UNIVERSITÉ DE SHERBROOKE

MODULATION OF TUMOR NECROSIS FACTOR RELATED APOPTOSIS-INDUCING LIGAND (TRAIL) RECEPTORS IN A HUMAN OSTEOCLAST MODEL *IN VITRO*

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Mémoire présenté à la Faculté de médecine et des sciences de la santé
en vue de l'obtention du grade de maître ès sciences (M. Sc.)
en Immunologie

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"Ni la contradiction n'est marque de fausseté ni l'incontradiction n'est marque de vérité"

-Blaise Pascal

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Il a été démontré dans notre laboratoire que les ostéoclastes (OCLs) provenant de patients atteints de myélome multiple (MM) présentaient une variation de l'expression des récepteurs de TRAIL en comparaison avec des ostéoclastes normaux. Il a également été montré que TRAIL (TNF-Related Apoptosis-Inducing Ligand), un membre de la famille TNF, était capable d'induire l'apoptose des cellules exprimant ses récepteurs, en liant les récepteurs DR4 et DR5, mais pas DcR1 et DcR2, qui sont des récepteurs "decoy", dont le domaine intracellulaire de mort est absent. La modulation observée de ces récepteurs pourrait créer une résistance à l'apoptose dans le microenvironment du MM et pourrait être associée au profil cytokinique de cette maladie, caractérisé par une forte expression du « Receptor Activator of NF-κB Ligand" (RANKL) et de "Macrophage Inflammatory Protein 1α" (MIP-1α), cytokines majeures de la résorption osseuse. Le but de notre étude a été de déterminer quelles cytokines présentes dans cette pathologie pourraient en être responsables. Lors de la maturation cellulaire, les précurseurs d'OCL provenant de sang cordon et mis en présence de M-CSF et RANKL se différencient en cellules multinucléées (MNCs) qui expriment des marqueurs OCL et sont capables de résorber l'os. Par immunocytochimie, il avait été montré que ces cellules OCL exprimaient les 4 récepteurs de TRAIL. En stimulant de telles cultures d'OCLs avec différentes cytokines (RANKL, MIP-1α, Transforming Factor β (TGF-β), osteoprotegerin (OPG), TRAIL) et la parathormone (PTH), nous avons observé une modulation de l'expression de ces récepteurs de TRAIL, au niveau mARN par RT-PCR, au niveau protéique par immunobuvardage de type western et au niveau de la membrane cellulaire par immunocytochimie. Afin de voir si ces changements conduisaient à une modulation du niveau de résistance à l'apoptose, des MNCs, cultivées en présence de ces différentes cytokines pendant 5 jours, ont été stimulées avec TRAIL pendant 24h. L'apoptose des OCLs a été évaluée par la méthode TUNEL. Aucune corrélation n'a pu être établie entre la modification de l'expression de ces récepteurs et le niveau d'apoptose, mais le nombre d'expériences est encore trop faible pour conclure, et de nouvelles expériences doivent être réalisées afin de pouvoir analyser cette corrélation de manière adéquate. Nos résultats suggèrent néanmoins la possibilité qu'une modulation des récepteurs de TRAIL à la surface des OCLs soit induite par certaines cytokines fortement exprimées dans les maladies osseuses avec hyperrésorption osseuse, et puisse ainsi induire une résistance à l'apoptose de ces cellules. En prolongeant la vie des OCLs, ces influences régulatrices pourraient rendre compte en partie de l'augmentation de la résorption dans le micro-

environnement osseux des maladies comme l'ostéoporose, la maladie de Paget, ou le myélome multiple. **MOTS CLÉS**: Osteoclaste, Apoptose, Resorption, TRAIL

Modulation of Tumor Necrosis Factor Related Apoptosis-Inducing Ligand (TRAIL) Receptors in a Human Osteoclast Model *In Vitro*

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We have previously shown that osteoclasts (OCLs) from multiple myeloma (MM) specimens vary from healthy OCLs in their expression of the TRAIL receptors. TRAIL (TNF-Related Apoptosis-Inducing Ligand), a member of the TNF superfamily, has been shown to induce apoptosis in cells by binding receptors DR4 and DR5, but not DcR1 and DcR2, its decoy receptors, which lack the necessary internal death domain. The observed modulation of these receptors may confer a resistance to apoptosis in the MM environment, and could be related to the cytokine pattern that primarily involves the resorption promoting Receptor Activator of NF-κB Ligand (RANKL) and Macrophage Inflammatory Protein 1 (MIP-1α). The aim of our study was to determine which cytokines present in the disease might be responsible for this modulation. In long term cultures of OCL precursors from cord blood in the presence of M-CSF and RANKL, multinucleated cells (MNCs) that express OCL markers form, and can resorb bone. Through immunocytochemistry we showed that these MNCs can express all four TRAIL receptors. By stimulating with various cytokines (RANKL, MIP-1α, Transforming Factor β (TGFβ), osteoprotegerin (OPG), TRAIL), and parathyroid hormone (PTH) in OCL cultures, we were able to observe receptor modulation at the mRNA level using real time PCR, the protein level using Western blot analysis, and cell surface expression via immunocytochemistry. To determine if these changes translated to a difference in resistance to apoptosis, cells treated with with apoptosis-inducing levels of TRAIL after 5 days of stimulation with the selected cytokines were evaluated via TUNEL to quantify apoptosis. While no correlation has yet been established between the observed receptor modification and apoptosis induction, sample size is a factor, and further tests will be performed. Our results suggest the possibility that TRAIL receptor modification is induced by multiple cytokines present in bone diseases, capable of altering both the susceptibility and resistance pathways in osteoclasts. By potentially prolonging the lifespan of the OCL, these regulatory influences may ultimately be contributory factors to the augmentation of resorption in the micro-environment of bone resorptive diseases like multiple myeloma, Paget's disease of bone, or osteoporosis.

KEY WORDS: Osteoclast, Apoptosis, TRAIL, Resorption

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LIST OF ABBREVIATIONS

BAD..... bcl-2-associated death promoter
BAK..... bcl-2-homologous antagonist killer
BAX..... bcl-2-associated x protein
BID..... BH3 interacting domain death agonist
BMP..... bone morphogenic protein
BMSC..... bone marrow stromal cells
BMU..... bone multicellular units
BSP..... bone sialoprotein
CA..... carbonic anhydrase
CAD..... caspase-activated DNAse
CBP..... CREB-binding protein
CCR..... c-c motif receptor
CFU-M..... macrophage colony forming unit
CLC..... chloride channel
CTR..... calcitonin receptor
CTX..... carboxy-terminal collagen crosslinks
DC-STAMP..... dendritic cell-specific transmembrane protein
DD..... death domain
DIABLO..... direct IAP binding protein with low pI
DISC..... death-inducing signaling complex
EAE..... experimental autoimmune encephalomyelitis
ERK..... extracellular signal-regulated kinase
FADD..... fas-associated protein with death domain
FGF..... fibroblast growth factor
FLICE..... FADD-like interleukin-1 beta-converting enzyme (caspase 8)
FLIP..... FLICE-like inhibitory protein
GM-CSF..... granulocyte macrophage colony-stimulating factor
GSK3..... glycogen synthase kinase-3
HGF..... hepatocyte growth factor
HSC..... hematopoietic stem cell
IAP..... apoptosis inhibitory protein
ICTP..... carboxyterminal telopeptide of type 1 collagen
IGF..... insulin-like growth factor
IKK..... inhibitor of kappa B kinase
ISEL..... in situ end-labeling
LIT..... lymphocyte inhibitor of TRAIL

M-CSF..... macrophage colony stimulating factor
MCP..... monocyte chemotactic protein
MGP..... matrix gla protein
MITF..... microphthalmia-associated transcription factor
MIP-1α..... macrophage inflammatory protein-1 alpha
MM..... multiple myeloma
MTOR..... mammalian target of rampamycin
NCX..... sodium-calcium exchanger
NFAT..... nuclear factor of activated t-cells
NF-kB..... nuclear factor kappa-light-chain-enhancer of activated B cells
OC(L)..... osteoclast
OCN..... osteocalcin
OPG..... osteoprotegerin
OPN..... osteopontin
OSCAR..... osteoclast-associated, immunoglobulin-like receptor
OSTM..... osteopetrosis-associated transmembrane protein
PBMC..... peripheral blood mononuclear cell
PI3K..... phosphoinositide 3-kinase
PGE2..... prostaglandin E2
PKA..... protein kinase A
PLC..... phospholipase c
PTH..... parathyroid hormone
PTHrP..... parathyroid hormone-related protein
RANKL..... receptor activated nuclear factor kappa ligand
RANTES..... regulated upon activation normal T-cell expressed and secreted
RIP..... receptor interacting protein
RYR..... ryanodine receptor
SMAC..... second mitochondria-derived activator of caspases
TCR..... t-cell receptor
TGF-β..... transforming growth factor- beta
TM4SF..... transmembrane-4-superfamily protein
TNF..... tumor necrosis factor
TRAF-6..... TNF receptor associated factor 6
TRAIL..... tumor necrosis factor-related apoptosis-inducing ligand
TRAP..... tartrate resistant acid phosphatase
TRPV5..... transient receptor potential cation channel subfamily V 5
TRUNDD..... TRAIL receptor with a truncated death domain
TUNEL..... terminal deoxynucleotidyl transferase dUTP nick end labeling
VCAM..... vascular cell adhesion molecule-1

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INTRODUCTION:

BONE: SKELETAL SYSTEM

The adult human skeletal system is a dynamic organ, responsible for many important functions; among them are providing support, protection, and allowing for movement. Equally importantly, the bones serve as a reservoir of growth factors and cytokines, maintain acid-base balance and mineral homeostasis, and provide the space for hematopoiesis. The composition of bone is 50 to 70% mineral, another 20 to 40% organic matrix, and 5 to 10% water (Clarke, 2008). Bone, like other connective tissues, is composed of cells, and extracellular matrix. This matrix is comprised of both collagen fibers (mainly Type I collagen, making up 90% of the total protein composition), and non-collagenous proteins (including osteocalcin, osteopontin, bone sialoprotein, accounting for the other 10%). In bone, however, the extracellular matrix is physiologically mineralized, through deposition of carbonated hydroxyapatite. It is this mineral component that gives bone its characteristic mechanical rigidity and strength, while the organic matrix provides elasticity and flexibility.

There are two major kinds of bone, trabecular (spongy) and cortical. Depending on their intended function, the ratio of cortical to trabecular bone differs between skeletal sites. Cortical bone is dense and hard, whereas trabecular bone is mesh-like; a honeycomb network of plates and rods in the bone marrow compartment. Both cortical and trabecular bone are normally formed in a lamellar pattern; where collagen fibers are very highly ordered and deposited in alternating orientations. This layered pattern is integral in providing bone with much of its mechanical strength (Eriksen et al., 1994).

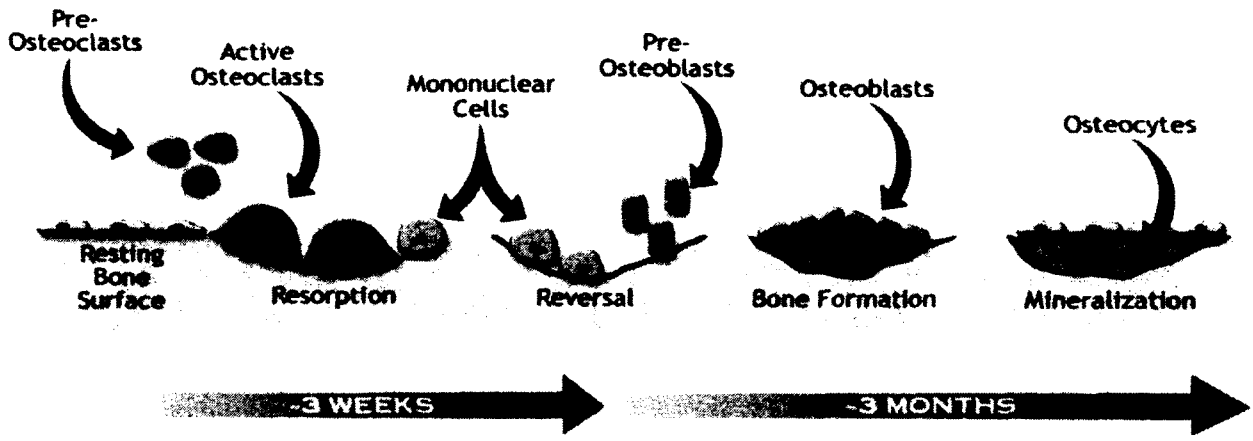


Figure 1: The Remodeling Cycle

The remodeling process requires approximately 4 months to complete a cycle with all four stages of remodeling. The recruitment and activation of osteoclasts marks the beginning of resorption, followed by their apoptosis and the recruitment of mononuclear cells and pre-osteoblasts during subsequent reversal. The chronologically limiting steps are bone formation by the osteoblasts, and their development into osteocytes during the mineralization of the new bone (Michigan, 2005)

The skeleton is constantly undergoing the remodeling process, removing old, structurally weakened bone, and replacing it with new layers of bone to maintain integrity and improve strength. According to Wolff's Law, the skeleton also constantly undergoes modeling so that it can adapt to changes in biomechanical forces (Chen et al., 2009). Like any other physiological system, this is one rooted in controlled balance, dependent on synergy between the mechanisms responsible for resorption and those responsible for formation. The three major cell types forming the foundation of bone activity are the osteoclasts that break down bone, osteoblasts that build new bone, and osteocytes that maintain living bone. Bone remodeling relies on these activities to be carried out by independent (yet ultimately synergistic) action of osteoblasts and osteoclasts in response to stimuli that can be biomechanical or strictly biological, depending on the circumstance.

BONE: THE REMODELING CYCLE

Bone remodeling is the mechanism of bone replacement in the skeleton. Bone replacement is initiated by osteoclastic resorption followed soon after by osteoblastic formation. The remodeling cycle is composed of four phases, which are necessarily sequential: resting/activation, followed by resorption/remodeling, then reversal and finally formation (Figure 1). Resorption and formation are closely linked within discrete temporary anatomic structures, described as "basic multicellular units," or BMUs. A BMU is active in three dimensions; excavating and refilling a tunnel through cortical bone, or a trench across the surface of cancellous (also called trabecular) bone.

Remodeling activation corresponds to the conversion of a region of bone surface from quiescence to remodeling activity, a process requiring changes in lining cells as well as local recruitment of new osteoclast precursors from circulation (Parfitt et al., 2002). These cells eventually develop, bind to the bone matrix, and as mature osteoclasts, they enact the resorption process, as will be described in depth later. This leads to the formation of characteristic Howship's lacunae on trabecular bone, and Haversian canals on the surface of cortical bone; the traces of bone resorption. Resorption takes only approximately 2 to 4 weeks of each remodeling cycle.

Once the resorption phase is completed, the reversal stage begins with initiation of apoptosis of the resorbing osteoclasts and the action of local monocytes (Reddy, 2004). Cytokines produced by osteoclasts and released during the resorption of bone act as recruitment signals, growth factors, and other stimulatory signals (Martin et al., 2005). Among them, for example, is transforming growth factor-β (TGF-β); released from the bone matrix, it decreases osteoclast resorption by inhibiting RANKL production by osteoblasts and stromal cells in the bone microenvironment, and directly induces apoptosis in osteoclasts (Houde et al., 2009). The purpose of the reversal stage is to transition from resorption to formation. The mononuclear cells present on the bone surface may complete the resorption process, producing the signals that segue the BMU into formation.

Subsequently, osteoblast precursors can locally proliferate and differentiate into osteoblasts. During the formative phase, these cells deposit new bone matrix, which is initially unmineralized and called osteoid, filling the resorption lacunae. Once embedded in

osteoid, the osteoblasts may mature into terminally differentiated osteocytes, producing and maintaining a canalicular network connecting them to the surface lining cells and other osteocytes (Parfitt, 1994). The osteoblasts lying on the bone surface become quiescent (resting phase) lining cells (Hill, 1998). With the completion of normal bone formation, approximately 70% of osteoblasts can be expected to undergo apoptosis, with the rest developing into osteocytes or more frequently, the bone-lining cells (Cohen, 2006). These bone-lining, osteoblast-like cells regulate the flow of mineral ions to the bone extracellular fluid, making up the blood-bone barrier, and can even re-differentiate to osteoblasts in time of need (Seeman, 2009).

In relation to resorption, bone formation is a long process, taking between 4 and 6 months to complete. This is one of the main reasons that precise regulation between the two phases is so crucial for balance in the skeletal system. During formation, osteoblasts synthesize new collagenous organic matrix, while regulating the mineralization of that same matrix. This regulation of mineralization is achieved by the release of small membrane-bound matrix vesicles that concentrate calcium and phosphate, and will enzymatically destroy inhibitors of mineralization like pyrophosphate or proteoglycans (Anderson, 2003).

In a healthy system, the process of bone remodeling helps preserve bone mechanical strength by replacing aged and microdamaged bone with healthier bone; bone that is better able to maintain calcium and phosphate homeostasis, and form a more efficient support structure.

SYSTEMIC AND LOCAL FACTORS AFFECTING BONE REMODELING

Bone remodeling is modulated by two major calcium-regulating hormones, parathyroid hormone (PTH) and 1,25-dihydroxy vitamin D. But the most important systemic hormone in maintaining normal bone turnover is estrogen. The mechanisms of action of estrogen on the skeleton are the topic of continued debate. It is agreed that the principal action of estrogen in the bone environment is to inhibit bone resorption, and several studies have shown that it aids in bone formation. Studies in animals suggest that estrogen acts by altering either the production or activity of local factors that regulate osteoblast and osteoclast precursors (Trivedi et al., 2010). A number of cytokines and

growth factors are released in response to estrogenic stimulation; M-CSF, IL-1 and –6, TNF, prostaglandins, and IGF-1, all of which have effects on bone cells in vitro and in vivo (Riggs et al., 2002). Most, but not all of these cytokines can be produced by the bone cells, so some are produced by other cells in the bone microenvironment, inducing both paracrine and autocrine effects on remodeling.

Hormones don't just affect differentiation and growth factor/cytokine flux. One of the major routes by which estrogen, for example, exerts its pro-formation effect is through encouraging apoptosis in osteoclasts but preventing it in both osteoblasts and osteocytes (Balasch, 2003). Prevention of apoptosis is performed through activation of a Src kinase signal transduction pathway, but the exact mechanisms of increasing sensitivity to apoptosis is unknown (Kousteni et al., 2001).

PTH is a peptide secreted by the parathyroid glands in response to changes in serum Ca^{2+}, and acts as the principal regulator of calcium balance. PTH is able to stimulate bone resorption and bone formation through activation of osteoclast signaling (Rosen, 2004). However, it achieves this particular effect indirectly, as PTH-receptors are expressed on osteoclast precursors, but not mature osteoclasts. PTH is a potent stimulator of bone resorption and has a biphasic effect on bone formation. There is an acute inhibition of collagen synthesis when dosed with high concentrations of PTH, but prolonged intermittent administration of this hormone produces increased bone formation, a property for which it is currently being explored clinically as an anabolic agent (Dempster, 2001)

1,25-Dihydroxy vitamin D has its greatest effect on intestinal calcium and phosphate absorption, but it also has direct effects on bone and other tissues. Vitamin D is critical for the differentiation of both osteoblasts and osteoclasts and can stimulate bone resorption and formation under some experimental conditions (Raiz, 1999).

Bone also contains a large number of growth factors. Among the most abundant are the IGFs, which, with their associated binding proteins, are thought to be important modulators of local bone remodeling. Transforming growth factor ß and its related family of bone morphogenetic proteins are present in the skeleton and have important functions not only in remodeling, but also in skeletal development. Many members of this superfamily have been well characterized in osteogenesis, but are also beginning to be

understood in the context of bone resorption. The bone morphogenic proteins (BMPs) are a group of signaling molecules originally characterized for their ability to induce bone formation (Cao et al., 2005). Most of the BMPs are expressed in skeletal tissue, and many are detectable in osteoblasts as well (Anderson et al., 2000). While the majority are recognized for promotion of bone formation, BMP-3, for example, is a negative regulator of bone formation (Daluiski et al., 2001). Transforming growth factor- β (TGF-β) is a major player in bone biology, directly inducing effects in both osteoclasts and osteoblasts. In fact, TGF-β1 will be discussed further in the apoptosis section as a potent influence on the bone balance.

A large number of cytokines and growth factors that can affect bone cell functions have now been identified. It is now well established that virtually all the cytokines and hormonal factors that influence bone resorption act via a common final pathway involving the RANKL/RANK/OPG balance. RANKL is the main stimulator of osteoclast differentiation and activation (Boyle, 2003, Hofbauer, 2000). RANKL is a TNF-family member expressed by osteoblast/stromal cells in the vicinity of osteoclasts. *In-vitro* and *in-vivo* studies have clearly shown that RANKL, by binding to its membrane-bound receptor RANK, is crucial in the formation, survival, and bone-resorbing activity of osteoclasts (Hsu, 1999, Lacey, 1998, Quinn, 1998) (Figure 2). The fine-tuning of bone resorption also involves osteoprotegerin (OPG), another member of the TNF receptor family; one that has no transmembrane domain. This secreted decoy receptor recognizes RANKL, and therefore competes with RANK, leading to the inhibition of osteoclast differentiation and bone-resorbing functions (Simonet, 1997).

