exposure of myeloma cells to zoledronic acid, the TRAIL transcript was increased in both zoledronic acid-sensitive and resistant cell lines. Of interest, the flow cytometric analysis indicated that the zoledronic acid-sensitive cell line RPMI 8226 had increased TRAIL receptors DR4 and DR5 with the decreased or unchanged level of decoy receptor DcR1 and DcR2. However, the increased DR4 and DR5 coupled with the increased DcR1 and DcR2 respectively, were noted in resistant cell line, WL2, when the cells were exposed to zoledronic acid. Therefore, the coupled reaction of TRAIL receptors and decoy receptors may be decisive for the cell sensitivity to the zoledronic acid. The uncoupled DR4, DR5 and DcR1, DcR2 in the sensitive cell line RPMI 8226 may be responsible for the drug sensitivity. This suggest that TRAIL-induced apoptosis is not regulated simply through either the competitive binding of TRAIL by DcR1 and DcR2 or by the transduction of protective signals mediated by either of these receptors. It does not preclude the possibility that changes of surface expression of TRAIL receptor(s) in each particular cell lines may modulate its

TRAIL sensitivity. On the other hand, it also suggests that zoledronic acid may act on the myeloma cells partially by activating TRAIL and its receptor pathway.

Since myeloma cells are located in the bone marrow microenvironment, the cytokines secreted by stromal cells in the vicinity should be considered. It was found that OPG, a secreted homologue of the tumour necrosis factor receptor, represented a fifth TRAIL receptor (Emery *et al.*, 1998). Therefore, it is possible that OPG could block the effect of zoledronic acid on the sensitive myeloma cells since it has been reported that OPG could inhibit TRAIL-induced apoptosis of Jurkat cells (Emery *et al.*, 1998). Future studies examining the role of OPG in blocking the effect of zoledronic acid would enable this question to be addressed.

Taken together, our *in vitro* studies indicate that zoledronic acid might produce the therapeutic action on myeloma cells (1) through direct anti-proliferative and apoptotic effects, (2) by inhibiting the mevalonate pathway, and (3) activating TRAIL and its receptor pathway. The results shown here also highlight the potential clinical value of the recently developed BP, zoledronic acid, which in combination with TRAIL may result in additive anti-myeloma effects, and yield benefit in the treatment of MM. Further studies are required to examine whether BPs with different chemical structure may have different effects on myeloma cell growth and apoptosis.

CHAPTER 4
IN VITRO STUDIES EXAMINING THE EFFECT OF THE NITROGEN CONTAINING BISPHOSPHONATE, ZOLEDRONIC ACID, ON OSTEOBLAST-LIKE CELLS

4.1 INTRODUCTION

It is widely believed that the increased bone mineral density in patients receiving BPs can be attributed to decreased bone turnover (Balena *et al.*, 1993; Chavassieux *et al.*, 1997; Storm *et al.*, 1993). This observed decrease in bone turnover appears to be due to BP-mediated inhibition of proliferation and recruitment of OC precursors (Hughes *et al.*, 1989; Schmidt *et al.*, 1996) as well as apoptosis of mature OC (Hughes *et al.*, 1995; Murakami *et al.*, 1995; Sato and Grasser, 1990; van Beek *et al.*, 1997). In recent years, it has been proposed that BPs may also act indirectly by modulating the expression of osteoclastogenic factors synthesised by OB (Derenne *et al.*, 1999; Giuliani *et al.*, 1998b; Nishikawa *et al.*, 1996; Sahni *et al.*, 1993; Vitte *et al.*, 1996). However few studies have been reported detailing the direct effects of BPs on OB (Giuliani *et al.*, 1998a; Reinholz *et al.*, 2000; Tenenbaum *et al.*, 1992; Toolan *et al.*, 1992; Tsuchimoto *et al.*, 1994). Therefore, the increase in bone mass observed following BP treatment may not be fully explained by the inhibition of bone resorption (Pataki *et al.*, 1997), but may, in part, be due to increased bone formation by OB.

Osteoblasts play a pivotal role during the biological response of bone to agents including hormones, polypeptide growth factors, and cytokines that stimulate bone resorption and/or inhibit bone formation. The proliferation and differentiation of OB precursors (or preosteoblasts) is an essential step in the bone formation process. It has been reported that pamidronate and zoledronic acid decrease OB proliferation and enhance the differentiation and bone-forming activities of OB (Reinholz *et al.*, 2000). However, the underlying mechanisms of action remain unclear. Therefore, to clarify why BPs inhibit OB proliferation and stimulate the differentiation of these cells, could provide additional clues with respect to the action and molecular targets of BPs.

Our understanding of OB differentiation and function has been hampered by a relative lack of markers which identify the earliest cells of this lineage (Gronthos *et al.*, 1999). Despite this, a

number of studies now demonstrate that the OB lineage shares a common stromal cell precursor with adipocytes, reticular cells, fibroblasts and chondrocytes (Gronthos *et al.*, 1994). Moreover, a number of independent studies have shown that the OB lineage at different stages of maturation can be separated, based on their expression of the stromal precursor cell marker, STRO-1 and the osteoblastic marker, alkaline phosphatase (Gronthos *et al.*, 1999; Stewart *et al.*, 1999). The majority of the NHBCs, which express the STRO-1^- /ALP^+ and STRO-1^-/ALP^- phenotypes, appear to represent fully differentiated OB. In contrast, the cells that express STRO-1^+/ALP^+ phenotype are considered to be representative of osteoprogenitors, whilst the cells, which only express STRO-1 antigen, represent stromal precursors.

Zoledronic acid is a new generation, nitrogen-containing BP and the most potent inhibitor of bone resorption currently available. In this study, we have investigated the effect of zoledronic acid on cultures of human OB with respect to (a) its ability to regulate the expression of molecules with roles in osteoclastogenesis and (b) its ability to promote bone formation, using an established model of human OB differentiation and function.

4.2 RESULTS

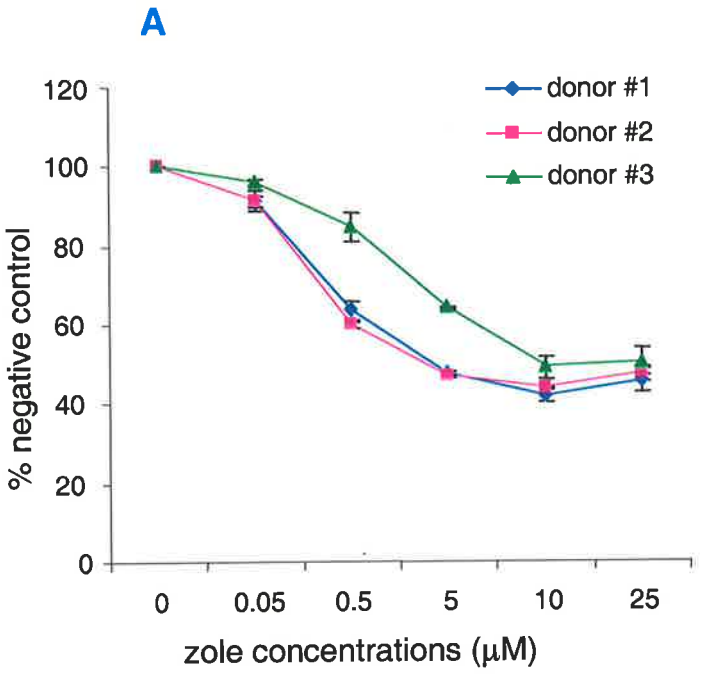
4.2.1 The Effect of Zoledronic Acid on Osteoblast-Like Cell Proliferation and Survival

Osteoblast-like cells derived from 3 donors were cultured in increasing concentrations of zoledronic acid. As shown in figure 4.1, zoledronic acid induced a significant decrease in cell number at concentrations of 0.05μM or greater at d3 in all three donors ($p<0.05$). Furthermore, zoledronic acid induced a decrease in cell number in a dose-dependent manner at both d3 and d5. Surprisingly, a low concentration (0.05μM) of zoledronic acid was found to increase cell number at d5, although there was no significant difference compared with negative control ($p>0.05$).

The actual concentration range of BPs that OB are exposed to under pharmacological conditions *in vivo* is unknown. Therefore, it is difficult to design *in vitro* experiments that can be directly correlated to *in vivo* physiological conditions. Five μM of zoledronic acid, a concentration having an effect in terms of inducing measurable cellular responses, was chosen for most of the experiments in this study. Osteoblast donor cells were cultured in the presence of 5μM zoledronic acid and the cell number and viability was assessed at the different time points using the colorimetric dye WST-1. Zoledronic acid was found to inhibit cell proliferation and induce cell death in a proportion of the cells. At day 3, a significant difference between negative control and zoledronic acid treatment groups was observed in cells from all three donors ($p<0.05$). In this study, donor #1 responded to zoledronic acid with the least sensitivity, whereas donor #3 responded to the drug with the most sensitivity (figure 4.2).

To differentiate between cytostatic and cytotoxic effects of zoledronic acid, studies were performed to examine if zoledronic acid induced apoptosis. Using the nuclear fluorochrome, DAPI, morphological examination revealed that treatment of OB-like cells with zoledronic acid led to morphological changes characteristic of apoptosis, including chromatin condensation, nuclear fragmentation and the formation of dense, rounded apoptotic bodies (figure 4.3). The proportion of apoptotic cells was low in all donor OB-like cell cultures and it was donor dependent. Donor #3 had more apoptotic nuclei compared with Donor #1 and #2 (refer to figure 4.1). In contrast, analysis by gel electrophoresis showed that zoledronic acid

Figure 4.1. The effect of increasing concentrations of zoledronic acid on the proliferation of human OB-like cells. Osteoblast-like cells derived from three different donors were seeded in 96-well plates at a cell density of 4000 cells/well. Cells were cultured in media supplemented with zoledronic acid at the different concentrations. Cell number and viability was quantitated at d3 and d5 using WST-1 as described in section 2.1.2.1 and the results are presented as the mean percentage of negative control ± SEM (triplicate cultures). Zoledronic acid induced a significant decrease of cell number at a concentration of 0.05μM at d3 (A) in all three donors ($p<0.05$, *t*-test). Furthermore, zoledronic acid induced a cytoreductive effect in a dose-dependent manner. A similar phenomenon was observed at d5 (B), however, zoledronic acid at a concentration of 0.05μM was found to increase cell number at d5, although this was not significantly different when compared with untreated cells ($p>0.05$, *t*-test). This study also revealed that OB-like cells derived from donor #3 proliferated faster than those derived from donor #1 and #2.


A
donor #1
donor #2
donor #3
% negative control
120
100
80
60
40
20
0
0
0.05
0.5
5
10
25
zole concentrations (μM)

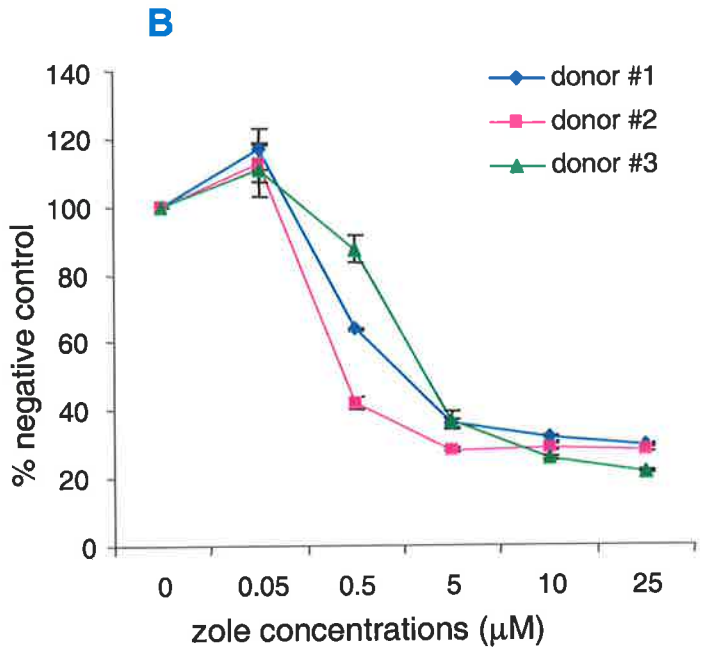
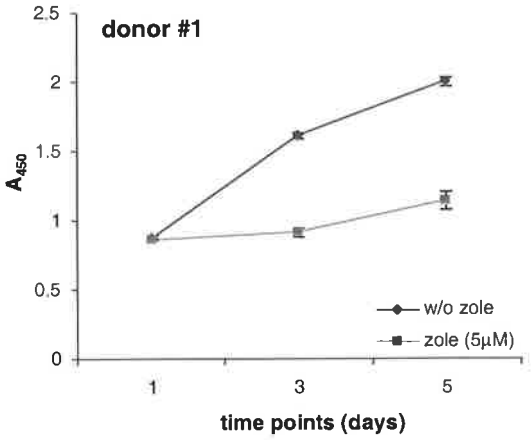
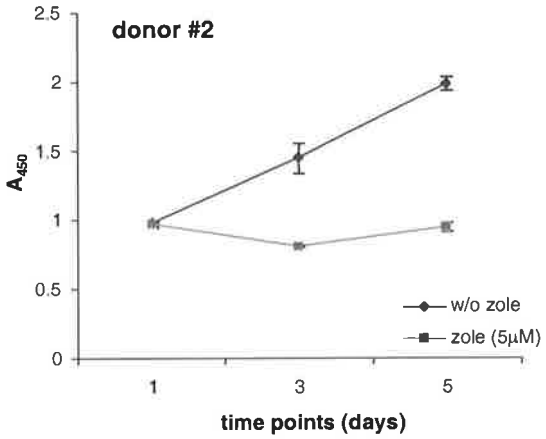

B
donor #1
donor #2
donor #3
% negative control
140
120
100
80
60
40
20
0
0
0.05
0.5
5
10
25
zole concentrations (μM)

Figure 4.2. Effects of 5 μM zoledronic acid on the proliferation of OB-like cells. Osteoblast-like cells derived from three different donors were seeded at a cell density of 4000 cells/well in 96-well plates using FACStar^{PLUS} cell sorter. Cells were cultured in medium in the presence or absence of zoledronic acid for the period of 5 days. The cell number and viability was assessed by WST-1 as described in the methods, and the data are presented as the mean absorbance of colorimetric reaction ± SEM (triplicate cultures). These studies demonstrate that zoledronic acid induces an anti-proliferative effect at a concentration of 5 μM in OB-like cells derived from all three donors. A significant difference between negative control and zoledronic acid treated groups was achieved at day 3 ($p<0.01$, *t*-test), and the sensitivity of OB-like cells to zoledronic acid was donor dependent. In this study, donor #1 (A) responded to zoledronic acid with the least sensitivity, whilst donor #3 (C) responded to the drug with the most sensitivity. The sensitivity of reaction of donor #2 (B) to zoledronic acid was found to be intermediate.

A


donor #1
2.5
2
1.5
1
0.5
0
A450
w/o zole
zole (5μM)
1
3
5
time points (days)

B


donor #2
2.5
2
1.5
1
0.5
0
A450
w/o zole
zole (5μM)
1
3
5
time points (days)

C

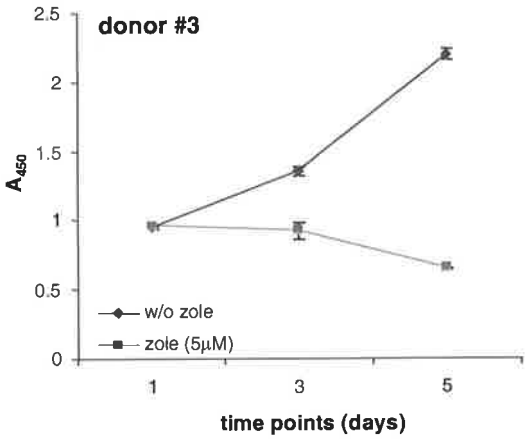
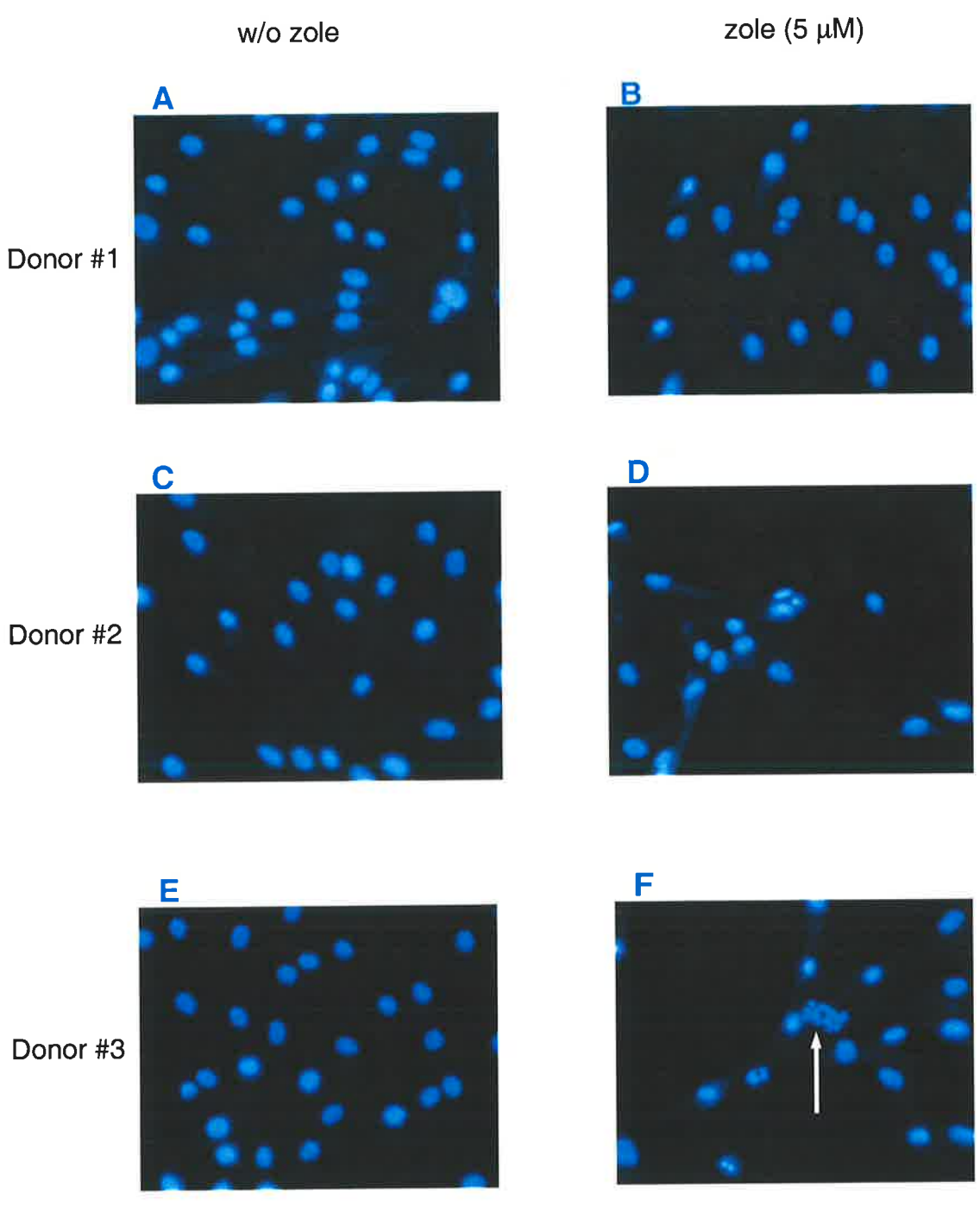

donor #3
2.5
2
1.5
1
0.5
0
A450
w/o zole
zole (5μM)
1
3
5
time points (days)

Figure 4.3. Effect of zoledronic acid on apoptosis of OB-like cells in culture. The OB-like cells were seeded in 8 chamber slides at a cell density of 7,500/well and cultured in the presence of 5 μM of zoledronic acid for 72 hrs. Cells were stained with DAPI and examined by fluorescence microscopy. Morphological changes characteristic of apoptosis, including condensation and the formation of dense, rounded apoptotic bodies were clearly evident in a small percentage of OB-like cells in all donor-derived cells (B, D, F) when compared with untreated cells (A, C, E). Consistent with the cell proliferation results (fig 4.1/4.2), fewer apoptotic nuclei were noted in donor #1 (B) and #2 (D) compared with donor #3 (F).


w/o zole
zole (5 μM)
A
B
Donor #1
C
D
Donor #2
E
F
Donor #3

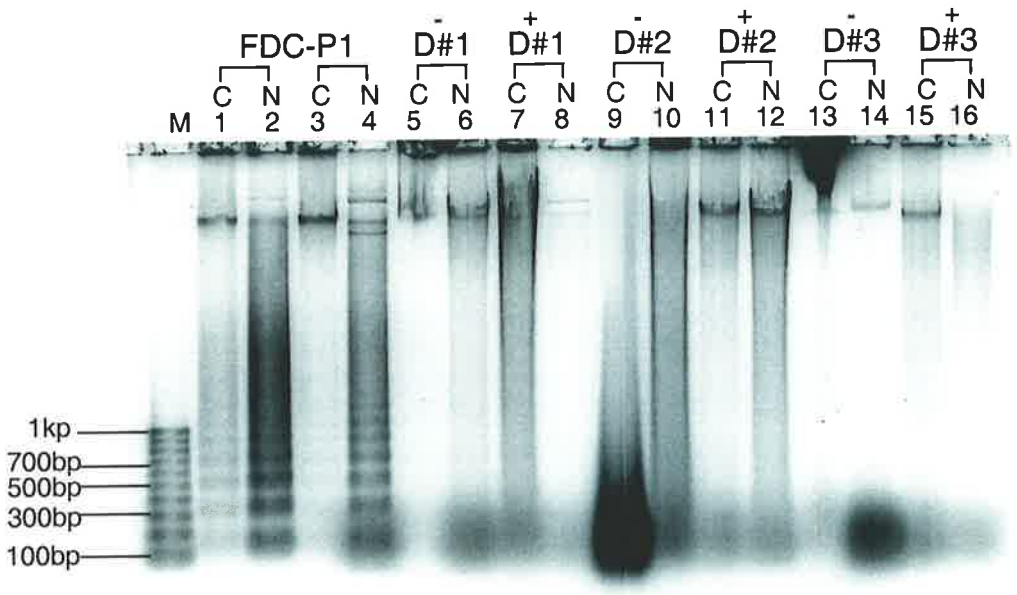
treatment failed to induce appreciable levels of apoptosis as evidenced by the lack of low molecular weight DNA fragments in all donors (figure 4.4).

It has been demonstrated in other cell types that BP-induced apoptosis is proceeded by activation of the caspase cascade (Benford *et al.*, 2001; Fromigue *et al.*, 2000; Hiraga *et al.*, 2001). To assess whether the cell death observed in OB-like cells might also be mediated by caspase activation, we assessed caspase 3 activity in all 3 donor OB cultures following zoledronic acid treatment (figure 4.5). Using the fluorometric caspase 3 substrate DEVD as described in the methods, it was clear that untreated OB-like cells possessed low caspase activity compared with other cell types, such as the myeloma cell line RPMI 8226. However, zoledronic acid did increase caspase activity in OB-like cells derived from all three donors. The extent of this increase varied in different donors, with a significant increase of caspase activity noted in donor #2 ($p<0.001$) and donor 3# ($p<0.005$).

4.2.2 Zoledronic Acid Mediates the Differentiation of Osteoblast-Like Cells

Using multi-parameter immunofluorescence and flow cytometry, cultured human OB-like cells can be separated according to their stage of differentiation. Using monoclonal antibodies (mAbs) STRO-1 and B4-78 (anti-bone/liver/kidney isoenzyme of AP), the OB lineage at different stages of maturation can be identified. Consistent with the previous findings (Gronthos *et al.*, 1999), the OB-like cell cultures were found to have a heterogeneous but highly reproducible pattern of expression of both STRO-1 and ALP (figure 4.6). Notably, zoledronic acid was found to alter the proportion of cells expressing STRO-1 and ALP cell surface markers when compared with the untreated control. There was an inverse reciprocal relationship between the alteration of cell proportion of $STRO\text{-}1^+/AP^-$ and $STRO\text{-}1^-/AP^-$ subpopulations. Zoledronic acid increased the percentage of cells expressing $STRO\text{-}1^-/AP^-$ phenotype, and decreased the percentage of cells expressing the STRO-1 antigen. At d1 there was a minor change in the relative proportion of $STRO\text{-}1^+/AP^-$ cells, whilst at d3, there was a significant decrease in the number of $STRO\text{-}1^+$ and AP^+ cells, with a concomitant increase in the number of $STRO\text{-}1^-/AP^-$ cells. At d5, the number of $STRO\text{-}1^+$ cells were found to increase, and assumed a phenotype similar to the untreated control. These observations were qualitatively similar in cells from each of four donors ($p<0.05$), and the effect of zoledronic acid appeared partially reversible.

Figure 4.4. Zoledronic acid does not induce detectable levels of intranucleosomal genomic DNA fragmentation. Osteoblast-like cells were treated for 72 hours with 5 μM zoledronic acid, intranuclear and genomic DNA fragmentation was assessed as described in the materials and methods (section 2.1.5). No low-molecular weight DNA fragments were observed in donor #1 (lanes 5-8), donor #2 (lanes 9-12) and donor #3 (lanes 13-16) either in the presence or absence of zoledronic acid. The murine cytokine-dependent myeloid cell line, FDC-P1 cells were used as a positive control as factor deprivation leads to the induction of cell death as shown in lanes 1-4.



lane 1,2: FDC-P1 without GM-CSF

lane 3,4: FDC-P1 with GM-CSF

lane 5,6: donor#1 w/o zole

lane 7,8: donor#1 zole (5μM)

lane 9,10: donor#2 w/o zole

lane 11,12: donor#2 zole (5μM)

lane 13,14: donor#3 w/o zole

lane 15,16: donor#3 zole (5μM)

C: cytoplasmic fraction

N: nuclear fraction

Figure 4.5. Zoledronic acid induces low levels of caspase activity in OB-like cells. Osteoblast-like cells derived from three different donors were seeded in 24-well plates with a cell density of 10,000 cells/well and cultured in the presence or absence of zoledronic acid at a concentration of 5 μM. Caspase-3 activity was measured by measuring the degradation of the fluorometric substrate DEVD as described in the methods. Osteoblast-like cells displayed relatively low caspase activity compared with the myeloma cell line RPMI 8226. Treatment resulted in an increase in caspase activity in the OB-like cells derived from all three donors. Although relatively resistant to zoledronic acid, cells from donor #1 displayed a small but measurable ($p<0.01$, *t*-test) increase in caspase activity. In comparision, caspase activity in donor #2 was increased dramatically ($p<0.001$, *t*-test) whilst there was an intermediate increase ($p<0.01$, *t*-test) in caspase activity in donor #3.

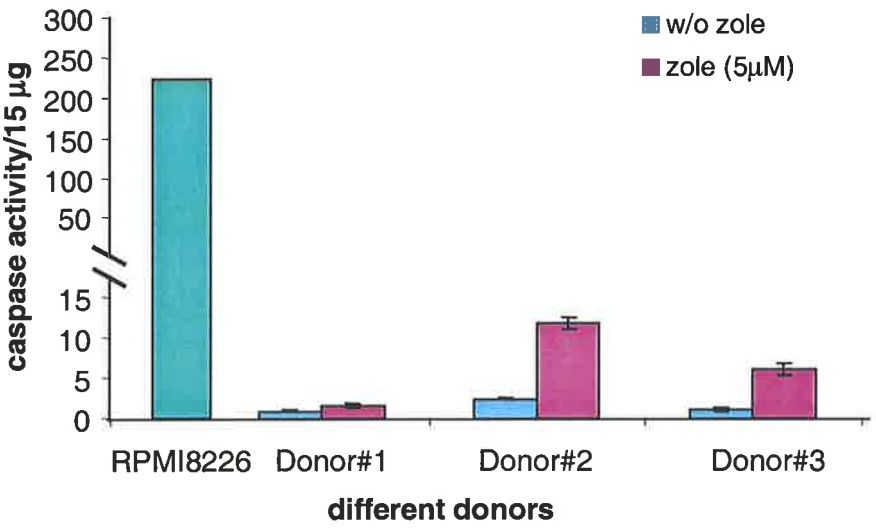
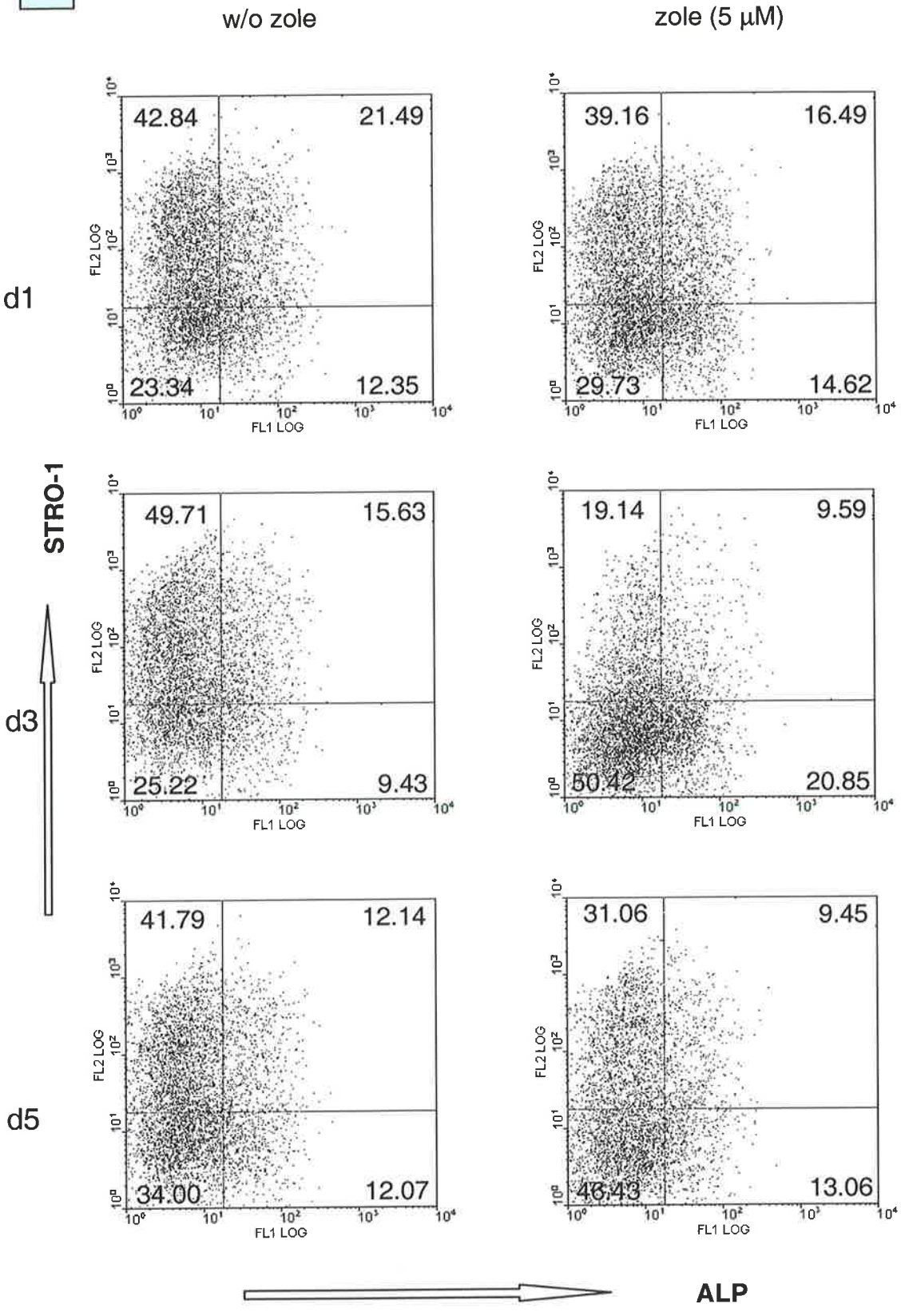

caspase activity/15 µg
300
250
200
150
100
50
15
10
5
0
w/o zole
zole (5µM)
RPMI8226
Donor#1
Donor#2
Donor#3
different donors

Figure 4.6. Modulation of STRO-1 and ALP expression by OB-like cells treated with zoledronic acid. A model of bone cell development has been previously described where cultures of normal human bone cells can be separated on the basis of their expression of the stromal precursor cell marker STRO-1 and the osteoblastic marker, alkaline phosphotase (AP). According to this model, cells with the surface phenotype STRO-1^+/AP^-, STRO-1^+/AP^+, STRO-1^-/AP^+ and STRO-1^-/AP^- represent preosteoblasts, committed OB, mature OB and osteocytes, respectively. Dual-colour immunofluorescence and flow cytometric analysis was performed using OB-like cells stained with the mAbs STRO-1 (anti-stromal precursors marker) and B4-78 (anti-ALP Ab). Immunoreactivity was revealed by incubation with an anti IgM-PE (y-axis) and IgG-FITC (x-axis), as described in the methods. The dot plot histogram represents 10,000 events collected as listmode data. The quadrant lines were established with reference to staining observed with isotype-matched control antibodies, 1B5 (FITC) and 1A6.12 (PE), respectively. The results show that zoledronic acid alters the proportion of cells expressing STRO-1 and ALP cell surface markers. There was an inverse reciprocal relationship between the alteration of cell proportion of STRO-1^+/AP^- and STRO-1^-/AP^- phenotype subpopulations. In brief, zoledronic acid increased the percentage of cells expressing STRO-1^-/AP^- phenotype, and decreased the percentage of cells expressing the STRO-1 antigen. At d1 there was a minor change in the relative proportion of STRO-1^+/AP^- cells, whilst at d3, there was a significant decrease in the number of STRO-1^+ and AP^+ cells, with a concomitant increase in the number of STRO-1^-/AP^- cells. At d5, the number of STRO-1^+ cells increased compared with d3. As seen in figure 4.6 (B), these observations were consistent in each of the four donors. The data for donor #2 are presented as a typical example of results obtained (A). The changes that occurred at d3 are observed for all four donors. The percentage of cell subpopulations, for all four donors, is shown in table 4.1.