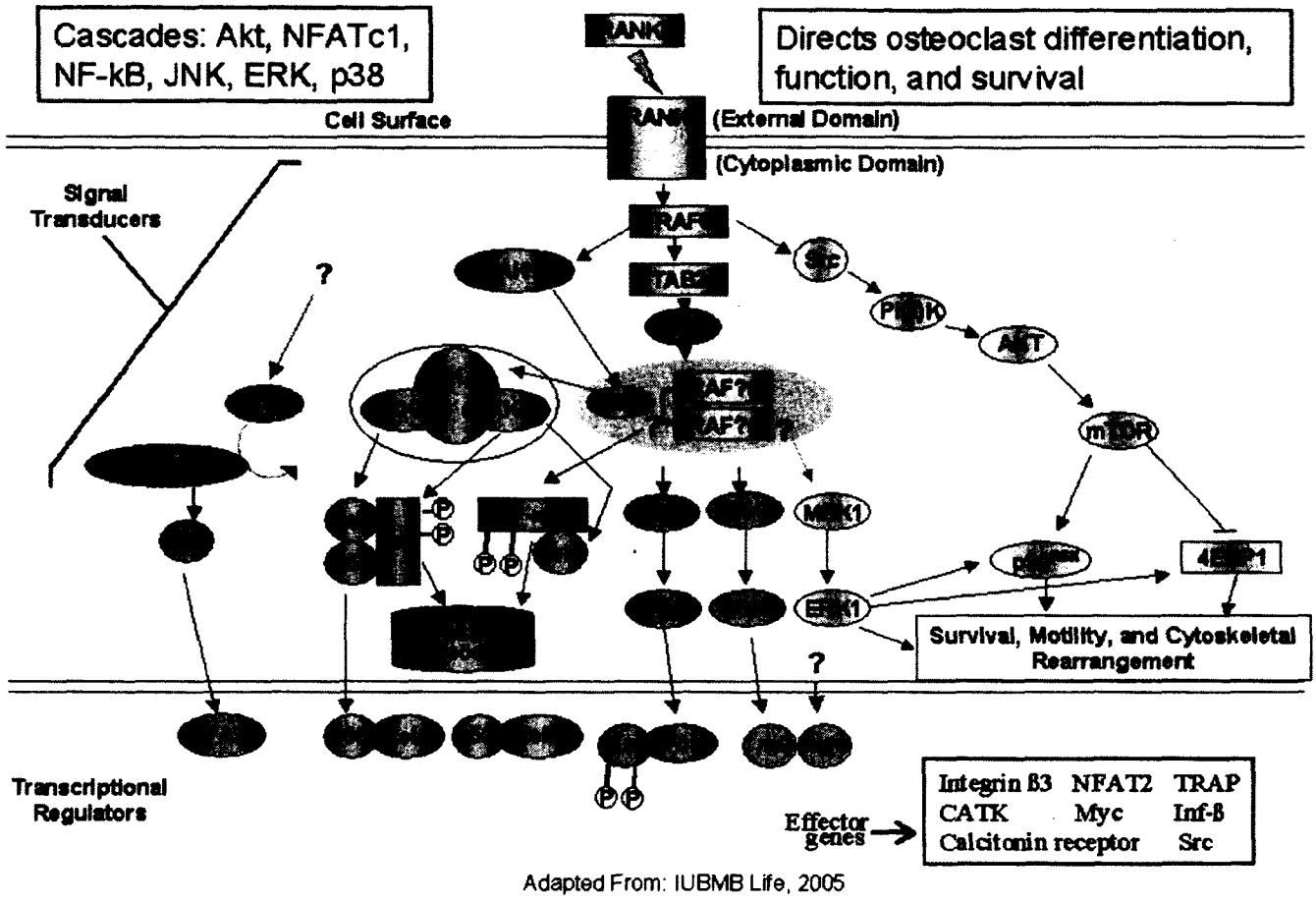


Figure 2: The RANKL/RANK signaling

RANKL is the principal cytokine in osteoclast differentiation, activation, and survival. RANKL activates the receptor RANK in a trimeric symmetric complex with TRAF6. Downstream signaling includes activation of IKK, NF- B, and MKK and subsequently stimulation of p38, MAPK, ERK, and JNK. Activation of the MAP kinases leads to activation and nuclear translocation of the transcription factors, c-Fos, c-Jun, and NFATc1. The c-src-dependent activation of PI3K results in activation of Akt, which then phosphorylates and inactivates the pro-apoptotic protein Bad.

Adapted from: IUEMB Life, 2003

Bone resorbing factors, hormones such as PTH or $1,25(OH)_2D_3$ or cytokines such as IL1, TNFα, and IL17, act mainly by increasing the RANKL/OPG ratio in the bone microenvironment. In contrast, inhibitors of bone resorption lower the RANKL/OPG ratio or block RANK signaling (Kong, 2000, Lubberts, 2000, Taganayagi, 2000). The major role of the RANKL pathway in bone resorption has been proven decisively in animal models, as severe osteoporosis is observed in OPG knockout mice, and osteopetrosis in RANKL KO mice (Blair, et al., 2005). Equally convincing is the similar study demonstrating development of osteopetrosis in mice over-expressing OPG (Bucay et al., 1998). In fact, the principle of this mechanism has already even been applied to therapy in humans, by blocking RANKL by OPG or more commonly, anti-RANKL antibodies to prevent osteoclast activation and bone resorption.

THE OSTEOCLAST

The osteoclast is derived from hematopoietic cells of monocyte-macrophage lineage. It is a cell uniquely responsible for the resorption of bone. The fully differentiated osteoclast is a large cell, typically containing between five and eight nuclei. Osteoclastogenesis, the production of these cells, involves a complex line of development including commitment, differentiation, multinucleation, and activation of immature osteoclasts. Osteoclast differentiation and eventual function rely on a variety of signals coming both from systemic hormones and those cytokines produced by neighboring cells (like T and B lymphocytes, marrow stromal cells, as well as osteoblasts and osteocytes) in the bone microenvironment. Since osteoclasts are secretory cells, they are to a degree, responsible for stimulation or inhibition of their own activity (Yavropoulo, 2008).

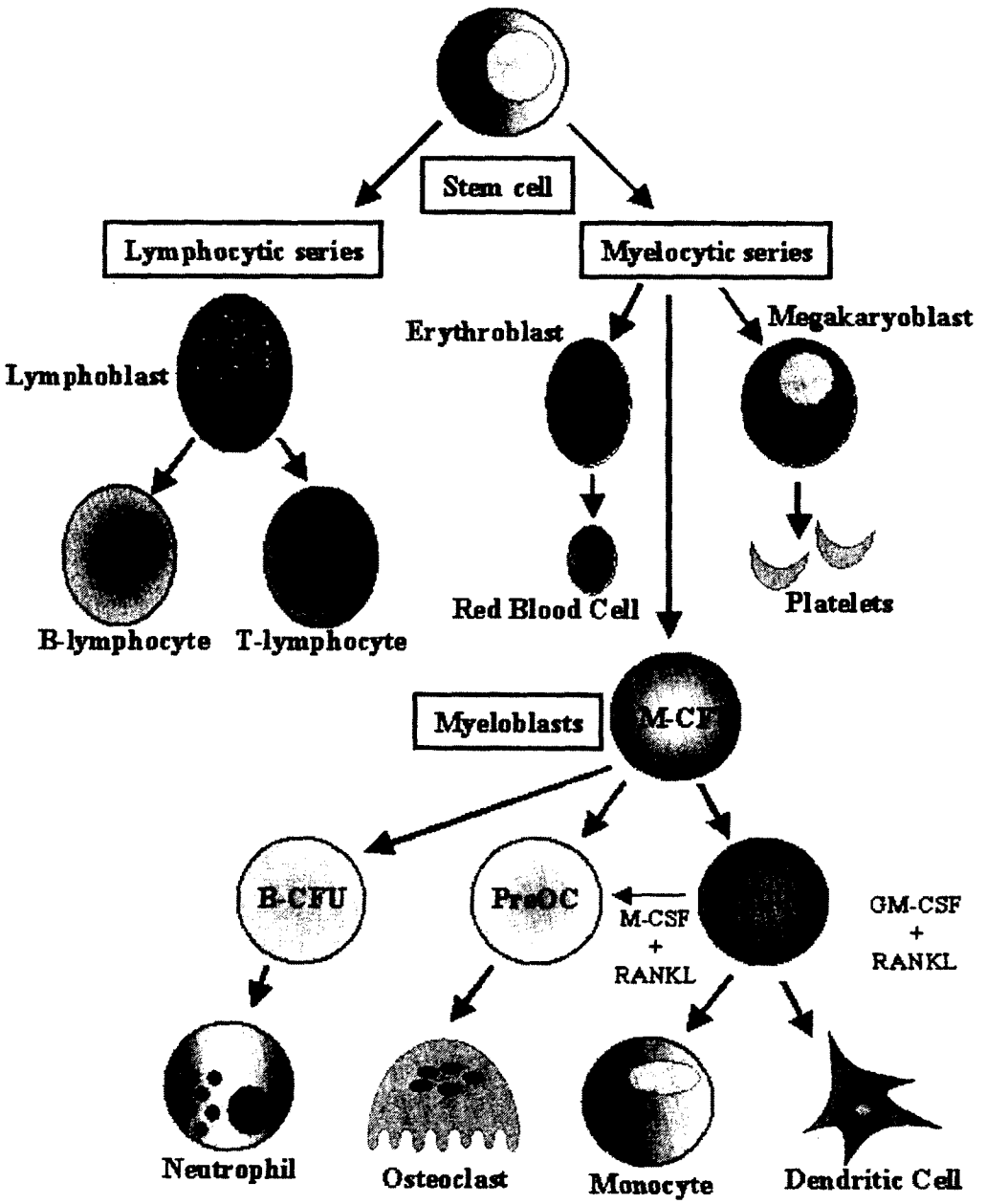
Of course, while it is perhaps the most complex step, osteoclastogenesis is just the beginning. To best understand the process of osteoclastogenesis, it will be discussed in the four sections mentioned above; commitment, differentiation, multinucleation, and maturation.

Commitment

The first cell type in the line (or tree, given its different possible outcomes) of osteoclast development is the pluripotent hematopoietic stem cell. This stem cell can further differentiate into granulocytes, megakaryocytes, monocytes/macrophages, and of course, osteoclasts (Figure 3). The earliest identifiable osteoclast-forming precursor is the granulocyte-macrophage colony forming unit (GFU-GM) (Roodman et al., 2000). The principle transcription factors involved in the early stages are the PU.1, the Microphthalmia-associated transcription factor (MITF) and c-FOS. The cytokine M-CSF is required to stimulate proliferation and prevent apoptosis of early osteoclast precursors.

The PU.1 transcription factor belongs to the Ets family of transcription factors and is responsible for the earliest events in osteoclastogenesis. PU.1 null mice do not just lack osteoclasts, but also macrophages, while still producing monocytic cells (Tondravi et al., 1997). PU.1 regulates the transcription of RANK gene in myeloid progenitors, allowing for RANK ligand signaling; RANKL being the key osteoclastogenic cytokine (Kwon et al., 2005).

Microphthalmia-associated transcription factor (MITF) is a leucine zipper protein implicated in the differentiation and survival of many cell types, even those developmentally unrelated to the osteoclasts (Kawaguchi et al., 2000). Like PU.1, MITF is expressed in macrophages, osteoclasts, and their myeloid precursors. Interaction with PU.1 allows MITF to regulate target genes like cathepsin K, acid phosphatase, and the osteoclast-associated receptor (OSCAR) in early osteoclast differentiation (Hu et al., 2007). Other MITF target genes include chloride channel 7 (Clcn7), necessary for bone resorbing activity through acidification, and Ostm1, a membrane protein necessary for chloride channel stability in osteoclasts (Meadows et al., 2007)



Adapted From: J Musculoskelet Neuronal Interact 2008

Figure 3: Osteoclastic differentiation

The pluripotent stem cell gives rise to a myeloid stem cell, which can in turn further differentiate to megakaryocytes, granulocytes, monocytes/macrophages and osteoclasts. The osteoclast is derived from the GM-CFU linage of the myeloblasts, receiving the signal to differentiate after contact with M-CSF and RANKL. **Adapted from**: J. Musculoskelet Neuronal Interact, 2008

Studies have shown that c-FOS is another key mediator of the lineage commitment between osteoclasts and dendritic cells; another cell type derived from the monocyte/macrophage precursors (Miyamoto et al., 2001). Differentiation in this respect is mediated by GM-CSF and M-CSF. While M-CSF and RANKL will induce osteoclastogenesis in the precursor cells, GM-CSF and RANKL are activating factors of dendritic cells (Anderson et al., 1997). However, after transduction of the M-CSF differentiation signal, leading to c-FOS expression, cells are no longer competent to respond to GM-CSF (Miyamoto et al., 2001)

M-CSF, required for proliferation and osteoclast prevention, has one receptor, transcribed by the macrophage c-FMS gene (Ross, 2006). This transcription depends on PU.1 as well (Anderson et al., 1997). M-CSF can also induce its own receptor expression by forming an autocrine loop to amplify M-CSF-mediated signals, and by stimulating PU.1 (Yao et al., 2006). Loss of function in the M-CSF gene in mice has been shown to result in a decrease in macrophage numbers and a lack of osteoclasts, leading to osteopetrosis (Dai, 2002). In cells deprived of M-CSF, MITF is sequestered to the cell cytoplasm through interactions with 14-3-3 proteins, preventing the translation of its numerous target genes required for osteoclastogenesis (Bronisz et al., 2006). Several different cell types, including T lymphocytes, stromal cells, and osteoblasts, produce M-CSF constitutively in the bone microenvironment. This production can be induced by elevated serum PTH levels, or inflammatory molecules like TNF-α and IL-10 (Weir, 1996). Recent publications indicate that TNF-α can also induce c-FMS expression (Yao et al., 2006). M-CSF/RANKL signaling activates expression of the osteoclastogenic genes by two mechanisms; by down-regulating Eos expression at mRNA and protein levels, leading to disassociation of co repressors from PU.1 and MITF genes, and by phosphorylation and activation of MITF by the ERK and p38 MAPK pathways, allowing for recruitment of co-activators CBP/p300 and BRG1 (Mansky et al., 2002). BRG1 (or SNF2L4) is used to unwind chromatin repressing gene activation, while CBP and p300 help increase gene expression by relaxing

the chromatin structure at the promoter site, recruiting transcriptional machinery like RNA polymerase II, and acting as adaptor molecules (Goodman et al., 2000).

Downstream signals PI3K, p43/44 ERK, PLCA, and the proto-oncogene c-Cb1 are the key signal transducers of M-CSF (Ross, 2006). The PI3K/AKT cascades regulate proliferation of the osteoclast precursors though GSK3 and FOXO. By phosphorylating these two proteins, their ability to inhibit entry into the cell cycle is suppressed, allowing the cells to respond to pro-proliferative stimuli (Hu et al., 2007).

To date, none of these osteoclastogenic pathways have been considered for therapeutic intervention, given that many of the transcription factors and associated cytokines are ubiquitous, regulating multiple cell lineages. To block one or more of these pathways would affect more than just osteoclastic selection and development, almost assuredly causing more harm than potential good.

Differentiation

The most important cytokine involved in osteoclastic differentiation is receptor activated nuclear factor κ-B ligand (RANKL), a member of the TNF superfamily. Under ordinary physiological conditions, RANKL is produced by stromal cells in the bone marrow, and by osteoblasts in the periosteum. In the instance of skeletal inflammation like that seen in rheumatoid arthritis, RANKL is also produced in great quantity by the T lymphocytes (Kong et al., 1999). In this example, RANKL can be cleaved from the membrane of the cell and interact with its receptor RANK as a soluble ligand. Many cell types express RANK, but PU.1 allows the induction of RANK on the cell surface of the precursor monocytes (Kido, 2008). Deletion of RANKL or its receptor results in an absence of osteoclasts, arresting osteoclastogenesis immediately after the stage of M-CSF-induced expansion of osteoclast progenitor cells. RANKL also has a decoy receptor; osteoprotegerin (OPG), which is produced primarily by osteoblasts and competitively binds RANKL to prevent RANK signaling (Yasuda et al., 1998). Deletion of the TNFRSF11B gene coding for OPG is associated with profiles of high osteoclastic activity and increased osteoclast numbers (Buckley et al., 2003). RANKL expression is regulated by a variety of hormones like PTH, PGE2, and forskolin, all of which act through the

cyclic AMP/PKA pathway, and though Vitamin D3, via the VDR-mediated pathway (Lerner, 2000)). RANKL production is also promoted by IL-1 expression, which can be upregulated by TNF-α (Wei et al., 2005).

RANKL signaling begins with the binding, symmetric trimerization and subsequent activation of the receptor RANK that interacts with the adaptor molecule TNF-receptor-associated factor 6 (TRAF6). The recruitment of TRAF6 is necessary, as TRAF6-deficient mice have shown to be severely osteopetrotic, due to an impairment of osteoclastogenesis, preventing their resorptive activity (Kobayashi et al., 2003). However, while the TRAF6-dependent signaling cascade is used by other receptors like CD40 and TLR family members, it can only induce osteoclastogenesis via RANKL signaling (Ye et al., 2002). Current research is incomplete, but suggests that the difference in signaling pathways employed by RANKL involves a different degree of downstream p38 kinase activation and higher recruitment of TRAF6 to the surface receptor (Kadono et al., 2005). Other downstream intracellular signaling pathways involve activation of IkB kinases (IKK) a and b, and other MAP kinases including p38. Once TRAF-6 activates the IkB kinase, the IKK induces phosphorylation of the inhibitory protein IkB. Ordinarily IkB is bound to the inactive form of NF-κB in the cytoplasm, but after its phosphorylation, this complex dissociates (May et al., 1997). Upon release from IkB, NF-κB translocates to the nucleus, binding to the DNA and inducing transcription of a variety of genes necessary for osteoclast differentiation and survival. Studies have shown that mice lacking NF-κB subunits are osteopetrotic and missing osteoclasts in a similar manner to those lacking TRAF-6 or RANKL (Iotsova et al., 1997). Without NF-κB there can be no osteoclast formation or resorption, as it initiates transcription of a vast number of osteoclast-related genes.

Over-expression studies of RANK have shown elevation of intracellular Ca^{2+} via PLC, which accelerates NF-κB translocation, and activation of JNK signaling (Komarova et al., 2003). Activation of MAP kinases by RANKL leads to activation and translocation of many other transcription factors, including ATF2, c-FOS, c-JUN, and members of the NFAT family, which leads to gene transcription necessary for osteoclast differentiation and activation (Matsumoto et al., 2000). Another related signaling pathway downstream of RANK is TRAF6 mediated PI3K (Arron et al., 2001). While the PI3K/AKT pathway is

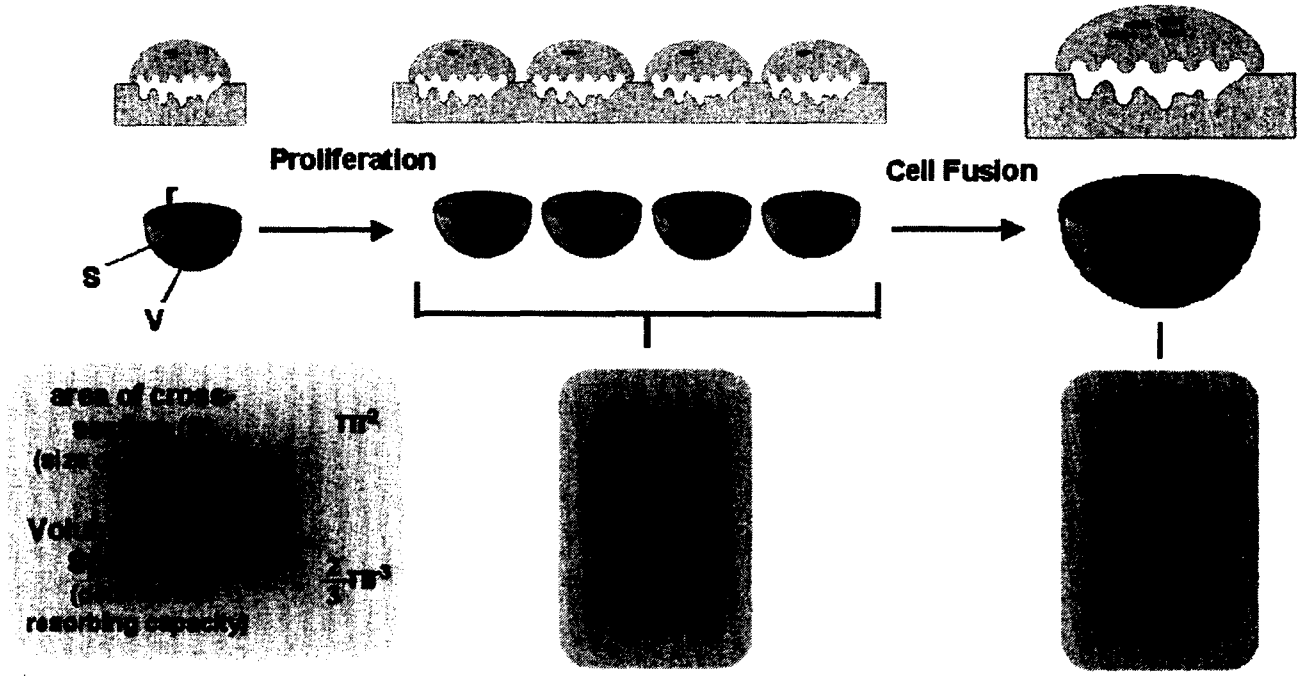
triggered by M-CSF to aid in cell proliferation, it also generates a protective anti-apoptotic mechanism. PI3K triggers generation of a lipid second messenger than subsequently activates AKT. AKT then inactivates the pro-apoptotic BAD via phosphorylation (Toker et al., 1997). Finally, it has also been shown that contact between the pre-osteoclast and the osteoblast or stromal cell is important to RANK/RANKL signaling (Garcia-Palacios et al., 2007). The importance of cell contact for osteoclast development and activation will be elaborated further in the section on multinucleation.

The NFAT family is necessary for the transcription downstream of NF-κB of a number of genes crucial to osteoclast formation. There are five members, with the most active being NFATc1, with studies showing that induction of expression of NFATc1 causes precursor cells to undergo differentiation even in absence of RANKL (Takayanagi et al., 2002). In the same study, deletion of the NFATc1 gene was shown to fully stop osteoclast formation downstream of RANKL (Takayanagi et al., 2002). In early stages of osteoclast development, NFATc2 is recruited to the NFATc1 promoter, but alone is not sufficient to activate the promoter. It is in co-operation with NF-κB that induction of NFATc1 is generated, followed by an auto-amplification phase (Asagiri et al., 2005). NFATc1 regulates many genes specific to the osteoclast, including cathepsin K, TRAP, CTR, and OSCAR (Matsumoto et al., 2004, Kim et al., 2005). To optimize some transcription it forms complexes with PU.1, MITF, and c-FOS, however these complexes are not necessary for osteoclastogenesis to take place (Crotti et al., 2008). In addition to being an osteoclastogenic transcription factor, NFAT negatively regulates osteoblastic differentiation by regulating fos-related protein (FRA)-2 expression (Zayzafoon, 2005)

Multinucleation

One of the most characteristic activities of the developing osteoclast is cell fusion and multinucleation. Although there are other types of cell fusion in the body, the cell fusion that takes place during osteoclastogenesis is easily distinguishable (Ishii et al., 2008). The most obvious effect of multinucleation is the increase the cell size of the osteoclast, allowing for greater overall resorptive effect. This is necessary because unlike a macrophage or a neutrophil, osteoclasts do not degrade their targets internally in lysosomes. The osteoclast must create a seal at the bone surface (described in greater detail

later), and form an "extracellular lysosome" (Teitelbaum et al., 2003). As shown in figure 4, while the area of the cross section taken rests the same proportionally in several non-fused cells and a fused multikaryon, the volume increases. This is important given that it is volume that determines resorbing capacity, by determining how far the osteoclast can spread. In addition, fusion not only increases the size of the osteoclast, but also transfers the RANKL signal cascade and its effects to the additional nuclei that now comprise the cell. Without the step of multi-nucleation in the differentiation of osteoclasts, resorption is greatly impaired, as it has been shown that mono-nucleated osteoclasts are inefficient resorbers of bone.



Adapted From: Mod Reumatol 2008

Figure 4: Osteoclast cell fusion and their resorptive bone capacity

The resorptive capacity of the osteoclast is represented as a volume of hemisphere whose cross section is identical to the cell size. The fusion of four mononuclear osteoclasts allows for a doubling of total volume, producing a more effective resorbing unit. In general, when n cells are fused, the volume of bone they can impact jumps to *H*n-fold. This means that osteoclasts can do their work more efficiently when they become multinucleated giant cells. **Adapted from**: Mod Rheumatol, 2008

Upon receiving the RANKL signal, the osteoclast precursor cell initiates a cascade of gene expression including those already discussed, as well as those producing chemokines MCP-1 and RANTES, both of which are chemotactic signals for monocytes (Kim et al., 2005). RANKL also induces the MCP-1 receptors CCR2 and CCR4, which when activated stimulate the PI3K signaling pathway (Hayashida et al., 2001). In addition, after production of these cytokines that attract monocytes, RANKL via NFATc1 induces gene expression of molecules that mediate cell-fusion, like vacuolar ATPase Vo domain d2 isoform and the dendritic cell-specific transmembrane protein (DC-STAMP). It does so by binding directly their promoter regions to initiate transcription (Kim K et al 2006). Mice deficient in either DC-STAMP or v-ATPase Vod2 present impaired osteoclast fusion and a clear reduction in resorption (Lee et al., 2001).

Studies have also shown that RANKL induces a translocation of membrane bound CD9 (a transmembrane-4 superfamily protein [TM4SF or tetraspanins]) to lipid raft microdomains from non-raft microdomains (Ishii et al., 2006). This could suggest that CD9 is interacting with other membrane-associated molecules that are responsible for osteoclastogenic cell fusion, and bring them to the lipid raft microdomain (Claas et al., 2001). Induction of a change in membrane composition (like that seen with lipid rafts,) is important as well in the sense that the lipid composition of the plasma membrane plays a large role in membrane fusion (Chernomordik et al., 2003). Interestingly, CD9 expression is elevated in cancellous bone tissue in osteoporosis, linking the molecule to bone resorptive diseases (Iwai et al., 2008). Effectively, the mechanism of fusion enables the osteoclast to be an efficient remodeling force, despite constituting a small fraction of the bone cell population.

Maturation and Remodeling

One of the final steps in the development of a mature, multinucleated, bone-resorbing osteoclast is the polarization of the cell membrane before it can effectively generate the resorptive pits. This requires the production of two structures; the ruffled membrane, a villous organelle that covers the area in contact with bone, and an actin ring forming the "sealing zone", isolating the resorptive microenvironment from the exterior.

Without polarization and the cytoskeletal re-organization involved in forming the extracellular lysosome, proper osteoclast function is lost (Novack et al., 2008). While the precise mechanisms for induction of polarization are unknown, there are several pathways and key molecules that have been identified.

Before polarization, the osteoclast needs to confirm that it is indeed in contact with the bone surface. The integrin $\alpha_v\beta_3$ (the principal osteoclast integrin) is responsible for the recognition of mineralized matrix, and does so by targeting the RGD (arginine-glycine-aspartic acid) sequence conserved in osteopontin and bone sialoprotein (Sharp et al., 1999). Once activated, integrins can transduce a variety of intracellular signaling pathways, the activation of which can be mediated directly by the ligand itself, or by signaling downstream of growth factor receptors (Haas et al., 1994). Deletion of the $\beta3$ integrin subunit leads to osteopetrosis in mice, and has been shown to be necessary for osteoclast motility, adhesion to the bone matrix, and polarization of the resorptive machinery (McHugh et al., 2000, Novack et al., 2008).

Integrin contact with osteoclasts is unique when compared to the mechanism employed by most other cells. Typically, integrins mediate matrix contact through focal adhesions, containing signaling and cytoskeletal molecules, and lead to the formation of stress fibers. The osteoclast, however, organizes its fibrillar actin into sealing zones rather than forming stress fibers, and forms podosomes, instead of focal adhesions (Faccio et al., 2003).

In motile osteoclasts, the sealing zone is frequently being disassembled, and other, non-podosomal integrins move to lamellipodia (membrane extensions) at the leading edge of the cell. For efficient bone resorption, the osteoclast must be able to detach, migrate, and re-attach to form a sealing zone. It is for this reason that resorptive pits do not resemble isolated holes, but rather a chain of overlapping circular indentations. The integrins, and particularly $\alpha_v\beta_3$ are absolutely necessary for this process. c-Src is one of the best-identified associated signaling molecules, having first been studied in 1991 where c-Src -/- mice were severely osteopetrotic, yet expressing large numbers of osteoclasts. The problem these mice developed was that their osteoclasts lacked ruffled membranes and actin rings (Soriano et al., 1991, Boyce et al., 1992). Now it is known that c-Src regulates

osteoclasts both through its role as a kinase and as an adaptor molecule (Miyazaki et al., 2004)

Also notable is that the osteoclast cytoskeleton is influenced by the interaction between $\alpha_v\beta_3$ and M-CSF/c-Fms pathways. Through c-Fms, M-CSF activates the integrin by targeting its cytoplasmic domain, altering the conformation of the extracellular, ligand-binding region (Faccio et al., 2003). This can emulate to a degree the activation of the $\alpha_v\beta_3$ integrin.

Ion Channels and Transporters

The establishment of the sealing zone and ruffled membrane makes the ultimate goal of the osteoclast possible: to secrete acid and acidic hydrolases into these sealing zones that become the resorption lacuna. The high amount of acid secreted by the osteoclast is necessary to dissolve the hydroxyapatite that makes up the majority of the bone structure. It's also necessary for the acidic hydrolases, particularly cathepsin K, to become active and degrade the remaining organic matrix (Teitelbaum et al., 2003). The motor powering acid secretion in osteoclasts are the vacuolar (v-) type H+-ATPases. In addition, to prevent alkalinization of the cytoplasm, the protons and chloride ions that are being transported through the channels must be replenished. For this the osteoclast depends on the concerted actions of carbonic anhydrase, responsible for the conversion of CO_2 to bicarbonate and protons, and an anion exchanger. The development and utilization of ion transporters and channels is the hallmark of the adult osteoclast; the manifestation of weeks of development in response to cell signals.

The resorbing osteoclast also must have a mechanism in place for the regulation of transport of the breakdown products in the sealing zone. The acid and proteases it secretes solubilize large amounts of calcium (up to 40 mM), and other organic breakdown products (Silver et al., 1988). While the majority of this material is moved by trancytosis by the osteoclast to the other side of the cell, it is not possible to fully prevent some calcium and phosphate ions from entering the osteoclast (Salo et al., 1997). To cope with this, the osteoclast is known to employ several calcium transporting membrane proteins (Figure 5). Among these are voltage operated and ligand-gated Ca^{2+} channels, (opened at depolarized membrane potentials), Na+-Ca^{2+}-exchangers, and ryanodine receptor Ca^{2+} channels (Datta

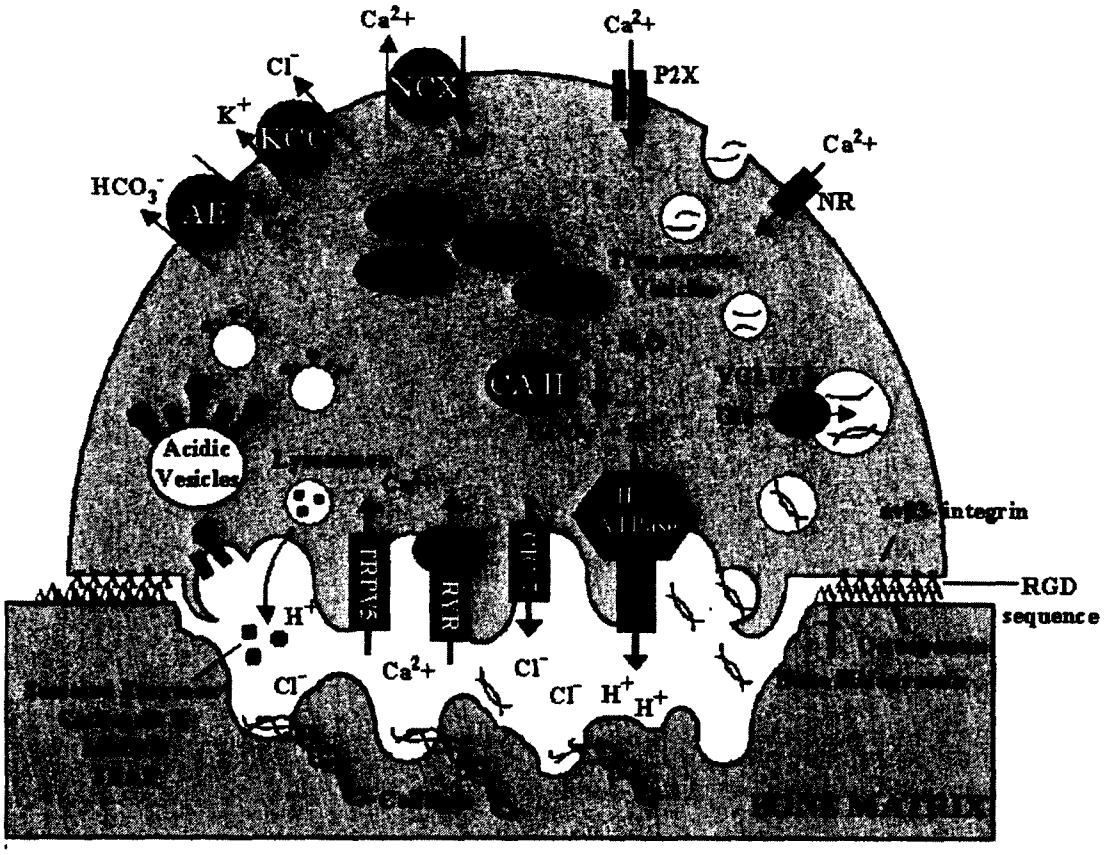
et al., 2003). In short, without the function of the calcium channels, osteoclasts are considerably less effective.

Osteoclastic bone resorption

The characteristic functional feature of the osteoclast is its capacity to dissolve bone mineral. The acidification process is initiated by carbonic anhydrase (CA) activity (Riihonen et al., 2007). The protons generated by this activity are transported into the resorptive microenvironment by the electrogenic proton pump (H+ATPase). It is the insertion of the acidified vesicles containing H+ATPase into the plasma membrane that causes the formation of the ruffled membrane, a phenotypically defining characteristic (Mattsson et al., 1994). A chloride/bicarbonate exchanger maintains the pH within the osteoclast, while electroneutrality is maintained by the presence of Cl^- channels. Thus, osteoclast-generated HCl is responsible for the acidification of the resorptive microenvironment. Once the mineral degradation is underway, the degradation of bone organic matrix (mostly type 1 collagen) can take place. This function is performed by many degradative factors including acid phosphatases (TRAP), matrix metalloproteinases (MMPs) and the lysosomal enzyme cathepsin K, which, like the ion channels necessary for acidification, polarize to the ruffled membrane after osteoclast attachment to the bone, to be as close as possible to the site of action (Mulari et al., 2003) (Figure 5).

Regulation of Activity

A panoply of diseases are associated with bone resorptive imbalance, related to both limited OC activity, as seen in osteopetrosis, or to excess, as with postmenopausal osteoporosis or chronic inflammatory bone diseases. Often, the osteoclast is the cell type targeted in treatment, by drugs classified as "anti-resorptive." Treatment with estrogen, raloxifene, and bisphosphonates is known to increase apoptosis of osteoclasts, and thusly inhibit their bone resorbing activity. Unfortunately, since the formation of bone is linked so tightly to signals received post-resorption, the complete termination of osteoclast activity still leads to reduced bone formation. Still, it is often a better alternative to accept this side effect than to leave the osteoclasts unchecked, like in the case of multiple myeloma, where massive resorption would otherwise take place. Therefore, detailed knowledge of the downstream signaling pathways and their molecular mechanisms, as



Adapted From: Rev Endocr Metab Disord 2006

Figure 5: Mechanisms of bone resorption by osteoclasts

The ruffled border, a resorptive organelle, is formed by the transport of vesicles containing H+ATPase to the bone-apposed surface of the cell following avb3 mediated cell attachment. Acidification is initiated by carbonic anhydrase, generating H and HCO3. H is transported out of the cell and into the resorption lacuna by an electrogenic proton pump (H+ATPase) located only in the ruffled border. Intracellular ph is maintained by a Cl/HCO_3 exchanger at the antiresorptive surface. Electroneutrality is maintained by a chloride channel (CLC7) located in the ruffled membrane that transports Cl into the resorptive lacuna. Calcium balance is maintained both by transporters at the antiresorptive surface and the bone-apposed surface (TRPV5, RYR, NCX, P2X, NR) The organic bone matrix, composed largely of type 1 collagen, is degraded by cathepsin K, an acid protease secreted into the resorptive space. **Adapted from**: Rev Endocr Metab Disord, 2006

well as the signaling molecules involved in osteoclastogenesis and even osteoblast activating signaling by osteoclasts is important. This knowledge may allow for generation of therapeutic agents that can affect osteoclast activity without overly disrupting bone formation. Recent studies have even shown through inhibition of c-SRC, the v-ATPase, or CLC-7 in osteoclasts that bone resorption decreases without interfering with subsequent bone formation (Boyce et al., 2006). Another relatively unexplored area is the role of Wnt signaling in osteoclasts, with nearly all publication on this subject coming in the last two years. Indirect Wnt pathways are better studied; for example, it is known that Wnt3a regulates osteoclast differentiation by down-regulation of RANKL expression and induction on osteoblasts, swinging the balance of RANKL and OPG against osteoclastogenesis (Spencer et al., 2005). However, these alternative treatments have only been performed and proven in animals, so induction of apoptosis in osteoclasts remains the most viable and proven treatment at the moment. It is in cases of disease, however, that we often see a modification in cellular response to apoptotic stimulation, often resulting in resistance to apoptosis. To further understand potential treatment methods, we must first understand the apoptotic process in health and disease, particularly in the bone microenvironment.

BONE: OSTEOBLAST

The major event preceding osteogenesis is the development of mesenchymal stem cells into differentiating, bone forming osteoblasts. This heritage separates the osteoblasts from their counterbalance, the bone-resorbing osteoclast. The osteoclasts arise from hematopoietic stem cells while osteoblasts are members of the same family tree under mesenchymal cells that give rise to bone, cartilage, fat, and fibrous connective tissue. Once osteoblasts, these cells eventually also give rise to bone-lining cells and osteocytes, depending on cytokine and biomechanical signals.

There are an abundance of factors and signaling pathways involved in osteoblast differentiation, including ATF4, transcription factor Runx2, Hedgehog, and FGF, but the two focal pathways are the Wnt and TGF-β signaling networks. There are three major stages of osteoblastogenesis: proliferation, matrix maturation, and mineralization, and each stage determines the function of the osteoblast at that time.

The cytokines involved in modulating osteoblast differentiation are extremely numerous, including TGF-β, several BMPs and their respective inhibitors like noggin, chordin, and others (Huang et al., 2007). Likewise, osteoblast function is known to be regulated by a number of hormones (and undoubtedly others yet unknown), including IGF-1, $1,25(OH)_2D$, PTH, PTHrP, IL-6, and the Notch pathway. Many of these cytokines and growth factors have contrary effects on osteoclasts, and cross talk of osteoblast/osteoclast signaling pathways remains a hot topic in research.

Signaling pathways in osteoblasts indirectly affect surrounding cells like the osteoclasts, as in the case of Wnt signaling, where Wnt affects fully differentiated osteoblasts by inducing OPG expression, thus regulating osteoclast function (Glass et al., 2005). Wnt in osteoblasts is known to be involved in crosstalk with TGF-β, an osteoclast-apoptosis inducer, as well (Houde et al., 2009, Mbalaviele et al., 2005).

Osteoblast function

Besides resorption, bone mass is determined by both the number of mature osteoblasts and their bone-forming activity/capability. The function of these osteoblasts can be separated into three bone-specific roles.

First is bone formation; the synthesis and subsequent secretion of the proteins that make up the extracellular matrix of bone (ECM), and following this, the expression of genes that are responsible for induction of mineralization of the ECM, the process that gives bone its characteristic hardened quality. The name for the unmineralized, organic portion of the bone matrix secreted by the osteoblast is osteoid, and it will eventually account for approximately 50% of bone volume. It is made up primarily of type 1 collagen, but also osteocalcin (OCN), chondroitin sulfate, matrix gla protein (MGP), osteopontin (OPN), bone sialoprotein (BSP), and growth factors like bone morphogenic proteins (BMPs) and TGF-β (Huang et al., 2007). As a way of regulation, the osteoblast will only deposit this osteoid on pre-existing mineralized matrix. While none of these molecules are unique to the osteoblast, it is the only cell type in the body that co-expresses their genes, making it a unique bone-depositing cell.

The second function of the osteoblast involves hematopoietic stem cell (HSC) expansion in the bone marrow, as osteoblasts are regulators of stem cell population (Calvi

et al., 2003). The third of these functions is the role played by osteoblasts in osteoclast differentiation, as previously discussed. In short, the two main cytokines necessary to trigger the signaling pathways for osteoclastogenesis and osteoclast activation, M-CSF and RANKL, are expressed in osteoblasts, particularly those of the active variety. Expression of these cytokines vary depending on the environment and maturity of the osteoblast. Likewise, expression of chemoattractants like CCL8, CCL6 and CCL12 (all of which are osteoclast-precursor recruiters) is modulated by calcineurin/NFAT signaling in osteoblasts, signaling that is increased post-bone deposition (Winslow et al., 2006). In a healthy system, the osteoblast and osteoclast preserve a delicate balance, each helping to regulate the presence and activity of the other so as to maintain bone homeostasis.

MULTIPLE MYELOMA: A DISEASE ASSOCIATED WITH A HIGH LEVEL OF BONE RESORPTION

Multiple myeloma is a B-cell malignancy characterized by the presence of a monoclonal population of plasma cells in the bone marrow. One prominent feature in multiple myeloma is the occurrence of skeletal events, including bone pain, lytic bone lesions, hypercalcemia, osteoporosis and pathological fractures (Bataille et al., 2004). The osteolytic lesions can extend from the inner bone marrow to the outer surface of the bone, weakening the bone, causing pain, and increasing the risk of fractures. They are typically localized at the spine, ribs, skull, and pelvis, but any site on the skeleton can exhibit lesions in cases of MM. The excessive bone resorption is generally observed in the vicinity of myeloma cells, and is linked to an increase in the number and activity of the osteoclasts (Bataille et al., 1991, Chappard et al., 1991). In turn, bone resorption and osteoclast activity further activate the myeloma cells, creating a "vicious cycle", as the proliferation and survival of myeloma cell has been shown to depend on osteoclast activity (Yaccoby et al., 2002). This excessive resorption is combined with tumor infiltration and correlates with the tumor burden (Roodman et al., 1997, Taube et al., 1992). These findings suggest that myeloma cells may stimulate bone resorption and thus are responsible for the increased bone resorption observed in multiple myeloma.

While it has been known for some time that media from human myeloma cells stimulates osteoclast activity, the particular osteoclast activating factors (OAFs) are not all known (Roodman, 2001). Likely candidates include IL-6, IL-1β, TNF-α, HGF, PTHrP, MIP-1α, and RANKL in particular (Roux et al., 2004). However, few of these have been identified in vivo due to other cells in the bone microenvironment that may also be responsible for cytokine production. For example, IL-6 has shown to not be expressed in highly purified myeloma cells (Chauhan et al., 1996). However, even while this is the case, it is under MM conditions that the cytokines are produced in excess, illustrating clearly the powerful effect of MM on the bone microenvironment in directing osteoclast activity. In fact, in the case of the same cytokine, IL-6, it has been shown that myeloma cells directly induce its release from stromal/osteoblastic cells (Barille et al., 1995)

One of the most critical pathways modulated in myeloma is the ratio of RANKL/RANK/OPG, a balance critical in regulation of osteoclast activity in both health and disease (Roux et al., 2004). Indeed, given that RANKL and OPG are established as critical role players in bone resorption regulation, many studies have been performed attempting to determine their role in MM bone destruction. The accumulation of myeloma cells in the bone marrow is associated with increased RANKL expression in the bone-marrow microenvironment. However, the cause of this RANKL over-expression is still controversial. Several studies, using immunohistochemistry performed on human bone-marrow specimens, biopsies or aspirates, have demonstrated an over-expression of RANKL in myeloma (Guiliani et al., 2001, Roodman & Dougall, 2008). RANKL expression has been detected in stromal cells, but not in myeloma plasma cells (Giuliani et al., 2001, Pearse et al., 2001, Roux et al., 2002). Furthermore, in cocultures of bone-marrow cells and myeloma plasma cells, adding tumor cells increased RANKL expression and inhibited OPG expression by the stromal component (Giuliani et al., 2001, Pearse et al., 2001). However, a few studies using human primary cells have reported that RANKL is expressed by the myeloma cells themselves (Sezer et al., 2002, Farrugia et al., 2003, Heider et al., 2003). Overexpression of RANKL and decreased OPG expression in myeloma are illustrated by the high levels of sRANKL and the low level of OPG measured by ELISA in the serum of patients with myeloma compared with their respective controls (Terpos et al., 2003). One interesting point to note is that induction of these responses was dependent on cell-to-cell contact, as another transwell system showed no effect on RANKL expression (Giuliani et al, 2002).