A


w/o zole
zole (5 µM)
d1
d3
d5
STRO-1
ALP
42.84
21.49
23.34
12.35
39.16
16.49
29.73
14.62
49.71
15.63
25.22
9.43
19.14
9.59
50.42
20.85
41.79
12.14
34.00
12.07
31.06
9.45
46.43
13.06
FL1 LOG
FL2 LOG

B

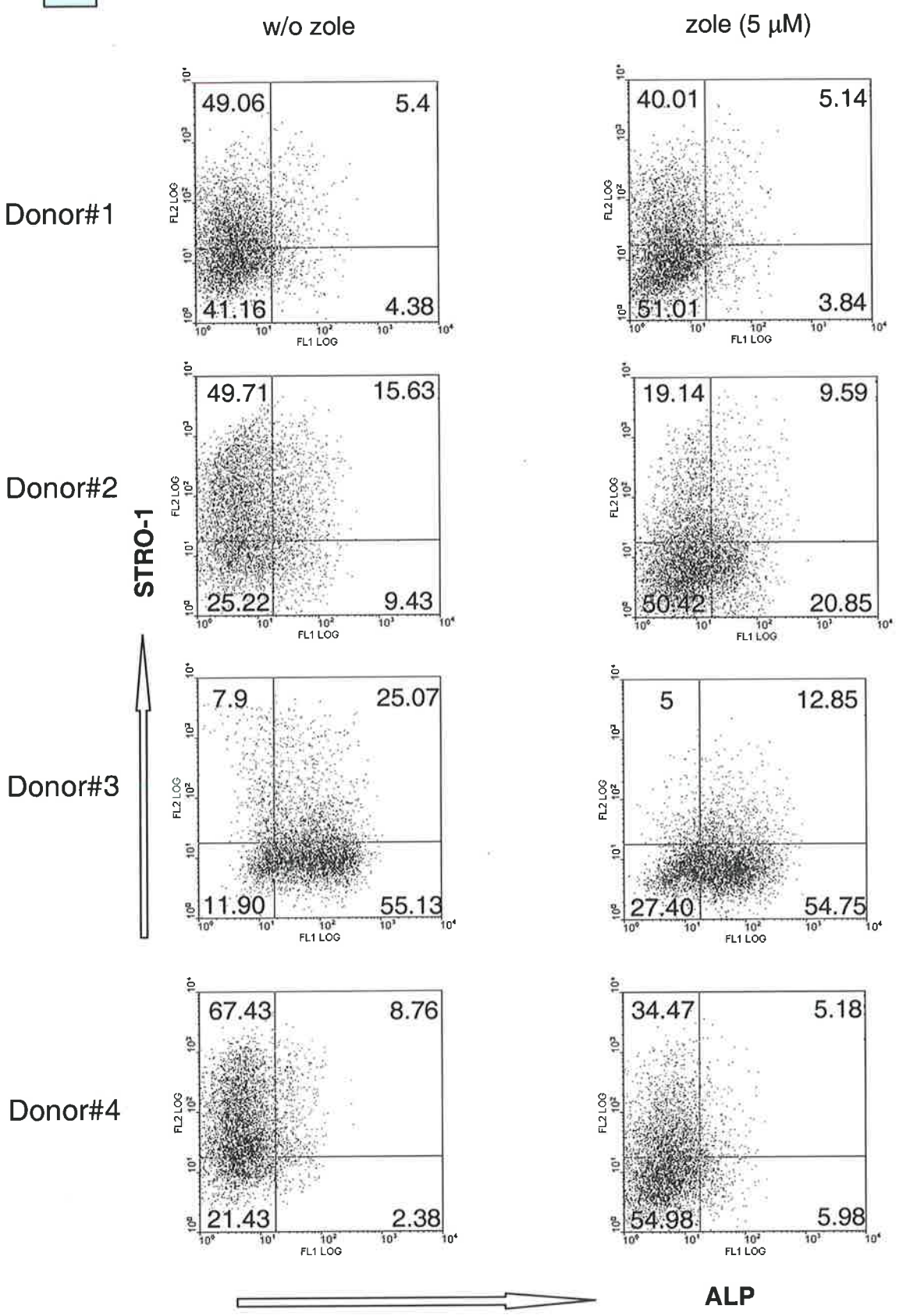

w/o zole
zole (5 μM)
Donor#1
Donor#2
Donor#3
Donor#4
STRO-1
ALP
FL1 LOG
FL2 LOG
49.06
5.4
41.16
4.38
40.01
5.14
51.01
3.84
49.71
15.63
25.22
9.43
19.14
9.59
50.42
20.85
7.9
25.07
11.90
55.13
5
12.85
27.40
54.75
67.43
8.76
21.43
2.38
34.47
5.18
54.98
5.98

Table 4.1. Summary of results of dual-colour immunofluorescence and flow cytometric analysis of osteoblast-like cells derived from four different donors treated with zoledronic acid. The percentages of osteoblast-like cells, which have the various phenotypes, are displayed. Spearman Rank Correlation analysis revealed that there was a negative correlation between the alteration of STRO-1^+/AP^- and STRO-1^-/AP^- subpopulation induced by zoledronic acid ($p<0.05$), suggesting that zoledronic acid treatment mediates differentiation of the osteoblast-like cells, or selectively affects the less mature cells.

	STRO-1^+/ALP^-		STRO-1^+/ALP^+		STRO-1^-/ALP^+		**STRO-1^-/ALP^-**	
	w/o zole	zole (5μM)	w/o zole	zole (5μM)	w/o zole	zole (5μM)	w/o zole	zole (5μM)
Donor #1, d1 (%)	46.37	**47.36**	8.34	8.96	5.47	5.48	39.83	**38.20**
Donor #1, d3 (%)	49.06	**40.01**	5.40	5.14	4.38	3.84	41.16	**51.01**
Donor #1, d5 (%)	50.66	**42.26**	6.21	5.11	4.78	4.47	38.35	**48.15**
Donor #2, d1 (%)	42.84	**39.16**	21.49	16.49	12.35	14.62	23.34	**29.73**
Donor #2, d3 (%)	49.71	**19.14**	15.63	9.59	9.43	20.85	25.22	**50.42**
Donor #2, d5 (%)	41.79	**31.06**	12.14	9.45	12.07	13.06	34.00	**46.43**
Donor #3, d1 (%)	5.83	**3.73**	21.55	8.49	62.17	69.62	10.45	**18.15**
Donor #3, d3 (%)	7.90	**5.00**	25.07	12.85	55.13	54.75	11.90	**27.40**
Donor #3, d5 (%)	7.69	**2.39**	44.77	18.77	42.21	65.52	5.33	**13.32**
Donor #4, d1 (%)	55.03	**50.98**	12.68	11.81	6.37	7.19	25.92	**30.01**
Donor #4, d3 (%)	67.43	**34.47**	8.76	5.18	2.38	5.98	21.43	**54.98**
Donor #4, d5 (%)	38.95	**35.51**	2.74	8.53	2.51	5.86	55.79	**50.10**

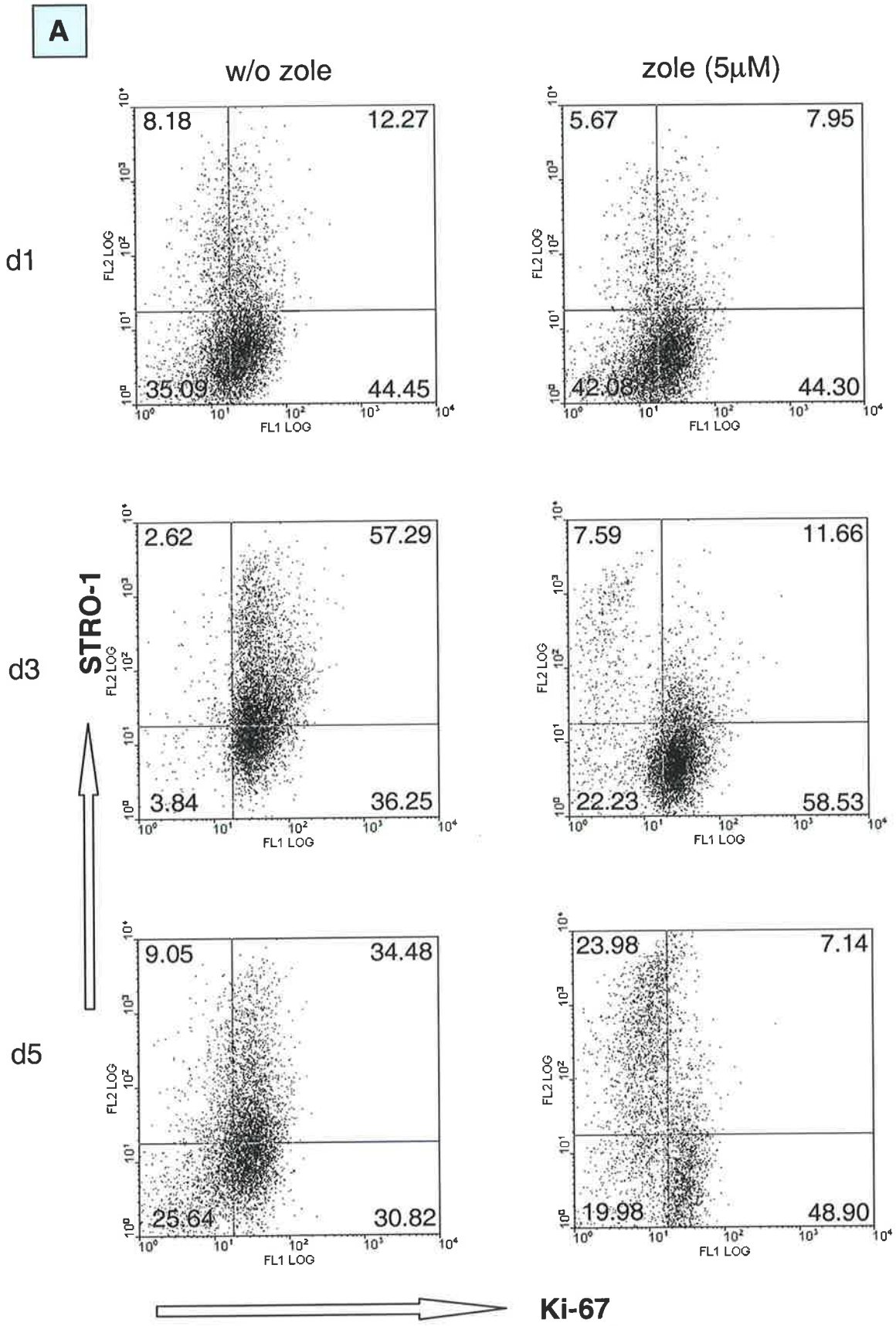
4.2.3 *STRO-1^{bright} Osteoblast-Like Cells Proliferate More Rapidly and Are More Sensitive to Zoledronic Acid*

To determine if a difference in the cell cycling status between different subpopulations may play a role in the differential sensitivity of these cells to zoledronic acid, dual-colour FACS analysis of OB-like cells was employed, examining the expression of STRO-1 and the cell cycle-specific antigen Ki-67. As seen in figure 4.7, the majority of the OB-like cells were actively dividing, as indicated by their expression of the Ki-67 antigen. At d1, approximately 60% of cells were found to express appreciable levels of the Ki-67 antigen, suggesting that most of the cells were in either G_1, S or G_2/M phase of the cell cycle, whilst the remainder were in G_0 phase. Consistent with the data in figure 4.6, zoledronic acid treatment resulted in the diminution in STRO-1 expression. The reduction in STRO-1 expression was associated with the induction of quiescence in the STRO-1^{bright} population as evidenced by the appearance of a STRO-1^{bright}/Ki-67^{negative} population. The data also revealed that zoledronic acid appeared to inhibit cell division when compared with the control. Moreover, following exposure of the cells to zoledronic acid, a distinct population of STRO-1^{bright} cells, which exhibited low forward and side scatter, were generated by d3, indicating that the STRO-1^{bright} cells may be undergoing zoledronic acid-induced cell death.

To further clarify the effects of zoledronic acid on the different subpopulation of OB-like cells, three-colour FACS analysis was employed using mAbs to STRO-1, ALP and the DNA fluorochrome, 7-AAD. As seen in figure 4.8, the cell cycle status was not related to the extent of ALP expression by the OB-like cells derived from donor #2. In contrast, the majority of cells expressing STRO-1 antigen at high levels were distributed in the S and G_2/M phase. Consistent with previous findings, treatment of OB-like cells resulted in a time-dependent increase in the apoptotic sub-G_0/G_1 peak, consistent with the onset of apoptosis.

To confirm whether zoledronic acid induced changes in STRO-1 and ALP expression was due to a difference in the rate of cell death in the different subpopulations, the STRO-1^{bright} and STRO-1^{negative} subpopulations of OB-like cells were sorted using the FACStar^{PLUS} cell sorter and the cell proliferation rate assessed using WST-1. Although there was no significant difference in the growth rate of STRO-1^{bright} and STRO-1^{negative} cells (figure 4.9), the STRO-1^{bright} cells were slightly more sensitive than STRO-1^{negative} cells to zoledronic acid ($p<0.05$).

Figure 4.7. The cycling status of STRO-1^{+} OB-like cells treated with zoledronic acid. Osteoblast-like cells were cultured in the presence or absence of zoledronic acid over the period of 5 days. The cells were harvested at different time points and stained with the mAb STRO-1 followed by IgM-PE, fixed in 70% cold ethanol on ice for 10 mins and subsequently immunoreacted with the FITC conjugated mAb Ki-67. The samples were analysed by dual-colour immunofluorescence and flow cytometry. The dot plot represents 10,000 events collected as listmode data. The quad-stat markers were established with reference to the reactivity obtained with the isotype-matched control antibodies, IgG1(FITC, X axis) and 1A6.12(PE, Y axis). At d1, approximately 60% of cells were found to express appreciable levels of the Ki-67 antigen, suggesting that most of the cells were in either G_1, S or G_2M of the cell cycle, whilst the remainder were in G_0 phase. Consistent with the data in figure 4.6, zoledronic acid treatment resulted in the diminution in STRO-1 expression. The reduction in STRO-1 expression was associated with the induction of quiescence in the STRO-1^{bright} population as evidenced by the appearance of STRO-1^{bright}/Ki-$67^{negative}$ population (A). The data also revealed that the STRO-1^{bright} cells, in the presence of zoledronic acid, assume low forward and side scatter (highlighted in red, on B and C) indicating that the STRO-1^{bright} cells may be undergoing zoledronic acid-induced cell death.

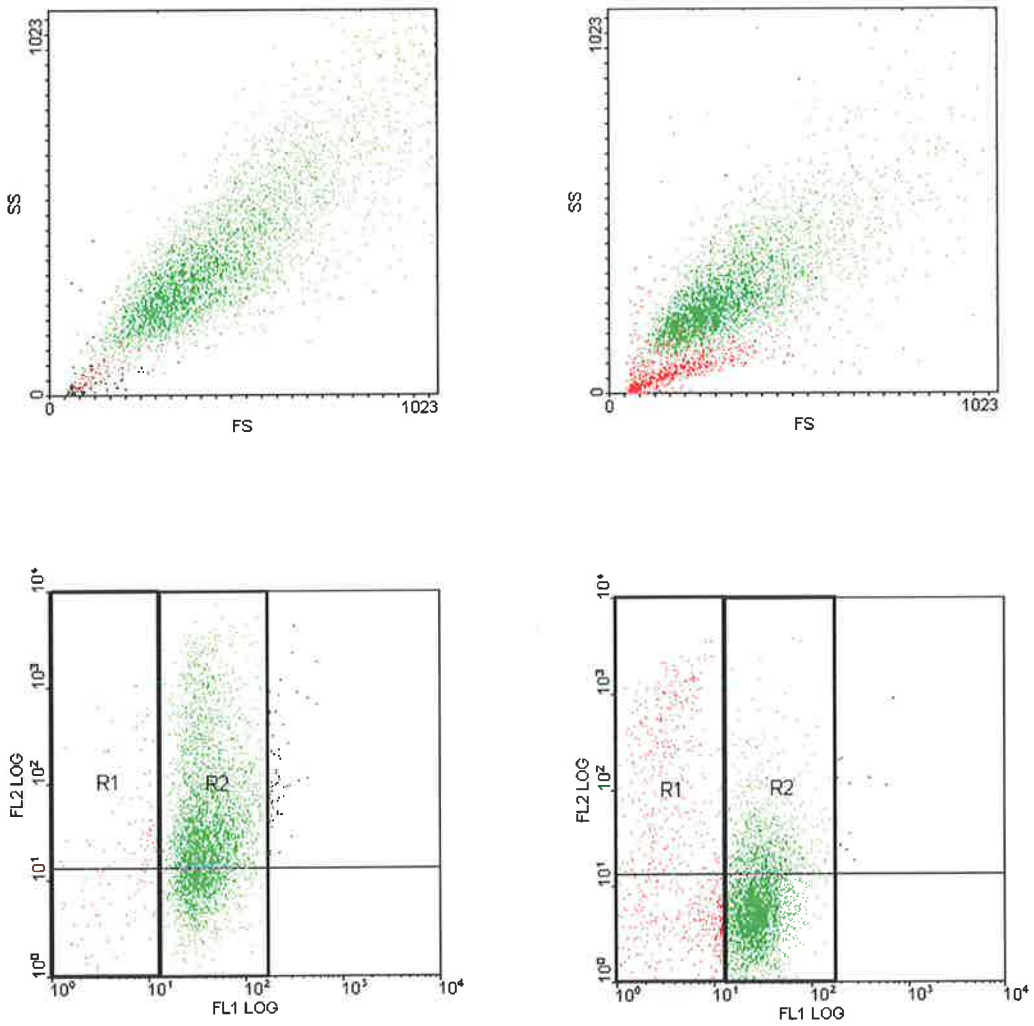

A
w/o zole
zole (5μM)
d1
d3
d5
STRO-1
Ki-67
FL1 LOG
FL2 LOG
8.18
12.27
35.09
44.45
5.67
7.95
42.08
44.30
2.62
57.29
3.84
36.25
7.59
11.66
22.23
58.53
9.05
34.48
25.64
30.82
23.98
7.14
19.98
48.90

B

Day 3

w/o zole

zole (5µM)



C

Day 5

w/o zole

zole (5µM)

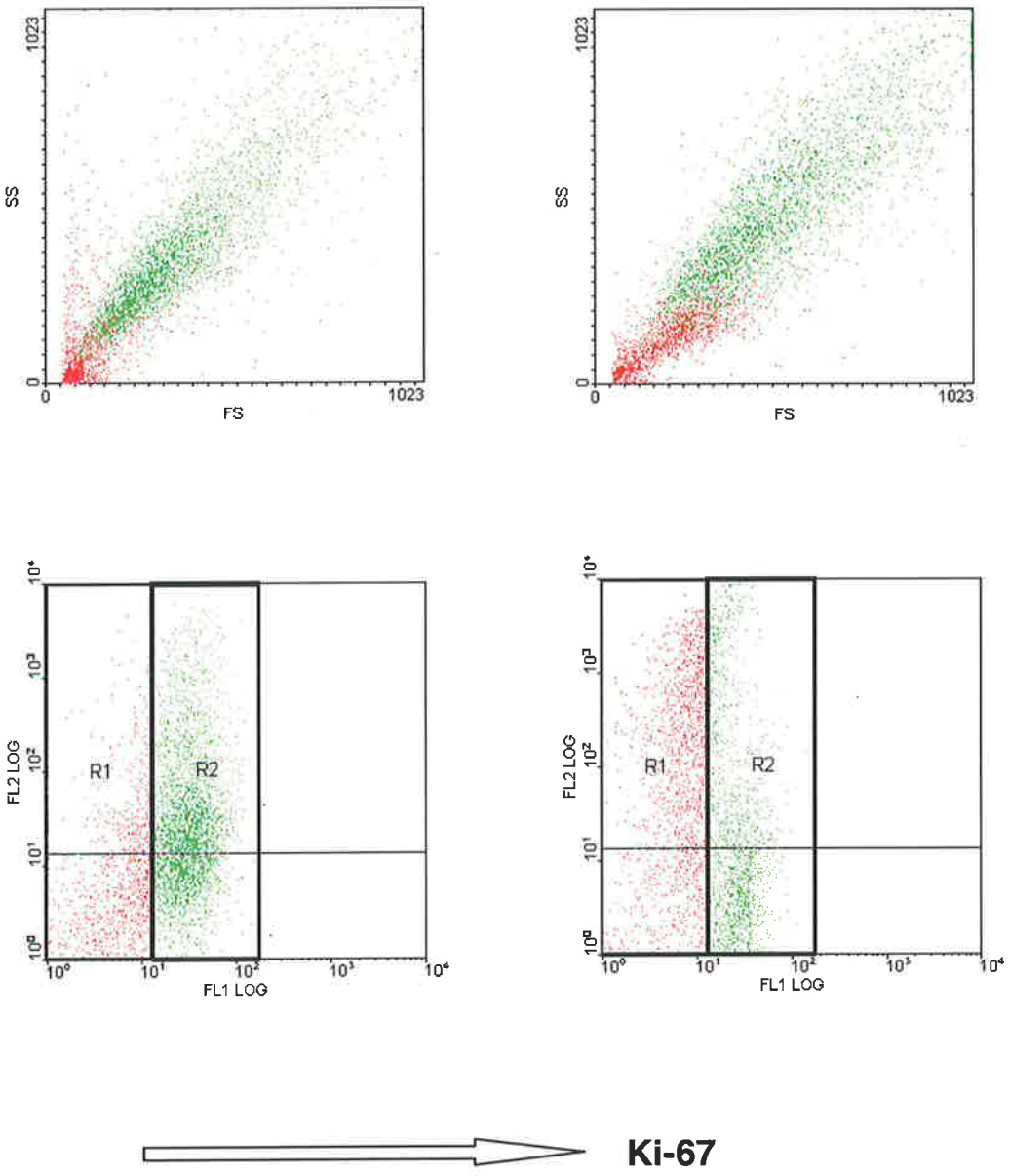
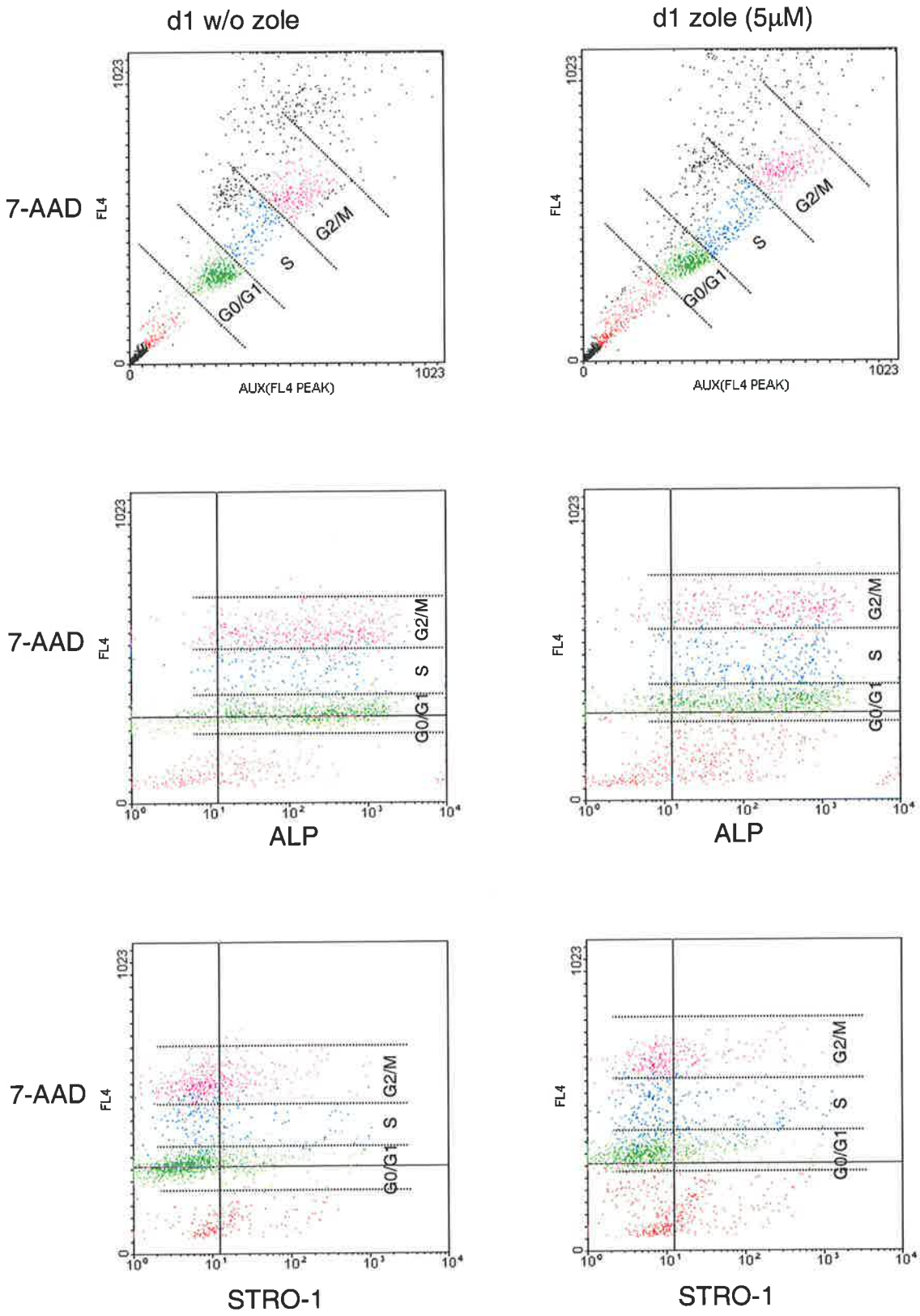
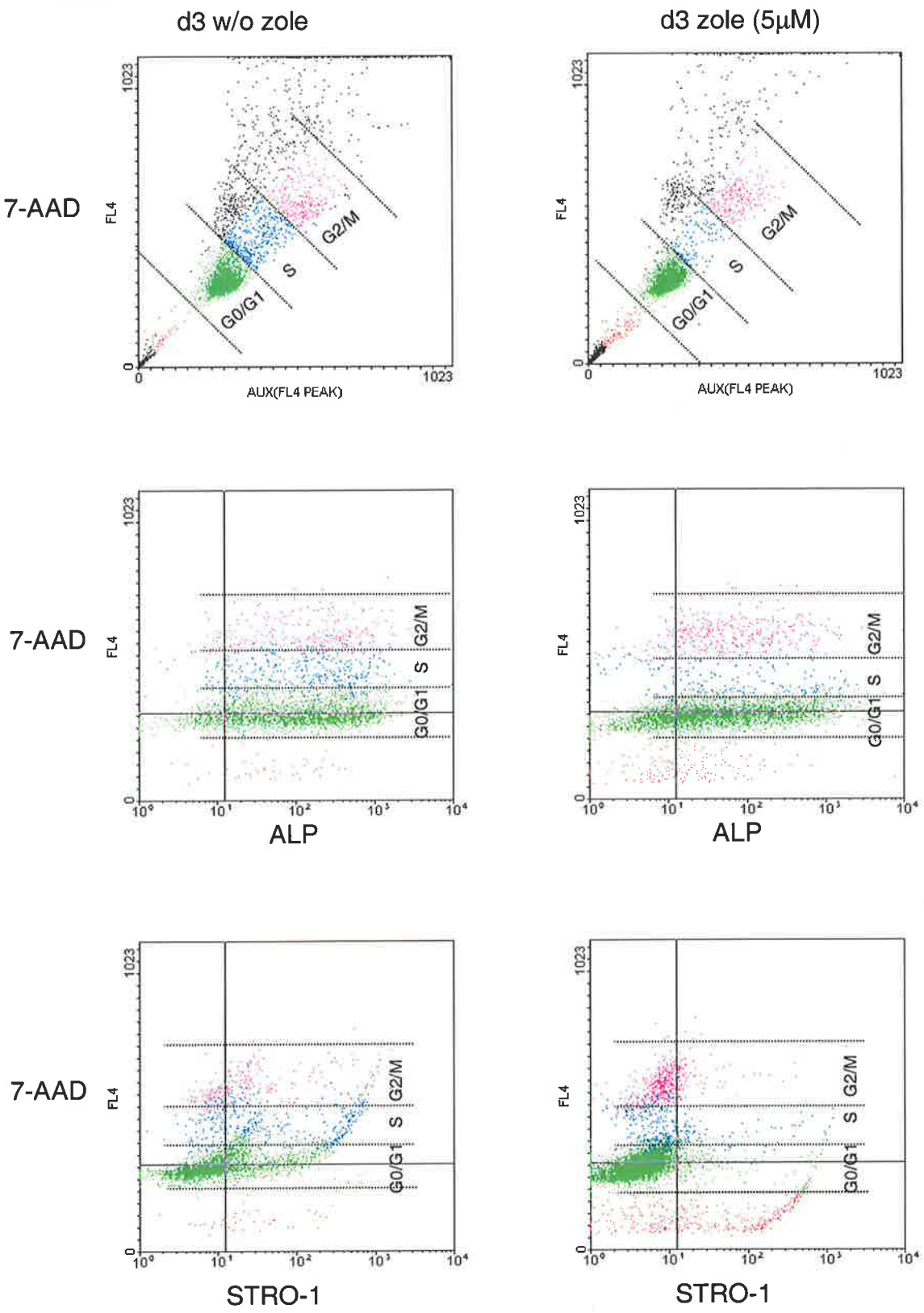


Figure 4.8. Three-colour immunofluorescence and flow cytometric analysis reveals that zoledronic acid induces S-phase arrest and cell death of the STRO-1^{bright} OB-like cells. Osteoblast-like cells derived from donor #2 were cultured in the presence or absence of zoledronic acid for 72 hrs. The cells were stained with the mAbs STRO-1, B4-78 coupled to FITC, and the DNA fluorochrome, 7-AAD, as described in the methods. The dot plot represents 10,000 events collected as listmode data. The auxiliary channel was used to discriminate between single cells and cell doublets. The latter were excluded and the single cells were gated in accordance to their stage of the cell cycle, including hypo-diploid sub-G_0/G_1, G_0/G_1, S and G_2/M. Cells expressing ALP antigen were analysed with respect to their cell cycle status (FL4). Similarly, cells expressing STRO-1 antigen were also analysed with respect to their cell cycle status (FL4). The results suggest that cell cycle status was not related to the extent of ALP expression on the OB-like cells derived from donor #2. In contrast, the majority of cells expressing STRO-1 antigen at high levels were distributed in the S and G_2/M phase. Consistent with previous findings (refer to figure 4.2), treatment of OB-like cells resulted in a time-dependent increase in the apoptotic sub-G_0/G_1 peak, consistent with the onset of apoptosis.

A


d1 w/o zole
d1 zole (5μM)
7-AAD
FL4
AUX(FL4 PEAK)
G0/G1
S
G2/M
ALP
STRO-1
0
1023

B


d3 w/o zole
d3 zole (5μM)
7-AAD
FL4
AUX(FL4 PEAK)
G0/G1
S
G2/M
ALP
STRO-1
0
1023

C

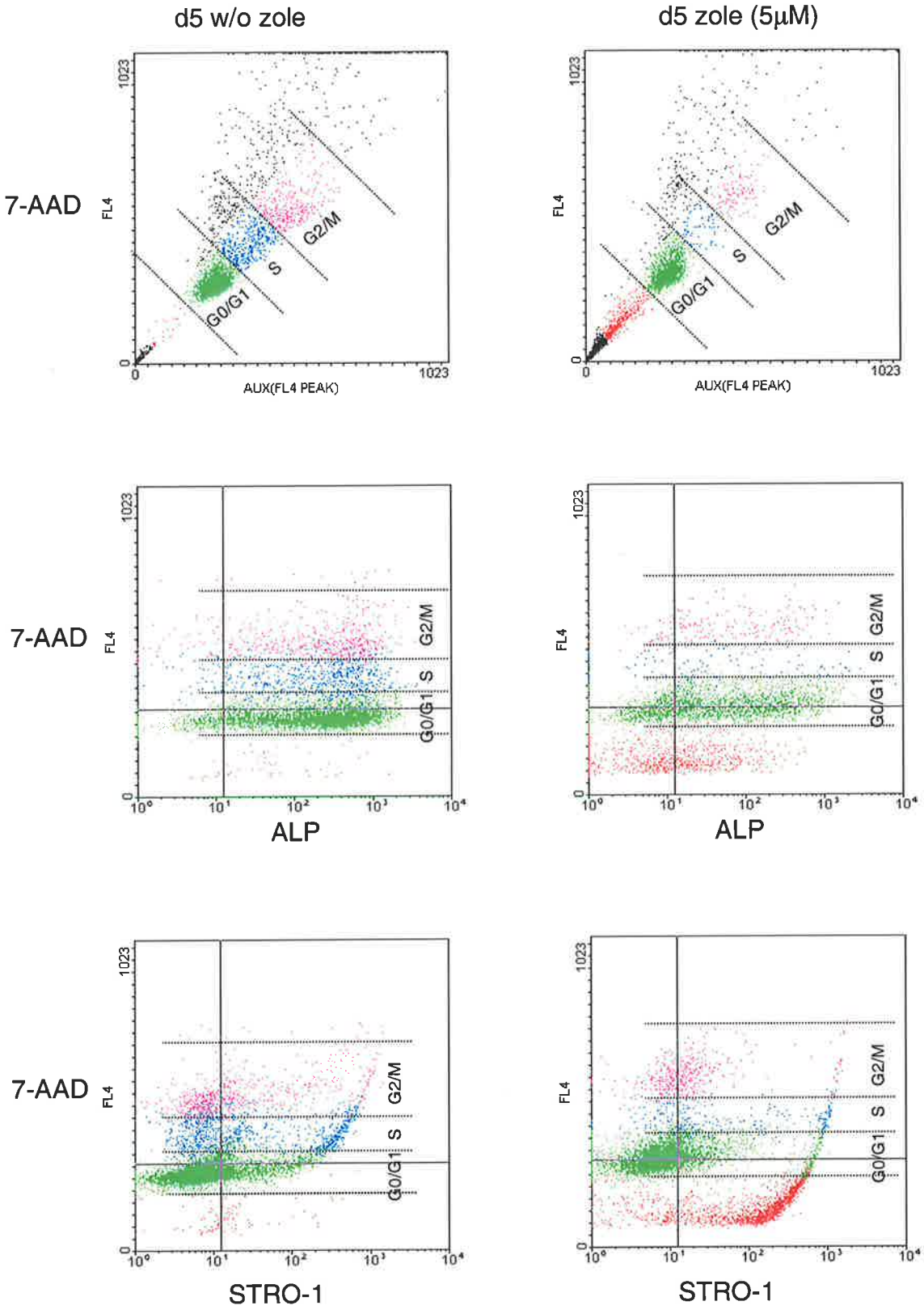
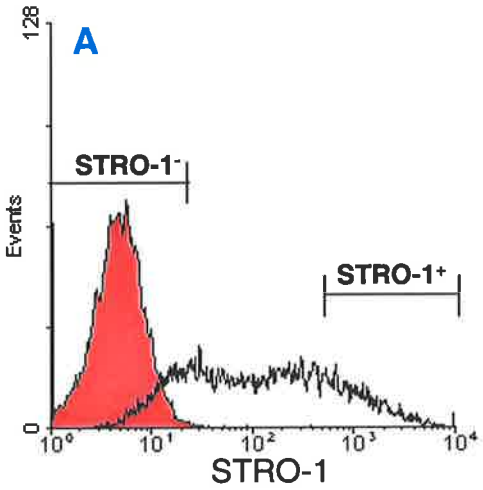
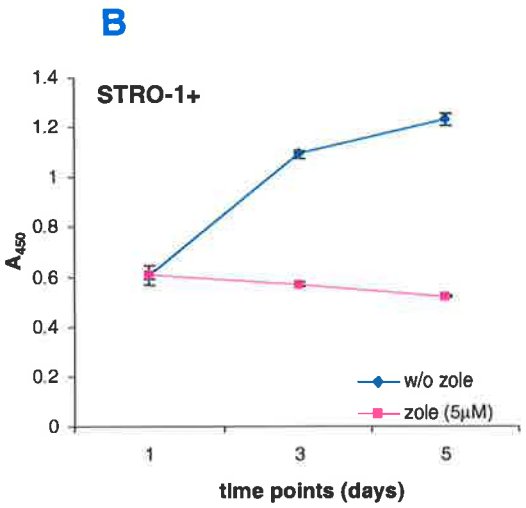
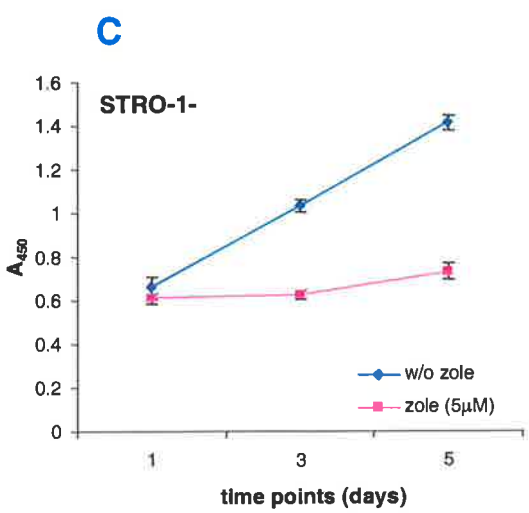
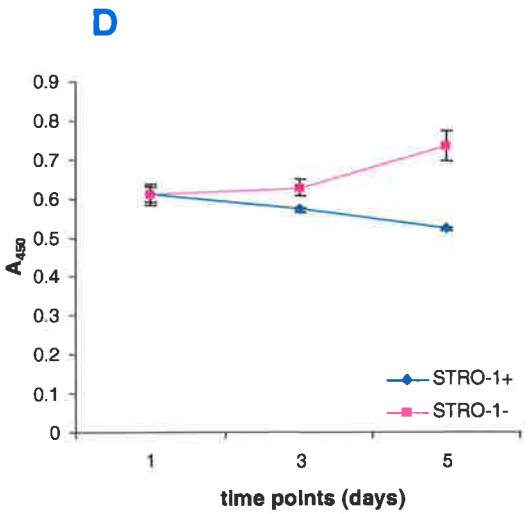

d5 w/o zole
d5 zole (5μM)
7-AAD
FL4
AUX(FL4 PEAK)
G0/G1
S
G2/M
ALP
STRO-1
0
1023

Figure 4.9. The STRO-1^{bright} population of OB-like cells is more sensitive to zoledronic acid induced cytostasis and cell death. Osteoblast-like cells derived from donor #2 were sorted into STRO-1^{bright} and STRO-1^{negative} subpopulations using FACStar^{PLUS} cell sorter. The cells were seeded in 96-well plates at a concentration of 4000 cells/well. Cells were cultured in the media in the presence or absence of zoledronic acid for the period of 5 days. The cell proliferation rate was assessed using WST-1 at different time points as previously described. The results demonstrated that although both STRO-1 ^{bright} (B) and STRO-1^{negative} (C) OB-like cells were sensitive to zoledronic acid treatment ($p<0.001$ at d3 and d5, *t*-test), STRO-1^{bright} cells were more sensitive than the STRO-1^{negative} cells to zoledronic acid (D) ($p<0.05$, *t*-test).


A
128
Events
0
STRO-1-
STRO-1+
10^0
10^1
10^2
10^3
10^4
STRO-1


B
STRO-1+
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A_{450}
w/o zole
zole (5μM)
1
3
5
time points (days)


C
STRO-1-
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A_{450}
w/o zole
zole (5μM)
1
3
5
time points (days)


D
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A_{450}
STRO-1+
STRO-1-
1
3
5
time points (days)

To further investigate differences in cell proliferation between STRO-1^{bright} and STRO-1$^{negative/dim}$ cells, Carboxyfluorescein diacetate succinimidyl ester (CFSE) was used. CFSE is a cell permeant fluorescein based dye and can be used for tracking cell division *in vitro* and *in vivo* (Lyons, 1999; Lyons, 2000). Using two-colour flow cytometric analysis, we examined the proliferative rate of the different subpopulations based on their expression of the STRO-1 and ALP cell surface markers. Unlike the cell proliferation results using WST-1, STRO-1^{bright} cells were found to have a significantly enhanced capacity to proliferate compared with the STRO-1$^{negative/dim}$ population (figure 4.10). Furthermore, zoledronic acid inhibited cell division in a dose dependent manner, and totally inhibited cell division at a concentration of 5 μM at d1 and d3 (figure 4.11). Although the expression of STRO-1 was marginally increased in the presence of 0.5 μM of zoledronic acid, it dramatically decreased in the presence of 5 μM of zoledronic acid (figure 4.11). However, following 7 days culture in the present of 5 μM zoledronic acid, the cells began to divide and this was accompanied with re-expression of STRO-1 antigen at the cell surface. Therefore, the decrease in STRO-1 expression may correspond to the inhibition of cell division, suggesting that the STRO-1 cell surface molecule, is strongly associated with OB-like cells which are actively dividing.

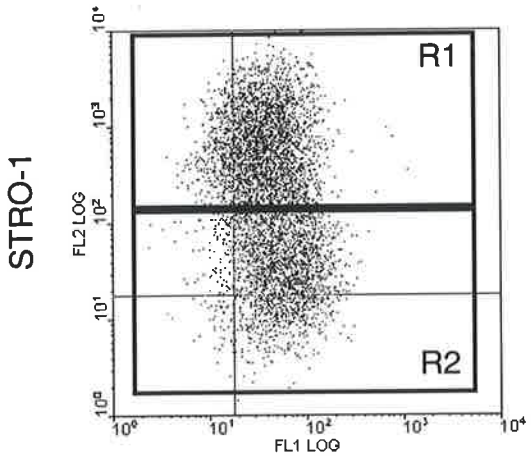
4.2.4 Zoledronic Acid Regulates the Expression of Osteoclast and Osteoblast-Related Genes

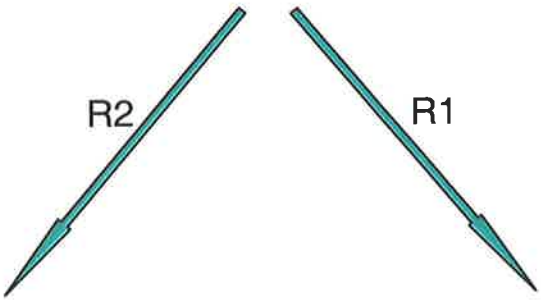
The alteration of gene expression was also detected in zoledronic acid-treated OB-like cells utilising semi-quantitative RT-PCR (figure 4.12). The mRNA integrity in each of the groups was confirmed by reproducible and consistent amplification of the "house keeping" gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Among the various genes investigated in this study, TNF-α and IL-1β were upregulated in donor #2 and donor #3 by prolonged exposure to zoledronic acid (d3 and d5). TNF-α gene expression increased 2-fold at d3 in donor #2, and in donor #3, it was increased 5- and 22-fold at d3 and d5, respectively ($p \leq 0.005$). Similar effects on IL-1β gene expression were also noted ($p < 0.005$). However, zoledronic acid did not alter the expression of other OAFs, including IL-6 and M-CSF.

In addition, the expression of the TNF-family molecules RANKL and OPG, which play a crucial role in osteoclastogenesis and bone resorption in normal bone remodelling, was also examined. The expression of OPG was unaltered over treatment periods of 5 days. There was no significant difference of the relative ratio of RANKL/OPG between the negative control and the cells exposed to zoledronic acid (figure 4.13).

Figure 4.10. The STRO1^{bright} fraction of OB-like cells have a greater proliferative capacity than the STRO-1^{negative} fraction. Osteoblast-like cells derived from donor #2 were labelled with CFSE as described in the methods. Osteoblast-like cells without CFSE labelling were used to establish a negative control (auto-fluorescence). Colchicine (100 ng/ml) was used to inhibit cell division and provided an input labelling index to establish a positive control. Cells were subsequently stained with STRO-1-PE as described above and the cell proliferation was analysed using the ModFit LT for win 32 (Version 2.0). The STRO-1^{bright} cells (R1) were found to have a significantly enhanced capacity to proliferate when compared with the STRO-1$^{negative/dim}$ population (R2) at d3 (B) and d5 (C).

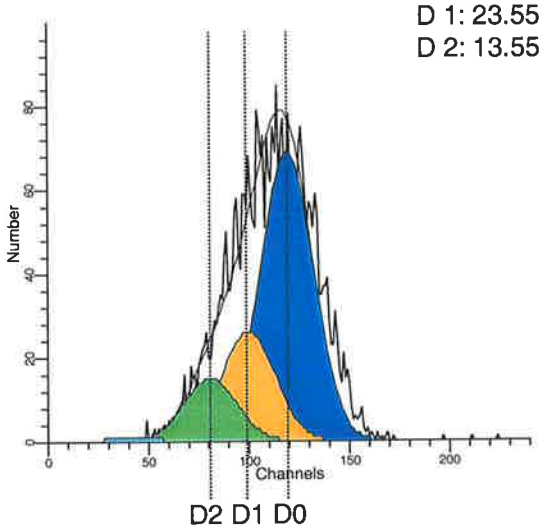
A





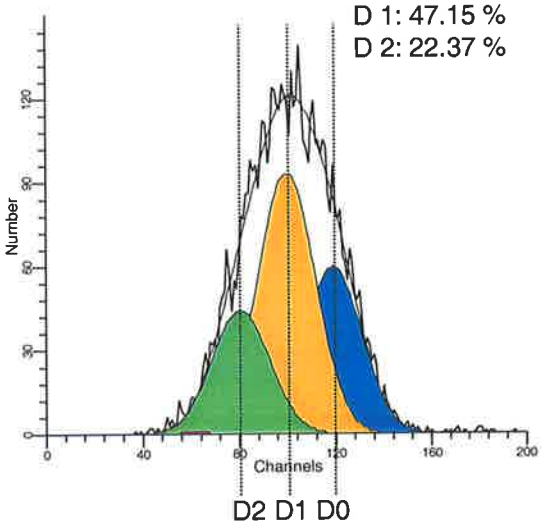
STRO-1 dim

D 0: 61.89 %
D 1: 23.55 %
D 2: 13.55 %



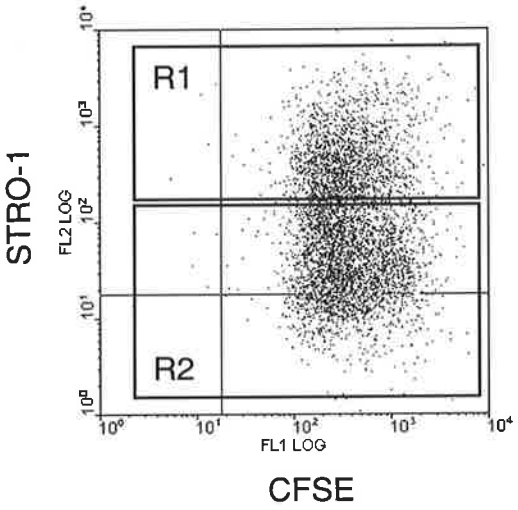
STRO-1 bright

D 0: 30.00 %
D 1: 47.15 %
D 2: 22.37 %

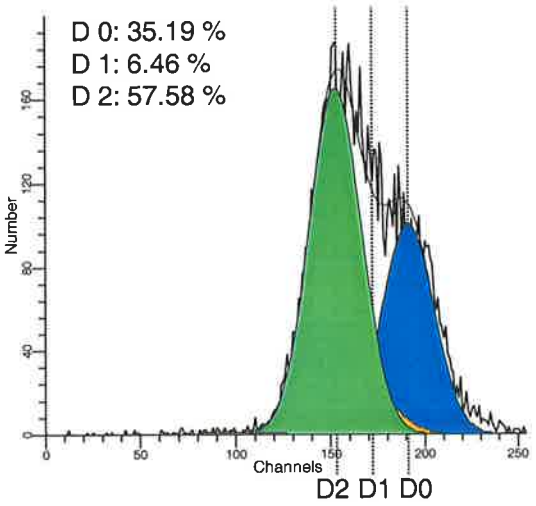


B

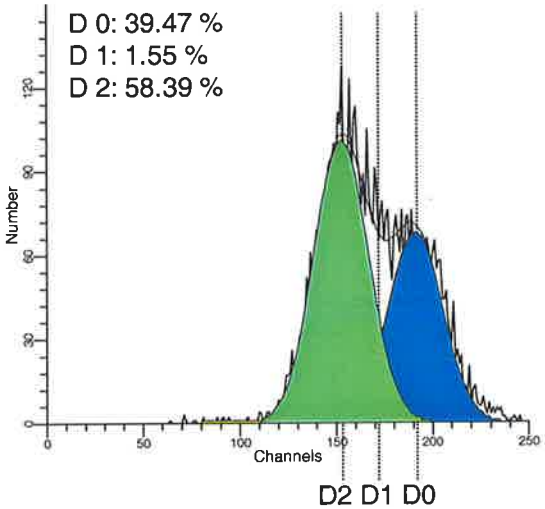
Day 3



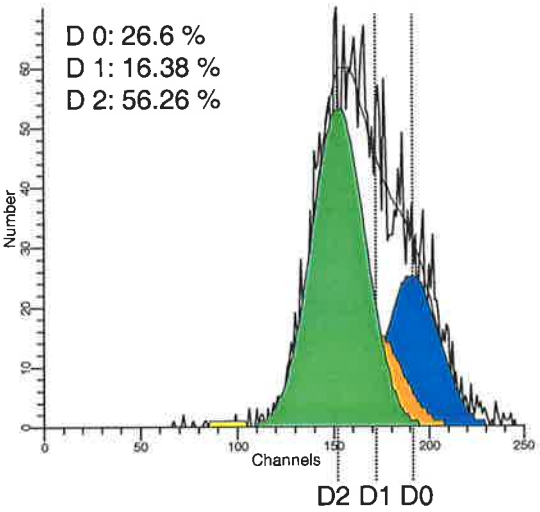
Cell divisions of total population



STRO-1 dim

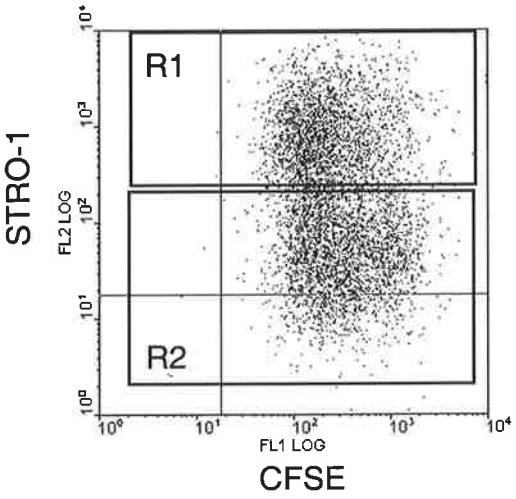


STRO-1 bright

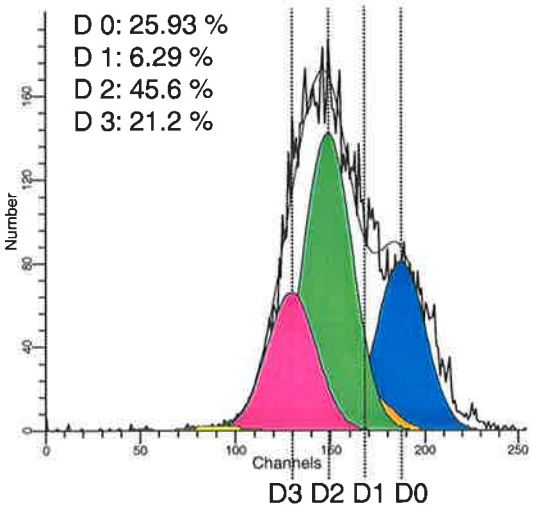


C

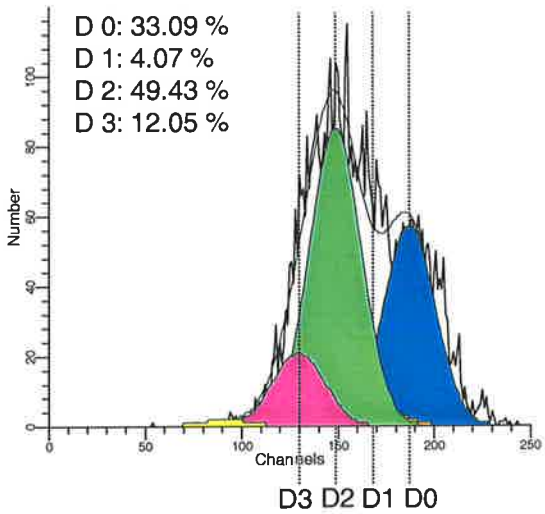
Day 5



Cell divisions of total population



STRO-1 dim



STRO-1 bright

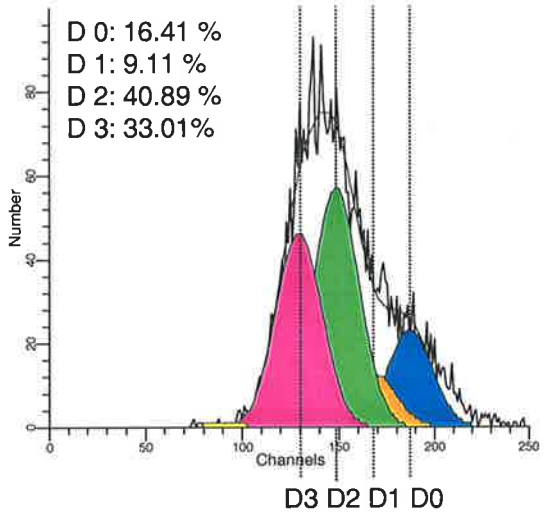
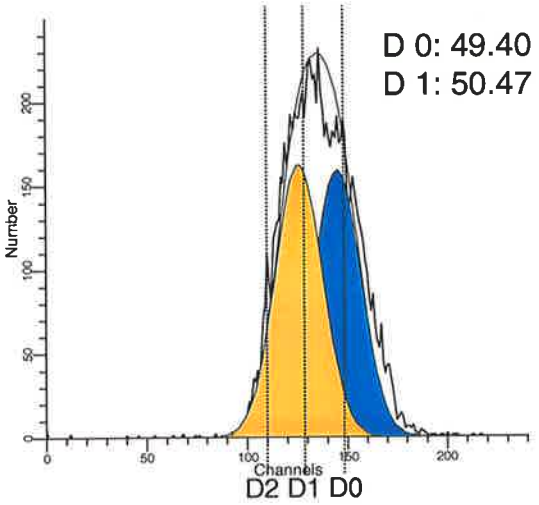


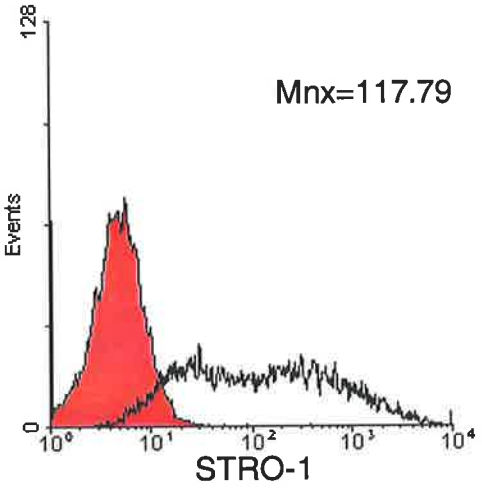
Figure 4.11. Zoledronic acid inhibits the proliferation of OB-like cells in a dose-dependent manner. Osteoblast-like cells derived from donor #2 were labelled with CFSE as described in the methods. Cells were subsequently cultured in the presence of zoledronic acid (0.5 & 5 μM) for 3 (A), 5 (B) and 7 (C) days, stained with STRO-1 and analysed as above. Zoledronic acid was found to inhibit cell division in a dose-dependent manner. In accordance with previous studies, zoledronic acid treatment resulted in a decrease in STRO-1 expression, which was reacquired upon cell division at d7 (C).

A

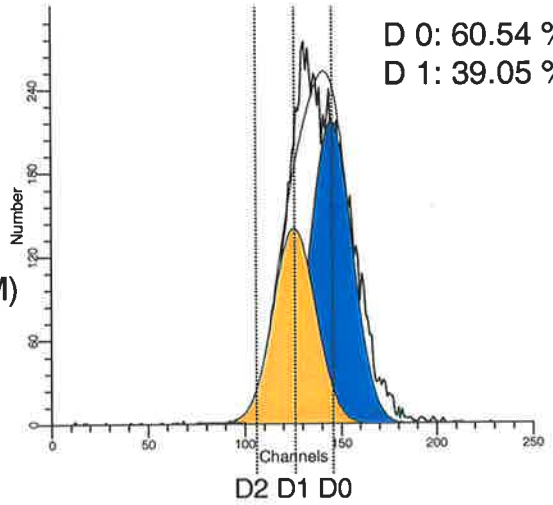
Day 3

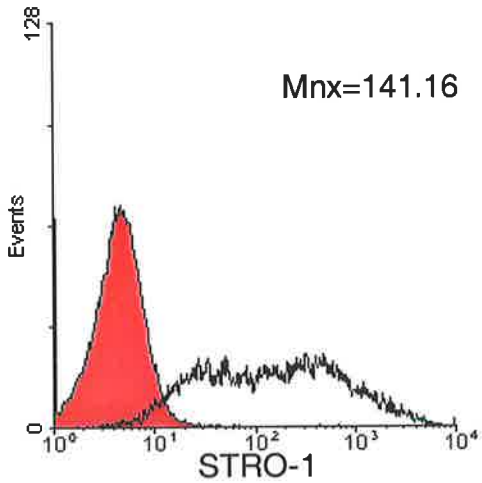
w/o zole



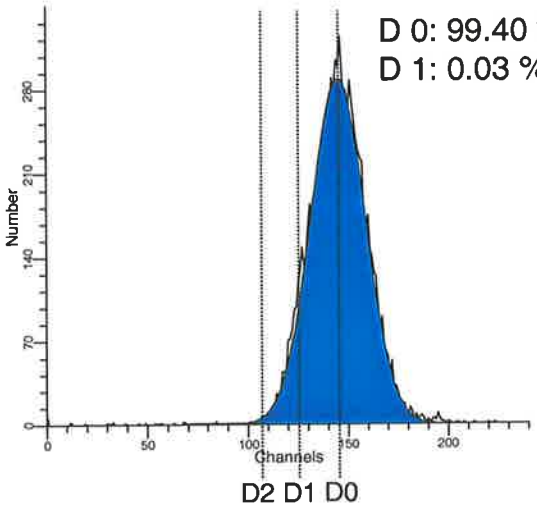


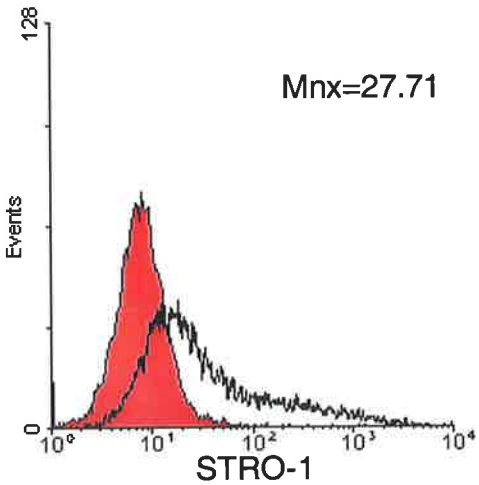
zole (0.5 μM)





zole (5 μM)

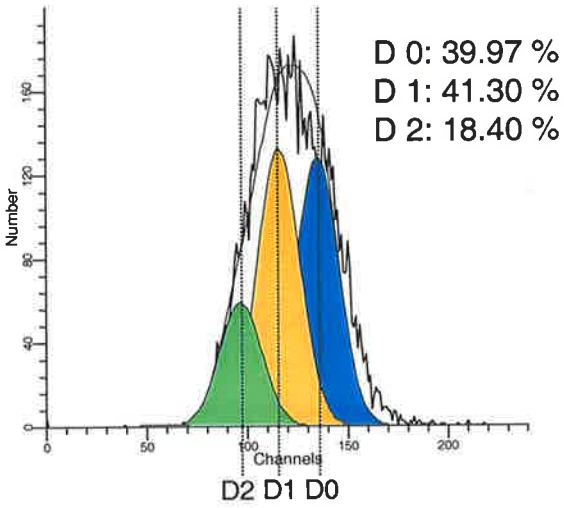


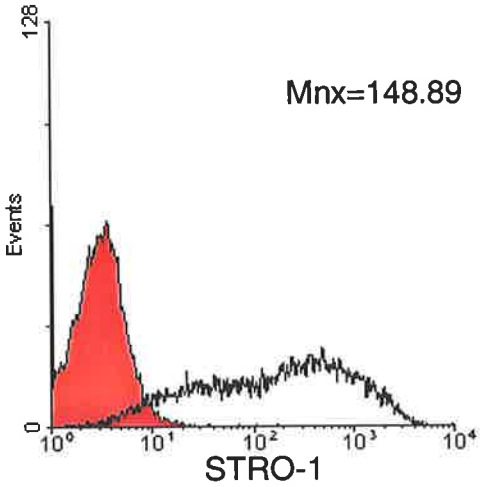


B

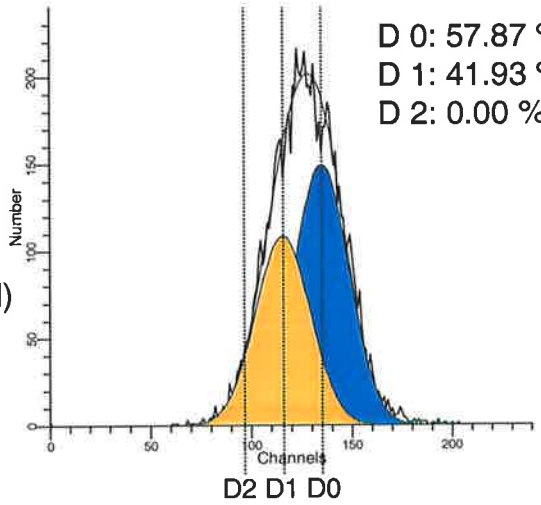
Day 5

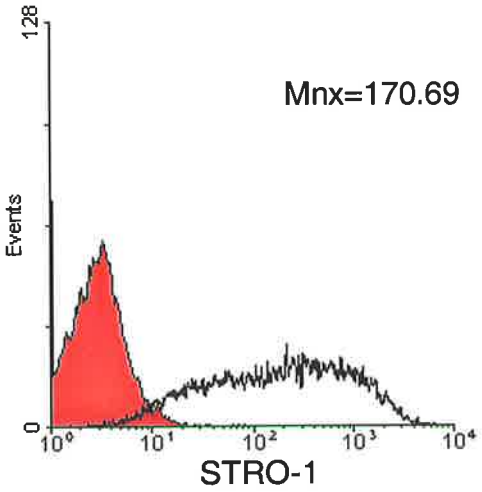
w/o zole



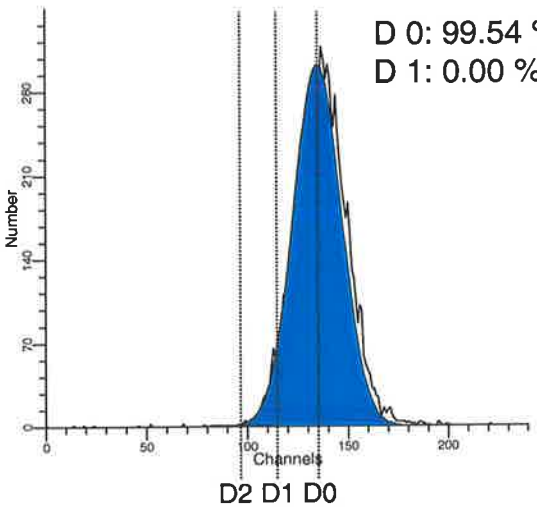


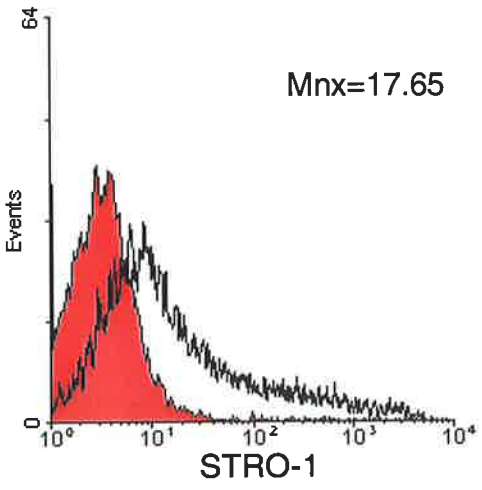
zole (0.5 μM)





zole (5 μM)

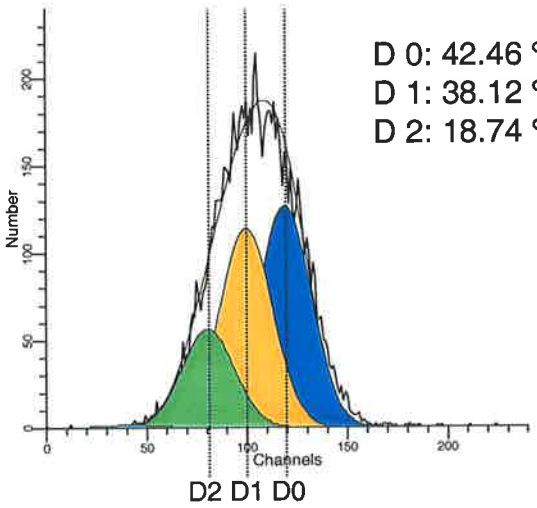




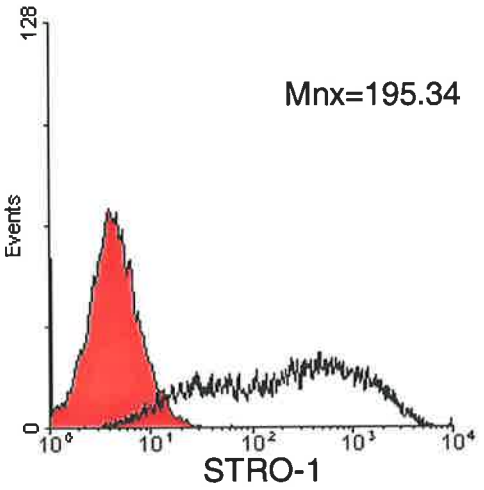
C

Day 7

w/o zole

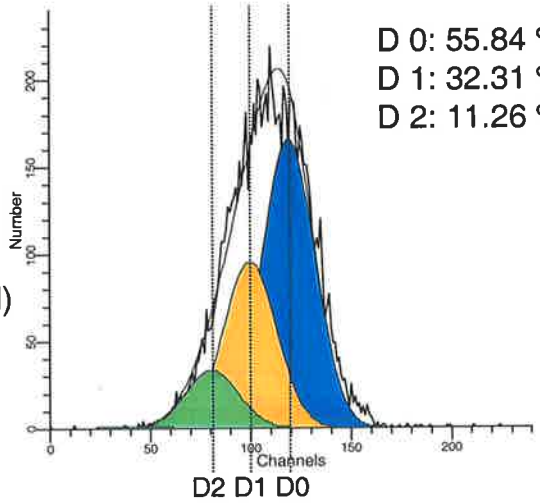


D 0: 42.46 %
D 1: 38.12 %
D 2: 18.74 %

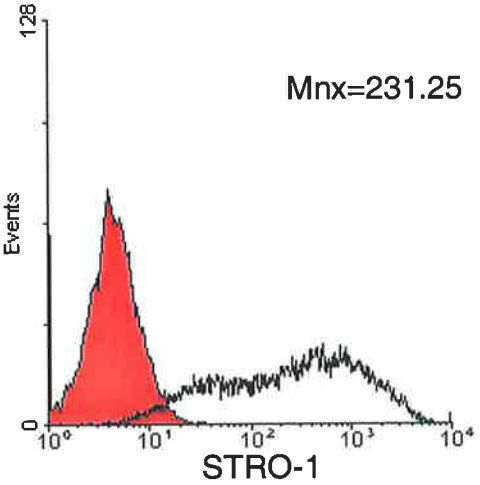


Mnx=195.34

zole (0.5 μM)

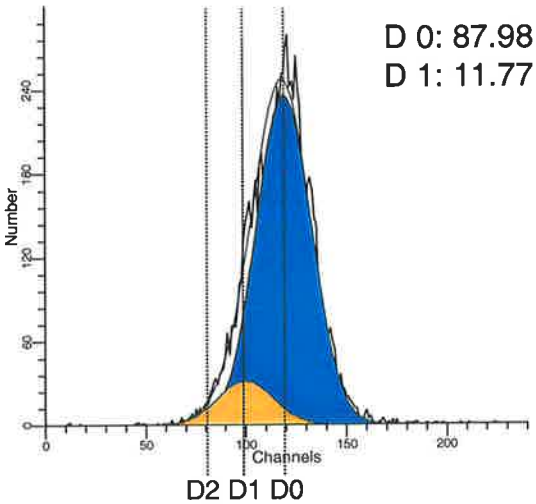


D 0: 55.84 %
D 1: 32.31 %
D 2: 11.26 %

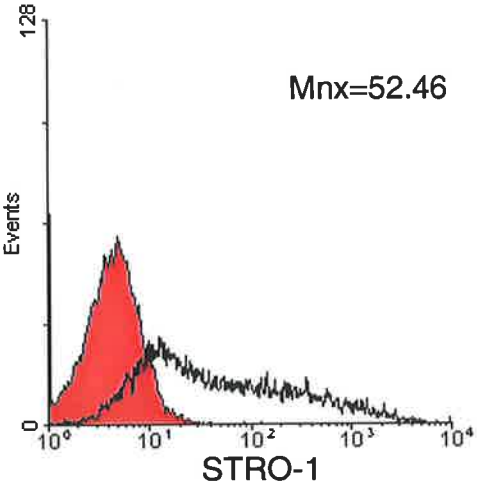


Mnx=231.25

zole (5 μM)



D 0: 87.98 %
D 1: 11.77 %

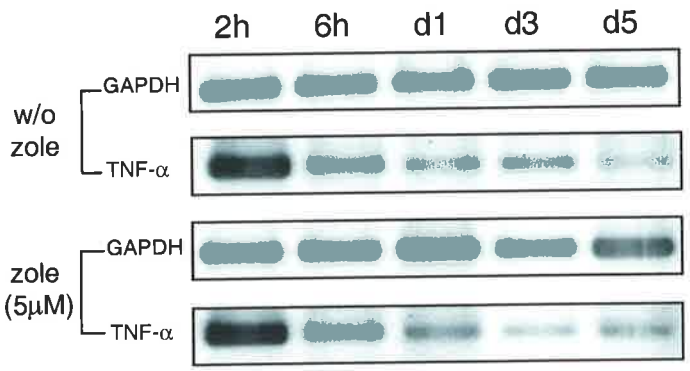


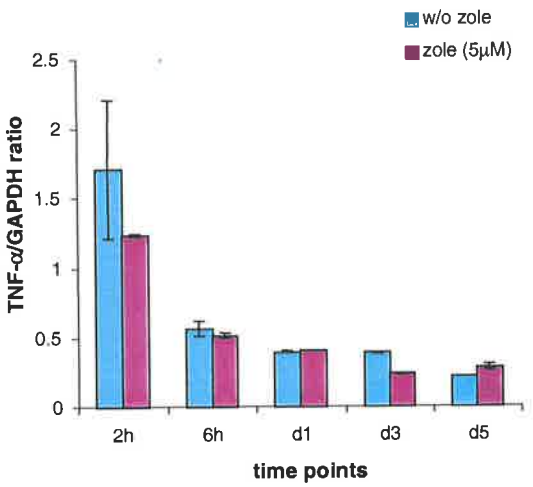
Mnx=52.46

Figure 4.12. The effect of zoledronic acid on the temporal expression of OC-related genes. Osteoblast-like cells derived from three different donors were seeded in 6-well plates at a cell density of 1.5×10^5 cells/well and cultured in the presence/absence of zoledronic acid. The cells were harvested at the five different time points, total RNA was isolated, and semi-quantitative RT-PCR was performed examining the expression of the OC-related molecules TNF-α (A), IL-1β (B), IL-6 (C), M-CSF (D), RANKL (E) and OPG (F) as described in the methods. The house keeping gene, GAPDH was used as an internal control for mRNA integrity and enabled relative gene expression to be determined. PCR products were subjected to electrophoresis on 2% agarose gel, stained with SYBR gold, visualised using a Fluorimager. The product bands were semi-quantitated and plotted as a histogram of the ratio of specific gene expression relative to the expression of GAPDH. These data suggest that the gene expression of TNF-α ($p=0.005$ in donor #2, $p<0.005$ in donor #3, Wilcoxon test), IL-1β ($p<0.005$ in donor #3, Wilcoxon test) is significantly upregulated in OB-like cells in response to 5 μM zoledronic acid.