Finally, profound depression of bone formation is associated with osteolysis in myeloma bone disease (Giuliani et al., 2006). Myeloma cells could suppress osteoblast differentiation by decreasing the activity of Runx2/Cbfa1, the principal master transcription factor for osteoblast differentiation and bone formation (Giuliani et al., 2005). In addition, overproduction by myeloma cells of Dkk1, an inhibitor of the Wnt signaling pathway, has also been reported (Tian et al., 2003). The Wnt signaling pathway is best known for playing a major role in bone formation, but is also a strong inhibitor of bone resorption, acting by stimulating OPG expression in osteoblasts (Glass et al., 2006). Thus,

through production of Dkk1 and subsequent depression of OPG production, myeloma cells are able to further disrupt the RANKL/OPG balance (Figure 6)

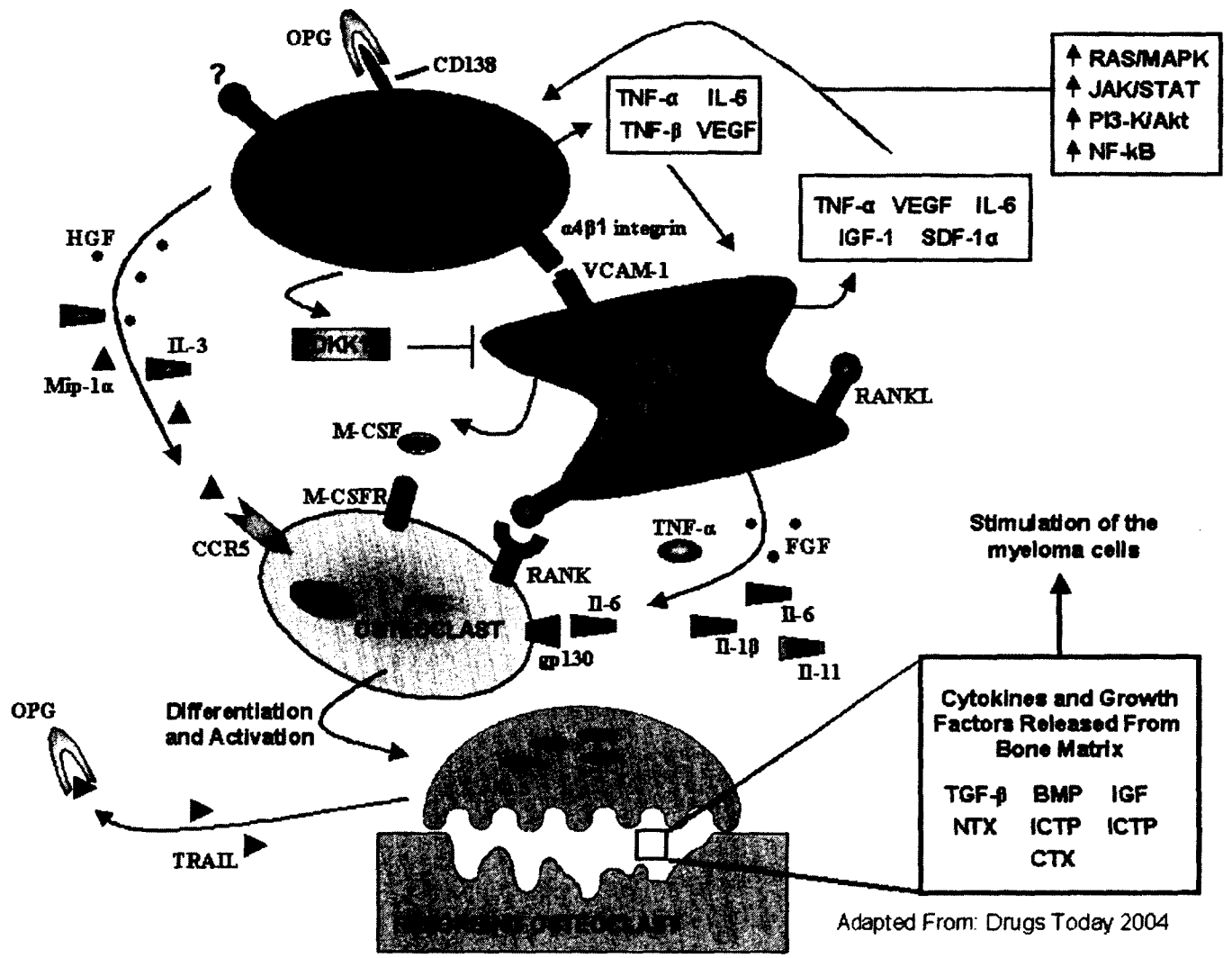


Figure 6: Interactions between myeloma cells, stromal cells, and osteoclasts in the myeloma bone micro-environment

Myeloma cells adhere to bone marrow stromal cells (BMSCs) through binding of α4β1 integrin (present on the surface of MM cells) to vascular cell adhesion molecule-1(VCAM-1) that is expressed on stromal cells. The adherence of MM cells to BMSCs/osteoblasts enhances the production of RANKL, M-CSF and other cytokines with osteoclast activity (IL-6, IL-11, IL-1b, TNFs, bFGF), while it suppresses the production of OPG (the decoy receptor of RANKL). The above cytokines also modify the bone marrow micro-environment, up-regulating RANKL expression and secretion by both stromal cells and

osteoblasts. Furthermore, myeloma cells produce MIP-1α, HGF and VEGF that enhance the proliferation and differentiation of osteoclast precursors. MIP-1α can also activate integrins to further induce cell adhesion. All these interactions lead to osteoclast differentiation, proliferation and activation and to increased bone resorption, reflected by the increased levels of bone resorption markers (TRACP-5b, NTX, ICTP, CTX). Summarized, these results emphasize the multiple complex interactions between myeloma cells, BMSCs, and the osteoclast. **Adapted from**: Drugs Today, 2004

Regardless of the pathway taken, the results of advanced multiple myeloma on the bone microenvironment are devastating, and treatment depends on either disrupting the signaling molecules involved or terminating the cells responsible for the increase in resorption, the MM cells and the osteoclasts.

APOPTOSIS: GENERAL

Apoptosis, or programmed cell destruction, is characterized by distinct morphological changes, and regulated by a specifically ordered series of biochemical events leading to cell death. The morphological changes include several major characteristics; condensation and fragmentation of nuclear chromatin, shrinkage of the cell, and changes to the plasma membrane that result in recognition and phagocytosis of the apoptotic cell (Arends et al., 1991). The biochemical events in apoptosis have been split into two phases that are less distinct than one might think; the initial commitment phase in which the apoptotic signal is received and the signal is first propagated, and the execution phase during which the characteristic morphological changes take place and the cell is irrevocably committed biochemically (Takahashi et al., 1996)

Cells in health and in disease both constantly maintain a balance between pro- and anti-apoptotic gene expression. The signal initiating the death of a cell, whether it comes from internally (intrinsic) or externally (extrinsic), calls for the co-operation of a cascade of downstream signaling events before apoptosis can take place. It is a tightly regulated process because it is not one that the body would want to execute unintentionally, leading to great waste. However, it is extremely important, as the system often needs to rid itself of cells in excess, in the way, malfunctioning, invading, or otherwise potentially dangerous.

This dedicated molecular program allows the organism to control cell numbers and types, helping foster a healthy, properly functioning environment.

The major workers in apoptosis are a group of cysteine proteases that are homologous to each other, part of a protein family known as the caspases (Budihardjo et al., 1999). Eliminating caspase activity slows significantly or stops altogether the process of apoptosis (Earnshaw et al., 1999). All of the caspases possess an active-site cysteine, and work by cleaving substrates at bonds after aspartic acid residues. The four amino-acid residues terminal to the cleavage site determines the substrate specificity of each caspase, and thusly the subfamilies into which they are divided (Thornberry, 1997). Interestingly, the activation of one or more type of caspase is not the direct source of degradation of cellular proteins, but instead the cleavage of a restricted set of target proteins (Hengartner, 2000). In many cases, this leads to an inactivation of the target protein, by splitting or total disassembly. But just as importantly, caspases can also activate proteins (especially other caspases), either directly by cleaving off a negative regulatory domain, or indirectly by simply cleaving off a regulatory subunit. Their work is responsible for most of the characteristic features of apoptosis; the cleavage of nuclear lamins necessary for nuclear shrinking and budding, loss of cell shape caused by cleavage of certain specific cytoskeletal proteins, and cleavage activation of the DNA ladder nuclease (CAD) (Enari, 1998, Rao et al., 1996, Buendia et al., 1999). These are just a few of the mechanisms put into place by caspase activation, with over a hundred already identified (Hengartner, 2000)

The Extrinsic Pathway

The death-receptor (or extrinsic) pathway is first activated by the binding of a member of the death receptor superfamily; for example CD95 ligand (Fas) to the CD95 receptor, or TRAIL to TRAIL-R1. Binding of the receptor induces a clustering (typically a trimerization), and subsequent formation of a death inducing signal complex, dependent on uniting the intracellular death domains of each receptor subunit (Curtin et al., 2003). Via the adaptor molecule Fas-associated death domain protein (FADD), the death complex recruits multiple procaspase-8 molecules, bringing them close enough together to induce auto-cleavage by proximity (Zhang et al., 2004). This step can be blocked by recruitment of the caspase homologue c-FLIP (Scaffidi et al., 1999). From here, the activated caspase-8

cleaves procaspase-3, activating it and beginning the caspase cascade to apoptosis (Figure 7)

The Intrinsic Pathway

The mitochondrial (or intrinsic) pathway is employed by cells often in response to extracellular stimuli and internal deterioration like DNA damage (Rich et al., 2000). Though there are many possible pathways, they each converge on the mitochondria, usually through activation of one of the pro-apoptotic members of the Bcl-2 family. Pro-apoptotic signals redirect and activate (via proteolysis, dephosphorylation, and other mechanisms,) the other Bcl-2 family members like Bax, Bim, Bad, and Bid from the cytoplasm to the mitochondria, where they have the chance to play a role in propagating the apoptotic signal (Gross et al., 1999, Adams et al., 1998). At the surface of the mitochondria, pro- and anti-apoptotic Bcl-2 family members will meet, competing to inactivate one another and regulate the mitochondrial response. Should the pro-apoptotic proteins be greater in number or effectiveness, an array of molecules is released from within the mitochondria (Rich et al., 2000). The most important among these is cytochrome c, which in turn associates with Apaf-1, changing its conformation and allowing it to form a heptamer, which binds to procaspase-9. Once in the complex, procaspase 9 is cleaved and activated, forming the active apoptosome (Czerski et al., 2004). It is at this point that the intrinsic and extrinsic pathways converge, with the activation of caspase-3, -6, or-7; the effector caspases which will cleave other vital intracellular proteins, advancing the cell through apoptosis. The activation and activity of caspase-3 can be antagonized by endogenous inhibitors of programmed cell death, members of the inhibitor of apoptosis (IAP) family. However, when the intrinsic pathway is activated, or with significant caspase activation post-apoptosome formation, the mitochondria releases the Smac/DIABLO protein, an inhibitor of the IAPs (Adrain et al., 2001). It is at this point that the cell is effectively committed to apoptosis.

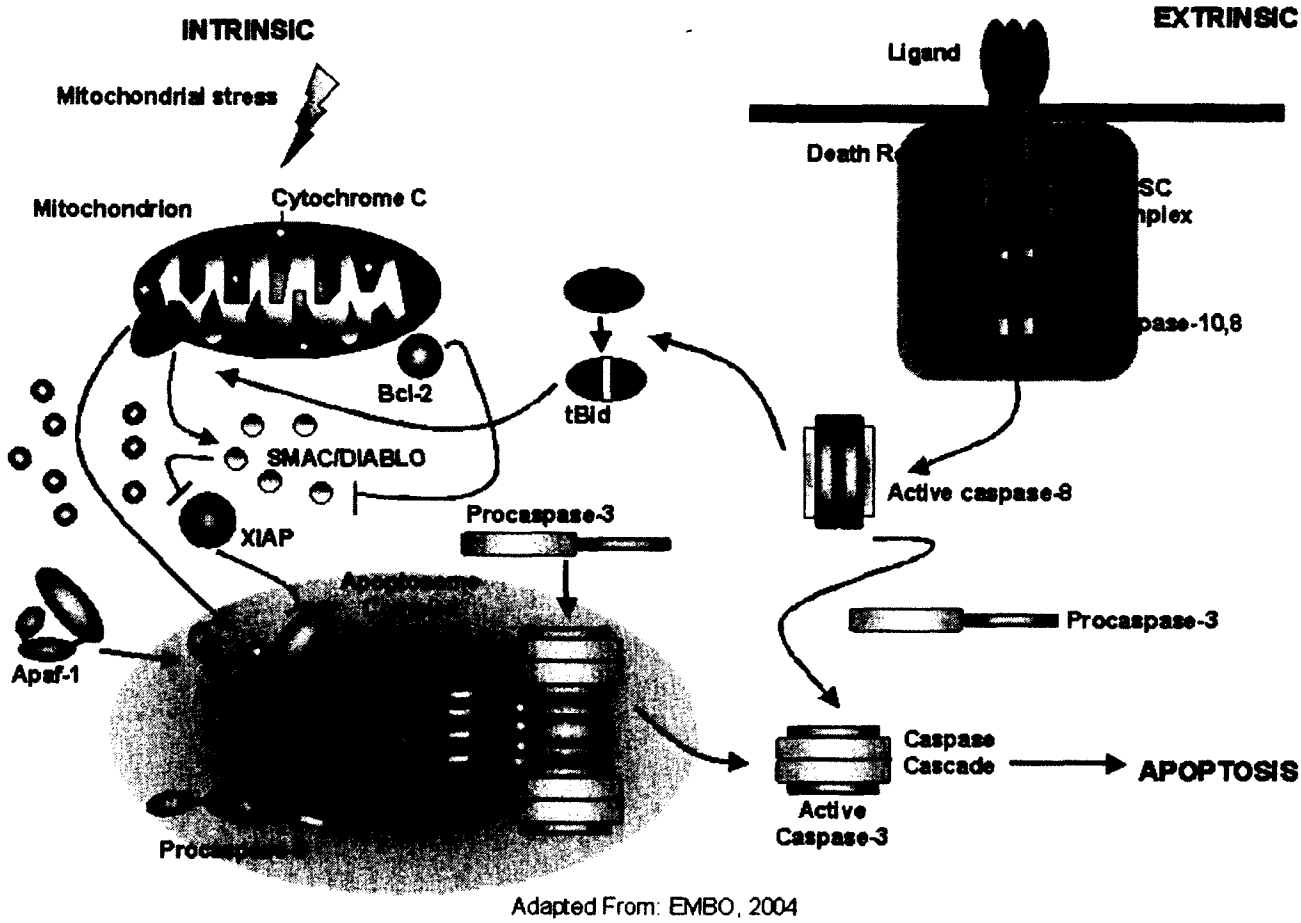


Figure 7: Apoptosis: intrinsic and extrinsic pathways to programmed cell death

The two major apoptotic pathways are illustrated: one activated via death receptor activation (extrinsic) and the other stress induced (intrinsic). Triggering of cell surface death receptors of the tumor necrosis factor (TNF) receptor superfamily, like CD95 or TRAIL-R1/-R2 results in activation of the initiator caspase 8 after its recruitment to a trimerized receptor-ligand complex (DISC) through the adaptor molecule Fas-associated death domain protein (FADD). In the intrinsic pathway, stress-induced apoptosis results in agitation of the mitochondria and the ensuing release of proteins, such as cytochrome *c*, from the inter-mitochondrial membrane space. The release of cytochrome *c* from mitochondria is partly regulated by Bcl2 family members, with both anti-apoptotic (Bcl2/ Bcl-XL/Mcl1) and pro-apoptotic (Bax, Bak and tBid) members. They inhibit or promote release of cytochrome c, respectively. Once released, cytochrome *c* binds to apoptotic protease-activating factor 1 (Apaf1), which results in formation of the Apaf1–caspase 9 apoptosome complex and activation of the initiator caspase 9. The activated initiator caspases 8 and 9 (depending on pathway of origin) then activate the effector caspases 3, 6 and 7, which are responsible for the cleavage of important cellular substrates, resulting in the classical biochemical and morphological changes associated with apoptosis. **Adapted from**: EMBO, 2004

APOPTOSIS: REGULATION OF REMODELING

Apoptosis is a key regulator of skeletal development and maintenance, and maintenance of those molecules that moderate it is critical. While it can be induced by growth factors, hormones and other cytokines, the effect of the stimulant depends on the cell to which it binds.

The numbers of osteoblasts are maintained in a steady state by progression to osteocytes and bone-lining cells, but even more frequently, simply by apoptosis. Unlike osteoclasts, despite the fact that nearly 70% of bone-depositing osteoblasts undergo this process, it is rare to observe apoptotic osteoblasts exhibiting chromatin clumping or nuclear fragmentation (Landry et al., 1997). This makes visual quantification and analysis of osteoblast apoptosis comparatively difficult. The osteoblasts must be identified by morphology and location, and specific techniques like TUNEL or ISEL are used to detect DNA breaks (Silvestrini et al., 1998). Apoptotic osteocytes are easier to identify, exhibiting condensed chromatin and degraded DNA, but naturally, more difficult to locate and observe. Unlike with osteoblasts, apoptosis of the osteocyte is indicative of the "death" of the region of bone for which it is responsible to maintain. New osteocytes cannot simply take the place of the old, unlike many other cell types.

It is known that CD95 and its ligand are both expressed in osteoblasts and osteocytes in vitro, as well as TNF and TRAIL receptors, and that stimulation of these cells with TNF or FasL induces apoptosis (Hatakeyama et al., 2000, Tsuboi et al., 1999). However, the exact roles of these receptors in normal physiologic apoptosis are unknown. An example of the importance of balance of apoptotic receptors is seen in osteoblasts in bone samples from postmenopausal women. These osteoblasts have increased expression of Fas mRNA and protein, as well as increased sensitivity to Fas ligand-induced cell death (Garcia-Moreno et al., 2004). Quickly it becomes clear that by modulating just osteoblast survival, the onset of osteoporosis can be pushed forward or back; one receptor can possibly directing an entire pathogenesis.

The osteoblasts can also be de-sensitized to apoptosis, notably by growth factors like FGF, TGF-β and others (Debiais et al., 2004, Jilka et al., 1998). The pathways can be rather direct; for example, IGFs upregulate the molecule calbindin-D28k, which binds to

caspase-3, inactivating it in the process (Wernyj et al., 1999). This blocks TNF-induced apoptosis in the osteoblast-lineage cells (Bellido et al., 2000). The pathway taken by FGF is less direct, involving PI3-kinase activity and promotion of the anti-apoptotic signal (Debiais et al., 2004). TGF-β, a molecule of interest, inhibits osteoblast apoptosis by decreasing the ratio of Bax to Bcl-2 (Bu R et al 2003). Highlighting TGF-β's importance, the deletion of Smad3, a necessary intracellular signaling protein associated with TGF-β, leads to decreased bone formation due to shorter osteocyte and osteoblast lifespan (Borton et al., 2001). TGF-β doesn't just regulate apoptosis by inhibiting it in bone modelers, it directly induces apoptosis in the resorbing osteoclasts through the Smad3 pathway (Houde et al., 2009). The matrix metalloproteinases (MMPs) that break down collagen have been associated with survival as well, probably through the release of growth factors associated with the collagenous matrix as bone is being processed (Zhao et al., 2000). In a healthy system, this helps maintain the rapid post-resorption modeling response. Other factors known to block or diminish the effects of TNF-induced apoptosis include fluid shear stress and certain BMPs (Chen et al., 2001, Pavalko et al., 2003)

However, not all growth factors regulate apoptosis in a positive manner for osteoblasts. BMP-2, for example, is a stimulator of bone formation. It is also capable of promoting osteoblast death by inducing expression of various caspases and the release of cytochrome-c from the mitochondria. This pathway has been shown to be dependent on PKC activation (Hay et al., 2001). Mechanical strain on the osteocytes can also induce apoptosis, leading to a call for resorption through site-specific repair signals. Once the osteocytes have detected changes in bone strength and microdamage through their canalicular system, it is believed that they self-induce apoptosis (Parfitt et al., 1996).

Osteoclast apoptosis is also heavily regulated, with many negative and positive regulators of their life span already identified. PTH and vitamin D3, for example, are associated with stimulation of bone resorption, and perhaps not coincidentally, are also associated with osteoclast survival and activation. The likely culprit here is their common mechanism, the elevation of expression of RANKL, the osteoclast activator, and decreased expression of osteoprotegerin (OPG), its regulatory partner (Gori et al., 2000). When PTH binds to osteoblasts, they increase RANKL expression. Likewise, D3 modulates

transcription of Runx2, the transcription factor in osteoblasts also responsible for RANKL expression.

Of course, RANKL is also associated with osteoclast activation, but M-CSF, IL-1 and TNF also support osteoclast survival. As described in the section on osteoclasts, after RANKL/RANK interaction, TRAF 6 recruits Src, PI3K, and Akt. Akt phosphorylates Bad and caspase-9, preventing apoptosis (Wong et al., 1999). Given that they are members of the same TNF family, IL-1 and TNF trigger a similar cascade to prevent apoptosis (Xing et al., 2001). This mechanism is not involved in M-CSF mediated survival though, despite the fact that M-CSF also activates PI3K, Akt, and Erk to propagate its signal (Bhatt et al., 2002). It is not fully understood, but believed that it is through Erk that M-CSF increases expression of anti-apoptotic genes for Bcl-2, Bcl-xL, and XIAP (Kanaoka et al., 2000). Since these genes are also upregulated by TNF signaling, it is possible that Erk is a modulator for cell survival in the other pathway as well.

Osteoclast apoptosis is not induced by calcitonin, which does induce their detachment and inhibit resorptive activity. This indicates that apoptosis is not the sole method by which osteoclastic resorption is stopped (Kallio et al., 1972). However, it can be induced by nitric oxide, through protein kinase A and eventual caspase-3 activation (Kanaoka et al., 2000). Apoptosis is also inducible by TNF-α signaling, though it is a much more potent osteoclastogenic factor.

There is now increasing evidence suggesting that changes in the regulation of osteoclast death may contribute to clinically important bone diseases, and that the induction of osteoclast apoptosis is a potential therapeutic tool for treating them (Roux, S. and Brown, JP., 2009). One intriguing apoptosis inducer in osteoclasts is TRAIL, or TNF-related apoptosis-inducing ligand (Roux et al., 2005). This mechanism is the next to be explained.