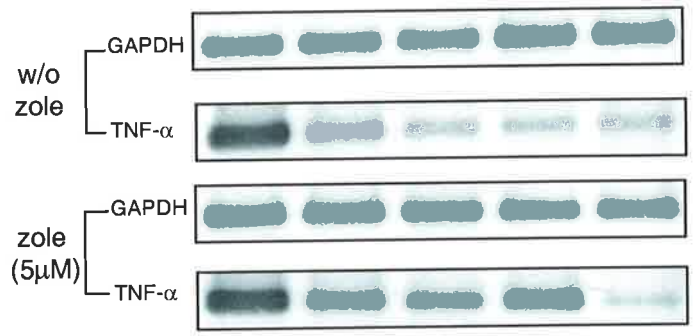
A

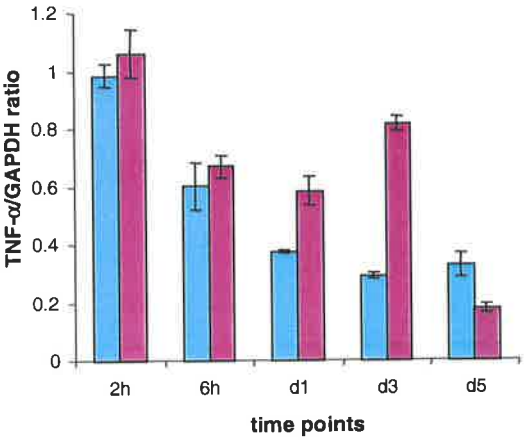
Donor #1





Donor #2





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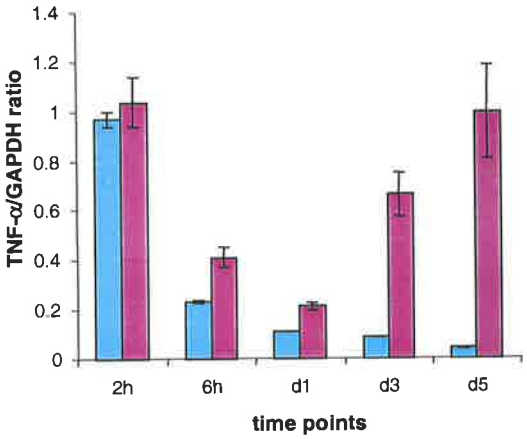

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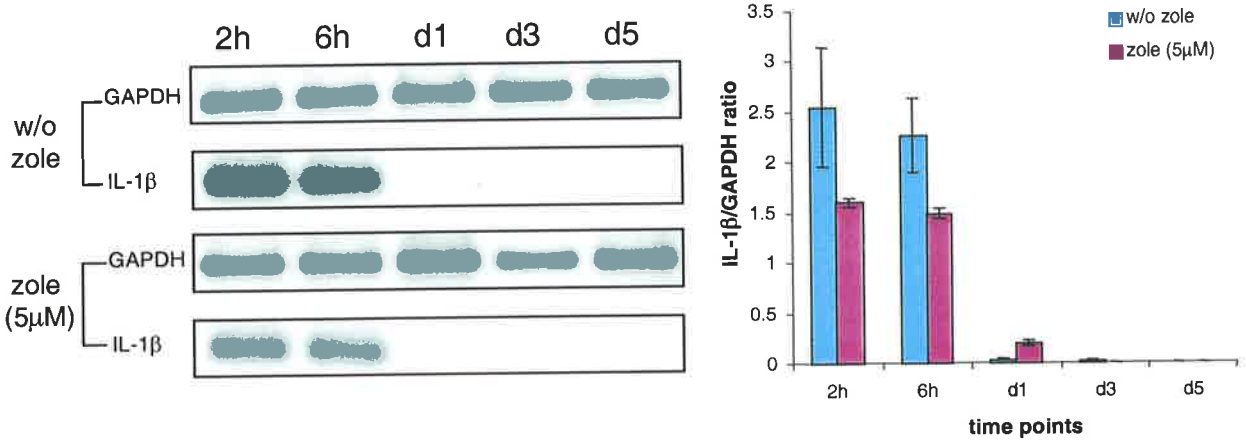

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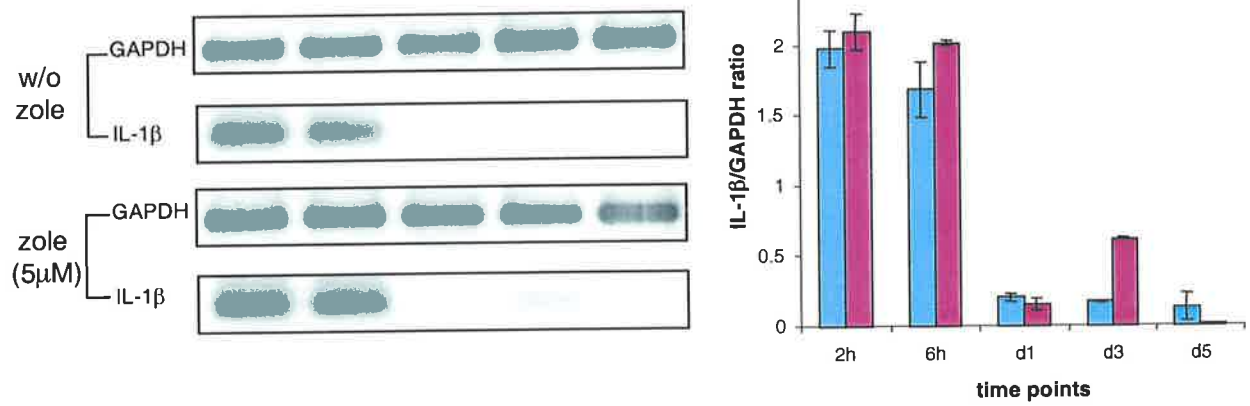

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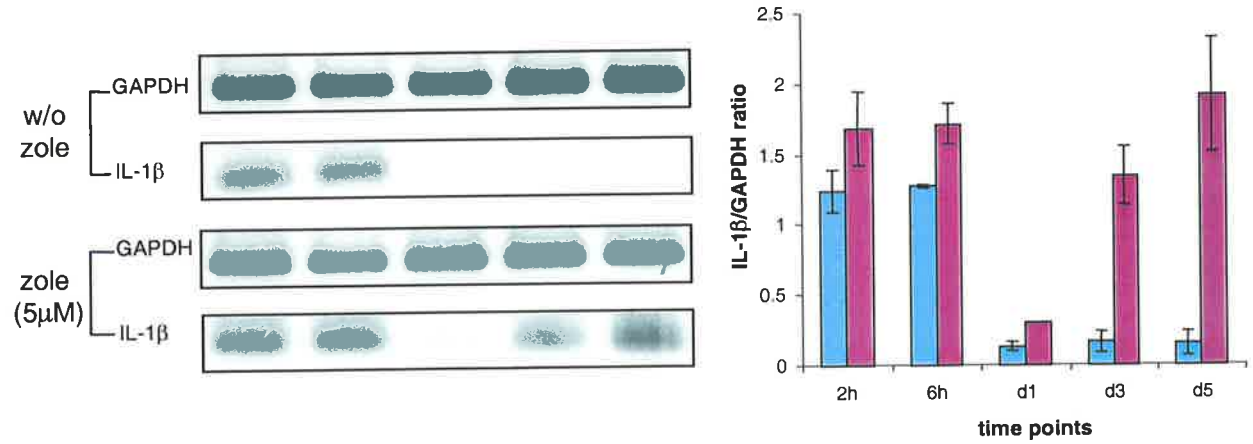

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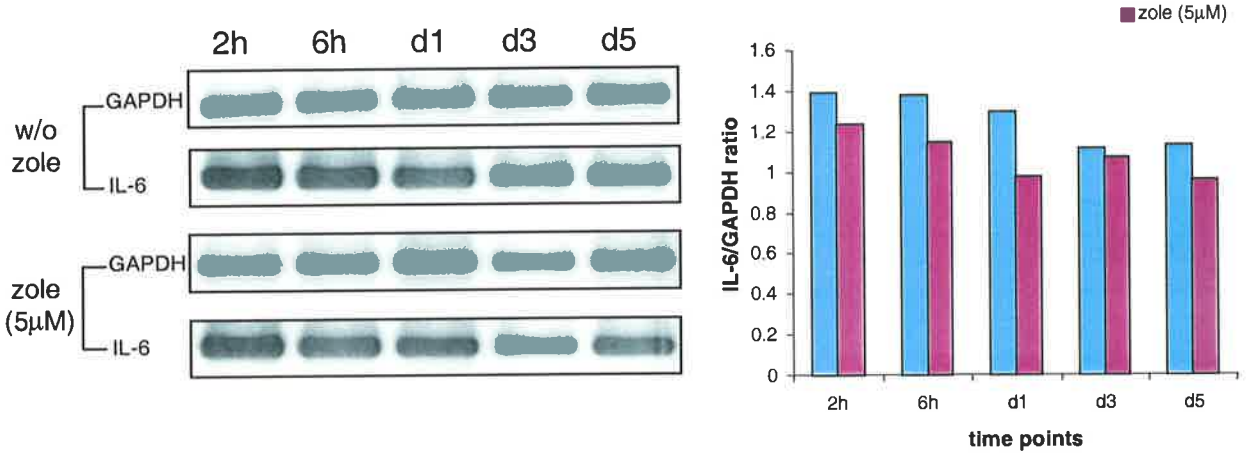

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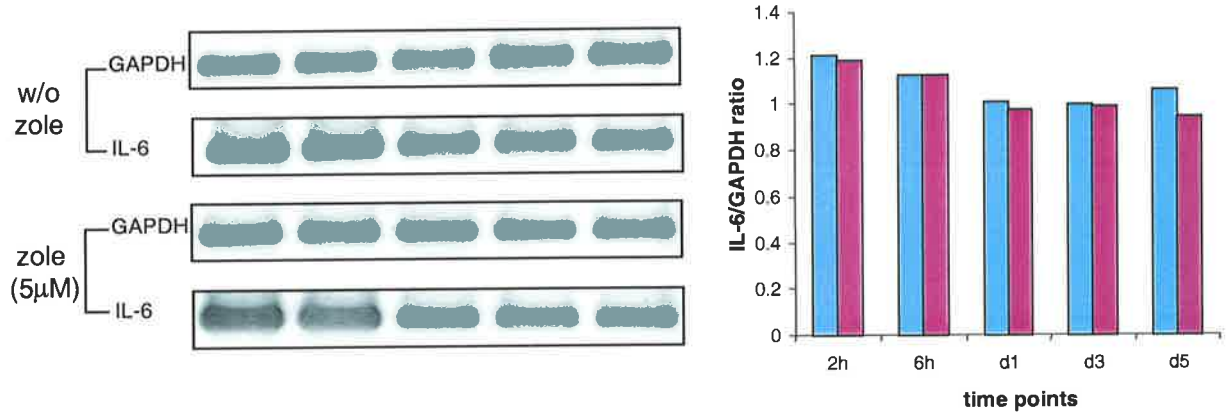

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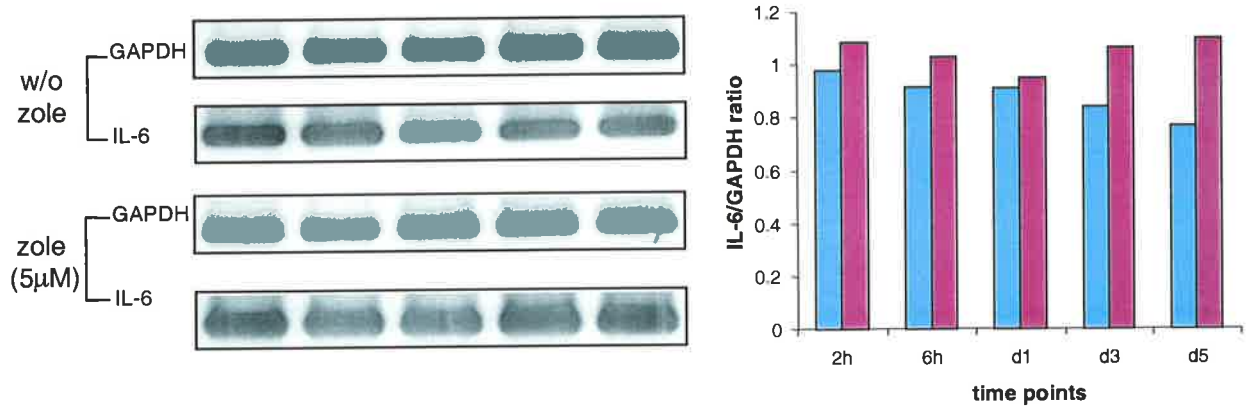

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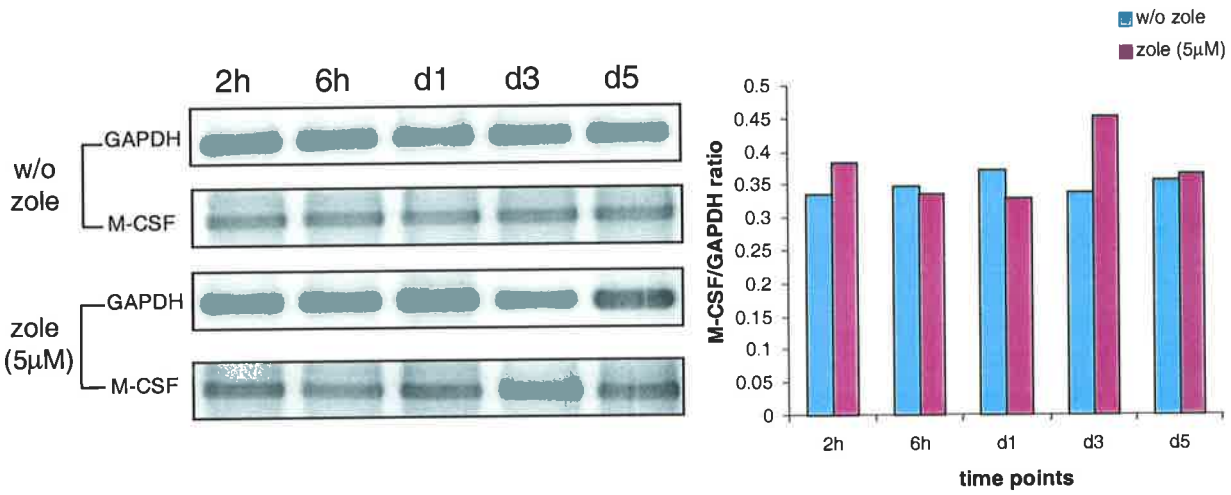

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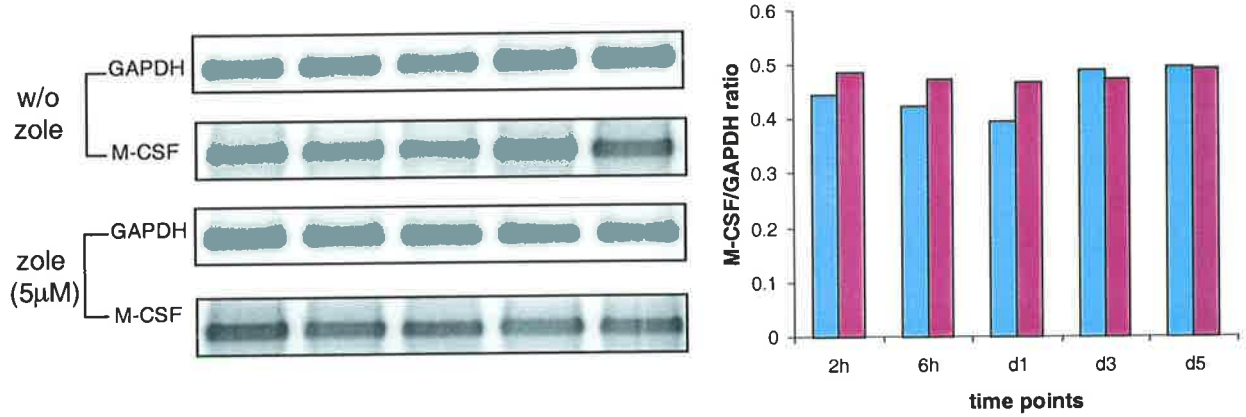

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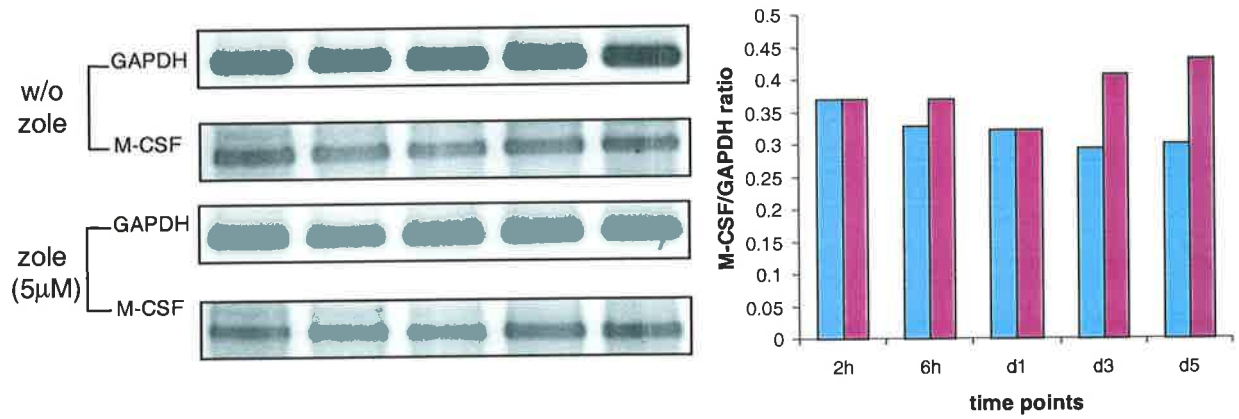

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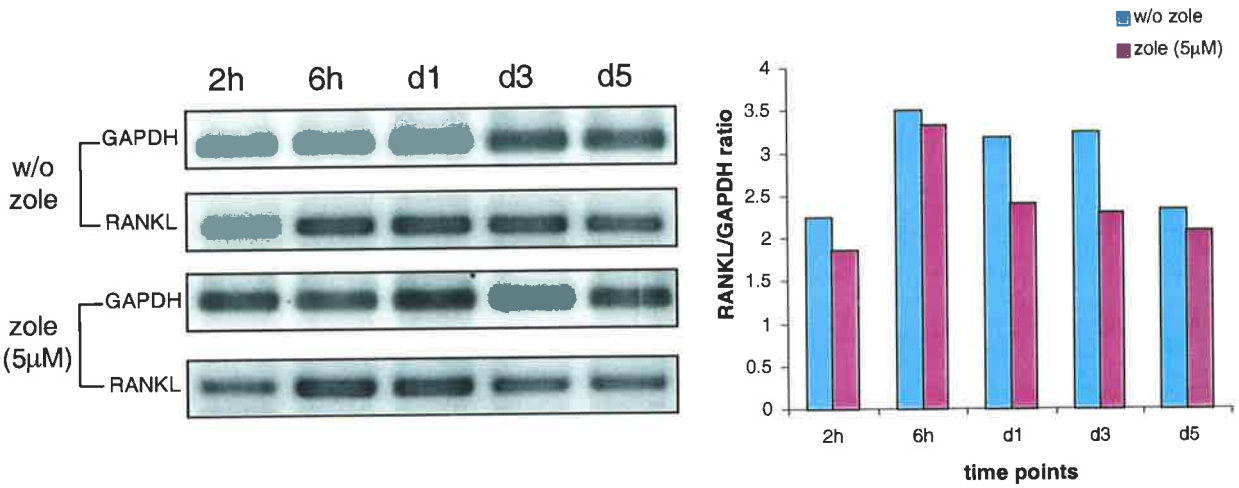

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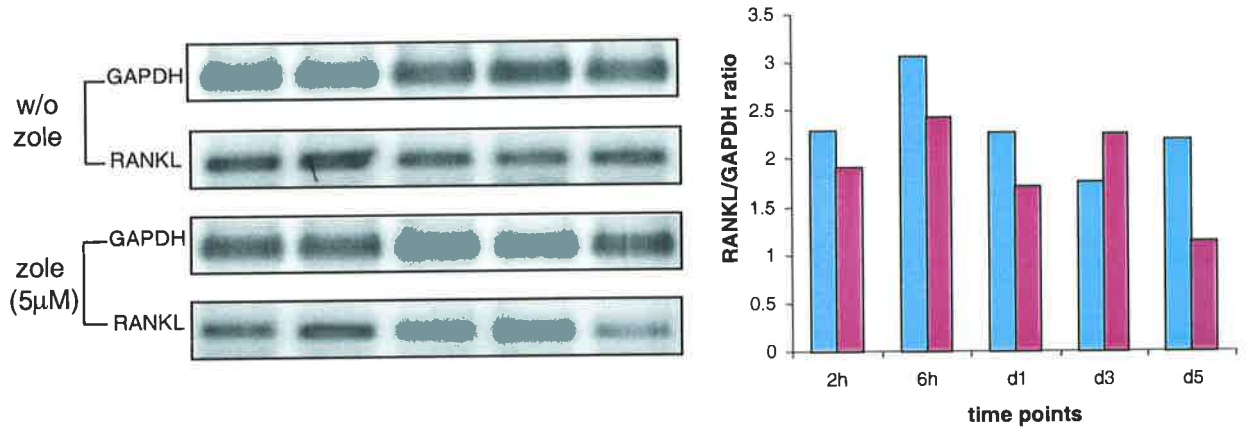

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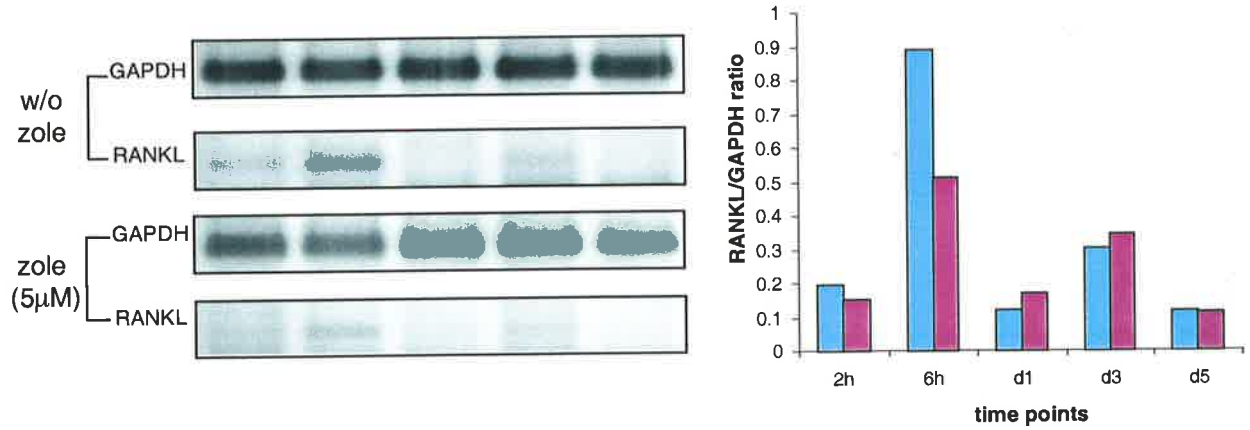

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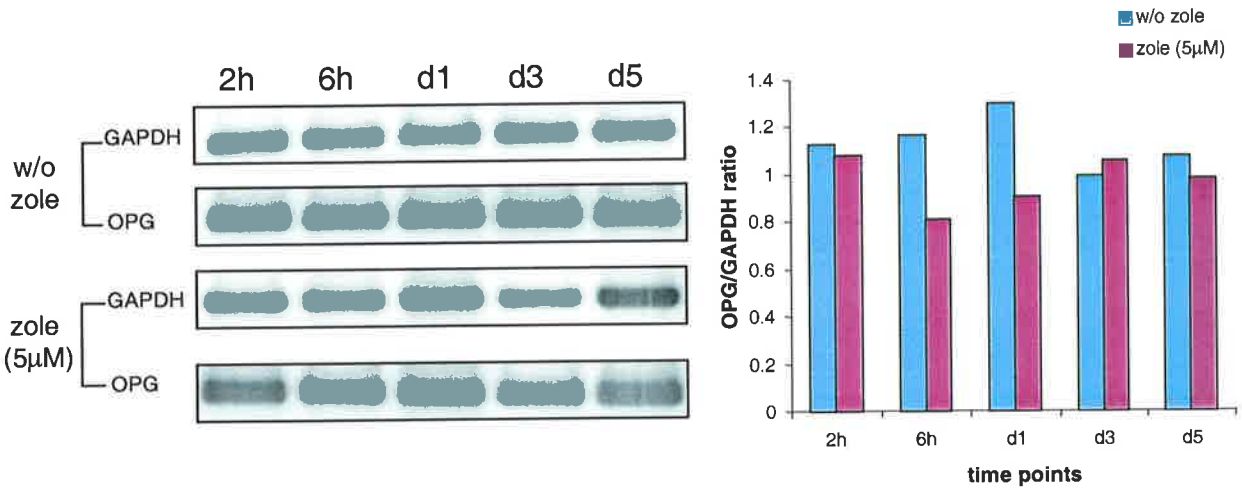

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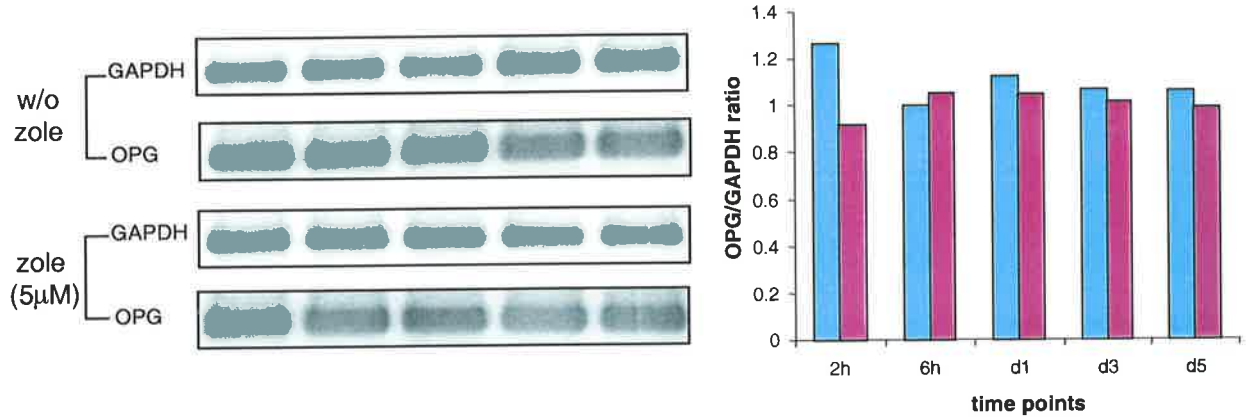

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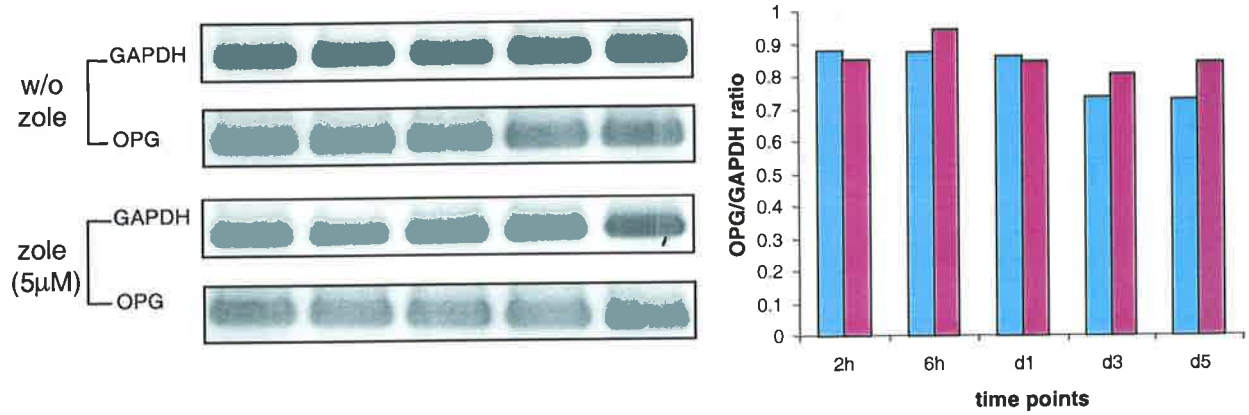
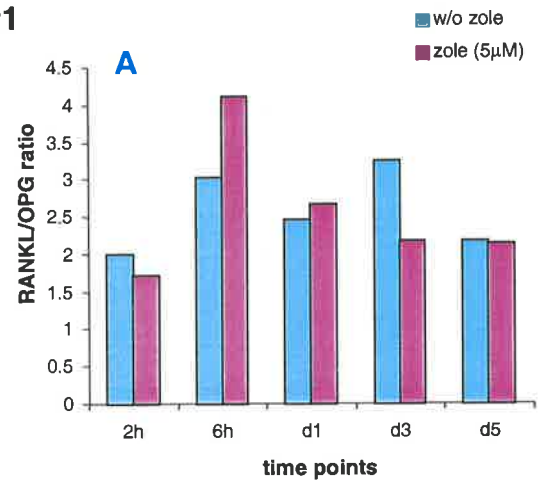


Figure 4.13. Zoledronic acid does not significantly alter the ratio of RANKL to OPG expression in OB-like cells. Semi-quantitative gene expression of RANKL and OPG was detected as described in figure 4.12. The results here showed that the relative ratio of RANKL/OPG, a key factor in OC formation, was not significantly altered in response to zoledronic acid.