TRAIL: GENERAL

Tumor **N**ecrosis **F**actor-**R**elated **A**poptosis-**I**nducing **L**igand (TRAIL) was first identified in 1995 based on sequence homology to other members of the TNF superfamily (Wiley et al., 1995). Also identifiable as Apo-2 Ligand, TRAIL is a type II transmembrane

protein showing high homology to CD95L, with 28% of its amino acid identity shared (Pitti et al., 1996). A couple of the more interesting and unique qualities of TRAIL were also identified in these early studies. Perhaps the most important of these was that TRAIL was shown to induce apoptosis in transformed or tumerogenic cells, but not in healthy, normal cells, making it a fascinating potential target for cancer treatment. The other surprising quality of TRAIL is related to its expression. While other members of the TNF family are often only transiently expressed on activated cells, and have tightly regulated expression, TRAIL was shown to have constitutively expressed mRNA in a wide range of tissues (Wiley et al., 1995). Because of this, first assumptions were that its receptor would be restrictively expressed, or capable of inducing apoptosis in only certain biological circumstances. This was partially true, but regulation of TRAIL-induced apoptosis has shown to be far more complex than just receptor or ligand regulation. TRAIL is now known to have five receptors, only two of which signal for apoptosis, and not consistently in all tissues. Much about TRAIL's apoptotic and non-apoptotic inducing signaling pathways and their biological effects remains to be uncovered.

While it is believed that TRAIL may have other secondary functions, its best-characterized feature is that of apoptotic induction (Figure 7). Of the TRAIL receptors, TRAIL-R1 (DR4) and –R2 (DR5) are the two who possess death domains (DD) in their intracellular segment. This is a six or seven (respectively) α-helix motif that can bind to other DDs through homotypic interactions (Lahm et al., 2003). This is necessary because trimeric TRAIL binds to the receptor, inducing a receptor homo- or heteromerical trimerization (Mongkolsapaya et al., 1999). Upon trimerization of the death domains, the Fas-associated death domain protein (FADD/Mort1) is recruited to them. FADD is able to act as an adaptor protein from this location, as binding at its DD causes a conformation change in the molecule, opening it up to allow for binding to its death effector domain (DED) (Newsom-Davis et al., 2009). This DED can now be bound by caspase-8 and/or –10, as well as cellular FLICE inhibitory protein (cFLIP; believed to function identically to caspase-8) to bind through their respective death domains. This results in a multi-protein complex called the Death Inducing Signaling Complex (DISC) (Kischkel et al., 1995, Sprick et al., 2000). Formation of the TRAIL DISC promotes the conversion of pro-caspases into their cleaved, active forms, so that they may in turn activate other

downstream caspases. While it is known that caspase-8 and –10 are cleaved in the same manner, the importance of caspase-10 to the DISC is still the subject of controversy (Kischkel et al., 2001). One study has shown that its expression can restore apoptosis sensitivity to caspase-8-deficient cells, while another has shown the opposite (Sprick et al., 2002). It is known, though, that through the DISC, caspase-8 is autocatalytically cleaved, generating a large and a small subunit. These subunits form active hetero-tetramers that activate the downstream effector caspases –3 and -7, inducing the destructive effects of apoptosis (Stennicke et al., 1998)

Some molecules in TRAIL signaling are anti-apoptotic regulators as well: while the two caspases present in the DISC are pro-apoptotic, cFLIP actually inhibits caspase activation at the DISC through competitive binding at the DED domain. cFLIP shares a very high sequence homology with these initiator caspases, yet does not necessarily initiate all the same actions. It contains two DED domains that are similar in structure to the N-terminal of pro-caspase-8, and a C-terminus with catalytically inactive caspase-like domains (Golks A et al 2005). It is the DED domain region that is recruited to the DISC, by homotypic DED interactions (Thome M et al 1997). By blocking availability to the receptor, cFLIP inhibits caspase-8 activation, and TRAIL induced apoptosis. However, there is one isoform of cFLIP is considered to be neither pro- nor anti-apoptotic, preventing binding of procaspase-8 but aiding in the autocatalytic activation of those procaspase-8 molecules already at the DISC (Micheau O et al 2002)

While this extrinsic route was the first pathway to apoptosis associated with TRAIL, more recent studies have also elaborated on its ability to trigger the mitochondrial release of other pro-apoptotic proteins (the intrinsic route). There is, in fact, a need for this alternate/additional activation as well. This is because cells can be grouped into two types, based on their reliance on mitochondria for the activation of caspases. The cells classed as Type II require DISC-mediated caspase activation followed by inactivation of the X-linked inhibitor of apoptosis protein (XIAP), which binds to caspases to prevent their action. The Type I cells do not require this extra step (Barnhart BC et al 2003)

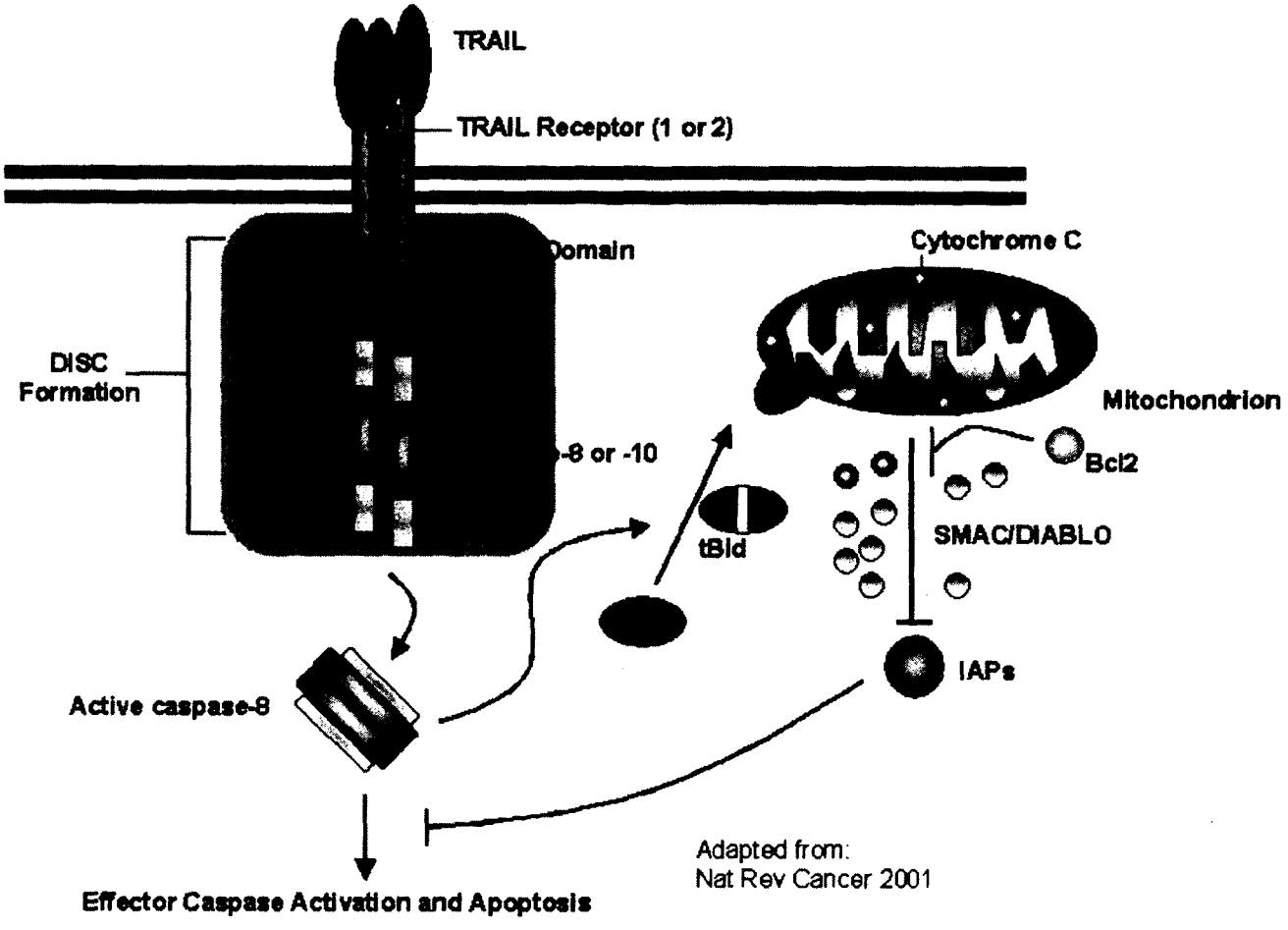


Figure 7: TRAIL and apoptotic induction

Trimerized TRAIL binds TRAIL-R1 or –R2, allowing for recruitment of FADD to the death domain (DD), which in turn recruits procaspase-8, forming the DISC, resulting in cleavage and activation of caspase-8. This action can also lead to the cleavage of Bid. TRAIL-induced truncated BID targets mitochondria, which causes rapid release of SMAC/DIABLO into the cytosol. Here, it binds to inhibitor of apoptosis protein (IAP) family members and reverses their inhibitory effects on activated caspase-3 and caspase-9. This simultaneously strengthens the extrinsic response and incorporates components of the intrinsic apoptotic pathway, making TRAIL a powerful apoptosis inducer. Release of SMAC/DIABLO from mitochondria can be inhibited by anti-apoptotic BCL2 family members. **Adapted from**: Nat Rev Cancer, 2001

The distinction between these two classifications of cells lies in the expression of anti-apoptotic molecules that may vary between cell types and lines, as well as differences in efficiency of the DISC formation (Newsom-Davis et al., 2009). Many cell lines can even

vary between type classifications, depending on their XIAP expression or the amount of the apoptotic agent in circulation (Jost et al., 2009)

To make TRAIL's double pathway activation possible in Type II cells, the Bcl-2 homology domain 3-interacting domain death agonist (Bid) plays a major role, connecting the direct apoptotic signal cascade from the extrinsic cascade to the starting point of the intrinsic pathway (Billen et al., 2008). Once caspase-8 is activated, it can cleave full length Bid, producing a truncated form of Bid (tBid). tBid in turn binds to the mitochondria, activating Bax and Bak to destabilize the integrity of the mitochondria's outer membrane. As already explained, this leads to release of cytochrome c, along with other pro-apoptotic proteins, importantly the natural antagonist of XIAP, known as second mitochondria-derived activator of caspases/direct IAP binding protein with low isoelectric point (Smac/DIABLO) (Waterhouse et al., 2002). Quickly the apoptosome is formed and XIAP neutralized. From this stage the progression of Type I and II cells is identical, with the activation of downstream effector caspases no longer put on hold.

NATURAL PREVENTION OF TRAIL-INDUCED APOPTOSIS

As previously noted, TRAIL possesses 3 other receptors that do not signal for apoptosis. Two of these are membrane bound proteins with surface homology similar to that of the two death receptors. TRAIL-R3 (LIT, decoy receptor R1 [DcR1]) binds to its ligand in the same manner as the death receptors, but lacks completely the DD, possessing a glycosyphosphatidylinositol (GPI) anchor in its place. TRAIL-R4 (TRUNDD, DcR2), on the other hand, is more complicated and perhaps the least well understood of the four membrane-bound receptors, expressing a truncated DD that allows alternate signaling. Studies in cells exhibiting over-expression of TRAIL-R4 have shown an induction of NF-κB signaling after stimulation with TRAIL (Degli-Esposti et al., 1997). It is for these qualities that the non-apoptosis inducing receptors are called "decoy" receptors, binding TRAIL molecules that would otherwise be available to target TRAIL-R1 and –R2, inducing apoptosis. In fact, they have also interfered with DISC formation in over-expression systems, going further than simple competitive binding to prevent apoptotic TRAIL signaling. In this study, TRAIL-R4 was co-recruited to TRAIL-R2, inhibiting DISC formation and subsequent caspase-8 activation (Merino et al., 2006). However, these

mechanisms have as yet only been observed in the aforementioned over-expression conditions, and remain to be confirmed in a true physiological setting. TRAIL resistance could be a symptom of impaired transport of DR4 and DR5 to the cell surface, effectively preventing the ligand from binding its death receptors (Fesik, 2005). It has been shown in one TRAIL-resistant cell line that DR4 is not recruited to the DISC and caspase-8 could not be activated following TRAIL treatment (Jin et al., 2004). TRAIL also bind the soluble receptor OPG, previously discussed as a major regulator of bone formation through its function as a decoy receptor for RANKL. However, whether the TRAIL/RANKL/OPG relationship has a physiologically important role appears less likely, as TRAIL knockout mice have a normal phenotype and proper bone density.

TRAIL: IMMUNOSURVEILLANCE

Innate and Adaptive Immune Systems:

Many members of the TNF and TNF-R superfamily have been shown to exert important functions in the immune system and immunosurveillance. Among these functions is the induction of apoptosis, necessary for the removal of autoreactive T and B cells, killing of infected cells by cytotoxic T lymphocytes, and down-modulating the immune response after an infection has been brought under control (Opferman et al., 2003). Interruption or reduction in apoptosis caused by these members of the TNF superfamily can mean severe consequences for the system as a whole, leading to disease spread, cancer or autoimmunity, to name a few potential problems. Not long after its discovery in 1996, TRAIL has been a molecule of interest in these models. It was quickly discovered that TRAIL is expressed on a variety of cells of the innate and adaptive immune systems. TRAIL and its receptors have been identified as one of the three death-receptor/ligand systems (FasL and TNF-α being the others) that regulate intercellular apoptosis in the immune system (Wu, 2009). In fact, in different systems, TRAIL was shown to have a huge variety of effects, between immunosuppression, immunoregulation, pro- or antiviral action, and tumor immunosurveillance (Lunemann et al., 2002, Song et al., 2000).

Up-regulation of TRAIL is a common and powerful mechanism employed by the immune system to induce apoptosis in a variety of cell types and situations. TRAIL

expression in the innate and adaptive immune systems varies depending on the stimulation status of the cells. Its up-regulation is seen on monocytes and macrophages after stimulation with lipopolysaccharide (LPS) as well as with interferon-b (IFN-b) (Ehrlich et al., 2003, Halaas et al., 2000). Not only that, interferon-c (INF-c) can induce expression of TRAIL on the cell surface of monocytes, dendritic cells (DCs) and natural killer (NK) cells (Fanger et al., 1999, Griffith et al., 1999). NK cells use surface-bound TRAIL as one of their effector mechanisms, proven by a study in which only the combined neutralization of TRAIL, CD95L and perforin could block the NK cell-mediated death of tumor cell lines (Kayagaki et al., 1999). These results have also been confirmed in vivo, showing TRAIL is critical in NK cell-mediated tumor cell-growth suppression. During development, TRAIL is predominantly expressed in liver NK cells. In the adult liver, there is also a subpopulation of NK cells as a result of the autocrine production of IFN-γ (Takeda et al., 2001). Synergistic activity between IFN-γ and TRAIL has been shown to down-regulate c-FLIP, sensitizing cells to apoptosis, making the two a potent combination (Stefanescu et al., 2008). TRAIL has also been found on IFN-γ-producing killer dendritic cells (IKDCs), linking IFN-γ TRAIL-induction to both the innate immune system and the adaptive immune system (Chan et al., 2006). Expression of TRAIL at the mRNA level has also been detected in peripheral lymphocytes after activation with monoclonal anti-CD3, an activator of T cells (Jeremias et al., 1998). In fact, Th1 cells are more sensitive to TRAIL-induced apoptosis than Th2, which may have to do with CD3-binding-mediated up-regulation of c-FLIP in Th2 cells (Roberts et al., 2003)

TRAIL and CD95L have been linked in several ways to T helper 1 and 2 (Th1 and Th2) responses. TRAIL has been detected on CD4+ and CD8+ T cells after T-cell receptor (TCR) stimulation in combination with type 1 IFNs, indicating that type I IFNs can regulate TRAIL-mediated T-cell toxicity (Kayagaki et al., 1999). This is an interferon dependent surface expression, making TRAIL unique from other apoptosis inducing ligands, like FasL (CD95L), which can be expressed by CD3/TCR stimulation alone (Kayagaki et al., 1999). Another difference between the two is that post-CD3 stimulation, Th1 cells will up-regulate CD95L, where Th2 cells express TRAIL. TRAIL regulates more than apoptosis, with gene disruption leading to inhibition of DC and Th2 cells to allergy

sites in mice (Weckmann et al., 2007). This led to reduction in Th2 cytokine release and a subsequent release in inflammation.

TRAIL has also been hypothesized to act as a regulator of hematopoiesis. Hematopoietic progenitor cells don't express TRAIL receptors, protecting them from TRAIL-induced apoptosis, while immature erythroblasts do (Zauli et al., 2006). In multiple myeloma, a decrease in the expression of TRAIL-R1, TRAIL-R2 and TRAIL itself help lead to stimulation of erythropoiesis (Grzasko et al., 2006). This effect is possibly a compensatory one, and does not significantly impact production, as one of the hallmarks of multiple myeloma is impaired erythropoiesis.

TRAIL and autoimmunity

It is possible that TRAIL is an important factor in several autoimmune diseases, and if nothing else, is a potent modulator of autoimmune responses. While TRAIL -/- and TRAIL-R -/- mice do not spontaneously develop autoimmunity, treatment of autoimmune diseases with TRAIL has been successful in several animal models (Falschlehner et al., 2009). In one study, gene transfer of TRAIL prevented collagen-induced arthritis, while blocking TRAIL led to an increase in severity (Song et al., 2000). In another arthritis study, mice receiving collagen-treated TRAIL-expressing DCs had diminished joint inflammation compared with controls. These mice exhibited a lowered T-cell proliferation and IFN-γ production, suppressing the arthritis (Liu et al., 2003). When TRAIL induces apoptosis in Th1 cells, there is a correlated reduction of IFN-γ, which in turn regulated chemokine production. With a reduction in these chemokines, there is a lesser infiltration of mononuclear cells and lymphocytes, reducing the inflammatory reaction. In rheumatoid arthritis, TRAIL has been shown to induce both apoptosis and proliferation of fibroblast-like synoviocytes, the characteristic cell type in the disease (Morel et al., 2005).

TRAIL -/- mice are also more susceptible to autoimmune arthritis and to diabetes (Lamhamedi-Cherradi et al., 2003). In non-obese diabetic (NOD) mice, diabetes development was accelerated in the TRAIL -/- mice (Lamhamedi-Cherradi et al., 2003 II). While it is known that for these diseases the participation of macrophages and T cells are required, it is not known which cells in a healthy system may be responsible for the anti-autoimmune effects of TRAIL, or if it even plays a part outside of disease models.

Interesting non-apoptotic effects that have been identified include the prevention of autoreactive T cells in experimental autoimmune encephalomyelitis (EAE). Blockage of TRAIL led to higher CNS inflammation levels, but apoptosis levels of inflammatory cells did not change, indicating that TRAIL's mechanism in this instance is prevention of their activation (Hilliard et al., 2001). Ironically, blockage of TRAIL led to a reduction in clinical severity, as TRAIL also contributes to neural damage during brain inflammation (Aktas et al., 2005). The dual role of TRAIL is not by any means exclusive to this model, as seen in many other systems where it has proven to be a double-edged sword.

TRAIL AND CANCER TREATMENT

TRAIL also plays a role in controlling tumor growth through its effects on the immune system. Exogenous TRAIL has been established as a potent potential anticancer therapeutic, capable of killing tumor cells selectively without negatively affecting normal cells (Walczak et al., 1999, Ashkenazi et al., 1999). TRAIL expressed on IFN-γ-stimulated NK cells has even been shown to be able to prevent experimental metastasis of tumor cells by apoptosis induction (Takeda et al., 2001). Lymphomas and carcinogen-induced tumors grow faster in TRAIL -/- or TRAIL-R -/- conditions (Finnberg et al., 2008).

TRAIL is potent in metastasis-specific tumor surveillance. In mouse models, lymph node metastases were greatly enhanced upon removal of TRAIL-R, as the TRAIL was ordinarily responsible for both sensitizing the cells to apoptosis by loss of adhesion, then inducing apoptosis directly (Grosse-Wilde et al., 2008)

However, even when the apoptosis-inducing TRAIL receptors can be detected, the majority of human primary tumor cells are only partially sensitive or completely desensitized to TRAIL-induced apoptosis. This presents a handicap in the use of TRAIL as a therapeutic molecule in many diseases. The good news is that in many cases, treatment by chemotherapy or with other biological agents can often sensitize the tumor cells while leaving normal cells largely unchanged (Koschny et al., 2007). In fact, the use of chemotherapeutic and biological agents to sensitize tumor cells to TRAIL-mediated apoptosis is now a common practice. The combination approach also has the additional advantage of potential synergistic activity, increasing treatment efficiency. The greatest

danger with combination treatment, however, is that some can induce apoptosis in non-malignant cells like hepatocytes, lymphocytes and osteoblasts (Koschny et al., 2007).

Several different types of cytotoxic chemotherapies have been shown to sensitize tumor cell lines to TRAIL-mediated apoptosis (Shankar et al., 2005). Of these, some have already entered clinical trials (Newsom-Davis et al., 2009). The mechanisms governing this adapted sensitization to TRAIL are complex. While TRAIL can engage the extrinsic and even the intrinsic apoptotic pathway on its own, chemotherapeutic agents and radiation preferentially induce cell death via the p53-dependent, intrinsic pathway (Newsom-Davis T et al 2009). Interestingly, they also induce transcriptional changes in the proteins involved in TRAIL signaling (Wajant et al., 2004). The sensitizing agents do so by lowering the apoptotic threshold through an augmentation in DISC formation. This is facilitated by an up-regulation of pro-apoptotic molecules like the death receptors, caspase-8, FADD, Bax and Bak, while simultaneously down-regulating anti-apoptotic molecules lie cFLIP-ligand, IAPs, Bcl-XL, Mcl-1 and Bcl-2 family members (Held et al., 2001).

TRAIL has also been combined with proteasome inhibitors to induce apoptosis (Sprick et al., 2002). It is believed that their combinatorial effect is achieved through TRAIL expression on NK cells, though this does not completely explain the increased sensitivity (Hallett et al., 2008). Combination of TRAIL and a proteasome inhibitor is currently undergoing clinical studies in treatment of advanced multiple myeloma, targeting the myeloma cells. Proteasome inhibition enhances mitochondrial (intrinsic) pathway activation, releasing cytochrome c as well as Smac/DIABLO to antagonize XIAP function. XIAP, briefly discussed above, is highly expressed in primary cells but significantly reduced in transformed cells, allowing for its inhibition to result in caspase-3 cleavage, helping trigger apoptotic cascades (Leverkus et al., 2003). Other potential mechanisms by which proteasome inhibitors could sensitize cells to TRAIL include repression of c-FLIP and/or p53, or by inducing caspase and Bax expression (Zhang et al., 2004). While studies have shown up-regulation of TRAIL-R1 and/or –R2 by chemotherapy as well as proteasome inhibition, this increase has not yet been proven to be the cause of sensitization. This was demonstrated in one study in which the absence of TRAIL-R1/R2 up-regulation did not reduce the sensitizing effect (Koschny et al., 2007)

TRAIL has also been combined with histone deacetylase inhibitors (HDACi), a group of compounds that can induce differentiation, cell cycle arrest, and apoptosis in tumor cells. This combinatorial treatment augments TRAIL-killing in sensitive lines and even sensitizes TRAIL-resistant tumor cell lines (Rosato et al., 2003, Nebbioso et al., 2005). The HDACi increase TRAIL-R and pro-apoptotic molecule expression while decreasing expression of anti-apoptotics, much like chemotherapeutics (Rosato et al., 2003). Another emerging treatment is with mimetics of Smac, molecules that target and inhibit XIAP, cIAP-1 and –2, helping strengthen the TRAIL signal. They have been shown to promote TNF-α-mediated cell death by promoting formation of a RIP-dependent caspase-8 cleaving complex, but have mixed results with TRAIL (Vince et al., 2007). Smac mimetics have been demonstrated to synergize with TRAIL, inducing apoptosis in cancerous cell lines, but have also been shown to activate canonical and non-canonical NF-κB pathways. While NF-κB signaling is strongly associated with survival, particularly in osteoclasts, this research indicates that perhaps activation of its non-canonical pathways could be pro-apoptotic (Varfolomeev et al., 2007). Another final combinatorial potential treatment is with PI3K-Akt inhibitors, given that signaling through this pathway negatively regulates TRAIL-induced apoptosis (Secchiero et al., 2003). However, despite its great promise, little data is currently available on the efficacy of this method.

Despite all of the information gathered in these treatments, the question as to why only tumor cells are typically sensitized to TRAIL-induced apoptosis remains. Fundamental differences between neoplastic and healthy cells, as well as their interaction with the cancellous microenvironment remain to be determined before a concrete answer can be given. Rather than focus on this, some research has concentrated on targeting TRAIL even further to the tumors. This is done by use of fusion proteins, where the extracellular domain of TRAIL is attached to a single chain variable antibody fragment selected for the tumor site (Bremer et al., 2004).

TRAIL: OTHER SIGNALING PATHWAYS

TRAIL is capable of inducing apoptosis in myeloma cells both in vitro, making it a solid candidate for therapy (Gazitt, 1999). While malignant plasma cells express TRAIL receptors, there appears to be no relation between TRAIL sensitivity and its pattern of

receptor expression (Lincz et al., 2001). While apoptosis was induced in vitro, the same profile is not observed in vivo in MM patients. It is possible that the increase in NF-κB signaling observed protects against TRAIL-induced apoptosis (Ni et al., 2001). While combination with proteasome inhibitors has already been discussed, it is possible that direct inhibition of the NF-κB signaling pathway may reverse myeloma cell TRAIL-resistance (Mitsiades et al., 2001). However, TRAIL's relation with NF-κB is even more direct than inhibition via other pathways.

The NF-κB family contains five proteins: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. These transcription factors specifically bind to DNA sites as dimers. Their activity from this point is regulated by interaction with a host of inhibitory IκB proteins, each having a different affinity for the separate members of the NF-κB family. The major cellular form of NF-κB is a heterodimer between p50 and p65, inhibited by IκBα, which masks its nuclear localization signal and holds it in the cytoplasm. In order to liberate NF-κB, an IκB kinase complex (IKK) phosphorylates IκBα. This phosphorylation leads to its poly-ubiquitinylation and subsequent proteasomal degradation (Zandi et al., 1997). NF-κB over-expression is seen in the progression of several cancers, including multiple myeloma (Dolcet et al., 2005). NF-κB blocks TNF-α-induced apoptosis by activation of c-FLIP either directly or through inhibition of JNK (Russo et al., 2001). While it has not been officially established, since TNF-α and TRAIL are members of the same family, it is very possible that NF-κB inhibits TRAIL through the same or a very similar pathway.

This makes it all the more intriguing that TRAIL is believed to induce NF-κB activation. TRAIL induced NF-κB activation in HeLa and 293 cells but not in MCF7 cells, indicating that TRAIL's influence is cell type dependent (Sheridan et al., 1997). This has been further resolved by over-expression of DR4 and DR5 in 293T cells, leading to an induction of NF-κB activation (Schneider et al., 1997). It is believed that after initial caspase activation through the extrinsic signaling route, parts of the primary signaling complex, FADD and caspase-8 join with RIP1, TRAF2, and IKKγ (or IKKα and caspase-10 in place of IKKγ), forming a secondary complex (Jin et al., 2006). This secondary complex is hypothesized to activate the IKK, allowing for NF-κB activation and transcription. In fact, besides activating the NF-κB pathway it is also believed to signal

through MAPK, JNK and p38 (Varfolomeev et al., 2005). The NF-κB induction has been correlated with TRAIL resistance in multiple cell lines, supporting the proposal of its activity as TRAIL-regulatory (Ravi et al., 2006, Zwacka et al., 2000).

Participation by the TRAIL-R4 (DcR2, TRUNDD) could also provide further pro-survival signaling through NF-κB, though this has not yet been studied extensively. It is known that both isoforms of TRAIL-R4 have a truncated death domain, and are incapable of propagating the apoptotic signal. However, it is possible that upon ligation with TRAIL, this domain may be capable of inducing the same IKK activity as is observed in DR4 and DR5 binding.

Notably, NF-κB is not the only method of TRAIL resistance, and has even been related to increased sensitivity, depending on the NF-κB transcription that was induced. One recent article demonstrates that melanoma cells' resistance to TRAIL is linked to their down-regulation of initiator caspases and DR4 (TRAIL-R1). However their findings did not correlate with induced NF-κB activation, indicating that this pathway was independent (Kurbanov et al., 2007). In this same study, several melanoma cell lines were studied, and those showing TRAIL-induced NF-κB activation were highly sensitive to TRAIL-induced apoptosis. In these cells, the NF-κB activation did not change expression levels of the antiapoptotic proteins like cFLIP and XIAP normally associated with NF-κB activation. The authors went as far as to demonstrate that acquisition of TRAIL resistance in these particular cell lines could be linked to reduced NF-κB activation, further reinforcing the earlier hypothesis that some TRAIL-induced NF-κB signaling could be non-canonical and pro-apoptotic (Kurbanov et al., 2007)

TRAIL's activation of the IKK complex likely also has further consequences, as the IKK complex has also been shown to be able to activate the rapamycin (mTOR) pathway by phosphorylating mTOR repressors (Plantivaux et al., 2009). mTOR is a PI3 kinase that regulates cell growth, proliferation, motility, survival, and other factors (Hanna et al., 2008). Like with NF-κB, activation of the mTOR pathway can lead to cFLIP over-expression, further augmenting TRAIL resistance (Panner et al., 2005). However, mTOR is also a negative feedback regulator of Akt and NF-κB, complicating its role as both a negative and positive regulator of TRAIL-induced apoptosis (Wu et al., 2007)

HYPOTHESIS AND AIM OF THE STUDY

TRAIL has been established as an effective killer of osteoblastic cells and myeloma cells in vitro and in vivo models. In 2005, the laboratory of Dr. Roux published the findings that human osteoclasts are susceptible to TRAIL-induced apoptosis, and that in vitro they express both TRAIL-R1 and TRAIL-R2, as well as TRAIL-R3 and –R4 decoy receptors (Roux et al., 2005).

In a previous study, TRAIL-R3 and TRAIL-R4 were found to be strongly expressed in osteoclasts from myeloma bone lesions, whereas the expression of TRAIL-R1 and TRAIL–R2 was weak or absent (Roux et al., 2005). It is of interest to note that none of these receptors were detected in normal bone, suggesting that *in vivo* the TRAIL pathway could be induced in osteoclasts under conditions characterized by a high level of osteoclast formation. The over-expression of the decoy receptors (TRAIL-R3 and TRAIL-R4) could possibly decrease osteoclast apoptosis, and contribute to the high level of osteoclast activity observed in myeloma. These findings are consistent with data showing that osteoclasts from PBMC generated in the presence of myeloma T-cells over-express TRAIL-R4, and display reduced expression of TRAIL-R1 (Colucci et al., 2004), suggesting induced-resistance to TRAIL apoptosis.

It can be assumed that some factor in MM is responsible for this shift; be it one or more cytokines working alone or in concert, cell-cell interaction, or some combination therein. Thus several candidate molecules, known to be overexpressed in MM and to contribute to the excessive bone resorption observed in the disease were chosen (Roux et al., 2004). Among them, RANKL and MIP-1α, major osteoclast-activating factors (OAF) involved in the pathogenesis of myeloma-induced osteolysis, were selected for experimentation in human osteoclasts. We also selected OPG and TGF-β for their role in osteoclast apoptosis (Houde et al., 2009, Chamoux et al., 2008), and PTH as a differential expression of TRAIL-R has been found in biopsies from patients presenting with an excessive production of PTH (unpublished results).

We have thus investigated the impact of adding these factors in osteoclast cultures, to determine whether they may be responsible for changes in TRAIL-receptor expression

at mRNA and protein levels, and if these changes would in fact impact their susceptibility to apoptosis. In addition, TRAIL expression was also evaluated, as TRAIL has been shown to be produced by osteoclasts (Chamoux et al., 2008), and to influence TRAIL-R expression (Colucci et al., 2007).

The goal of this study was to determine whether local factors or cytokines that are overexpressed in bone diseases associated with a high level of bone resorption such as multiple myeloma, might be responsible for modulation of TRAIL receptors in osteoclasts, which may in turn desensitize them to apoptosis. The acquisition of knowledge of the mechanisms of TRAIL-R induction and its modulation in human osteoclasts might better help us directly target and treat bone resorptive disorders through TRAIL treatment.

MATERIALS AND METHODS

Reagents

Cytokines, growth factors, recombinant human TRAIL (rhTRAIL), rh M-CSF, rhGM-CSF, and OPG were purchased from R & D Systems. Antibodies for DR4 and DR5 were furnished by Abcam, DCR1 by Millipore, HRP-anti-goat/sheep and DCR2 by Santa Cruz. Antibodies for β-actin and HRP-anti-rabbit were produced by Cell Signaling, while HRP-anti-mouse, the molecular ladder, and the Ficoll-Paque were from Amersham Biosciences. Finally, the DMEM, antibiotics, and Fetal Bovine Serum are Wisent products, while OPTI-MEM was purchased from Gibco.

Culture of Human Umbilical Derived Cord Blood Monocytes:

Consent is obtained from the mother before birth. Immediately after delivery, the blood resting in the umbilical cord is extracted to a sterile 50 mL tube containing heparin. The average yield of cord blood extracted is around 30 mL. After obtaining and mixing the blood, it is stored at 4° Celsius until a member of the laboratory can retrieve it. Given the sensitivity of the cells involved, this period of time rarely exceeds a few hours. After diluting at 1:1 volume in D-MEM, the mononuclear leukocytes are separated by gradient density centrifugation, using Ficoll-Plaque. The suspension of low density (compared with the red blood cells) contains the mononuclear cells (monocytes and lymphocytes) and is found at the interphase between the serum and Ficoll. This cellular fraction is removed and washed by centrifugation in D-MEM. Following this, the pellet of cells is re-suspended in D-MEM. The cells are again washed in a solution of D-MEM and the pellet is re-suspended in 3 ml of OPTI-MEM afterwards. The remaining MNCs are counted via Hemacytometer, after dilution of a sample, and addition of an 8% v/v solution of 80g of NH_4Cl in PBS 1X to lyse any remaining red blood cells and of Trypan blue for the exclusion of non-viable cells.

From this stage, the MNCs are re-suspended in a culture medium comprised of OPTI-MEM, with 2% Fetal Bovine Serum (FBS; Gibco), and an antibiotic/antifungal mix (PSF): 1% penicillin/streptomycin and 0.2% amphotericin B. The volume of this dilution is determined by the number of cells obtained, so as to give a concentration of $3x10^6$ cells/ml in suspension. This suspension is then plated and placed in the incubator at 37° C in a humidified atmosphere at 5% CO_2 overnight. The following day, the media is removed, taking with it the non-adherent cells, and is replaced with the same growth medium (OPTI-MEM, FBS 2%, PSF 1.2% dilution) described above, with the addition of GM-CSF 100 pg/ml. The cells are left to incubate in this media for a period of three days in the incubator. Afterwards, the media is exchanged for the growth medium with M-CSF 25 ng/ml and laboratory produced RANKL at 100ng/ml to induce osteoclastic development. This media is renewed twice a week over a period terminating 21 days after originally being placed in culture. At this time, there is a significant presence of well-differentiated, mature human osteoclasts

Expression and purification of RANKL:

The expression vector pGEX is transformed in competent E.coli BL21 bacteria. After obtaining a high quantity of bacteria capable of producing RANKL, a colony is inoculated in tubes containing 50 mL of LB medium with 50 μg/mL of Ampicillin. These tubes are incubated with agitation at 37° C until the following day. These cultures are then added to 500 mL of fresh LB medium supplemented with Ampicillin, and incubated at 37° C until the solution has reached an optic density (OD) between 0.6 and 0.8 at a wavelength of 595 nm. At this point, the addition of 2 mM of IPTG to the bacteria allows the induction of expression of the desired proteins. These cultures are incubated with agitation at 22° C until the following day. The bacteria are recovered by centrifugation at 3000 rpm for 25 minutes at 4° C. The pellet of cells is re-suspended in a lysis buffer at 4° C (PBS 1X, 1% Triton X-100, 1/1000 CLAP, 1/1000 PMSF, pH 7.4). The bacterial lysis is carried out for six cycles of sonication on ice for 30 seconds at 35% intensity. The cellular lysate is then centrifuged at 13000 rpm (20000 g) for 15 minutes at 4° C. The supernatant is recovered and glutathione-sepharose beads (GE Healthcare) are added to it. These beads

have been pre-rinsed with cold PBS 1X. The mix is agitated by rotation at 4° C for 30 minutes, followed by a centrifugation at 15000 rpm (20000 g) for 5 minutes at 4° C. Now, the supernatant is discarded and the beads are rinsed 8 times with cold PBS 1X. These washes are each performed identically- with 8 mL of cold PBS 1X added to the beads for 5 minutes of agitation, terminating with centrifugation so that the supernatant may be discarded. After the washes, the beads are transferred to an eppendorf tube with 250 μL of elution buffer (20 mM glutathione in 50 mM Tris-HCl, pH 8.0), and incubated under agitation for 20 minutes at room temperature. This tube is centrifuged at 5000 rpm (5000 g) for 5 minutes and the supernatant is recovered in another tube. The elution process is repeated 4 more times. All of the eluted fractions are analyzed by SDS-PAGE, and the protein concentration is determined with the aid of the RC DC protein assay kid (Bio-Rad). The fractions are combined and stored in dilutions of 100 μl at –80° C. The biological efficacy of the RANKL protein is verified in cell culture via resorption assays on both a plastic surface and bone slices.

Isolation and Differentiation of Pre-Osteoclasts:

The methocult growth suspension is a semi-solid medium used to stimulate the development of CFU-GM from the monocyte/macrophage precursors collected, and aids in the proliferation of mononuclear cells after extraction from the donor umbilical cord. The cells spend 11 days in this culture and approximately a 15-fold increase in numbers can be expected.

This procedure begins after the step in cell culture described above (page 58) in which the mononuclear cells are counted. The cells are re-diluted into a suspension of $1.25x10^6$ cells in 500 μL of OPTI-MEM 2% FBS, 1.2% PSF. Using a syringe and a 16 G$1^1/_2$ needle, 5 mL of methocult is placed in a 50 mL tube (Falcon), and then the suspension is transferred to the tube containing the media. The tube is sealed and vigorously vortexed so that the cells are equally distributed. Afterwards, the cap is removed and the tubes are left for 10 minutes to let the bubbles formed by vortexing disperse. With another syringe, 1.1 mL of methocult/cell suspension is plated in a 35 mm Petri dish. Two dishes are placed a 100 mm Petri dish along with a third 35 mm Petri

filled with H_2O so that the medium does not dry out, and the Petris are left to incubate at 37° C in a humidified atmosphere at 5% CO_2 for a period of 11 days.

For the extraction of the mononuclear cells from methocult, 3 ml of PBS 1X is added to each Petri and mixed to dissolve the media. This solution is removed to a 50 mL tube (Falcon), and each Petri is subsequently washed with another 2 mL of PBS 1X, which is also recovered to the same tube. Once is it determined that the recovered cells have been sufficiently well mixed, the tube is centrifuged at 1500 rpm for 10 minutes. The supernatant is discarded and the pellet is re-suspended in 50 mL of D-MEM 1.2% PSF, to be centrifuged for another 10 minutes at 1500 rpm. Now the cells are re-suspended in 3 mL of OPTI-MEM 2% FBS, 1.2% PSF with M-CSF 25 ng/mL and laboratory produced RANKL at 100ng/mL, counted following the same procedure outlined above (page 58) and plated at a concentration of 350,000 cells/mL, and the cells are treated henceforth as cells in the 11^{th} day of culture; to be changed each three days with the same concentration of cytokines (M-CSF 25 ng/mL, RANKL 100ng/mL). The culture contains mature, resorbing osteoclasts by the 10^{th} day.

Apoptosis evaluation via TUNEL:

For the detection and quantification of apoptosis in the osteoclasts, the TACS Blue Label kit from R&D Systems was used. This TUNEL-derived technique allows for the visualization of internucleosomal DNA degradation through cytochemistry.
The cells are cultured following the protocol described (page 58), initially 3x10^6 cells/mL in the chambered well slide (Lab-Tek), 0.4 ml per well. At the end of the culture period, and any stimulation that may have taken place during, the media is removed and the cellules are put into deprivation through the addition of a less rich growth medium (OPTI-MEM 1%FBS, 1.2% PSF) without the growth factors it had been treated with. The cells are left in the incubator under previous temperature and CO_2 conditions listed (page 58) overnight. At this time, the cells are treated with TRAIL at 15 ng/ml to induce apoptosis. After 24 hours of treatment, the cells are rinsed three times with cold PBS 1X, then fixed over a period of 10 minutes at –20° C with a solution of 2:1 (ethanol):(glacial acetic acid). This fixing solution also permeablizes the cell membranes. The fixation is followed by

eight 15-minute washes with HBS 1X to re-hydrate the cells. The positive control wells (2 μL Nuclease diluted in 100 μL of Nuclease buffer) are incubated for 20 minutes at 37° C. The other wells are filled with PBS 1X during this time. Following this step, the walls of the culture slide are removed and the slide is left for five minutes at room temperature in a peroxidase solution (5 mL H_2O_2 30% in 45 mL Methanol). Now, the slides are submerged again in PBS 1X for 3 repetitions at 5 minutes each, then treated with the TdT Labeling solution (5 mL of TdT Labeling Buffer 10X in 45 mL nanopure H_2O) for five minutes. Immediately following, the slide is placed in a humid chamber for a period of 2 hours at 37° C with 20 μL of the reaction mix (3.6 μL of TdT-dNTP, 3.6 μL of Manganese, and 3.6 μL of TdT enzyme in a volume of 180 μL of TdT Labeling Buffer.

After these 2 hours of incubation, the slide is left for five minutes in the STOP TdT solution (5 mL of STOP TdT Buffer 10X in 45 mL of nanopure H_2O. The slide is then dipped in nanopure H_2O three times to wash off excess solution, and the sides are dried so that the Streptavidin-HRP solution may be added (1 μL of Streptavidin-HRP in 375 μL of Blue-Streptavidin-HRP diluent). This incubation period lasts 30 minutes at room temperature, and is followed by three more washes in nanopure H_2O. At this stage, the sides of the slide are again dried, and the nuclear blue staining is performed by addition of the TdT Blue-label solution for 5 minutes. The marking takes place through an enzymatic reaction forming an insoluble, blue precipitate with fragmented DNA. The slide is washed once more and counter-stained with nuclear FAST-red for 30 minutes to give a pink color to the non-apoptotic nuclei that remain. To completely remove any traces of water before mounting the slide, it is dipped in a series of three solutions of ethanol at rising concentrations each time (75%, 95%, and 100%) and finally once in oxy-xylene. The slide is then mounted with the hydrophobic medium Permount (Fisher), and the cover is sealed with nail polish.

Now the staining can be observed by using the light microscope, and apoptotic quantification can take place by manually counting and comparing the multinucleated cells. For the purpose of this study, any cells with more than 3 nuclei are considered to be osteoclasts. Those with blue nuclei are considered apoptotic, allowing for a percentage comparison to be formed of apoptotic MNCs versus total MNCs.

Western Blot Protein Analysis:

The Western blot allows for the study of the expression of proteins throughout the cell after protein extraction. To recuperate the amount of protein desired the cells are cultured in larger wells (12-multiwell plate, Falcon) and 1.5 mL of the cell suspension is added on the first day. At the end of the cell culture period of 21 days, the cells are washed 3 times with cold PBS 1X and the wells are treated with 50 μL each of lysis buffer (1% NP-40, 50 mM Tris HCl, 0.25% Sodium Deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μg/ml Protease Inhibitors (Roche), 1 mM $NaVO_4$, 1 mM NaF, pH 7.4). After 10 minutes on ice the wells are scraped with a rubber policeman and the suspension is transferred to a 1.5 mL microcentrifuge tube. For the following 10 minutes the tubes are left on ice and mixed by inversion periodically. Afterwards, the tubes of lysate are centrifuged at 13000 rpm at 4° C, and the pellet is discarded. The proteins resting in the supernatant are transferred to another tube and dosed (Bio-Rad).

The dosage is performed as follows: In a 96 well plate a standard curve from 0 to 10 is made with BSA 1mg/mL. (0-1-2-3-4-6-8-10 in triplicate, in which the number represents the μL of BSA added, completing each volume of BSA to 10 μL with H_2O. 2 μL of each protein solution is added in triplicate to other wells on the plate, and also completed to 10 μL with H_2O. 20 μL of Reagent A (20 μL of solution S and 1 mL of Reagent A) is added to each well, followed by 200 μL of Reagent B. The plate is left to develop for five minutes then placed in a microplate reader set to measure absorbency of light at 595 nm (Titertek Multiskan MCC/330). From the standard curve the concentration of each protein sample can be calculated.