Donor #1



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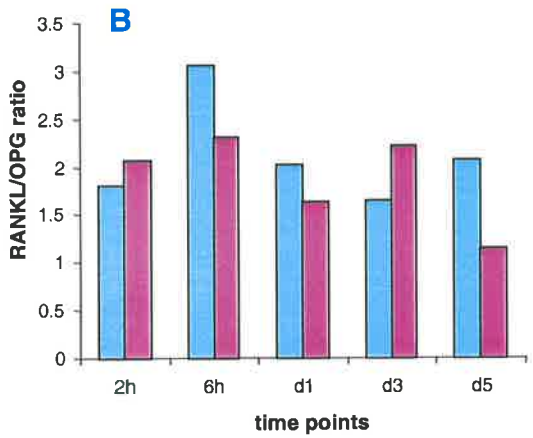

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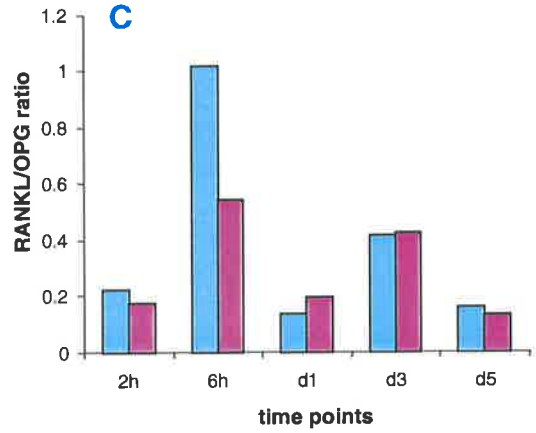


Exposure of OB-like cells to zoledronic acid also increased the expression of bone related genes (figure 4.14). In donor #3, the expression of OCN increased 6-, 11- and 8-fold at d1, d3 and d5, respectively. The expression of BSP was also found to be upregulated in donor #3. However, the exposure of zoledronic acid did not alter the gene expression of OCN and BSP in donor #1 and donor #2, or of the core binding factor alpha-1 (CBFA-1), a transcription factor that plays an integral role in OB differentiation.

4.2.5 The Up Regulation in Gene Expression Mediated by Zoledronic Acid Was Not Related to the Proportional Alteration of Osteoblast-Like Cell Subpopulations

As previously described (figure 4.6), culture of OB-like cells in 5 μM zoledronic acid, resulted in a decrease in the number of cells which expressed a preosteoblast phenotype ($STRO\text{-}1^{+}/AP^{-}$), with a concomitant increase in the number of cells with a mature OB phenotype ($STRO\text{-}1^{-}/AP^{-}$). This prompted us to examine if the alteration in gene expression observed, was related to the inherent differences in the gene expression profile of the $STRO\text{-}1^{bright}$ and $STRO\text{-}1^{negative}$ subpopulations. In an attempt to answer this question, total RNA was isolated from freshly sorted $STRO\text{-}1^{bright}$ and $STRO\text{-}1^{negative}$ OB-like cells derived from donor #3. Using semi-quantitative RT-PCR technique, gene expression was examined in both subpopulations. Interestingly, the gene expression profile detected in these two populations was comparable (figure 4.15). However, when the sorted cells were re-cultured either in the presence or absence of zoledronic acid for three days, significant changes in gene expresion were noted. As shown in figure 4.16, $STRO\text{-}1^{bright}$ cells expressed more IL-1β and TNF-α than $STRO\text{-}1^{negative}$ cells, whilst $STRO\text{-}1^{bright}$ cells express less OCN and BSP than $STRO\text{-}1^{negative}$ cells. Although zoledronic acid did not affect most of the genes expressed in $STRO\text{-}1^{bright}$ and $STRO\text{-}1^{negative}$ subpopulations, the expression of message for TNF-α was reduced in $STRO\text{-}1^{bright}$ OB-like cells and increased in $STRO\text{-}1^{negative}$ cells when exposed to zoledronic acid for three days. Furthermore, OCN gene expression in $STRO\text{-}1^{bright}$ cells was also found to be upregulated.

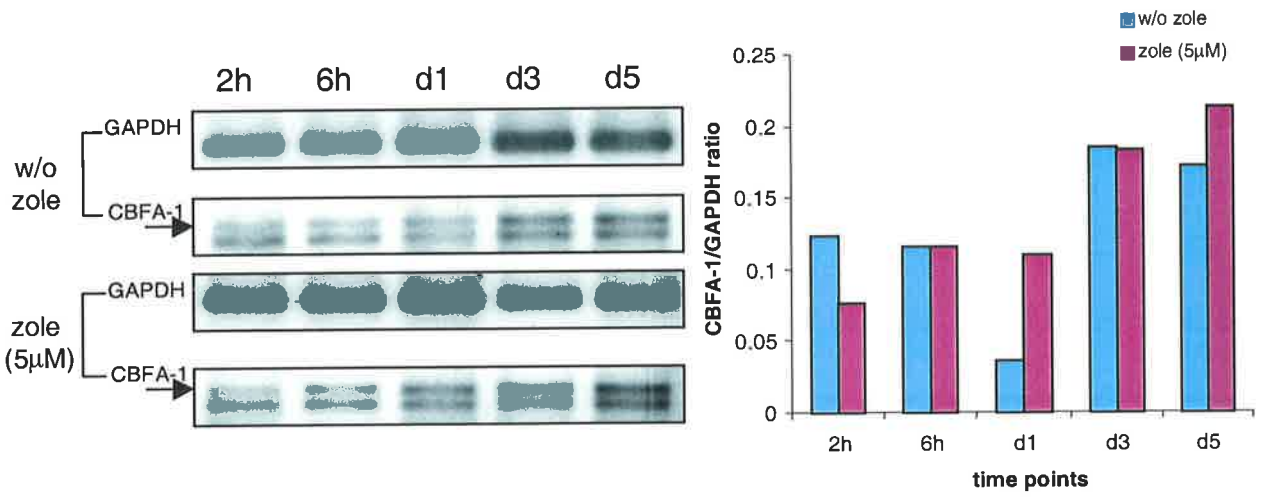
4.2.6 Zoledronic Acid Decreased Protein Expression on the Osteoblast-Like Cells

The expression of cell surface protein STRO-1, RANKL and ALP is important for OB maturation and function, therefore, these proteins were investigated using single-colour immunofluorescence and flow cytometric analysis. Culture of OB-like cells in zoledronic

Figure 4.14. The effect of zoledronic acid on the temporal expression of OB-related genes. As described in fig 4.12, the expression of OB-related genes including CBFA-1 (A), BSP (B), and OCN (C) was examined by semi-quantitative RT-PCR. These data suggest that although CBFA-1 and BSP gene expression does not appear to be altered in response to zoledronic acid, OCN gene expression is significantly ($p=0.005$ in donor #3, Wilcoxon test) upregulated in OB-like cells, partially in donor #3. The arrow indicates the position of an upper band for CBFA1.

A

Donor #1



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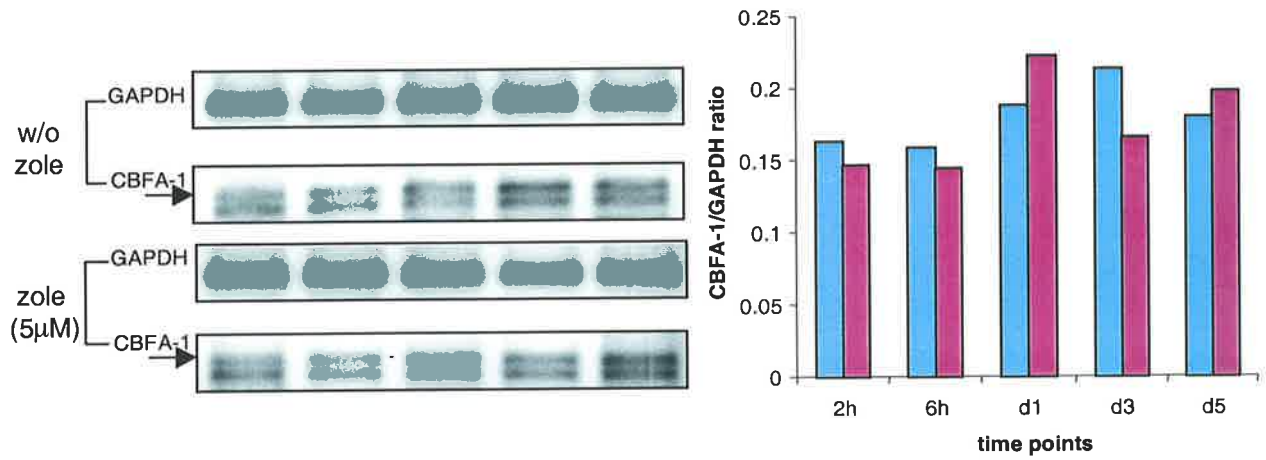

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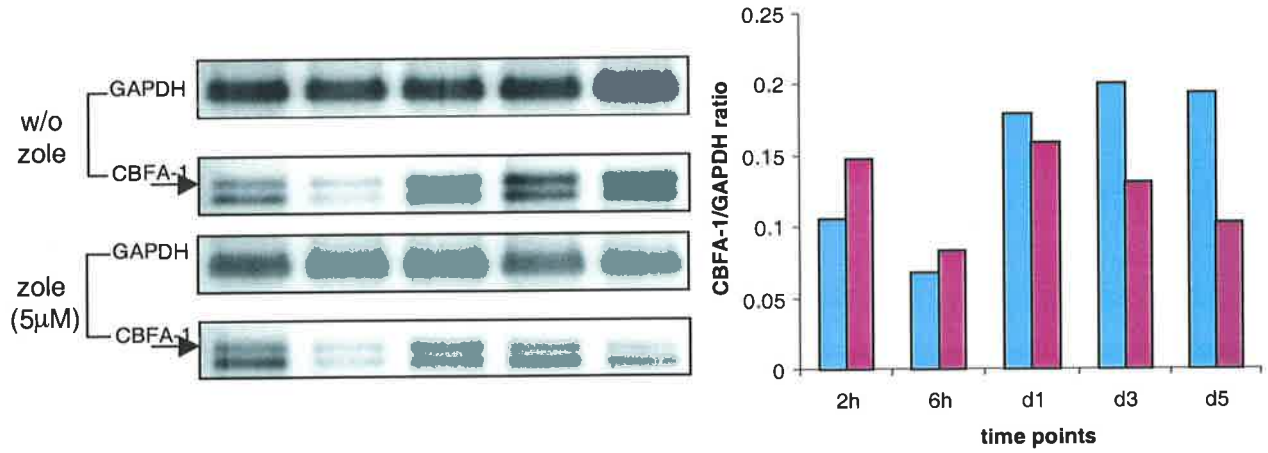

B

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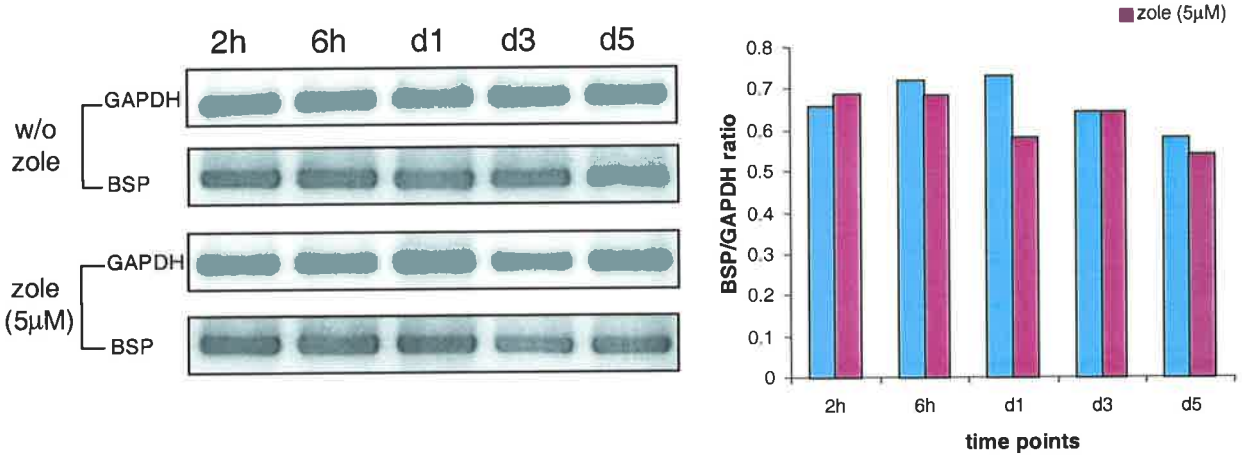

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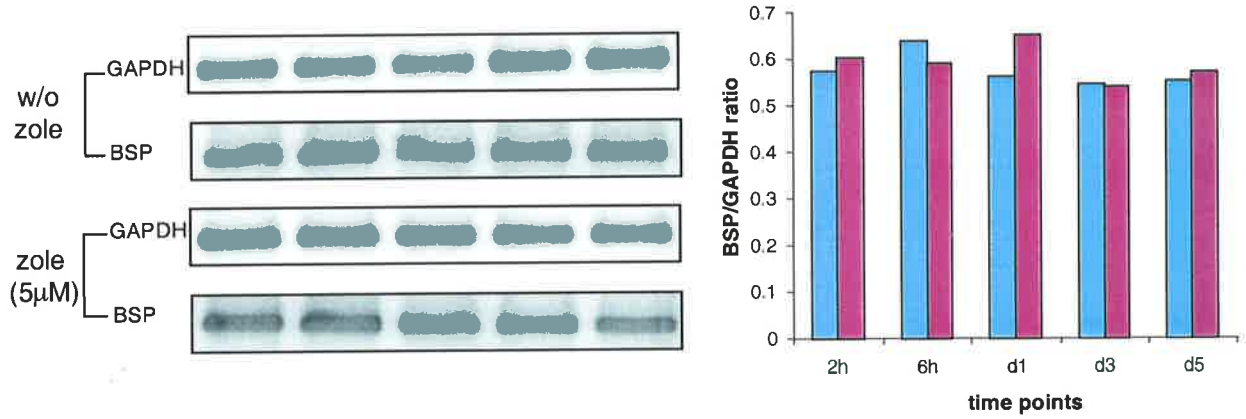

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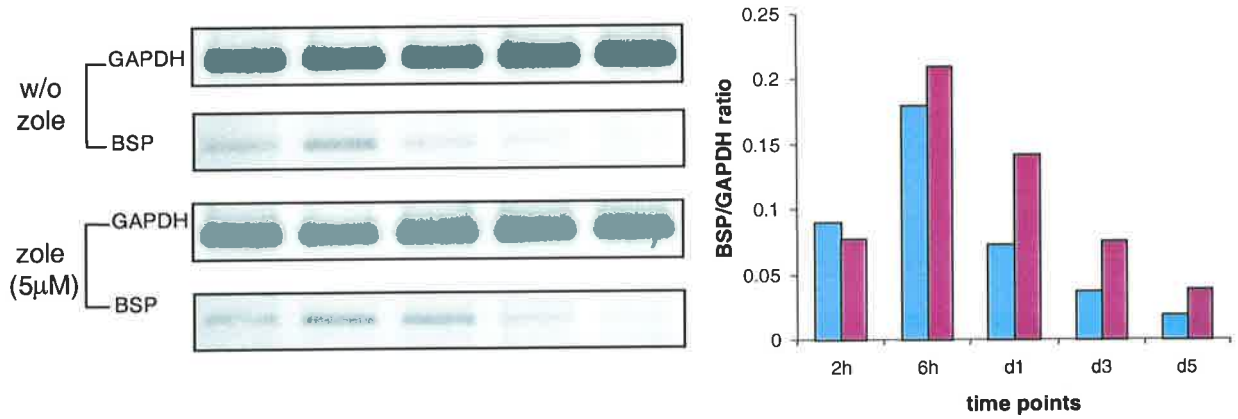

C

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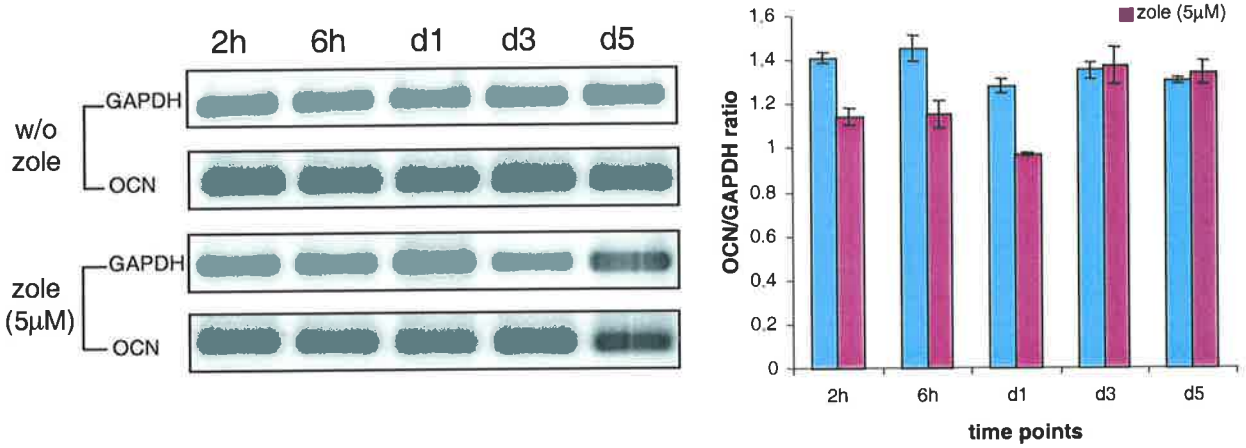

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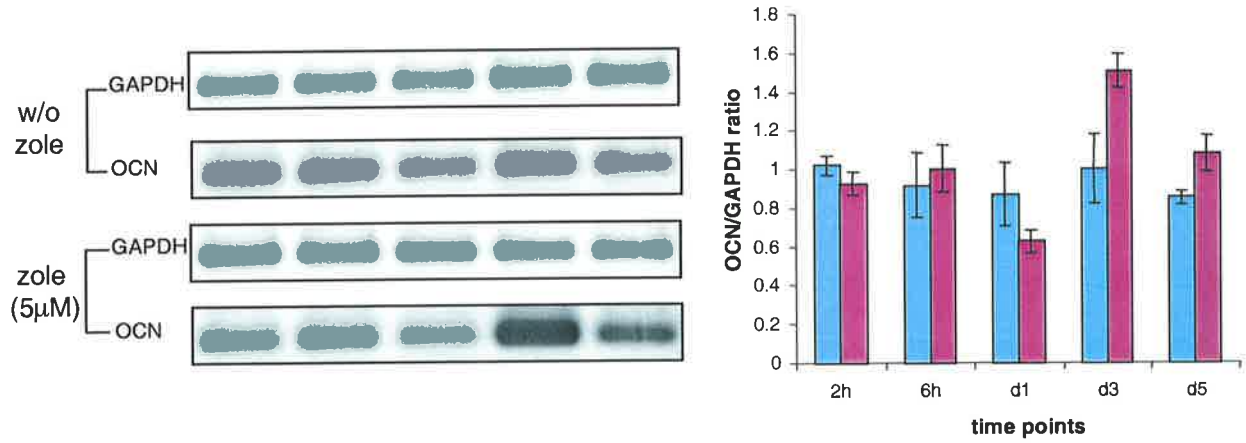

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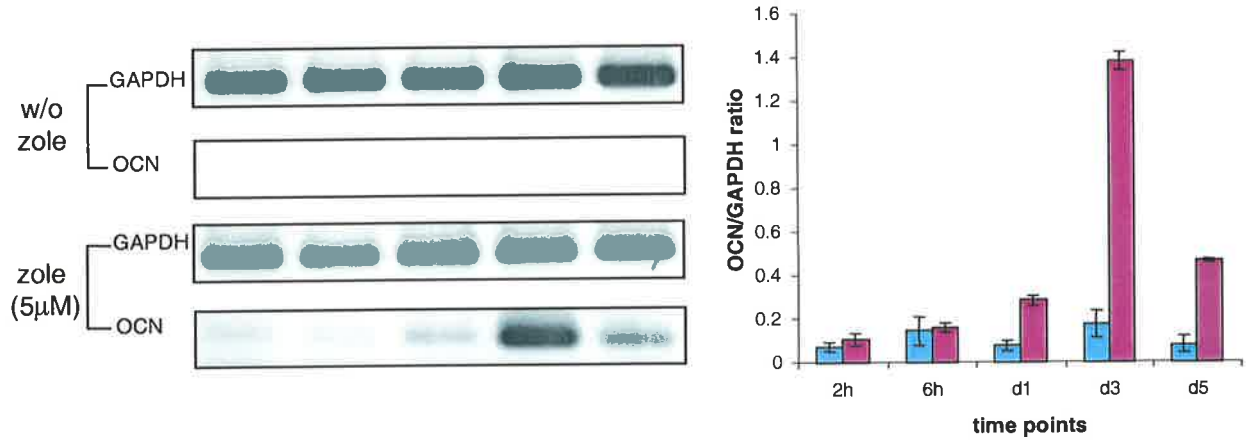
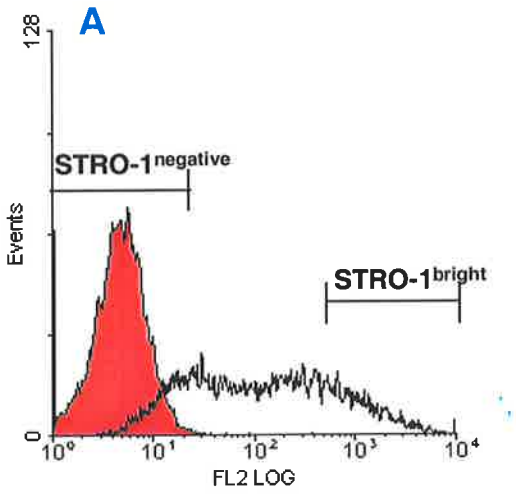
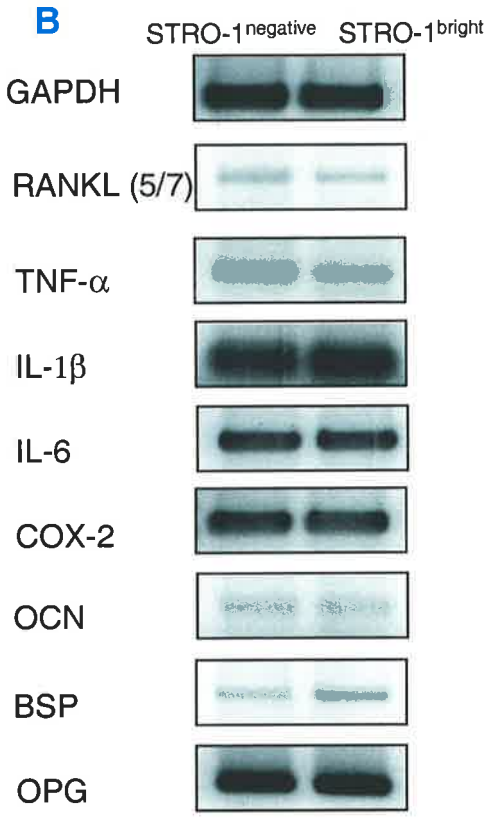


Figure 4.15. Freshly sorted STRO-1^{bright} and STRO-1^{negative} cells do not differ with respect to the expression of OC- and OB-related gene. Osteoblast-like cells derived from donor #3 were sorted by FACStar^{PLUS} cell sorter into STRO-1^{bright} and STRO-1^{negative} populations as previously described (A). Total RNA was isolated from the both populations, and gene was examined by semi-quantitative RT-PCR. The various transcripts examined in this study included: TNF-α, IL-1β, IL-6, RANKL, OPG, COX-2, BSP and OCN (B). The product bands were semi-quantitated and plotted as a histogram of the ratio of specific gene expression relative to the expression of GAPDH (C). There was no difference in the relative gene expression between two subpopulations ($p>0.05$, Wilcoxon test).


A
128
Events
0
STRO-1negative
STRO-1bright
10⁰
10¹
10²
10³
10⁴
FL2 LOG


B
STRO-1negative
STRO-1bright
GAPDH
RANKL (5/7)
TNF-α
IL-1β
IL-6
COX-2
OCN
BSP
OPG

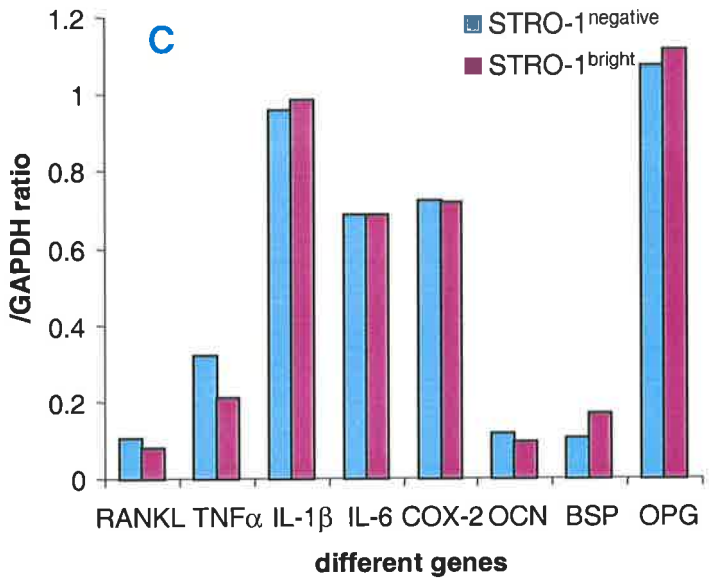
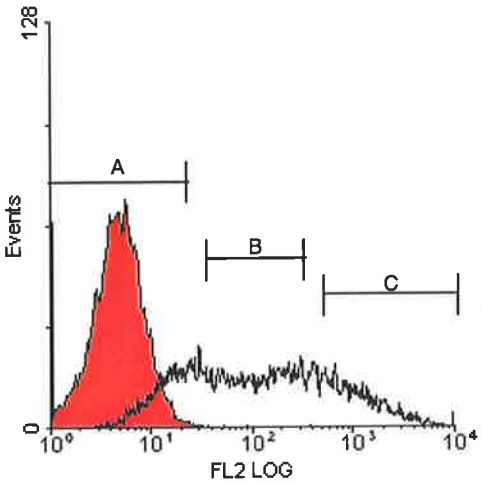
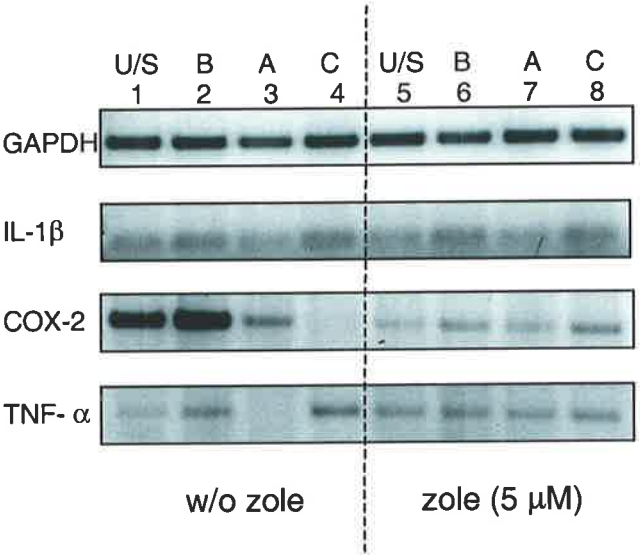

C
STRO-1negative
STRO-1bright
1.2
1
0.8
0.6
0.4
0.2
0
/GAPDH ratio
RANKL
TNFα
IL-1β
IL-6
COX-2
OCN
BSP
OPG
different genes

Figure 4.16. Gene expression in sorted STRO-1^{bright} and STRO-1^{negative} subpopulations of OB-like cells treated with zoledronic acid. Osteoblast-like cells derived from donor #2 were sorted using FACStar^{PLUS} cell sorter. Unsorted, STRO-1^{negative} (horizontal bar A), STRO-1^{intermediate} (horizontal bar B) and STRO-1^{bright} (horizontal bar C) cells were sorted as described previously. The sorted cells were seeded in the 6-well plates (2.5 $\times 10^5$ cells/well) and cultured for 72 hrs in the presence or absence of zoledronic acid. The cells were harvested and total RNA isolated, semi-quantitative RT-PCR was performed in order to detect any changes in gene expression. The product bands were quantitated using ImageQuant and the results plotted out as a histogram of gene expression relative to GAPDH. Interestingly, the expression of a majority of the genes examined was substantially different between the STRO-1^{negative}, STRO-1^{intermediate}, and STRO-1^{bright} fractions when the cells were cultured for 3 days in the absence of zoledronic acid. Of note, STRO-1^{bright} cells expressed more IL-1β and TNFα transcripts than STRO-1^{negative} cells. In contrast, STRO-1^{bright} cells expressed less OCN, COX-2 and BSP transcripts than STRO-1^{negative} cells. The STRO-1^{intermediate} population expressed very high levels of COX-2 transcript, which was reproducibly down regulated following 3 days culture in zoledronic acid. Although zoledronic acid did not alter the expression of most of the genes examined in STRO-1^{bright} and STRO-1^{negative} subpopulations, the expression of TNFα was reproducibly down regulated in STRO-1^{bright} OB-like cells and upregulated in the STRO-1^{negative} fraction. In contrast, the expression of OCN in STRO-1^{bright} was upregulated in response to zoledronic acid.