A quantity of 50 μg of the proteins is placed in a tube completed to 40 μL with H_2O, and 10 μL of Sample buffer 5X is added (20% Glycerol, 140 mM SDS, 2% β-mercaptoethanol, 150 mM bromophenol blue, 25% Concentrator buffer [35 mM SDS, 100 mM Trizma Hydrocholoride (Sigma), pH 6.8]). This solution is heated at 95° C on a Multi-Blok heater (Lab Line) for five minutes and immediately spun down then transferred to ice. The samples are then loaded into an SDS-PAGE 12% gel in an electrophoresis machine filled with Electrophoresis buffer (250 mM Trizma Base (Sigma), 2000 mM Glycine, 35 mM SDS), and run at 135 V for 90 minutes. At this time the gels are

transferred from the machine to a bath of Transfer buffer (25 mM Trizma Base (Sigma), 200 mM Glycine, 3.5 mM SDS, Methanol 20%) for ten minutes. During this same time frame, 6 sheets of 3 mm chromatography paper (Whatman) are also placed in the bath. The PVDF membrane (Roche) is activated for 30 seconds in methanol and placed in H2O for 2 minutes, then is also bathed in the transfer buffer. After the ten minutes have expired, the membrane and gel are placed in between the chromatography papers in the semi-dry transfer machine (Bio-Rad). The machine is set to an alternating current at 45 milli-Amps per membrane, and run for 70 minutes. At this time, the proteins have been transferred to the surface of the membrane, where detection via immuno-labeling will be possible. Now that the transfer is completed, the membrane is placed immediately into blocking solution comprised of TBS-Tween 1X(2.42g/l Tris base, 8 g/L NaCl, 0.1% Tween 20 (Amersham Biosciences), pH 7.5) and 5% of either powdered skim milk, BSA, or Gelatin, depending on the antibody used. After 2 hours of blocking to prevent background binding, the primary antibody directed towards the protein of interest is introduced at a specific dilution in the particular blocking solution. The concentrations of antibodies (primary and secondary) used are provided on Table 1. The primary antibody is left to incubate at 4° C overnight in a sealed pouch on a rotisserie shaker (Thermolyne). The following day, the membrane is washed 3 times with TBS-Tween 1X at room temperature for 15 minutes on a rocking machine (Stovall). After the third wash, the secondary antibody in a solution of blocking buffer is added to the membrane in a second pouch, and the membrane is rocked on the same machine for a period of 3 hours at room temperature. Now the membrane is removed and washed again 3 times following the same protocol. At this point, the protein-specific antibody binding is visualized by chemiluminescence (ECL, Amersham Bioscience). Enhanced Luminol and Oxidizing reagent are mixed in equal quantities to put 500 μL on the surface of the membrane, which is placed in the Versadoc (Bio-Rad). This measures light intensity produced by the binding of the reagents to secondary antibody bound to the membrane, indicating prescence of the target protein. The intensity of the bands is then quantified with the Quantity One program associated with the Versadoc Machine.

For the re-use of these membranes so that we might observe different protein expression through use of another antibody, the membrane must first be stripped. After

exposure in the Versadoc, the membrane is placed in TBS-Tween 1X and then transferred to a stripping solution (7.56 g/L Trizma Base, 20 g/L SDS, 7 m/L β-mercaptoethanol, pH 6.7). The stripping is carried out for 30 minutes in a water bath at 55° C. Afterwards, the membrane is washed a minimum of 6 times in TBS-Tween 1X for 10 minutes each. After the final washing, a new Western blot can be performed starting at the blocking step.

Table 1: Western blot antibody concentrations and solutions:

Antibody	Dilution	Blocking Solution
Anti-TRAIL R1	1/400	TBS-Tween/Milk 5%
Anti-TRAIL R2	1/350	TBS-Tween/Milk 5%
Anti-TRAIL R3	1/250	TBS-Tween/BSA 5%
Anti-TRAIL R4	1/100	TBS-Tween/Gelatin 5%
Anti-Rabbit-HRP	1/1000	TBS-Tween/Milk 5%
Anti-Mouse-HRP	1/500	TBS-Tween/Milk 5%
Anti-Goat/Sheep-HRP	1/1000	TBS-Tween/Milk 5%
Anti-Actin	1/500	TBS-Tween/Milk 5%

Immunocytochemistry:

For the detection of cell surface expression of receptors, the LSAB2 kit from Dako was used. The cells were not permeabilized so that any staining observed should be representative of cell surface expression only.

The cells are cultured following the protocol described (PAGE 58), initially 3x10^6 cells/mL in the chambered well slide (Lab-Tek), 0.4 ml per well. At the end of the culture period, and any stimulation that may have taken place during, the media is removed and the cells are rinsed 3 times with PBS 1X and fixed with PFA 3.7% for ten minutes at room temperature. Following this, the cells are rinsed 3 more times with PBS 1X and stored at 4° C with PBS 1X in a humid chamber until the following steps can be performed.

Before staining, the walls of the culture slide are removed and the slide is left for five minutes at room temperature in a peroxidase solution (5 mL H_2O_2 30% in 45 mL PBS 1X). Now, the slide is submerged in PBS 1X for 3 repetitions at 5 minutes each, and then

placed in a protein-blocking solution (Dako) for 90 minutes, so that non-specific binding can be avoided as much as possible. Immediately afterwards, the antibody of choice is diluted to its optimal target concentration (TABLE 2) in Antibody diluent (Dako) and applied to each well. One well is left untreated with the target antibody, and instead a concentration of equal molarity of an IgG of the host animal for the primary antibody is applied. This well serves as a negative control to show any basal reactivity with the secondary antibody. The slide is placed in a humid chamber at 4° C and left overnight to incubate. The following day, the slide is washed three times in PBS 1X for 10 minutes on a rocking machine (Stovall), then the slide edges are dried and two drops of secondary antibody (Biotinylated Link Universal, Dako) are placed on each well for 90 minutes. After this incubatory period, the slide is again washed 3 times in PBS 1X at 10 minutes intervals with rocking. Following this, each well is now covered with two drops of peroxidase-labeled streptavidin (Dako) for 30 minutes. The streptavidin binds to the secondary antibody to amplify the signal further over any background staining later. The slide is subjected to a final series of 3 washes with PBS 1X as described, and the edges are dried so that several drops of chromogen AEC (Dako) may be used to stain each well, for a period varying between 3 and 7 minutes. The chromogen binds to the streptavidin, staining red those cells that are positive for surface expression of the target protein. The slide is rinsed once in PBS 1X and covered in a 1:1 dilution of Mayer's Hematoxylin (Dako) and H_2O for 1 minute and promptly rinsed. The Hematoxylin stains bluish-purple the nuclei (and faintly some cell structures), allowing the differentiation between mono- and multi-nucleated cells. The slide is then mounted with a 1:1 dilution of PBS 1X and glycerin, and the cover slip is sealed with nail polish.

Now the staining can be observed by using the light microscope, and manual counting of positive and negative staining relative to the negative control. As with the apoptosis assays, cells of 3 or more nuclei were considered to be osteoclasts, and the others were excluded from the data.

Table 2: Immunocytochemistry Antibody Concentrations:

Antibody	Dilution	µg/µl
Anti-TRAIL R1	1/400	5 ng/ml
Anti-TRAIL R2	1/350	4.5 ng/ml
Anti-TRAIL R3	1/250	4 ng/ml
Anti-TRAIL R4	1/100	0.8 ng/ml
Anti-Rabbit-HRP		
Anti-Mouse-HRP		
Anti-Goat-HRP		

RNA extraction and Real-time PCR:

RNA extraction was performed with the RNeasy plus kit (Qiagen). RNA was kept at –80° C and sent to the Laboratoire de Génomique Fonctionnelle de l'Université de Sherbrooke for analysis. Controls of housekeeping genes *39S ribosomal protein L19, mitochondrial*, *pumilio homolog 1*, and *60S ribosomal protein L13a* were performed simultaneously to ensure accuracy.

Statistical Analysis:

The Wilcoxon statistic was used to determine the significance of the results. This test allows for paired analysis outside of a normal distribution (i.e. Gaussian curve), matching each sample's variance to its control sample from the same donor. Results within $P<0.05$ are considered significant. All of these statistics have been calculated on the GraphPad Prism program (v5).

RESULTS

TRAIL receptor expression in osteoclasts evaluated by real-time PCR and Western analysis

Receptor Activator of Nuclear Factor-kappa B Ligand (RANKL) is the major factor involved in osteoclast development, activation, and survival (Nakagowa et al., 1998). NF-κB signaling can inhibit the apoptotic cascade, giving the active osteoclast more time to enact bone destruction. In the case of multiple myeloma, in which RANKL expression is elevated in the bone microenvironment, we can expect a relative decrease in sensitivity to apoptosis in the osteoclasts, due to the increase in NF-κB signaling and other RANKL-induced survival pathways (Terpos et al., 2009). Given its anti-apoptotic properties and critical contributions to MM pathogenesis, it was thus reasonable to assume that RANKL may play a part in the modulation of TRAIL-R expression, another pathway by which it might inhibit osteoclast apoptosis.

To evaluate the potential effects of treatment with RANKL on human osteoclasts, while in their typical growth media, the osteoclasts were stimulated with 50, 100, and 200 ng/ml with each media change, starting before the final developmental changes take place in the osteoclasts, approximately one week before extraction, and terminating the day before extraction. The doses indicated are in fact added to the basal level of the culture media of 100 ng/ml, necessary for the differentiation of the monocytes/macrophages into resorbing osteoclasts in our model. Therefore, actual dosage was 150, 200, and 300 ng/ml.

We observed that increasing doses of RANKL did not significantly affect the expression of any of the TRAIL receptors (Figure 8).

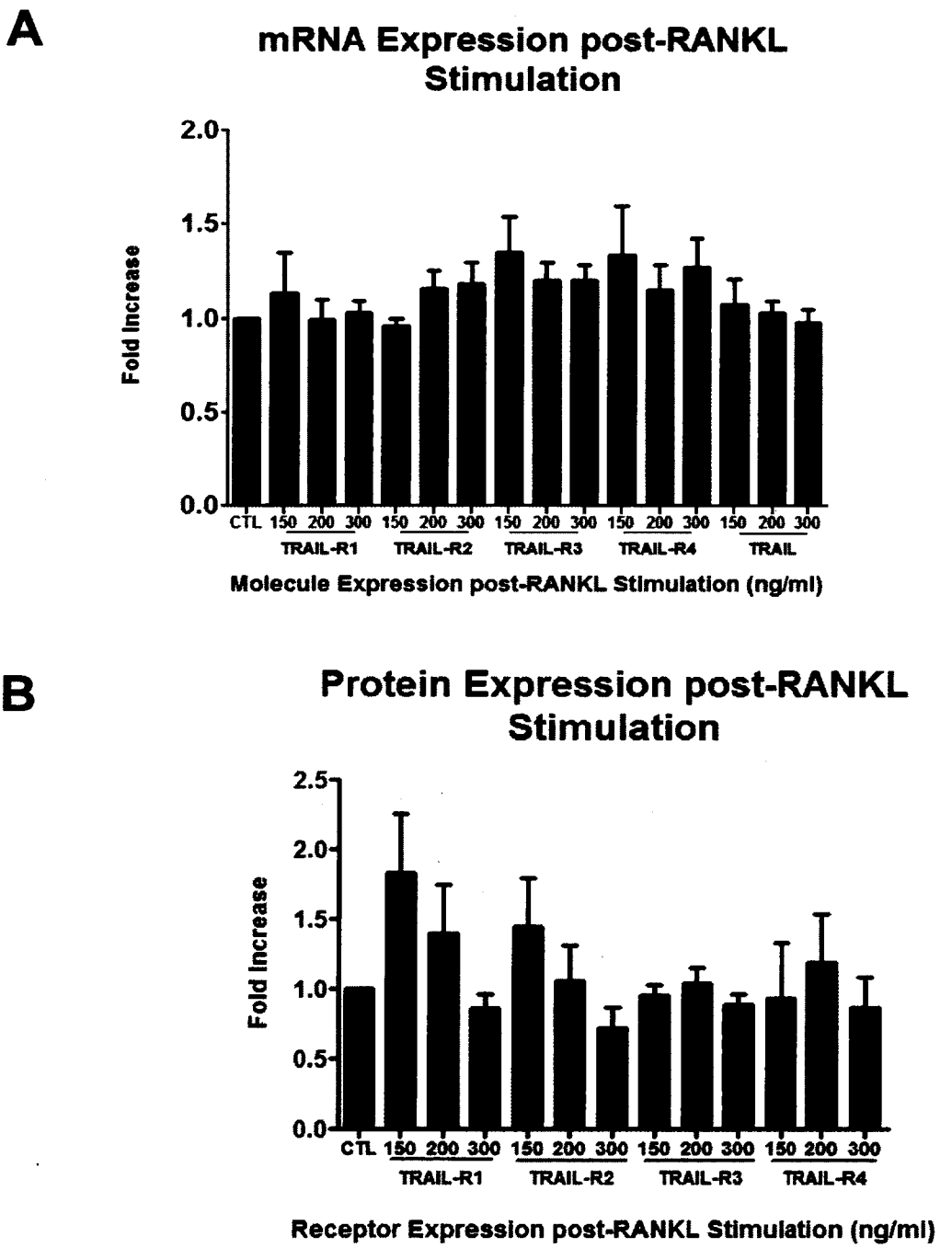


Figure 8: Protein and mRNA expression of death receptors post-RANKL stimulation evaluated by western blot and real time-PCR

The cells were treated over a period of six days at varying concentrations of RANKL (150, 200, and 300 ng/ml). TRAIL-R expression was evaluated at either the mRNA (A) or protein (B) level. Protein expression is normalized to actin expression. N=3-8

In mRNA, while the non-dose-dependent increasing trend of TRAIL-R3, and TRAIL-R4 were visible at n=4, statistical variance was too great to apply significance. Means range from 20-35% higher than the control, but variation meant no p-value was smaller than 0.1456. Therefore it can be observed that RANKL dosage does not significantly affect mRNA expression of TRAIL-R, or TRAIL itself. Variation was even greater in the analysis of protein extracts from treated osteoclasts. No trends, significant or otherwise, were observable from the data collected, with n ranging from 4 to 8 depending on receptor.

Effect on Osteoclasts upon Stimulation With MIP-1α:

MIP-1α (Macrophage inflammatory protein-1α), an OAF implicated in bone lytic diseases and particularly MM, was another potential candidate as modulator of TRAIL-R expression. MIP-1α is produced by myeloma cells, and is known to stimulate osteoclast formation and bone resorption (Choi et al., 2000). Interestingly, it has been shown that MIP-1α directly affects cell signaling pathways like activation of AKT/protein kinase B, and the MAPK pathway in myeloma cells through CCR5; mediating growth, survival and migration of myeloma cells (Tsubaki et al., 2007). However, it has not yet been shown if MIP-1α is capable of inducing the same survival effects in human osteoclasts. Likewise, it has yet to be established whether MIP-1α's survival-promoting effects extend to modulation of TRAIL-R in order to avoid apoptosis.

Stimulation of all cells in culture were performed following the same protocol as outlined in the section detailing results from RANKL stimulation. Doses of MIP-1α were 50, 100, and 200 ng/ml, respectively. While several trends were observed, stimulation with MIP-1α did not lead to any significant changes in mRNA expression of TRAIL- receptors

or TRAIL (Figure 9-A). However, TRAIL–R3 trended upwards without reaching statistical significance. With an n=5, the mean increases ranged from 4-25%. However, no p-value was determined to be lower than 0.1538, even while all but one sample showed increased mRNA production of TRAIL-R3. Therefore no significant mRNA augmentation could be concluded.

In protein expression assays (Figure 9-B) stimulation with MIP-1α at 100 ng/ml did in fact increase TRAIL-R1 protein expression by more than 75%. Its mean protein expression was 1.786 times that of the control cells, with a corresponding p value of 0.0265, placing it within the limits of $p<0.05$ to establish significance. A similar increase in TRAIL-R3 was observed at quantities of 100 and 200 ng/ml of MIP-1α. Dosage of 100 ng/ml produced an increase at a factor of 1.49, while 200 ng/ml increased TRAIL-R3 protein levels by 1.459. Each of these were statistically significant with p values of 0.0162 and 0.0183, respectively. This significant protein elevation matched the observed but statistically unconfirmable mRNA trends.

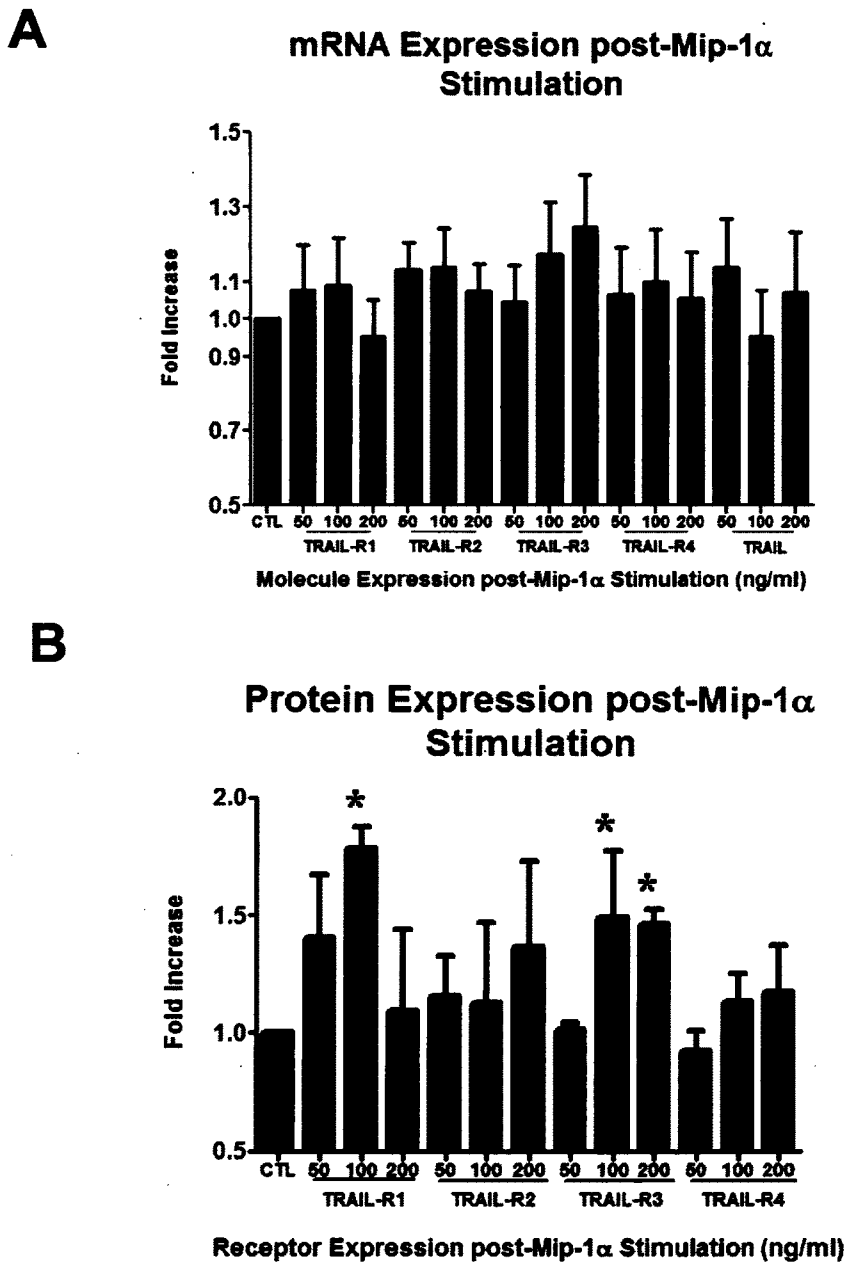


Figure 9: Protein and mRNA expression of death receptors post- MIP-1α stimulation evaluated by western blot and RT-PCR

The cells were treated over a period of six days at varying concentrations of MIP-1α (50, 100, and 200 ng/ml). TRAIL-R expression was evaluated at the mRNA (A) and protein (B) level. MIP-1α increased protein expression of both TRAIL-R1 and TRAIL-R3. N=3-7
*=$p<0.05$

Effect on Osteoclasts upon Stimulation With TRAIL:

TRAIL has been shown to be able to modify its own receptors' expression. It was demonstrated in 2007 that stimulation of human osteoclasts with massive quantities (500 ng/ml) of TRAIL directly led to DR5 (TRAIL-R2) mRNA and protein upregulation (Colucci et al., 2007). Serum TRAIL, for example, is found at 65 pg/ml (Secchiero et al., 2010). Smaller levels of TRAIL however, are more than enough to induce significant apoptosis in *in-vitro* human osteoclast cultures, as demonstrated by S. Roux et al in 2005. The aforementioned study by Colucci confirmed that in apoptosis induction, TRAIL functions the same in osteoclasts as in other cell lines, triggering activation of caspase-8 and Bid. It is known that MM cells produce TRAIL (and sensitize osteoblasts to it) as one of their bone loss mechanisms (Tinhofer et al., 2006). Thus one goal of the current study was to determine if lower levels of TRAIL, more physiologically realistic than 500 ng/ml, and thus closer to those that might be observed in the MM microenvironment, were also able to induce receptor modification and potential sensitization in osteoclasts (Figure 10).