A





1 unsorted osteoblast-like cells: w/o zole

2 STRO-1^{intermediate} : w/o zole

3 STRO-1^{negative} : w/o zole

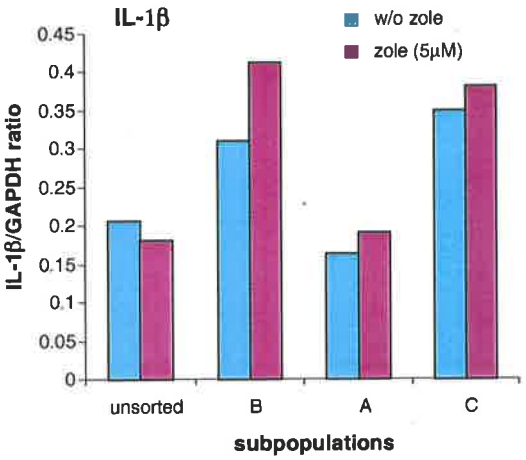
4 STRO-1^{bright} : w/o zole

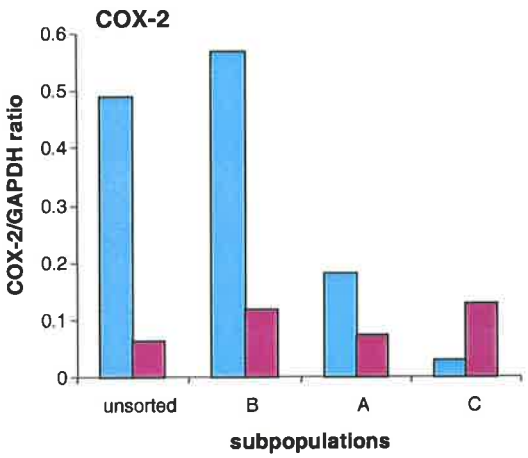
6 unsorted osteoblast-like cells: zole (5μM)

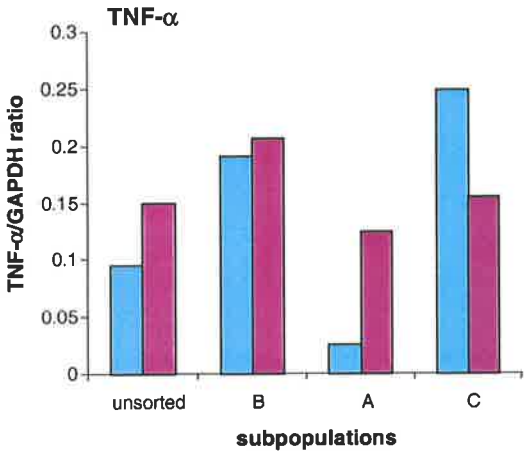
7 STRO-1^{intermediate} : zole (5μM)

8 STRO-1^{negative} : zole (5μM)

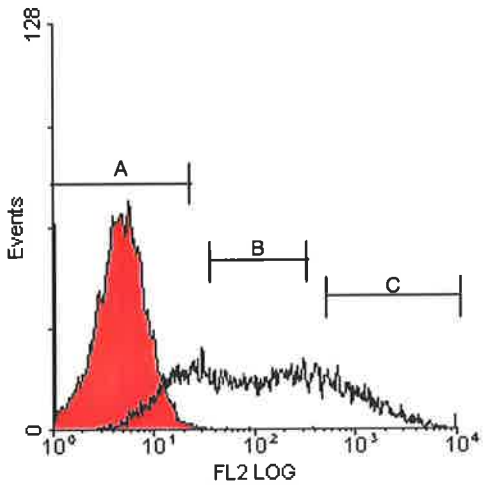
9 STRO-1^{bright} : zole (5μM)

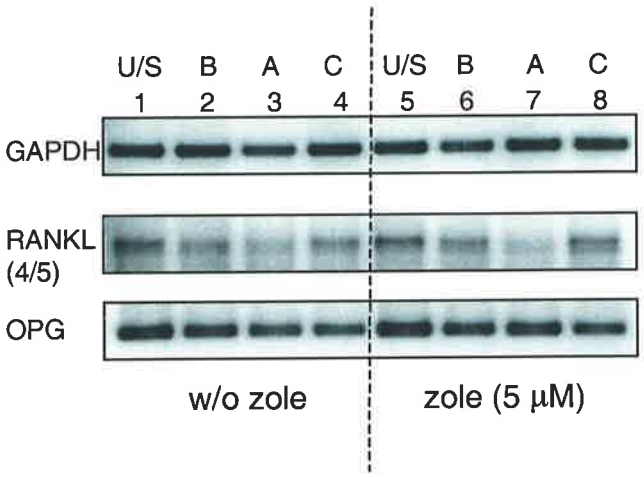


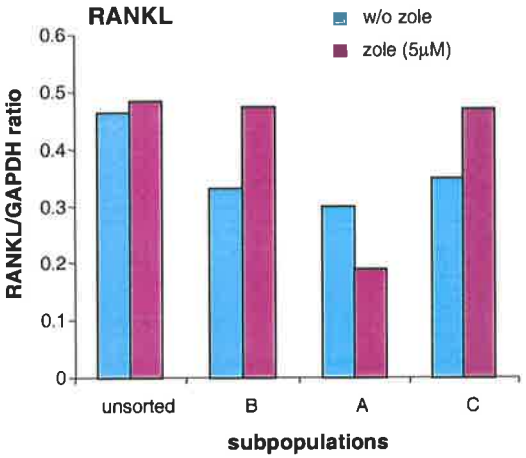


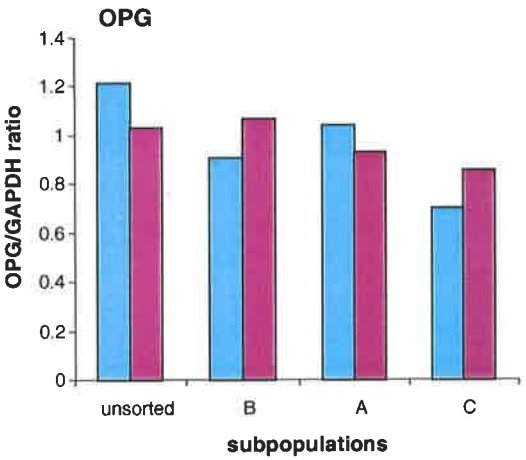


B









1 unsorted osteoblast-like cells: w/o zole

2 STRO-$1^{intermediate}$: w/o zole

3 STRO-$1^{negative}$: w/o zole

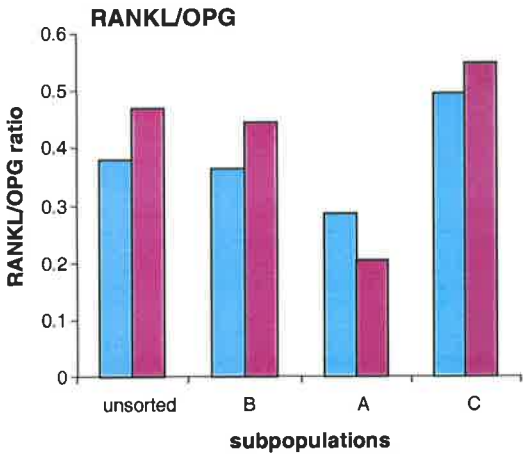
4 STRO-1^{bright} : w/o zole

6 unsorted osteoblast-like cells: zole (5µM)

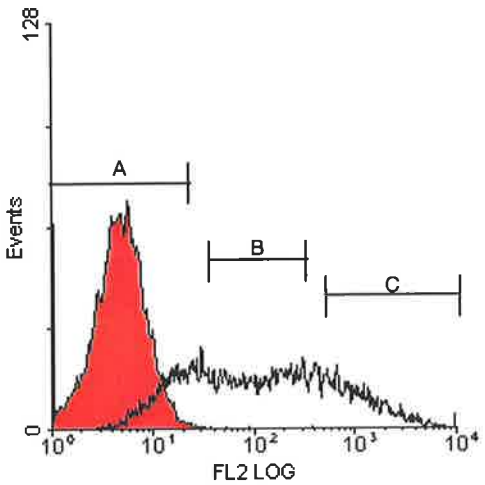
7 STRO-$1^{intermediate}$: zole (5µM)

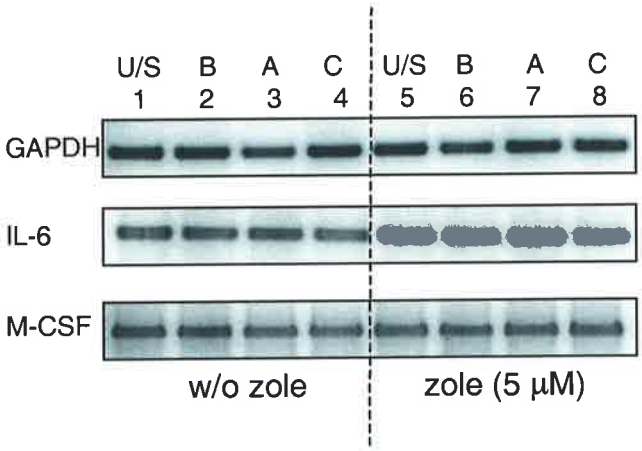
8 STRO-$1^{negative}$: zole (5µM)

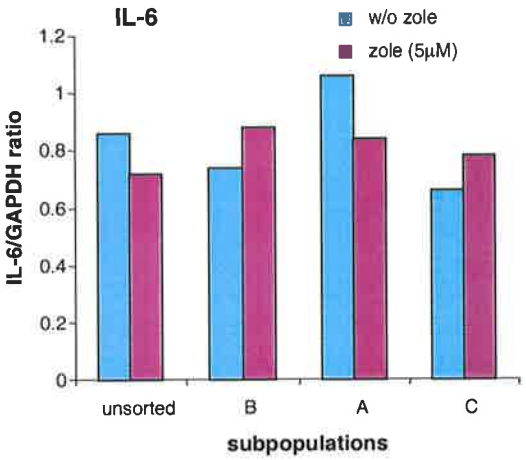
9 STRO-1^{bright} : zole (5µM)

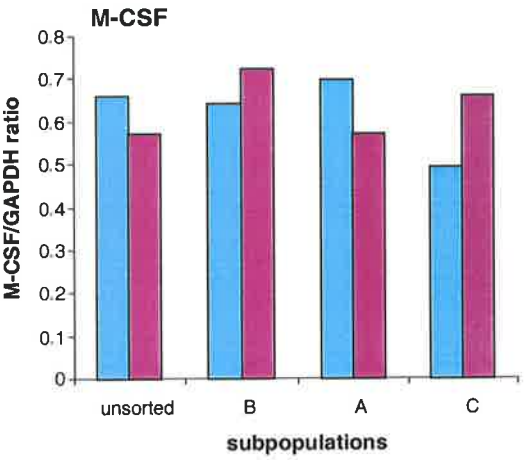


C









1 unsorted osteoblast-like cells: w/o zole

2 STRO-1^{intermediate} : w/o zole

3 STRO-1^{negative} : w/o zole

4 STRO-1^{bright} : w/o zole

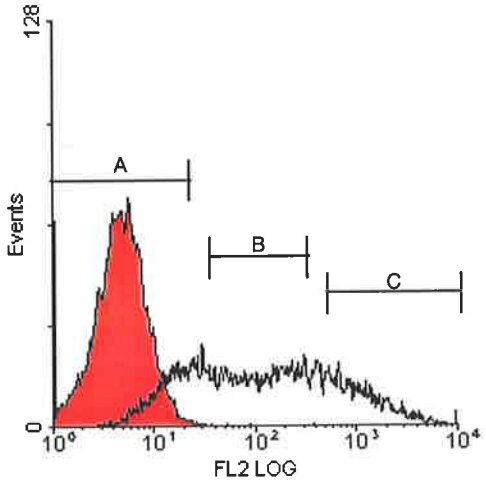
6 unsorted osteoblast-like cells: zole (5μM)

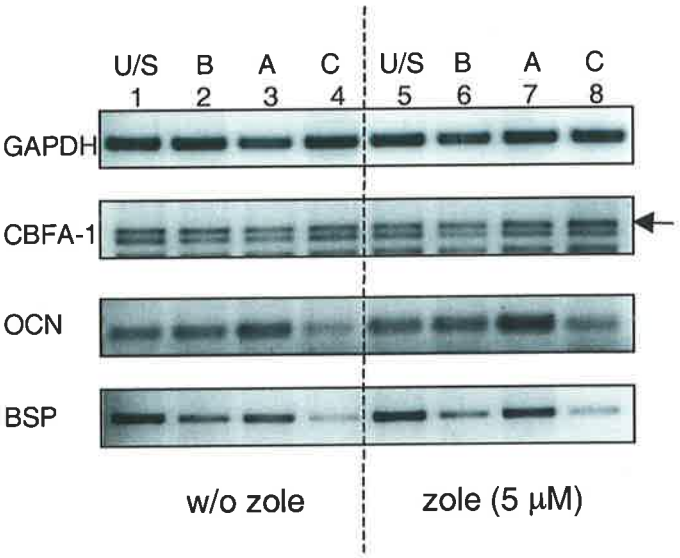
7 STRO-1^{intermediate} : zole (5μM)

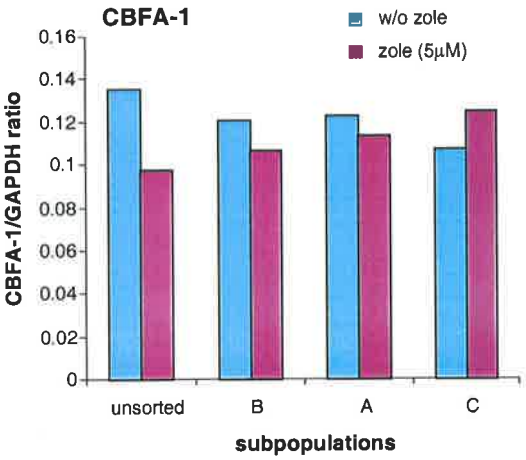
8 STRO-1^{negative} : zole (5μM)

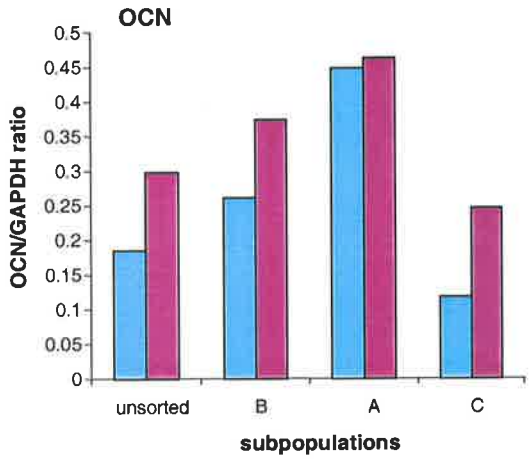
9 STRO-1^{bright} : zole (5μM)

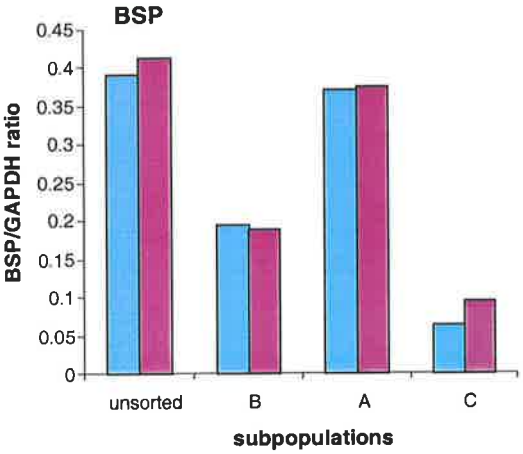
D











1 unsorted osteoblast-like cells: w/o zole

2 STRO-$1^{intermediate}$: w/o zole

3 STRO-$1^{negative}$: w/o zole

4 STRO-1^{bright} : w/o zole

6 unsorted osteoblast-like cells: zole (5μM)

7 STRO-$1^{intermediate}$: zole (5μM)

8 STRO-$1^{negative}$: zole (5μM)

9 STRO-1^{bright} : zole (5μM)

acid for three days, resulted in a decrease in the expression of membrane protein expression of STRO-1 (figure 4.17) and RANKL (figure 4.18) on the OB-like cells. In contrast, when cytoplasmic protein expression was examined, no significant changes were noted, suggesting that membrane expression was regulated by mechanisms independent of gene expression (figure 4.20). A comparable down regulation of ALP expression was observed for both membrane and cytoplasmic expression of this protein (figure 4.19).

4.2.7 Zoledronic Acid Upregulated TACE Gene Expression

Our studies suggest that zoledronic acid regulates the membrane expression of RANKL without demonstrably affecting the level of transcript expression. Recent reports suggest that membrane-bound RANKL is able to be cleaved by a member of the metalloprotease-disintegrin, TACE. In the absence of an antibody specific for TACE, we examined the regulation of TACE expression using semi-quantitative RT-PCR. As seen in figure 4.21, zoledronic acid treatment resulted in an up regulation of TACE gene expression at d1 and d3 in donor #2, at d3 in donor #3. The up regulation in TACE expression coincided with a reduction in RANKL and STRO-1 expression. Moreover, TACE gene expression declined to baseline levels at d5, temporally consistent with the re-expression of RANKL and STRO-1 expression.

4.2.8 TNF-α and IL-1β Augmented STRO-1$^+$/ALP$^+$ Subpopulation and Increased Cell-Dividing Potential of Osteoblast-Like Cells

As described previously, zoledronic acid treatment resulted in the up regulation of TNF-α and IL-1β in OB-like cells. To investigate whether TNF-α and IL-1β expression can overcome the cytostatic effects induced by zoledronic acid, OB-like cells were cultured with recombinant human TNF-α or IL-1β and the cell phenotypes were assessed by dual-colour immunofluorescence and flow cytometric analysis. As seen in figure 4.22, cells cultured in TNF-α and IL-1β resulted in an increase in the number of STRO-1$^+$/AP$^+$ cells.

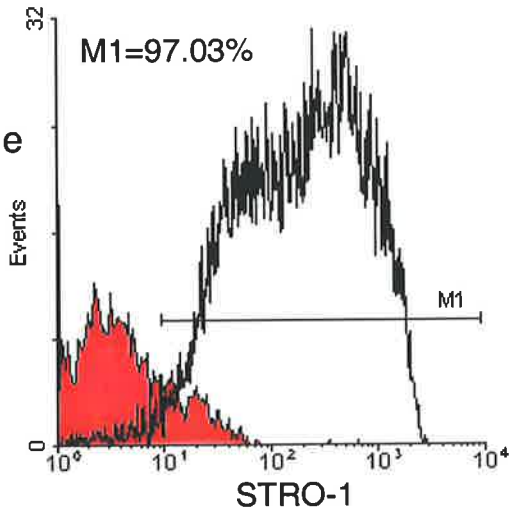
Consistent with previous findings, STRO-1^{bright} and ALP^{bright} cells possessed a greater proliferation capacity than the cells expressing low levels of the STRO-1 and ALP antigens. Of note, TNF-α had a greater capacity to increase the number of STRO-1^{bright} cells, whilst IL-1β had greater capacity to increase the number of ALP^{bright} cells. Moreover, both TNF-α

Figure 4.17. The zoledronic acid-mediated down regulation of STRO-1 expression occurs at the level of surface expression. Fluorescence histograms depicting the expression of STRO-1 protein at the surface (A) and within the cytoplasm (B) of OB-like cells derived from donor #2. The data is expressed as the relative cell count (y-axis) versus the intensity of STRO-1 expression (log scale). 10,000 events were collected as listmode data. The horizontal bar (region M_1) depicts the relative percentage of cells expressing STRO-1 antigen compared to the isotype matched negative control mAb, 1A6.12, coupled to FITC. The mean fluorescence of STRO-1 expression on the cell membrane was decreased 3-fold when the cells were exposed to zoledronic acid (from 130.43 to 30.95). In contrast, no difference was noted in permeablised cell staining, suggesting that zoledronic acid modulates the expression of STRO-1 at the cell surface.

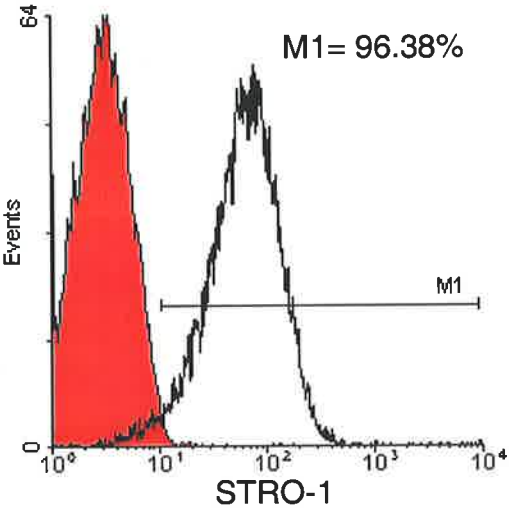
A Membrane protein expression

B Cytoplasmic protein expression

w/o zole

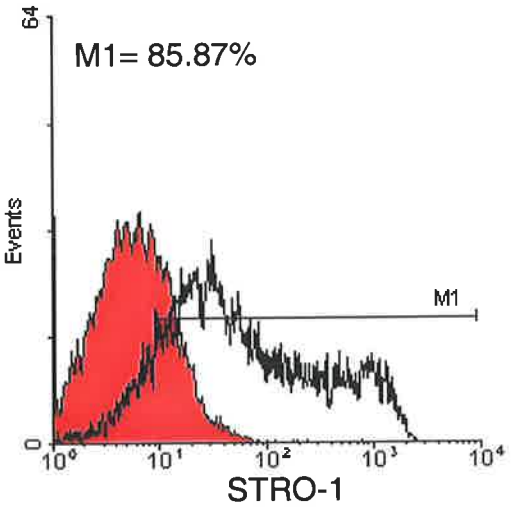


MnX=130.43

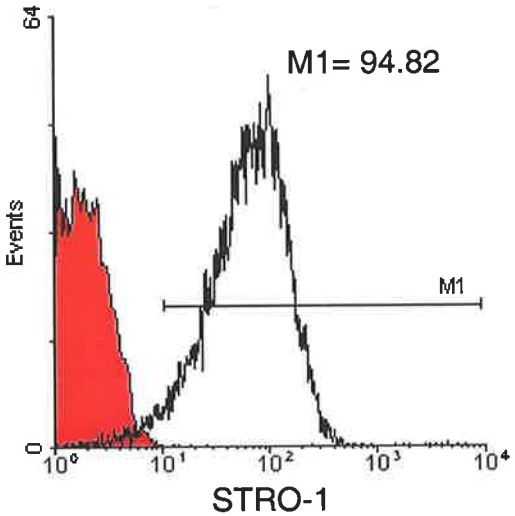


MnX=56.04

zole (5μM)



MnX=30.95



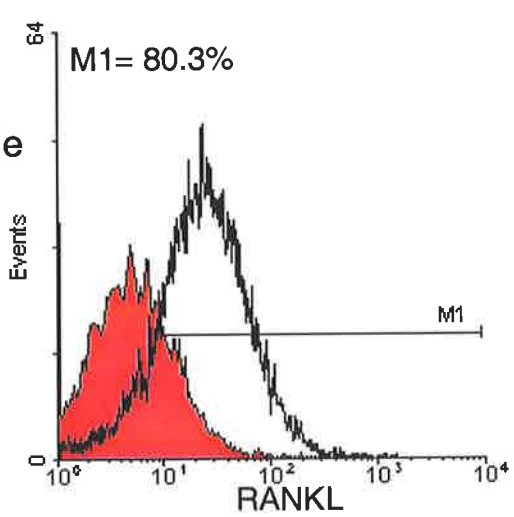
MnX=57.89

Figure 4.18. The zoledronic acid mediated down regulation of RANKL expression occurs at the level of surface and cytoplasmic expression. Fluorescence histograms depicting the expression of RANKL protein at the surface (A) and within the cytoplasm (B) of OB-like cells derived from donor #2. The data is expressed as the relative cell count (y-axis) versus the intensity of RANKL expression (log scale). 10,000 events were collected as listmode data. The horizontal bar (region M_1) depicts the relative percentage of cells expressing RANKL antigen compared to the isotype matched negative control mAb, 1A6.11 (for mAb TRANCE), or α-MAP (for anti-rabbit RANKL), coupled to FITC. A decrease in mean fluorescence of RANKL expression both in membrane and permeablised staining was observed when the cells were exposed to zoledronic acid, with a more profound decrease in membrane protein expression.

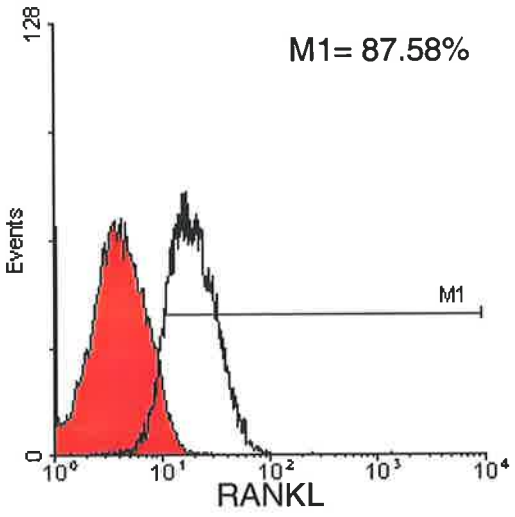
A Membrane protein expression detected by TRANCE Ab

B Cytoplasmic protein expression detected by anti-rabbit RANKL Ab

w/o zole

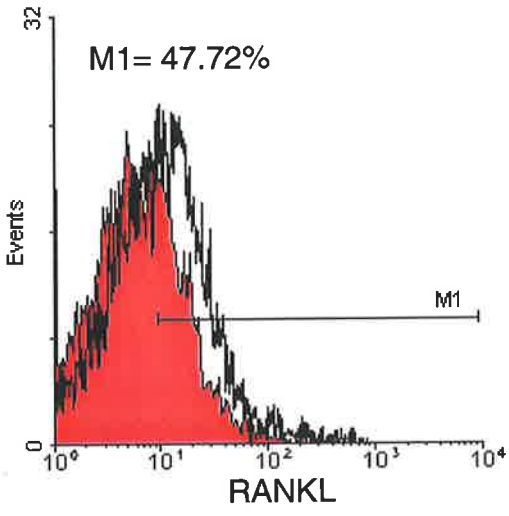


MnX=16.64

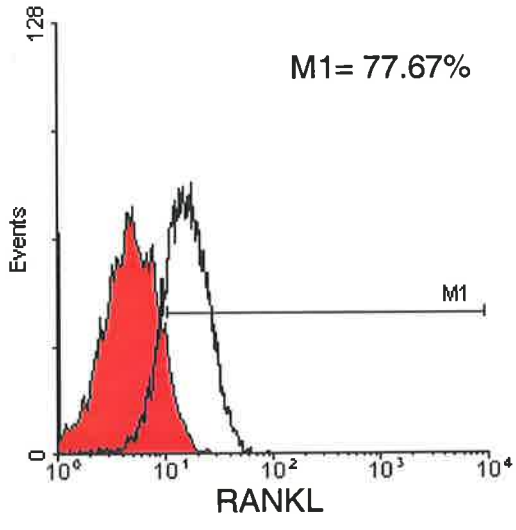


MnX=14.95

zole (5μM)



MnX=2.6



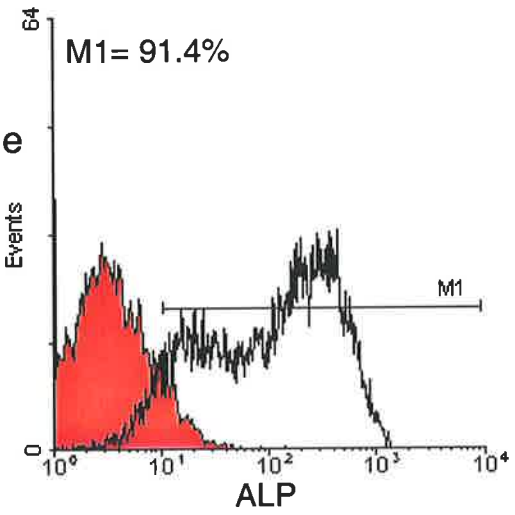
MnX=10.32

Figure 4.19. The zoledronic acid mediated down regulation of ALP expression occurs at the level of both the cytoplasmic and membrane expression. Fluorescence histograms depicting the expression of ALP protein at the surface (A) and within the cytoplasm (B) of OB-like cells derived from donor #2. The data is expressed as the relative cell count (y-axis) versus the intensity of ALP expression (log scale). 10,000 events were collected as listmode data. The horizontal bar (region M_1) depicts the relative percentage of cells expressing ALP antigen compared to the isotype matched negative control mAb, 1B5, coupled to FITC. A decrease of mean fluorescence of ALP expression both in membrane and permeablised staining was observed when the cells were exposed to zoledronic acid.

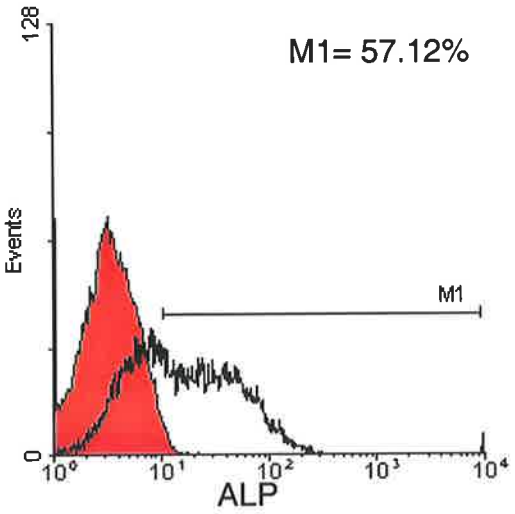
A Membrane protein expression

B Cytoplasmic protein expression

w/o zole

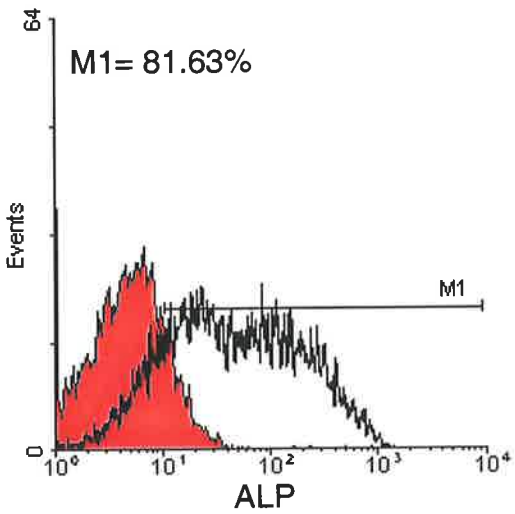


MnX=81.61

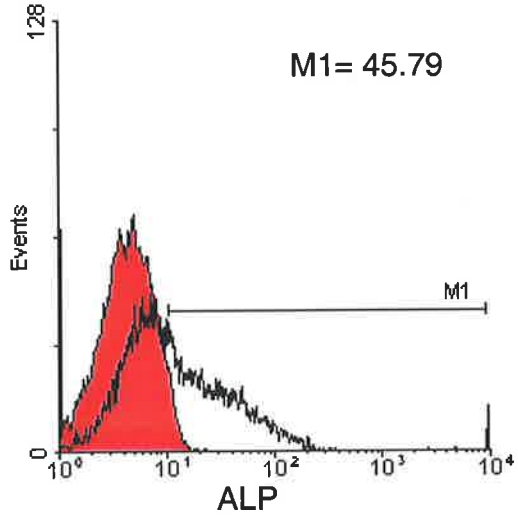


MnX=12.1

zole (5μM)

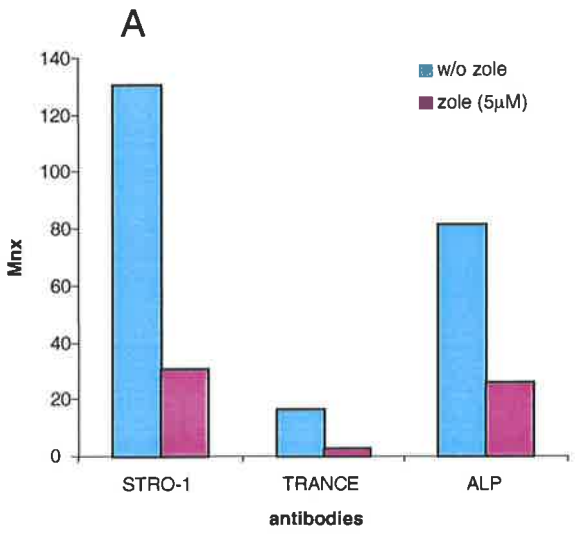


MnX=26.09



MnX=7.28

Figure 4.20. Summary of membrane and cytoplasmic protein expression by OB-like cells treated with zoledronic acid. As described above, zoledronic acid mediated a decrease in the expression of membrane protein expression (A) for STRO-1, RANKL and ALP on the OB-like cells. This decrease was not as significant when cytoplasmic expression was examined for both RANKL and STRO-1 (B). Data are displayed as the mean fluorescence of each protein from which the mean fluorescence value of the isotype-matched negative control has been subtracted.


A
140
120
100
80
60
40
20
0
Mnx
w/o zole
zole (5μM)
STRO-1
TRANCE
ALP
antibodies

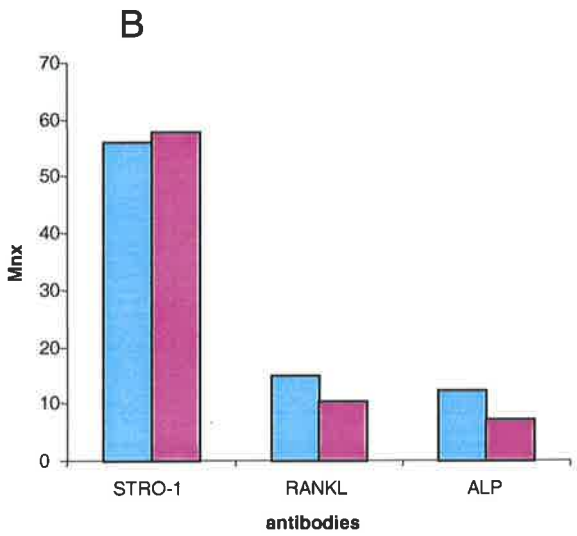
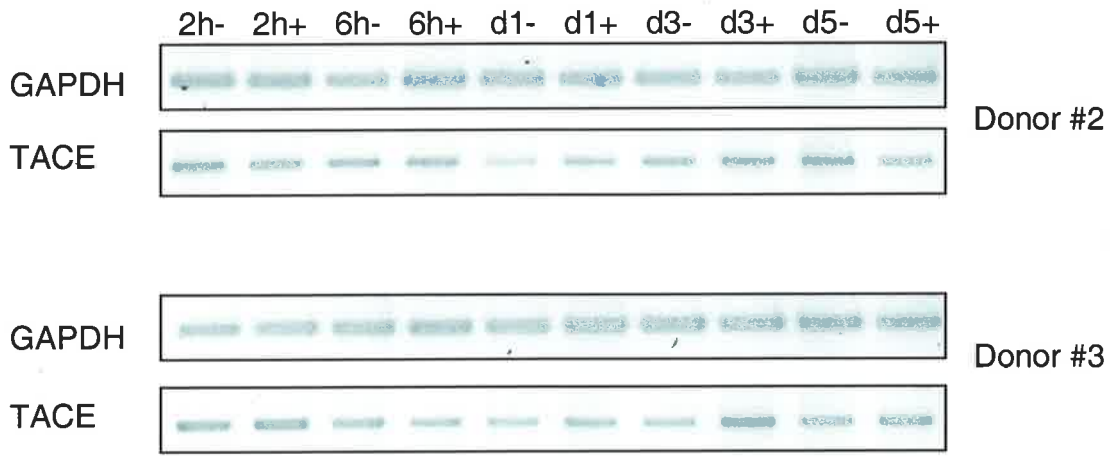

B
70
60
50
40
30
20
10
0
Mnx
STRO-1
RANKL
ALP
antibodies

Figure 4.21. Zoledronic acid upregulates TACE gene expression. As described in figure 4.12, the gene expression of TACE was examined by semi-quantitative RT-PCR. It can be seen that zoledronic acid upregulates TACE gene expression at d3 in donor #2 (A), and donor #3 (B).


2h-
2h+
6h-
6h+
d1-
d1+
d3-
d3+
d5-
d5+
GAPDH
TACE
Donor #2
GAPDH
TACE
Donor #3

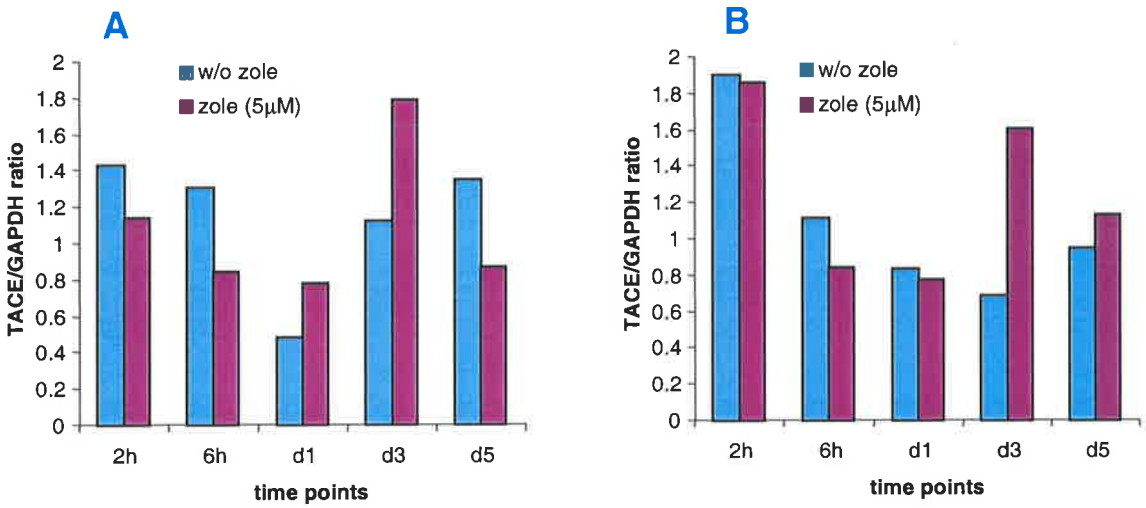
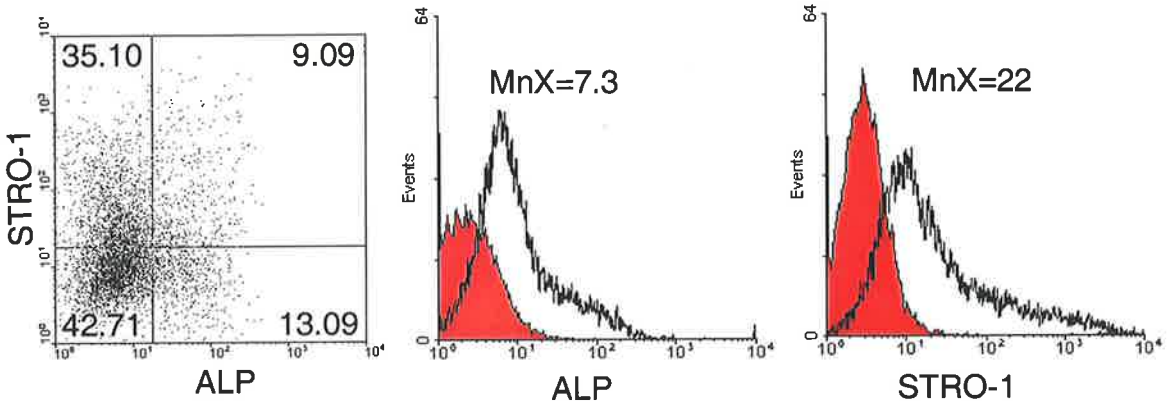
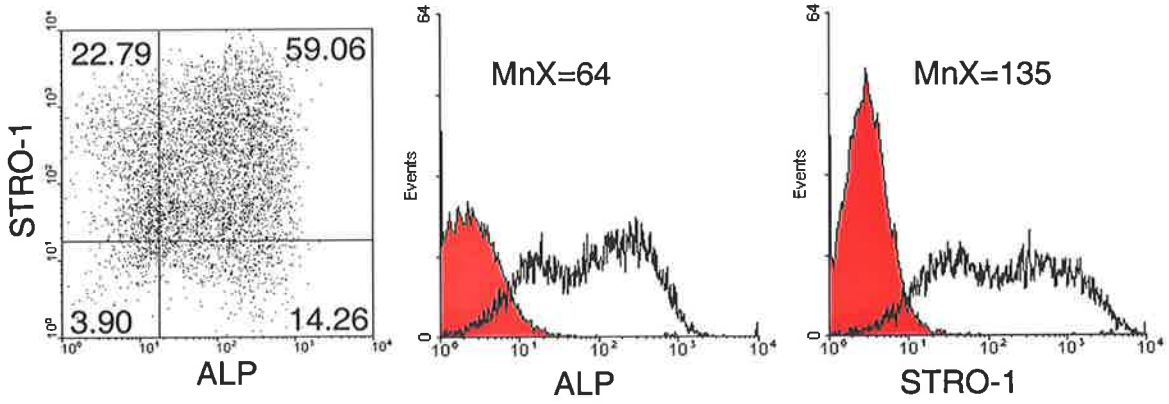

A
w/o zole
zole (5μM)
TACE/GAPDH ratio
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2h
6h
d1
d3
d5
time points
B
w/o zole
zole (5μM)
TACE/GAPDH ratio
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2h
6h
d1
d3
d5
time points

Figure 4.22. Recombinant human TNF-α and IL-1β increases the number of STRO-1^+/AP^+ osteoprogenitor-like cells. Osteoblast-like cells derived from donor #4 were cultured in the T75 flasks and treated with TNF-α and IL-1β at concentrations of 5 ng/ml and 10 ng/ml, respectively. The cells were harvested at d6 and stained with STRO-1 and ALP as described in figure 4.12. The results show that culture of OB-like cells in TNF-α and IL-1β increases the number of STRO-1^+/AP^+ osteoprogenitor cells.

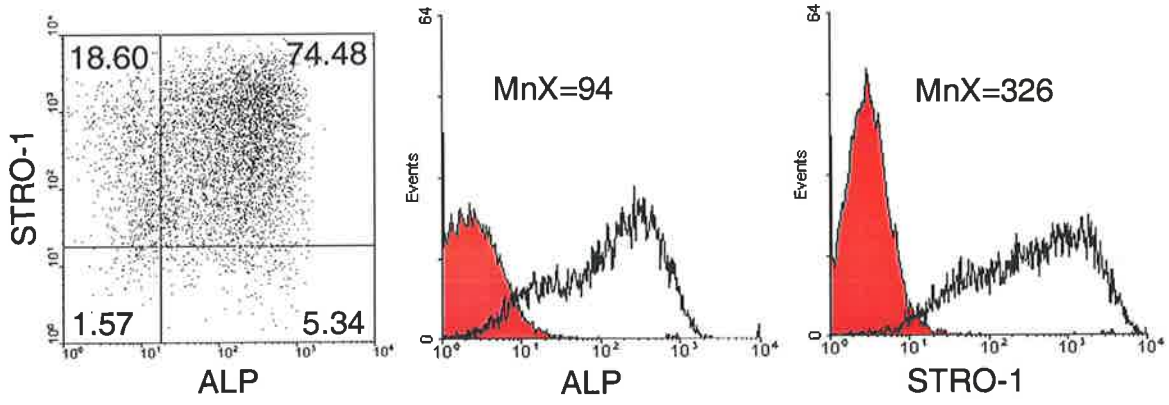
Neg.



IL-1β



TNF-α



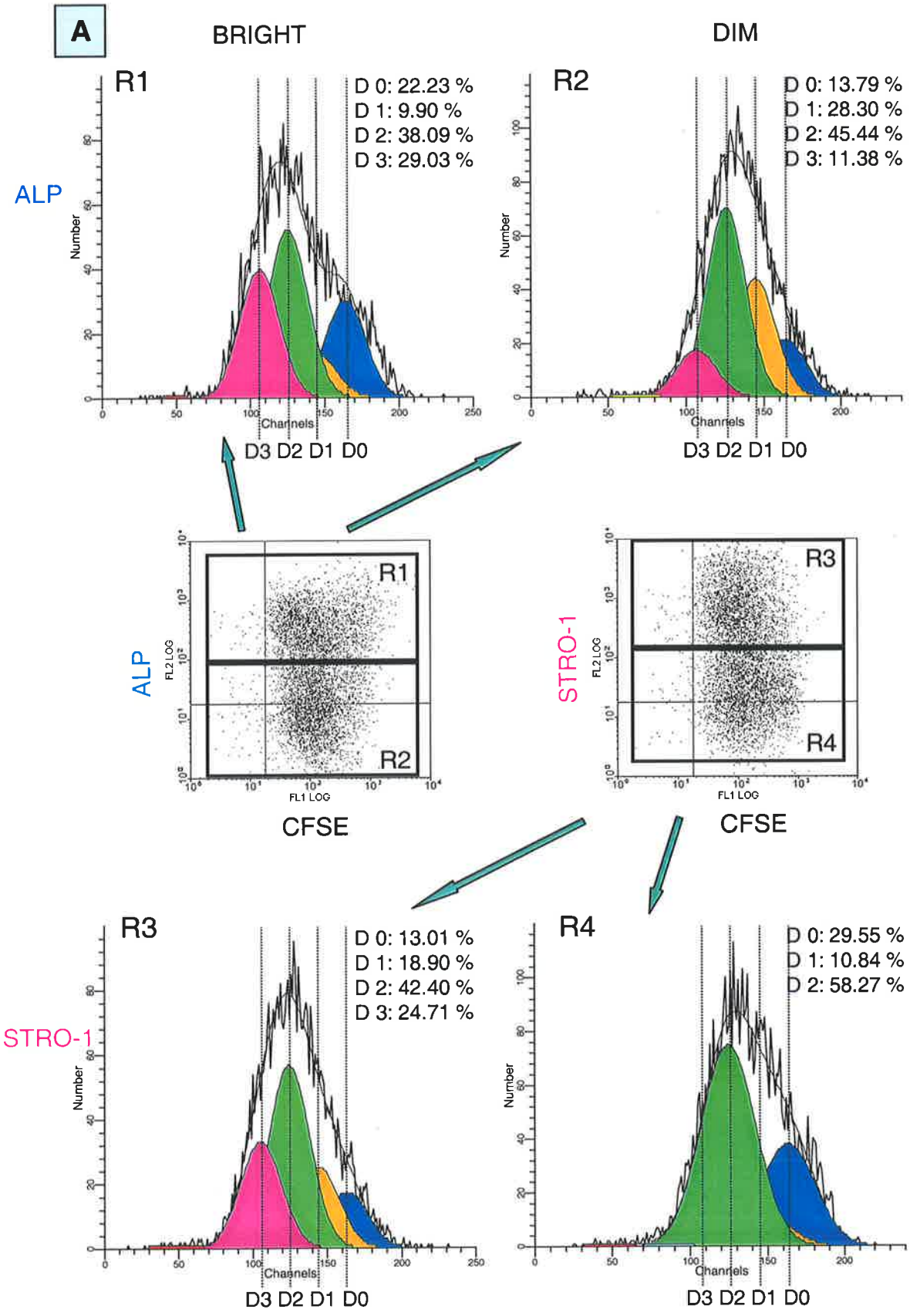
and IL-1β were found to significantly enhance the proliferative capacity of the OB-like cells in culture (figure 4.23).

4.2.9 Mineralisation Potential of Osteoblast-Like Cells is Enhanced by Zoledronic Acid Treatment

As demonstrated above, zoledronic acid treatment resulted in a decrease in the STRO-1^{bright} population of OB-like cells, which was due, in part, to the greater death rate of STRO-1^{bright} cells compared to the STRO-$1^{negative}$ population. It was also noted that zoledronic acid treatment resulted in the inhibition of cell proliferation, which was accompanied by a decrease in STRO-1 antigen expression. One possibility of the loss of STRO-1 expression was that the STRO-1^{bright} population differentiated into more mature OB-like cells. To investigate this possibility, the ability of the OB-like cells to form a mineralised bone matrix was assessed by culturing the cells in osteoinductive conditions in the presence of zoledronic acid at different concentrations (figure 4.24A). The level of mineral formation by the OB-like cells was dependent upon the concentrations of zoledronic acid used in cultures. Lower levels of mineral deposition were evident in the OB cultures treated with zoledronic acid relative to the negative controls before day 28. In contrast, at day 35 a significantly ($p<0.05$) higher level of mineral formation was observed in the OB treated with zoledronic acid at a concentration of 0.5 μM in donor #1 and donor #2. However, the equivalent levels of mineral deposition were noted at day 35 in donor #3 in the presence of 0.5 μM of zoledronic acid. The potential for mineral formation in OB-like cells was significantly reduced when the cells were cultured with zoledronic acid at concentrations higher than 5 μM in all three donors. As zoledronic acid treatment was shown to induce cell death of OB-like cells in a dose dependent manner (please refer to figure 4.1), we normalised the amount of calcium phosphate produced per viable cells. As shown in figure 4.24B, when cells were cultured in zoledronic acid at a concentration of 0.5 μM, this resulted in a significant increase in mineral formation in donor #1 and #2 ($p<0.001$).

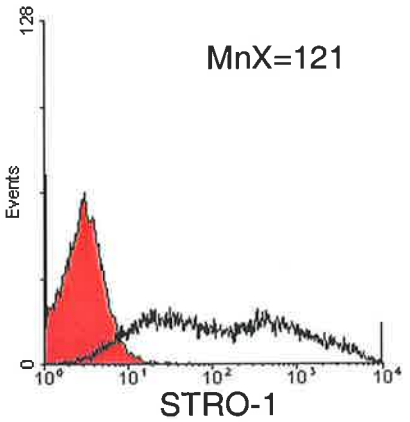
We noted that the high concentration of zoledronic acid decreased mineral formation of OB-like cells when treated with fresh drug at weekly intervals for the duration of the assay. To reduce the cell death caused by constant drug exposure, single dose zoledronic acid was administrated only at d0 of cultures and omitted in subsequent weekly feeding of the cultures. As seen in figure 4.25, zoledronic acid at very low concentrations (0.05 and 0.5 μM) did not enhance the mineral formation in the OB-like cell cultures. However, when zoledronic acid

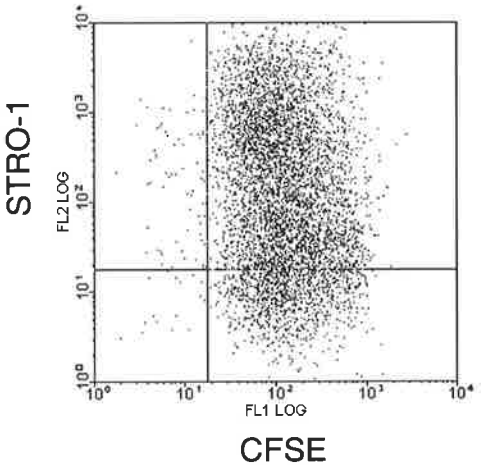
Figure 4.23. TNF-α and IL-1β increases the proliferative potential of OB-like cells. Osteoblast-like cells derived from donor #2 were labelled with CFSE as described in the methods. Cells were subsequently cultured in the presence of TNF-α (5 ng/ml) and IL-1β (10 ng/ml) for 3 and 5 days, stained with STRO-1 or ALP and analysed as above. Consistent with previous findings, STRO-1^{bright} and ALP ^{bright} cells had an enhanced proliferative potential than cells expressing negative/dim STRO-1 and ALP (A). Of note, TNF-α was more effective augmenting the growth of STRO-1^{bright} cells (B), whilst IL-1β was more able to augment the growth of ALP^{bright} cells (C). Both TNF-α and IL-1β were found to enhance cell division (D).


A
BRIGHT
DIM
R1
D 0: 22.23 %
D 1: 9.90 %
D 2: 38.09 %
D 3: 29.03 %
R2
D 0: 13.79 %
D 1: 28.30 %
D 2: 45.44 %
D 3: 11.38 %
ALP
Number
Channels
D3 D2 D1 D0
R1
R2
FL2 LOG
FL1 LOG
CFSE
STRO-1
R3
R4
R3
D 0: 13.01 %
D 1: 18.90 %
D 2: 42.40 %
D 3: 24.71 %
R4
D 0: 29.55 %
D 1: 10.84 %
D 2: 58.27 %

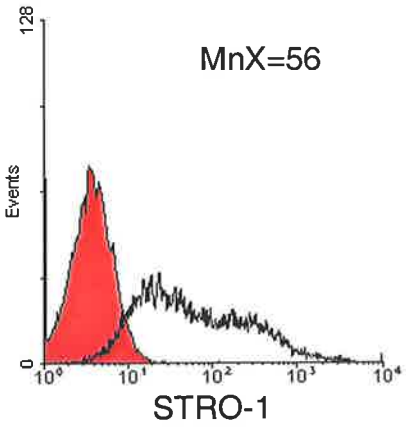
B

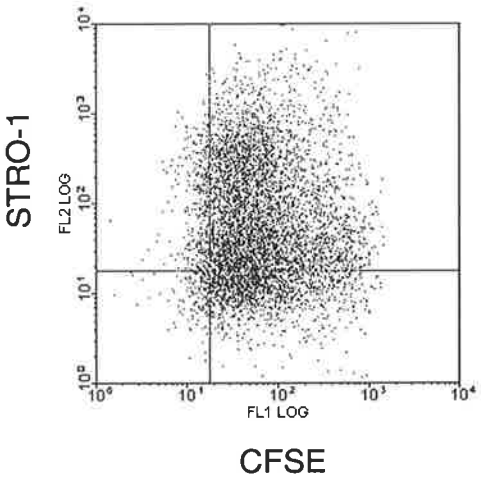
Neg.



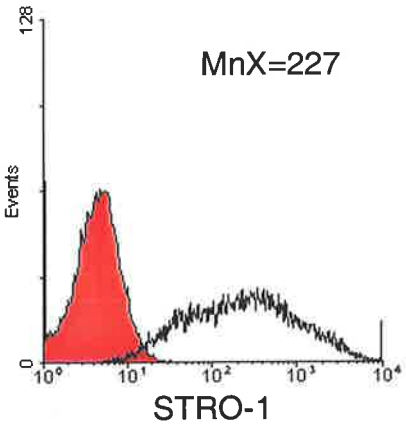


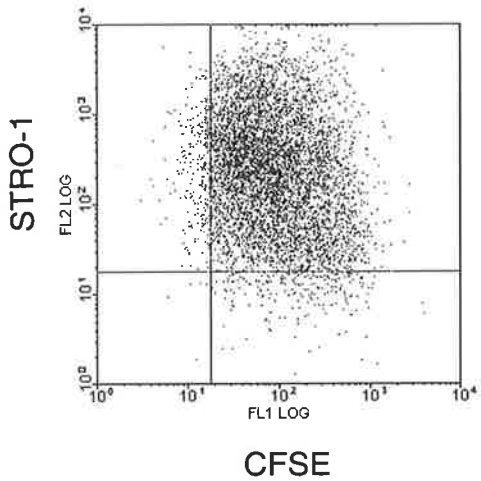
IL-1β





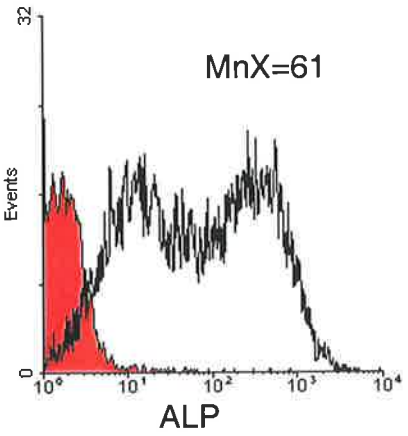
TNF-α

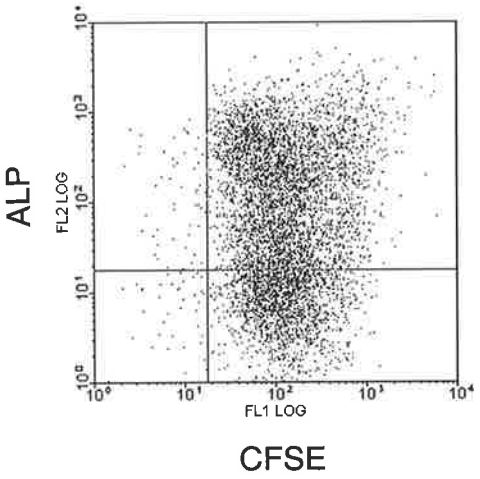




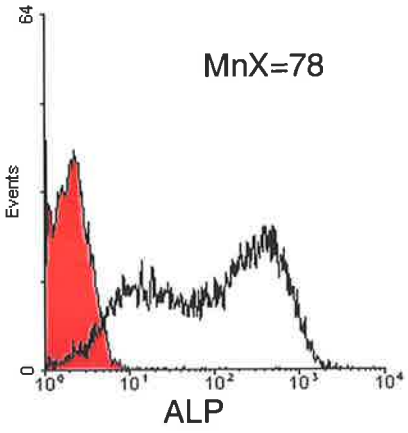
C

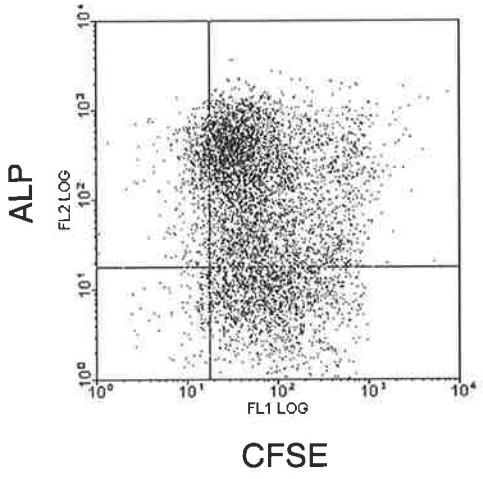
Neg.



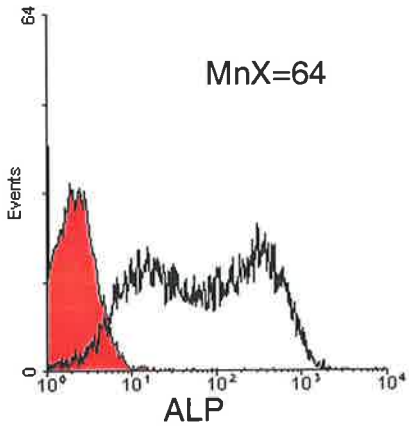


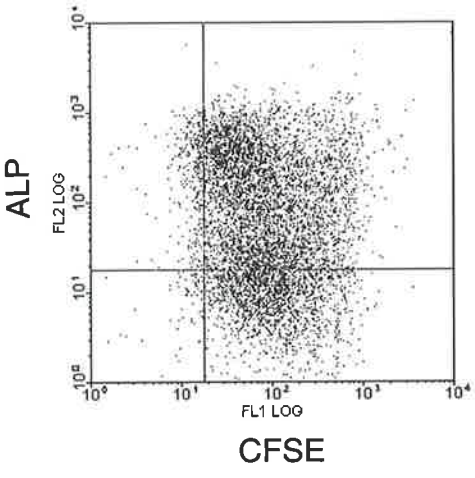
IL-1β





TNF-α





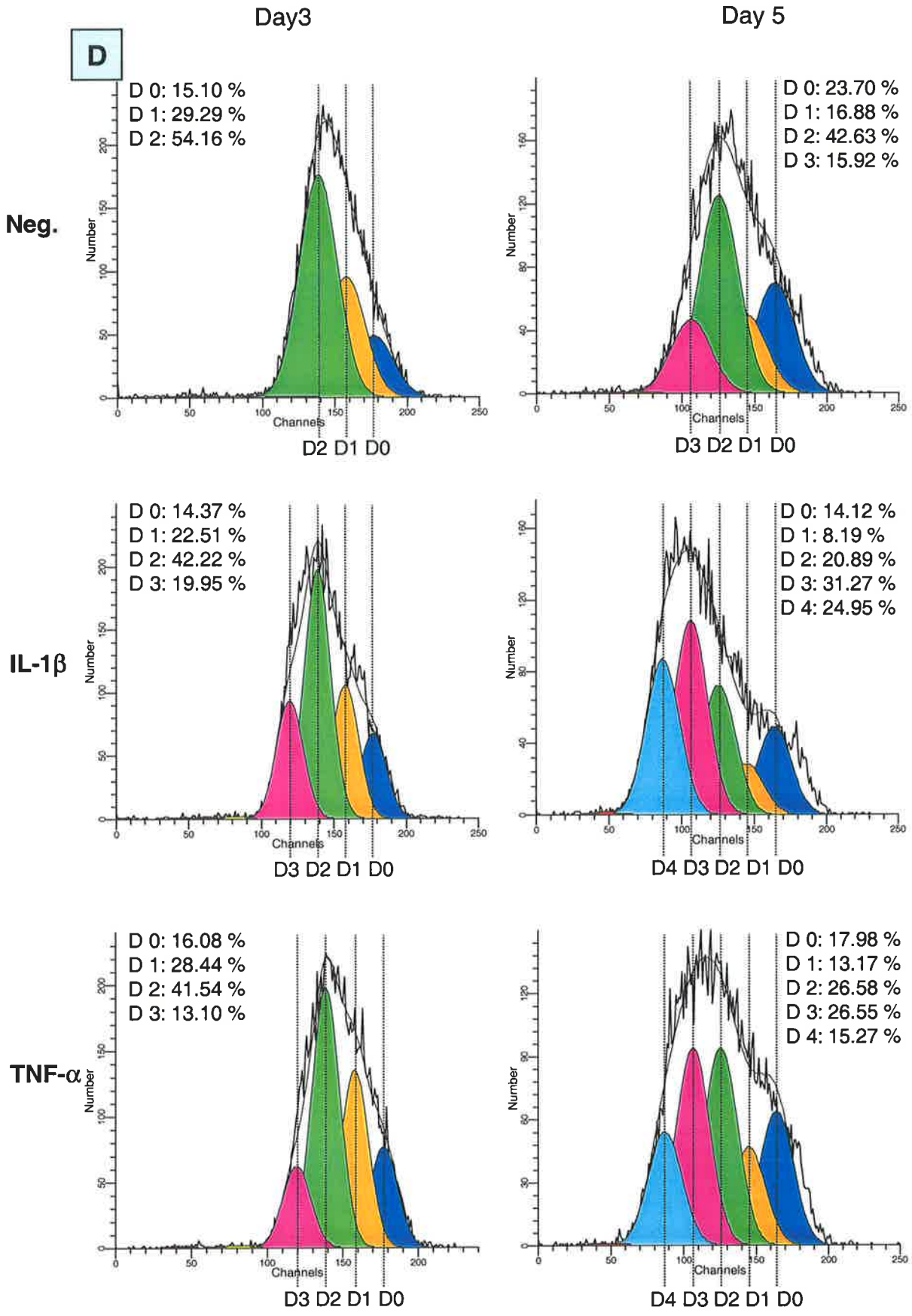
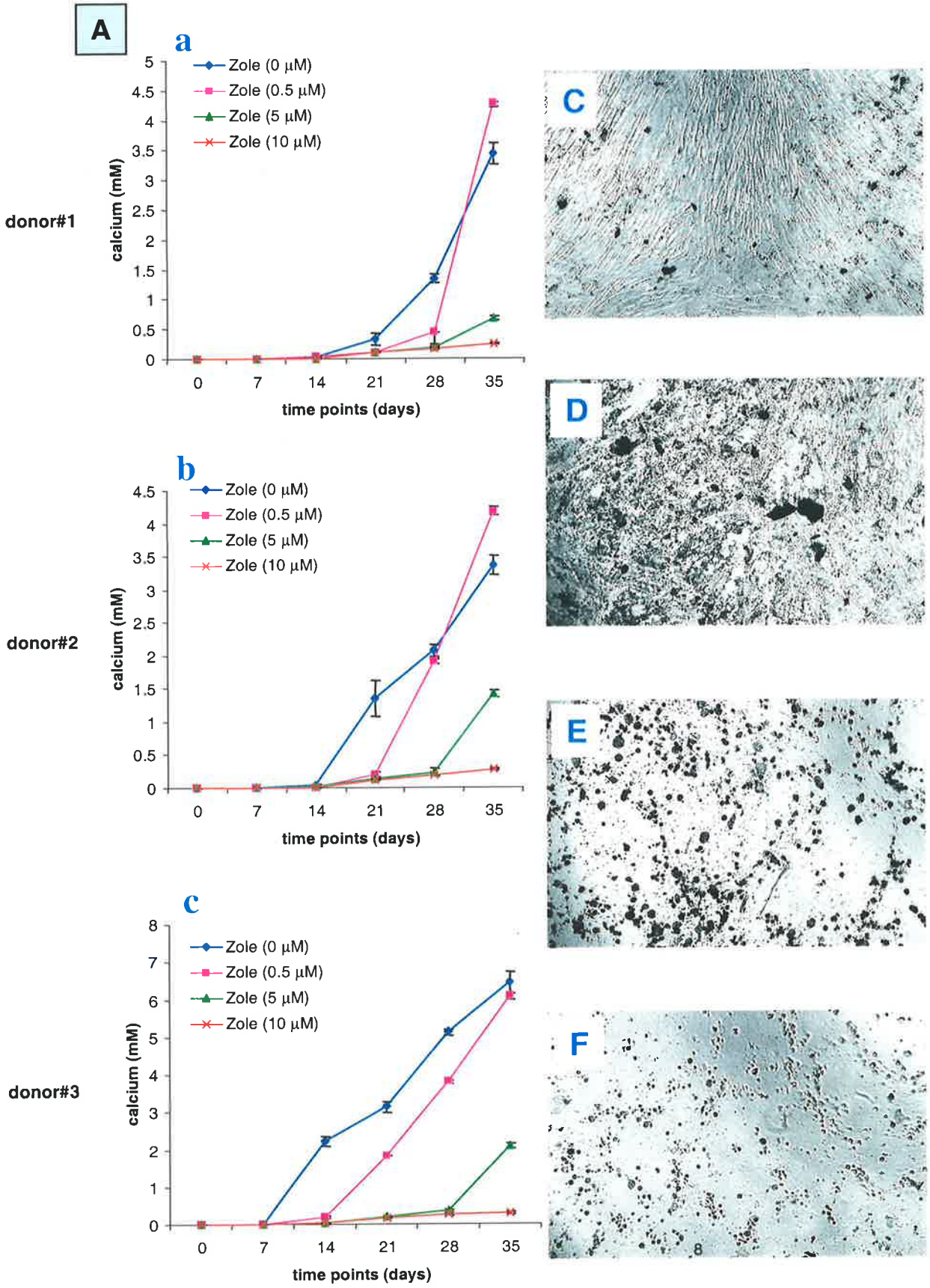
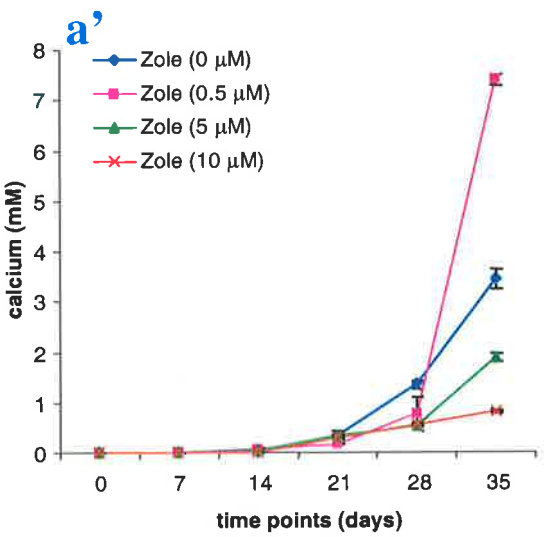

Day3
Day 5
D
Neg.
D 0: 15.10 %
D 1: 29.29 %
D 2: 54.16 %
D2 D1 D0
D 0: 23.70 %
D 1: 16.88 %
D 2: 42.63 %
D 3: 15.92 %
D3 D2 D1 D0
IL-1β
D 0: 14.37 %
D 1: 22.51 %
D 2: 42.22 %
D 3: 19.95 %
D3 D2 D1 D0
D 0: 14.12 %
D 1: 8.19 %
D 2: 20.89 %
D 3: 31.27 %
D 4: 24.95 %
D4 D3 D2 D1 D0
TNF-α
D 0: 16.08 %
D 1: 28.44 %
D 2: 41.54 %
D 3: 13.10 %
D3 D2 D1 D0
D 0: 17.98 %
D 1: 13.17 %
D 2: 26.58 %
D 3: 26.55 %
D 4: 15.27 %
D4 D3 D2 D1 D0
Number
Channels

Figure 4.24. Mineralisation potential of OB-like cells is enhanced by zoledronic acid treatment. Osteoblast-like cells derived from three different donors were seeded in 96-well plates at a cell density of 8,000 cells/well in triplicate, and cultured in osteoinductive conditions, as described in the methods. The cells were treated with zoledronic acid at different concentrations (from 0 to 50 μM) and cultures were "fed" weekly with fresh medium containing zoledronic acid at the noted concentrations. The release of free calcium from the matrix was achieved by treating the adherent cell layers under acidic condition as described in the methods. Samples then were reacted with o-cresol-phthalein-complexon and the colorimetric reaction was read at 570 nm. The absolute calcium concentration was determined according to a standard curve for calcium. Results were confirmed by measuring calcium levels using AAnalyst 300 Atomic Absorption Spectrometer. The results showed that lower levels of mineral deposition were evident in the OB-like cells treated with zoledronic acid compared to the negative control before day 28. In contrast, at day 35 a significantly ($p<0.05$, *t*-test) higher level of mineral formation was observed in the OB-like cells treated with zoledronic acid at a concentration of 0.5 μM in donor #1 and donor #2. However, the equivalent levels of mineral deposition were noted at day 35 in donor #3 in the presence of 0.5 μM zoledronic acid. The potential for mineral formation in OB-like cells was significantly reduced when cells were cultured with zoledronic acid at concentrations greater than 5 μM in all three donors. As zoledronic acid induced cell death of OB-like cells in a dose-dependent manner, calcium levels were normalised to viable cell number (panel B), and showed that zoledronic acid at a concentration of 0.5 μM significantly increased mineral formation in donor #1 and #2 ($p<0.001$, *t*-test). The morphology of cells in culture was assessed at d35, photographed, and represented as C (negative control), D (0.5 μM of zole), E (5 μM of zole) and F (10 μM of zole).

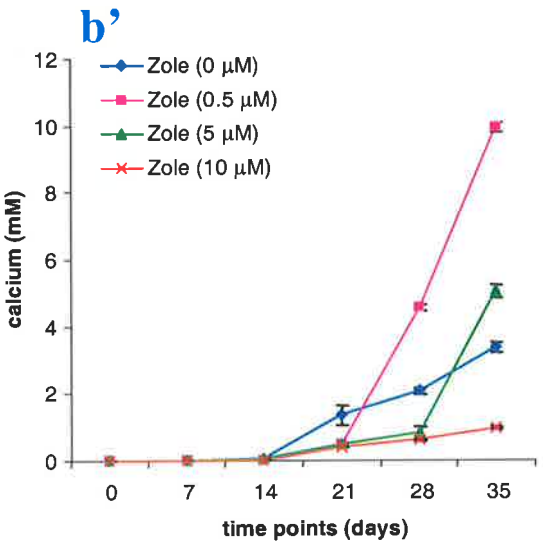

A
a
Zole (0 μM)
Zole (0.5 μM)
Zole (5 μM)
Zole (10 μM)
calcium (mM)
time points (days)
donor#1
b
donor#2
c
donor#3
C
D
E
F

B

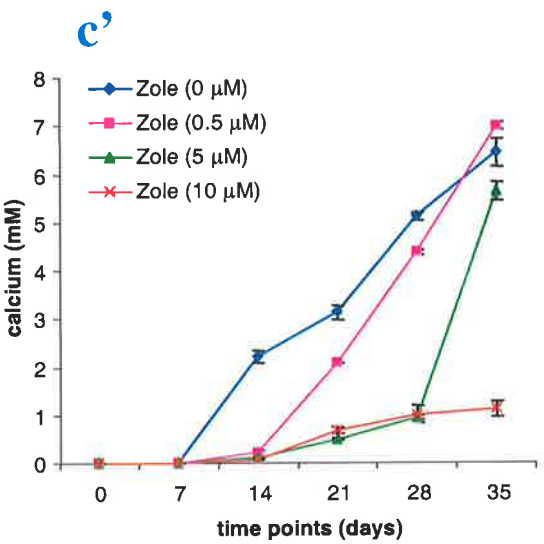
donor#1



donor#2

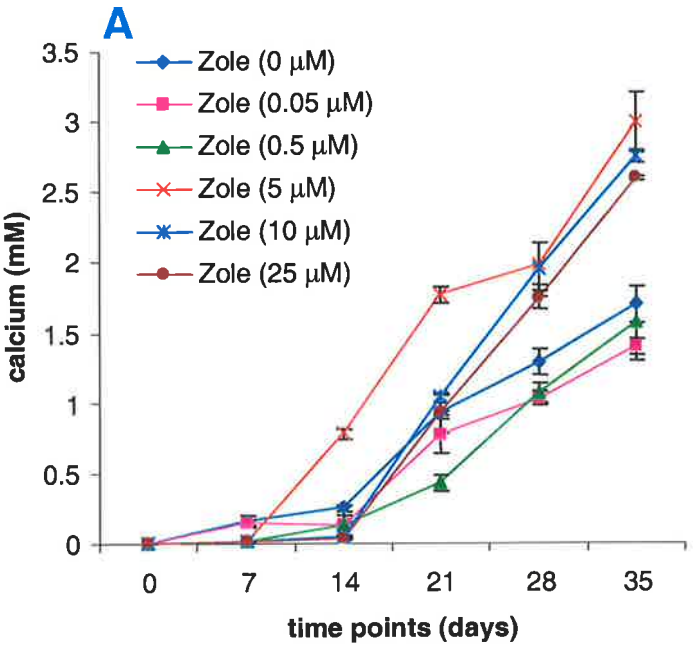


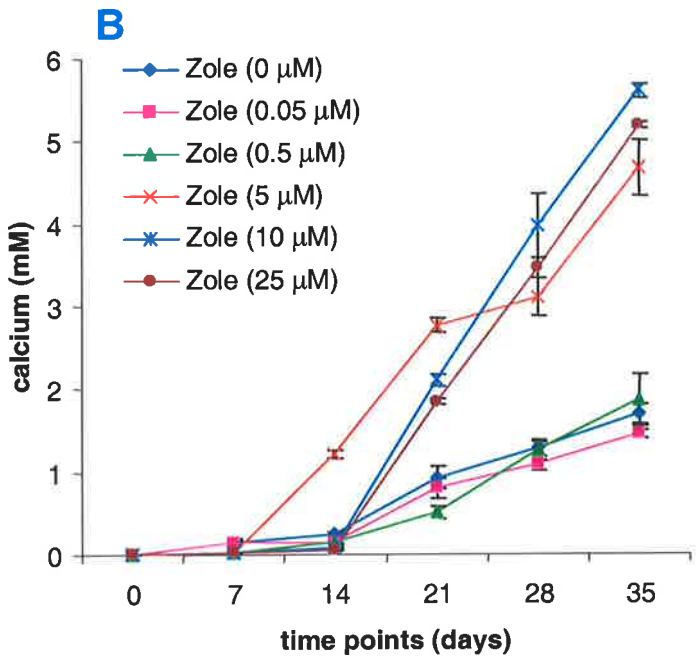
donor#3



was used at concentrations between 5 and 25 μM, this resulted in a significant increase in mineral formation ($p<0.05$). When the data were normalised to viable cells, the difference was even more significant ($p<0.005$) and the trend was the same in the all three donors. It also shown that a concentration of 10 μM of zoledronic acid is the most suitable concentration to enhance mineral formation *in vitro* ($p<0.05$).

Figure 4.25. A single high-dose treatment of zoledronic acid enhances mineral formation of OB-like cells. Osteoblast-like cells were established as described in figure 4.24. In contrast, cells were only treated with zoledronic acid at d0 at different concentrations. The results showed that zoledronic acid at very low concentrations (0.05 and 0.5 μM) did not affect the mineral formation of OB-like cells. Zoledronic acid at concentrations ranging between 5 and 25 μM significantly increased mineral formation ($p<0.05$, *t*-test). When the data were normalised to viable cell number, the difference was more significant ($p<0.005$, *t*-test) and the trend was the same in the all three donors. Zoledronic acid at a concentration of 10 μM was found to be the optimal concentration in augmenting mineral formation ($p<0.05$, *t*-test). The typical data of donor #3 before normalisation (A) and after normalisation (B) are presented.


A
Zole (0 μM)
Zole (0.05 μM)
Zole (0.5 μM)
Zole (5 μM)
Zole (10 μM)
Zole (25 μM)
calcium (mM)
3.5
3
2.5
2
1.5
1
0.5
0
0
7
14
21
28
35
time points (days)


B
Zole (0 μM)
Zole (0.05 μM)
Zole (0.5 μM)
Zole (5 μM)
Zole (10 μM)
Zole (25 μM)
calcium (mM)
6
5
4
3
2
1
0
0
7
14
21
28
35
time points (days)

4.3 DISCUSSION

To date, zoledronic acid represents the most potent BP in suppressing bone resorption and normalising serum calcium in patients with metastatic bone disease (Berenson *et al.*, 2001b; Body *et al.*, 1999; Major *et al.*, 2001). In animal model studies, zoledronic acid was found not only to mediate a dose-dependent suppression of cancellous bone turnover and resorption, but also augmented cancellous bone formation (Pataki *et al.*, 1997). Moreover, zoledronic acid was recently shown to enhance bone formation on a calcium sulphate bone graft substitutes in a rabbit model of tibial fracture repair (Sharpe *et al.*, 2002).

The zoledronic acid-mediated increase in bone formation seen in animals and humans could be attributed to the establishment of a positive bone balance between osteoblastic bone formation and osteoclastic bone resorption. This is thought to occur by (1) the diminution of bone resorption by the inhibition of OC recruitment, proliferation, differentiation and maturation, (2) the newly formed bone would be less likely to be remodelled and therefore have more time to complete mineralisation, and (3) if the decrease in resorption depth at individual remodelling sites is not coupled by a decrease in formation, the local bone balance will be positive. Despite this, the underlying molecular mechanism of this enhanced bone formation at the level of the OB, is not clearly understood. As such, this study was designed to explore the effect of zoledronic acid on cultures of human OB-like cells at both a molecular and cellular level.

Our *in vitro* studies show that zoledronic acid caused a dose and time-dependent decrease in human OB-like cells (refer to figure 4.1/4.2). This observation is consistent with the work of Reinholz and colleagues who demonstrated that zoledronic acid induced cell death and cytostasis in the immortalised human fetal OB (hFOB) cells (Reinholz *et al.*, 2000). Although our studies and studies by others (Reinholz *et al.*, 2000) show that BPs induce cytostasis and cell death, other studies have shown that BPs can inhibit apoptosis of murine osteocytes and OB mediated by etoposide, TNF-α or glucocorticoid (Plotkin *et al.*, 1999). Meaningful comparisons between these data are difficult, as the experimental systems used here are dramatically different and as such these opposing effects may be, in part, due to differences in the cell types, duration of treatment, the BP analogues, and the concentrations of BPs used in these experiments.

The zoledronic acid-induced reduction in OB-like cell number was due to the combination of cytostasis (inhibition of proliferation) and cell death. Staining of nuclei with the nuclear fluorescent stain DAPI, demonstrated morphological changes characteristic of apoptosis including chromatin condensation, nuclear fragmentation and formation of dense, round apoptotic bodies in a small number zoledronic acid treated OB-like cells (refer to figure 4.3). Despite this, the reduction in cell number was mainly due to an inhibition of cell proliferation (refer to figure 4.11). Assessment of the induction of intranucleosomal DNA fragmentation as assessed by gel electrophoresis (refer to figure 4.4), supported this observation. In addition, the induction of apoptosis in OB-like cells by zoledronic acid was assessed by monitoring caspase-3 activity. Although the caspase activity was relatively low compared to the other cell lines, zoledronic acid did significantly enhance caspase 3 activation (refer to figure 4.5). Therefore, it is conceivable that the cell death in all OB tested may be a late event following zoledronic acid treatment. Although it is established that caspase-regulated apoptosis is at least one entity of programmed cell death (PCD), it has also been reported that non-apoptotic PCD with necrotic-like cell appearance can be regulated independent of caspases by c-Myc, dexamethasone and ceramide (Mochizuki *et al.*, 2002). Therefore, it is likely that zoledronic acid induces a combination of apoptosis, and non-apoptotic PCD in the OB-like cells *in vitro*.

To date, numerous studies have failed to determine how BPs reach the OB *in vivo*. Although BPs may come directly from the general circulation after their administration, they are also released from bone into the microenvironment of OB, either by passive diffusion or following resorption. In addition, under pharmacological conditions, the concentration of BPs encountered by OB *in vivo* is unknown. As such, it is difficult to design *in vitro* experiments that can directly correlate to physiological conditions. Using a mouse model, Sato *et al* (1991) reported that half of the bone forming surface had a moderate deposition of BPs, with one fifth the density compared to the BP deposition on the bone resorption surface (Sato *et al.*, 1991). The high concentration of BPs in the space beneath resorbing OC, *in vivo*, is due to OC-induced acidification of the bone surface. The high local concentration declines immediately when the bone resorption is completed. Therefore, it is reasonable to assume that the concentration of BPs to which OB are exposed would be lower than that encountered by OC. In time, the BP containing bone will be "buried" under newly formed osteoid (Sato *et al.*, 1991), supporting the notion that osteoblastic exposure to BPs will be limited. As such,

most of the studies detailed here, made use of a concentration of 5 μM zoledronic acid, as this was shown to mediate a measurable effect on the OB phenotype.

Osteoblasts produce most of the bone matrix constituents and enhance new bone formation. As we have shown before, zoledronic acid induced OB-like cell death in a dose-dependent manner. Therefore, this decrease in OB cell number would conceivably result in a loss of bone mass. On the other hand, the increase in bone mass and strength in an animal model (Balena *et al.*, 1993) and human systems (Rossini *et al.*, 1994; Storm *et al.*, 1993) led to the assumption that BPs may enhance the maturity of OB, thereby increasing the bone density.

As described previously (refer to section 4.2.2), OB can be separated into four subpopulations based on their stage of cellular differentiation. Using dual-colour immunofluorescence and flow cytometric analysis, we examined the effect of zoledronic acid on the differentiation of OB-like cells. Treatment of OB-like cells with 5 μM zoledronic acid resulted in an increase in the number of STRO-1$^-$/AP$^-$ cells, with a concomitant decrease in the number of STRO-1$^+$/AP$^-$ cells in all cultures of OB-like cells tested (refer to figure 4.6). This suggests that zoledronic acid may augment the maturation of OB-like cells by either selectively inhibiting proliferation and inducing cell death in the OB precursor population and/or by mediating the differentiation of the preosteoblast population. Although the representation of each of the four subpopulations was found to be highly donor dependent, this could not be attributed to the donor age or gender of the individual, from whom the cells were derived. As described in the materials and methods, OB cultures from two donors were from individuals diagnosed with osteoarthritis, whilst the remainder were from healthy, young volunteers. Although there were differences in the response to zoledronic acid treatment, these differences were restricted to the magnitude of the response but not in its direction. These studies were confirmed by immunofluorescence and flow cytometric analysis, which showed that zoledronic acid decreased both membrane and total cell ALP protein expression (refer to figure 4.19). In addition, the dramatic decrease in cell surface STRO-1 expression following zoledronic acid treatment may be due to both a decrease in the number of cells that express STRO-1 and/or a decrease in the level of expression of this protein at the cell membrane (refer to figure 4.17). Interestingly, zoledronic acid did not alter the total STRO-1 protein expression in the cytoplasm, indicating that changes in membrane protein expression may be due to enzymatic cleavage of STRO-1 from the cell surface. In support of this notion, examination of the expression of TACE (ADAM 17), a membrane

metalloprotease, revealed that the loss of STRO-1 protein from the cell membrane was preceeded by an elevation in TACE expression (refer to figure 4.21). These data suggest that STRO-1 may represent a membrane protein belonging to a small cohort of proteins cleaved by this enzyme, which include TNF R1/II, IL-1RII, TNF-α, TGFα, amyloid-beta precursor protein (APP) and RANKL.

In order to investigate whether the apparent selective reduction in the STRO-1 population seen following zoledronic acid treatment, was due to inherent difference in the sensitivity of the STRO-1^{bright} OB-like cells to this agent, dual colour immumofluorescence and flow cytometric analysis was performed examining the expression of Ki-67 in combination with STRO-1. The expression of the human Ki-67 protein is strictly associated with cell proliferation, and during interphase the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. Ki-67 can be used to analyse those cells which are in the so-called growth fraction, as Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2 and mitosis), but is absent from resting cells (G_0, or so-called "quiescent" cells) (Scholzen and Gerdes, 2000). Our data revealed that the majority of the OB-like cells were actively dividing. When compared with the control, zoledronic acid appeared to inhibit cell division, and moreover resulted in the emergence of a population of cells with low forward and side scatter in a time-dependent manner. These cells were found to express STRO-1 antigen at high levels and lacked measurable Ki-67 expression (refer to figure 4.7). This suggested that cells that express STRO-1 at high levels, may be more sensitive to zoledronic acid and the Ki-67 negative cells in this case may represent quiescent cells or cells undergoing apoptosis. This was further examined using three-colour flow cytometric analysis.

The human OB-like cells at different stages of the cell cycle were analysed using the nuclear stain, 7-AAD. In combination with STRO-1 and ALP, this enabled us to examine the cell cycle status of the cells at different stages of osteoblastic maturation. One limitation encountered in these studies, was the inability to resolve those cells in G_0 or G_1, as these cells contain the same nuclear content. These studies revealed that zoledronic acid reduced the number of cells in S and G_2/M phase, and the stage of cell cycle was not related to the extent of ALP expression by the OB-like cells. In contrast, the majority of cells expressing STRO-1 antigen at high levels were distributed in the S and G_2/M phases, suggesting that zoledronic acid preferentially induced quiescence and cell death in these populations (refer to figure

4.8). These data therefore suggest that STRO-1^{bright} cells are more susceptible to zoledroic acid induced cell death due to their enhanced proliferative potential.

To further examine the preferential induction of cell death in the STRO-1^{bright} population, the STRO-1^{bright} and STRO-1^{negative} subpopulations of OB-like cells were sorted by a FACStar^{PLUS} cell sorter and the proliferative potential assessed using WST-1. As anticipated, the STRO-1^{bright} cells were more sensitive to zoledronic acid than STRO-1^{negative} cells (refer to figure 4.9).

To further examine if the growth rate of cells contributed to their sensitivity to zoledronic acid, OB-like cells were labelled with CFSE to track cell division over different periods of culture. To our knowledge, we have shown here for the first time that STRO-1^{bright} cells possess a greater proliferative potential than the STRO-1$^{negative/dim}$ subpopulation (refer to figure 4.10). These data confirmed our previous findings that the most STRO-1^{bright} cells had a greater proliferative potential and were mainly distributed in the S and G_2/M phase of the cell cycle. Furthermore, zoledronic acid inhibited cell division in a dose-dependent manner, accompanied by a dramatic decrease in STRO-1 expression on the cell surface (refer to figure 4.11). This reduction in STRO-1 expression may be due to the induction of quiescence. In support of this notion, when the cell proliferation was inhibited with the spindle poison, colchicine, the expression of STRO-1 was also decreased. This suggests that the cells may lose their STRO-1 surface expression when cell division is inhibited. Our results suggest that cell division was totally inhibited by zoledronic acid a the concentration of 5 μM at d3 and d5. Surprisingly, at d7, approximately 12% cells of these cells underwent their first division, which was accompanied by the resumption of STRO-1 expression. This suggests that OB-like cells may start to recover from the effect of zoledronic acid by either decreased drug exposure due to drug consumption/breakdown or by up regulating the expression of molecules, which are essential for cell proliferation and maturation.

For this reason, gene expression of both osteoclastogenesis and osteoblastogenesis-related molecules were investigated in our studies (refer to figure 4.12). Our data revealed that the expression of TNF-α and IL-1β was decreased in the negative control in a time-dependent manner. Interestingly, these genes were significantly upregulated in OB-like cells following treatment with 5 μM zoledronic acid, particularly in donor #3, which was the most sensitive donor. This increased expression of TNF-α and IL-1β may be due to the zoledronic acid-

induced decrease in cell density due to the induction of cytostasis and cell death. As described in the chapter 3, TNF-α is a multifunctional cytokine which can mediate apoptotic cell death or induce cell proliferation depending on the cell cycle status (Baxter *et al.*, 1999). Furthermore, TNF-α plays an essential and dual role in bone remodelling, both stimulating the proliferation of OB or inducing OB apoptosis (Hill *et al.*, 1997). The effects of TNF-α on osteoclastic resorption of mineralised matrix are dependent on the stage of OC development and the concentrations applied (van der Pluijm *et al.*, 1991). IL-1β is a key proinflammatory mediator implicated in the pathogenesis of various diseases associated with bone loss, such as osteoporosis, tumour-associated osteolysis, and rheumatoid arthritis. IL-1β, a survial factor for OC, acts through the release of soluble factors from OB and other marrow cells. The biological effect of IL-1β on bone, depends on complex interactions with many factors, including IL-1 receptor antagonist secreted by OB, and IL-1 receptors expressed by OC (Sunyer *et al.*, 1999).

Although TNF-α and IL-1β have been shown to stimulate OC-like formation in human marrow cultures (Lader and Flanagan, 1998), these cytokines are not capable of substituting M-CSF in stimulating human OC formation (Fujikawa *et al.*, 2001). Hence, it is more likely that these cytokines act in the marrow microenvironment to enhance M-CSF-stimulated OC formation. Two recent reports demonstrating that TNF-α was sufficient to stimulate cultures of bone marrow macrophages to differentiate into OC independent of RANKL-RANK interaction, may be confounded by contamination with minute concentrations of RANKL (Azuma *et al.*, 2000; Kobayashi *et al.*, 2000). In support of this, in stromal-free cultures of marrow derived macrophage precursors in the presence of saturating concentration of OPG, TNF-α by itself, failed to induce differentiation of OC (Lam *et al.*, 2000). Although previous studies have established the importance of OC activation by cytokines including IL-1β, TNF-α and IL-6, the role of these cytokines on OB development of bone loss requires further clarification.

In our studies, the roles of TNF-α and IL-1β induced by zoledronic acid are more likely to rescue OB and compensate for the decrease in cell number. Although some reports suggest that TNF-α directly mediates the apoptosis of murine OB (Kitajima *et al.*, 1996a; Kitajima *et al.*, 1996b), we could not detect any cell death in human OB-like cells after treatment with IL-1β and TNF-α, consistent with the findings of others (Kwon *et al.*, 1998; Tsuboi *et al.*,

1999). Furthermore, we found IL-1β and TNF-α at a concentration of 10 ng/ml and 5 ng/ml, respectively, gave rise to an increase in the STRO-1^+/ALP^+ subpopulation (refer to figure 4.22), which we have shown previously to possess greater proliferative rate than other subpopulations (refer to figure 4.23). This supported our hypothesis that the upregulation of TNF-α and IL-1β, induced by zoledronic acid represents a mechanism, by which OB-like cells may overcome the growth suppression effects of zoledronic acid.

It is well known that OB control the recruitment and activity of OC under physiological and pathological conditions. The inhibitory effect of BPs on osteoclastic bone resorption may be mediated, at least in part, by the regulation of the production of cytokines synthesised by OB. It was reported that BPs inhibit OC-stimulating factors such as IL-6 (Giuliani *et al.*, 1998b) and induce OC-inhibiting factors (Nishikawa *et al.*, 1996; Vitte *et al.*, 1996) released by the OB. Under physiological or pathological conditions, IL-6 is released mainly in response to stimulating factors, including IL-1β and TNF-α. Although there was an apparent increase in IL-1β and TNF-α gene expression observed in our studies, there was no significant change in IL-6 and M-CSF gene expression. It is important to consider that the studies reported here only examined expression at the genetic level. Future studies examing IL-6 and M-CSF protein expression are required and should provide us with a more complete understanding about the regulation of IL-6 and M-CSF expression by zoledronic acid.

The RANKL/RANK/OPG system is considered to mediate the final common step in OC formation. RANKL promotes the differentiation of OC precursor cells to OC through the RANK receptor expressed on the OC precursor cells. OPG acts as a decoy receptor that negatively regulates the signalling between RANKL and RANK. It was found that IL-1β and TNF-α, but not IL-6, could stimulate RANKL gene expression in human osteoblastic cells (Hofbauer *et al.*, 1999). Although zoledronic acid treatment led to an upregulation of IL-1β and TNF-α gene expression in our studies, we did not find a significant change in RANKL and OPG expression at the transcriptional level (refer to figure 4.13). In contrast, at the protein level, our data suggest that zoledronic acid treatment leads to a significant decrease in the membrane expression of RANKL on human OB-like cells, without a commensurate decrease in total protein expression (refer to figure 4.18). This is consistent with the findings of Mackie and colleagues who demonstrated that RANKL mRNA was down-regulated by the BP, pamidronate, in the rat osteosarcoma cell line UMR 106-01 (Mackie *et al.*, 2001). In

agreement with their findings, we also did not detect any alteration in OPG gene expression. Thus, we conclude that BPs also can exert an anti-resorptive effect *via* a decreased RANKL/OPG ratio.

As discussed previously, RANKL is a type II membrane-anchored polypeptide, which is released from the plasma membrane by tumour necrosis factor alpha converting enzyme (TACE) (Lum *et al.*, 1999). Soluble and truncated RANKL are important components in pathological bone loss (Quinn *et al.*, 1998; Udagawa *et al.*, 1999). Although the decreased RANKL expression in OB-like cell surface may due to the decreased sythesis of this protein, it is also possible that zoledronic acid treatment leads to the upregulation of TACE expression and activity leading to the loss of membrane RANKL expression. Examination of the expression of TACE (ADAM 17) revealed that the loss of RANKL protein from the membrane was preceeded by an elevation of TACE expression on d1, d3 following zoledronic acid treatment (refer to figure 4.21). Similary, expression of STRO-1 on the membrane was also significantly down regulated following zoledronic acid treatment (refer to figure 4.17). This was not associated with an alteration in total STRO-1 protein expression in the cytoplasm, indicating that like RANKL, changes in STRO-1 membrane expression may also be due to enzymatic cleavage. Interestingly, the resumption of STRO-1 expression at d5 and d7 following zoledronic acid treatment is correlated with a decrease in TACE expression to baseline levels of TACE expression at d5.

The discovery of the DNA-binding protein CBFA1 has provided a marker of early osteogenic commitment (Ducy *et al.*, 1997; Komori *et al.*, 1997; Rodan and Harada, 1997). CBFA1 belongs to the runt-domain gene family and has been specifically associated with osteogenesis in rodents. The precise function of CBFA1 is unknown, but it is thought to regulate the OB-specific genes. Although our results indicated that BSP and OCN gene expression was upregulated by zoledronic acid in the OB-like cells derived from donor #3, there was no alteration in CBFA-1 expression at the transcript level (refer to figure 4.14). At the protein level, zoledronic acid was found to upregulate the expression of OCN at d1 and d3 at both the transcript and protein level (data not shown).

As discussed above, OB can be separated into four subpopulations based on the stage of differentiation. Previous studies (Gronthos *et al.*, 1999) have shown that OB at diffeent stages of development exhibit differential gene expression. Our studies show that zoledronic acid

dramatically alters the expression of a number of genes, especially in the zoledronic acid sensitive donors. It was therefore thought that these large changes in gene expression may be due to changes in the relative proportions of these subpopulations following zoledronic acid treatment. To exclude this possibility, the STRO-1^{bright} and STRO-1^{negative} cells were sorted by FACStar^{PLUS} cell sorter and their incident gene expression profile was determined. Interestingly, the gene expressions in these two subpopulations are similar (refer to figure 4.15). However, the difference between the two populations emerged when the cells were cultured in the media for three days in the presence or absence of zoledronic acid (refer to figure 4.16). As expected, the STRO-1^{negative} subpopulation was found to express more OCN and BSP than the STRO-1^{bright} subpopulation, consistent with their stage of differentiation. Zoledronic acid increased OCN and BSP expression in STRO-1^{bright} cells indicating that zoledronic acid may "drive" STRO-1^{bright} cells to differentiate into more mature OB. Furthermore, STRO-1^{bright} cells were found to express more IL-1β and TNF-α than the STRO-1^{negative} subpopulation, suggesting that the upregulation of IL-1β and TNF-α in the whole population could not be attributed to a decrease in the proportion of STRO-1^{bright} cells. Of note, the middle population which display intermediate levels of STRO-1, express the highest level of COX-2, whilst the STRO-1^{bright} cells express the least amount of COX-2. Zoledronic acid only increased COX-2 expression in the STRO-1^{bright} subpopulation, which may facilitate the proliferation of STRO-1^{bright} cells. As expected, there was no significant difference in IL-6, M-CSF, OPG and CBFA-1 expression among the different subpopulations. In terms of RANKL, zoledronic acid increased RANKL expression in the STRO-1^{bright} subpopulation whilst it was decreased in the STRO-1^{negaive} subpopulation. It should be noted that the results presented herein were generated from cells isolated by FACS and cultured separately in the absence of cells at other stages of differentiation, which may significantly alter the pattern of gene expression seen in a mixed culture.

A previous report suggests that zoledronic acid can enhance the rate of mineralisation of viable immortalised human fetal OB (hFOB) cells (Reinholz *et al.*, 2000). Consistent with this finding, our data suggest that zoledronic acid at a concentration of 0.5 μM increases mineral formation in OB-like cells at day 35. However, the potential for mineral formation in OB-like cells was decreased when the cells were cultured with zoledronic acid at concentrations exceeding 5 μM. It should be noted that in these experiments, the cells were subjected to fresh zoledronic acid every week for the entire 35 day period. As we discussed previously, zoledronic acid induces cell death in a dose dependent manner, and therefore the

cell number in the zoledronic acid treated groups was substantially lower than that in the untreated control group. This suggests that the viable single cells have greater potential for mineral formation when cultured in the presence of zoledronic acid. When the data was normalised to the viable cells number, there was a significant increase in mineral formation when the cells were treated with 0.5 μM of zoledronic acid. The potential for mineral formation in OB-like cells was decreased when the cells were cultured with zoledronic acid at concentration exceeding 10 μM in all donors (refer to 4.24). However, when the cells were only treated with zoledronic acid once during the whole period of the mineralisation assay, it was evident that zoledronic acid at low concentrations (≤0.5 μM) had no significant effect on the mineral formation of human OB-like cells *in vitro*. In contrast, a significantly higher level of mineral formation was observed in the OB-like cells treated with zoledronic acid at a concentration between 5 μM and 25 μM. Of note, 10 μM zoledronic acid was found to be the optimal concentration which enhanced the mineral formation in the OB-like cells (refer to 4.25). Taken together, we can therefore conclude that zoledronic acid at concentrations between 5 μM and 25 μM can significantly augment mineral formation. It should be emphasised that zoledronic acids inhibits cell proliferation and induces cell death in a proportion of cells, therefore leading to the conclusion that the increased mineralisation potential is due to an increase in the number of mature OB. This may clarify an additional observation that BPs can mediate a biphasic effect on the formation of bone-like nodules, with a dose-dependent stimulation at lower concentrations and an inhibitory effect at higher concentrations (Giuliani *et al.*, 1998a).

Taken together, our studies show that in addition to its effects on OC, zoledronic acid also has direct effects on the proliferation and survival of OB-like cells *in vitro*. Our observations support the notion that zoledronic acid is anabolic in bone by increasing the proportion of differentiated OB and enhancing the bone-forming activities of these cells.

CONCLUSIONS

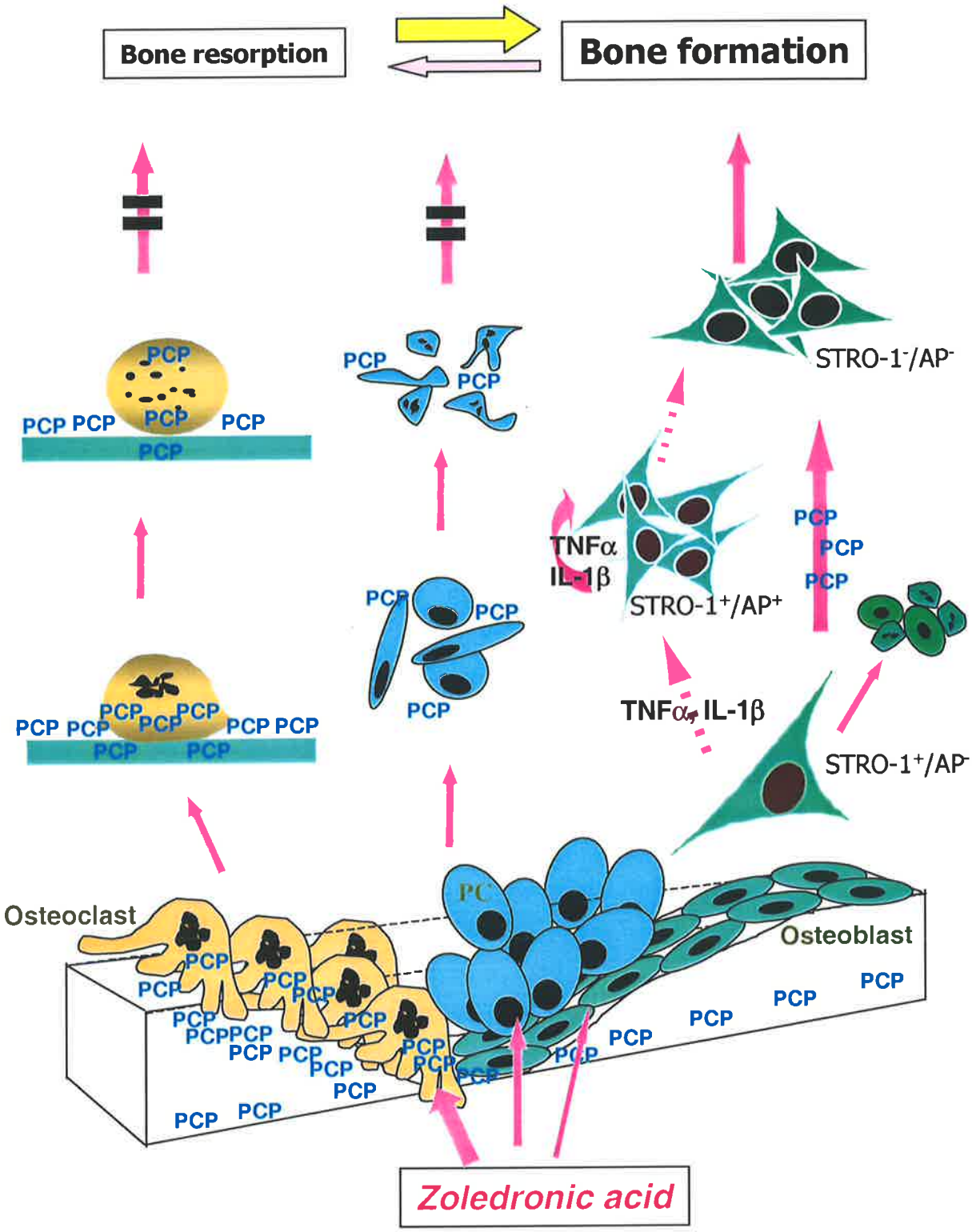
This thesis presents several novel observations of the molecular and cellular mechanisms of the way in which zoledronic acid effects myeloma cells and osteoblasts.

The reaction of the human body to a drug is complicated by the fact that different cell types have distinct responses based on the concentrations to which they are exposed. Bisphosphonates are uniquely distributed to the bone matrix, by virtue of their affinity for hydroxyapatite. Only cells in close proximity to the bone matrix will be affected by these compounds. Three major cell types, namely osteoclasts, myeloma cells and osteoblasts would therefore be affected in MM patients receiving bisphosphonate treatment. Our studies indicate that in addition to inhibiting bone resorption, zoledronic acid can enhance bone formation (figure 5.1).

Zoledronic acid induces apoptotic and non-apoptotic cell death in myeloma cells by inducing S phase arrest in a time and dose dependent manner and inhibiting the mevalonate pathway. In addition, zoledronic acid upregulates the expression of TRAIL in myeloma cells and induces cell death by uncoupling the regulation of the TRAIL death receptors and decoy receptors. The uncoupling of TRAIL receptors may ultimately determine the sensitivity of myeloma cells to zoledronic acid. The *in vitro* studies suggest that zoledronic acid represents a potent agent to reduce bone loss in patients with MM by inducing cell death in myeloma cells, a source of numerous osteoclast-activating factors.

Zoledronic acid induces cell death in the osteoblast-like cells in a dose dependent manner. Compared to the other subpopulations, STRO-1 ^{bight} osteoblast-like cells have a greater proliferative potential and this may account for a greater sensitivity to zoledronic acid by blocking cell replication in S and G_2/M phase in a dose-dependent manner. Zoledronic acid at the concentration of 5 μM leads to an increase in the number of STRO-1^-/AP^- (mature osteoblast/osteocyte-like) cells, with a concomitant decrease in the number of STRO-1^+/AP^- (stromal precursors) cells. Based on the ability of osteoblast-like cells to form mineralised bone matrix and their expression of bone-associated matrix proteins, we found that a proportion of STRO-1 ^{bright} osteoblast-like cells differentiate into maturing osteoblast in the presence of zoledronic acid. This is thought to account for the enhanced bone formation

Figure 5.1. Zoledronic acid augments bone formation by acting on three major cell types, namely osteoclasts, myeloma cells and osteoblasts in patients with MM. Zoledronic acid inhibits bone resorption not only by inducing apoptosis of osteoclasts but also by inhibiting osteoclast-activating factors synthesised by myeloma cells and osteoblasts. It also affects osteoblasts directly by expanding and maturing osteoblasts to enhance osteoblastic bone formation.


Bone resorption
Bone formation
PCP
STRO-1⁻/AP⁻
TNFα
IL-1β
STRO-1⁺/AP⁺
TNFα, IL-1β
STRO-1⁺/AP⁻
Osteoclast
Osteoblast
Zoledronic acid

observed. Our studies showed that zoledronic acid optimally increased bone formation at a concentration of 10 μM. We demonstrate that zoledronic acid is able to upregulate IL-1β and TNF-α expression in osteoblast-like cells. Culturing osteoblast-like cells in recombinant human IL-1β and TNF-α increase the number of STRO-1^+/AP^+ osteoprogenitors, which in the presence of zoledronic acid may also differentiate into maturing and functional osteoblasts. Furthermore, zoledronic acid was found to decrease the expression of RANKL on the osteoblast-like cell surface possibly by upregulating TACE expression, which may also be responsible for the decrease in STRO-1 expression on the cell surface.

Taken together, zoledronic acid inhibits bone resorption not only by inducing apoptosis of osteoclasts but also by inhibiting osteoclast-activating factors synthesised by myeloma cells and osteoblasts. It also affects osteoblasts directly by expanding and maturing osteoblasts to enhance osteoblastic bone formation. This represents the first study to provide evidence that zoledronic acid directly augments bone formation *in vitro*.

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