Given that apoptosis can be heavily induced at doses as low as 10 ng/ml, human osteoclasts in vitro were stimulated with 0.5, 2.5, and 25 ng/ml, respectively. No significant changes were observed in the mRNA expression 24hrs post-stimulation. Similarly, no significant changes occurred in protein expression of TRAIL's four surface receptors (Figure 9). While high doses of TRAIL (25 ng/ml) at n=3 appear to elevate protein quantities of TRAIL-R1 and –R2 by 92% and 70%, respectively, one sample t tests showed that the respective p-values of each (0.3081 and 0.6291), were too great to infer significance. Although non significant, it is difficult to completely rule out any

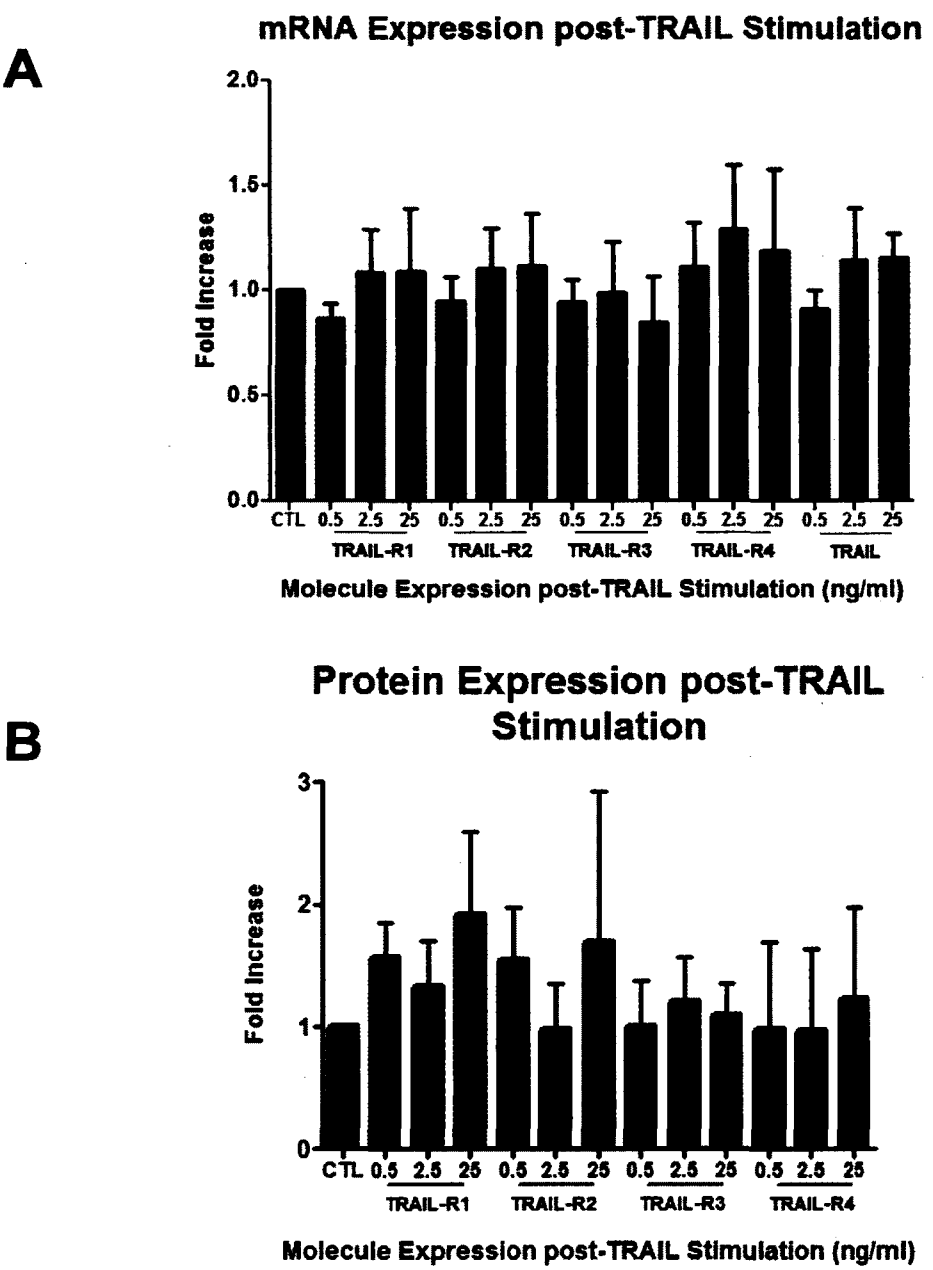


Figure 10: Protein and mRNA expression of death receptors post-TRAIL stimulation as evaluated by western blot and RT-PCR.

The cells were treated over a period of six days at varying concentrations of TRAIL (0.5, 2.5, and 25 ng/ml). No changes in TRAIL-R expression were observed at either the mRNA (A) or protein (B) level. N=3-4

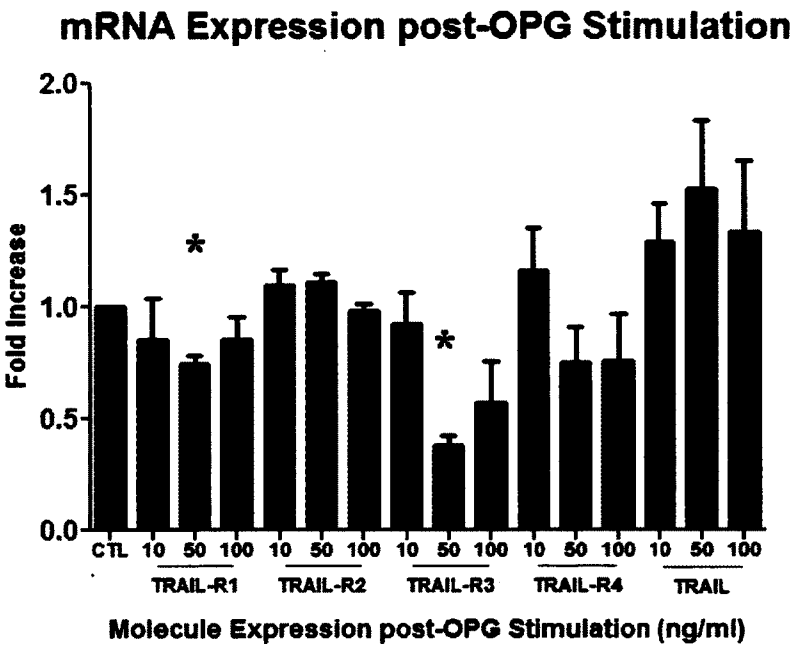
effect of TRAIL on its own receptor expression because of the few samples (n=3) and the high variability of the measurements.

Effect on Osteoclasts upon Stimulation With OPG:

Osteoprotegerin production, at least by osteoblasts, is known to be reduced in multiple myeloma, sensitizing the osteoblasts themselves further to TRAIL-induced apoptosis (Giuliani et al., 2001). OPG may decrease osteoclast survival by blocking RANKL, and may protect osteoclasts against apoptosis in MM where OPG levels are reduced and where RANKL is over-expressed. However, the presence of OPG has also been shown to prevent TRAIL-induced apoptosis in osteoclasts, as OPG is the decoy ligand of TRAIL as well as RANKL. Indeed, OPG may modulate osteoclast response to TRAIL-induced apoptosis by interfering with the local concentration of TRAIL which is decreased in the presence of OPG by direct binding (Chamoux et al., 2008). While OPG has not been identified as a molecule capable of directly inducing signaling, alternate roles for osteoprotegerin have not been ruled out. Indeed, besides its role in blocking RANKL actions, OPG may also have a direct effect on osteoclasts, potentially by creating multimeric tri-dimensional structures on RANK, and it has been shown that OPG could directly inhibit the expression of cathepsin K, a major enzyme involved in bone resorption (Theoleyre et al., 2004; Wittrant et al., 2002).

To evaluate the effects of OPG on TRAIL-R expression, OPG at concentration of 10, 50, and 100 ng/ml was added to osteoclast cultures over a period of six days (Figure 11).

A



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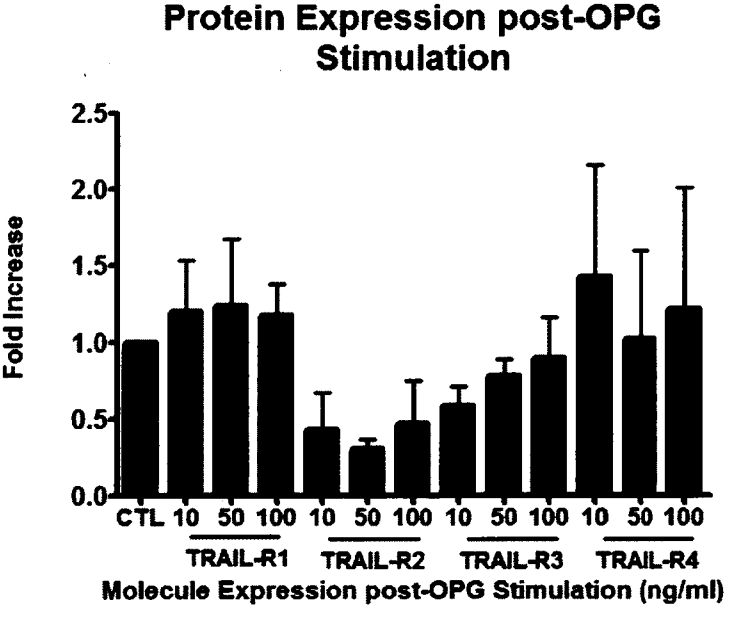


Figure 11: Protein and mRNA expression of death receptors post-OPG stimulation as evaluated by western blot and RT-PCR

The cells were treated over a period of six days at varying concentrations of OPG (10, 50, and 100 ng/ml). TRAIL receptors expression was evaluated by real time-PCR (A) and WB analysis (B). N=3-4 *=$p<0.05$

Real time RT-PCR showed that doses of 50 ng/ml of OPG significantly reduced both TRAIL-R1 and –R3. TRAIL-R1 mRNA levels were reduced by 25% (with a p value of 0.0247), and on average, TRAIL-R3 values decreased by 62% relative to the control (with a p-value of 0.0427) (Figure 11-A). Western blot protein analysis of human osteoclasts stimulated with OPG did not show the same trends as those expressed by mRNA (Figure 11-B). It is thus difficult to conclude to any effect of OPG on TRAIL-R expression.

Effect on Osteoclasts upon Stimulation With TGF beta

Transforming growth factor- β (TGF-β) is an important functional mediator of osteoclasts, playing a major role in proliferation, migration, differentiation, and survival of many different cell types (Rahimi et al., 2007). Human osteoclasts have been shown to express both type-I and type-II TGF-β receptors, each necessary for TGF-β signaling (Houde et al., 2009). TGF-β is one of the many cytokines embedded in the bone matrix released during resorption, and in cases of elevated resorption like seen in myeloma, large quantities can be introduced to the bone microenvironment. TGF-β1 is the most abundant TGF-β isoform detected in bone, and can induce osteoclast apoptosis (Roux et al., 2005). In addition, TGF-β limits osteoclast proliferation and fusion, and reduces osteoblastic RANKL expression, but simultaneously enhances RANKL-mediated differentiation (Takai et al., 1998, Fuller et al., 2000, Orcel et al., 1990)

TGF-β was added in osteoclast cultures during the third last week (TGF- β concentrations of 0.1, 1, and 10 ng/ml). In the presence of the highest dose of TGF-β (10 ng/ml), a quantity sufficient to induce osteoclast apoptosis in deprived cultures (Houde et al., 2009), a significant decrease of 30% in mRNA expression of TRAIL-R1 was observed

(p=0.0288) (Figure 12-A). No changes in TRAIL receptor expression were observed in total protein lysates (Figure 12-B).

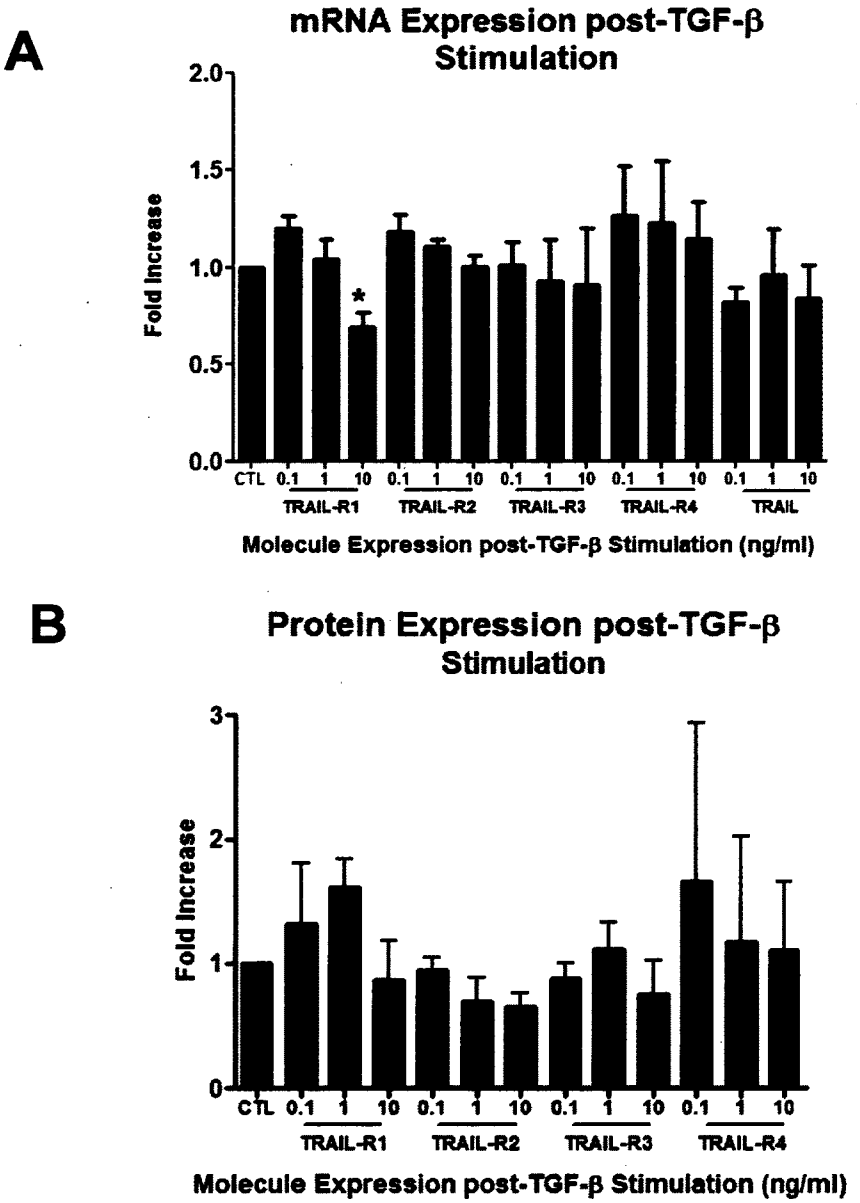


Figure 12: Protein and mRNA expression of death receptors post-TGF-β stimulation as evaluated by western blot and RT-PCR

The cells are treated over a period of six days at varying concentrations of TGF-β (0.1, 1, and 10 ng/ml). TRAIL receptors expression was evaluated at mRNA level by real-time PCR (A), and at protein level by WB (B). N=3-4 *=$p<0.05$

Effect on Osteoclasts upon Stimulation With PTH:

PTH induces an elevation in RANKL production by osteoblasts, allowing for the RANKL cascade to begin in osteoclasts and their precursors, inducing both differentiation and activation (Ma et al., 2001). Notably, PTH receptors can be identified on osteoblasts and pre-osteoclasts, but their expression on mature osteoclasts is controversial (Dempster et al., 2005). This problem is presumably circumvented in this experimental model by beginning stimulation at day 15 of cell culture, when the population is still immature, as osteoclast precursors do express PTH receptors. Theoretically, it is at this stage that stimulation with PTH could have its modulatory effect on TRAIL receptors. Immunohistochemistry performed by the laboratory of Dr. Roux on tissue samples from patients with hyperparathyroidism (characterized by overproduction of PTH) exhibited a reduction in decoy receptors on osteoclasts, and an augmentation of death receptors, the opposite of the profile observed in multiple myeloma (results not published). Thus it could be expected that PTH would have an effect on TRAIL-R expression in an in vitro model.

There were three separate concentrations with which the osteoclasts were periodically dosed: 10, 25, and 100 ng/ml (Figure 13). Real time-PCR evaluation showed that administration of 25 ng/ml significantly increased TRAIL-R2 (DR4) mRNA expression by a margin of 17% on average ($p=0.0121$). However, this trend was not supported by protein evaluation through western blot, suggesting that the slight mRNA increase did not lead to other changes.

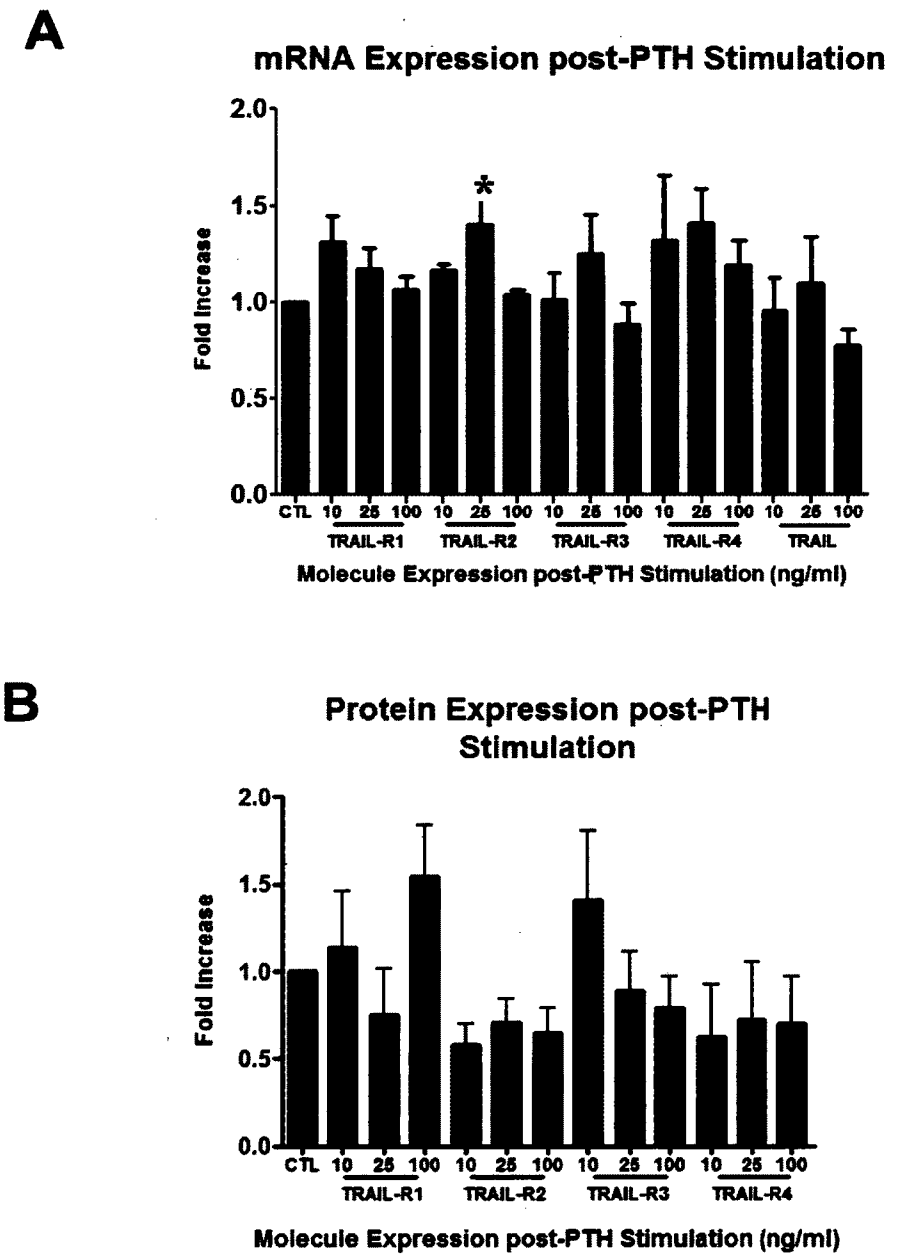


Figure 13: Protein and mRNA expression of death receptors post-PTH stimulation as evaluated by western blot and RT-PCR

The cells are treated over a period of six days at varying concentrations of PTH (10, 25, and 100 ng/ml). TRAIL-R expression was analyzed at either the mRNA (A) or protein (B) levels. N=3-4 *=$p<0.05$

EVALUATION OF PROTEIN EXPRESSION BY IMMUNOCYTOCHEMISTRY

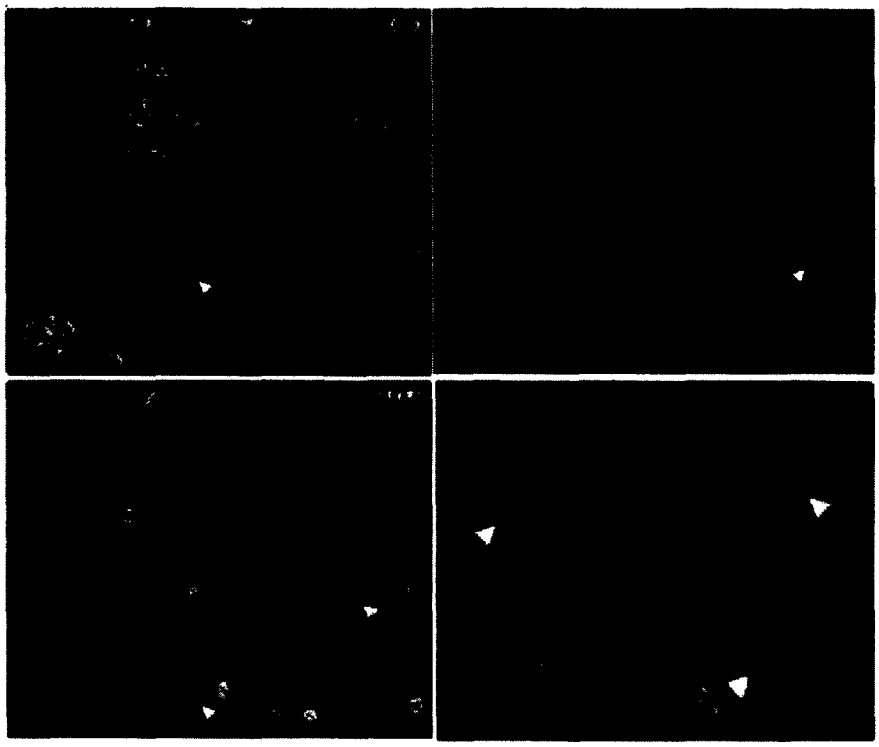
To confirm the WB results, and to determine if the protein expression was actually modulated in osteoclasts and not in non-osteoclastic cells also present in the cultures, immunostaining experiments were performed on osteoclast cultures (Figure 14).

Significant increases in TRAIL-receptors expression in osteoclasts were observed in the presence of TRAIL, but not in the presence of the other factors. In cultures treated TRAIL, the proportion of osteoclasts expressing TRAIL-R1 was significantly increased compared to untreated controls (77% with 0.5 ng/ml, 72% with 2.5 ng/ml vs 70% in controls, $p<0.05$). In cultures treated with 2.5 ng/ml of TRAIL, the proportion of osteoclasts expressing TRAIL-R2 expression was also significantly increased (59% vs 56% in controls, $p<0.05$). Although no changes in TRAIL-R expression were observed in the presence of TRAIL in the mRNA levels and only trends towards increase in the expression of TRAIL-R1 and R-2 were observed in WB analysis from the whole culture extracts, changes were observed in TRAIL-R1 and R-2 expression at the osteoclast surface. A larger number of experiments testing TRAIL in osteoclast cultures should be performed to validate this interesting data.

We also observed that MIP-1α increased the expression of TRAIL-R1 and TRAIL-R3 in WB analysis from proteins extracted from the whole cultures, however no significant increases were observed in immunocytochemistry experiments. There again, these discrepancies may be related to the lower number of experiments performed (n=3) and should be validated by larger number of experiments.

This ICC data is very important to verify, as osteoclast cultures are composed of osteoclasts and non-osteoclastic cells (macrophage polykaryons and monocytes), and this

A



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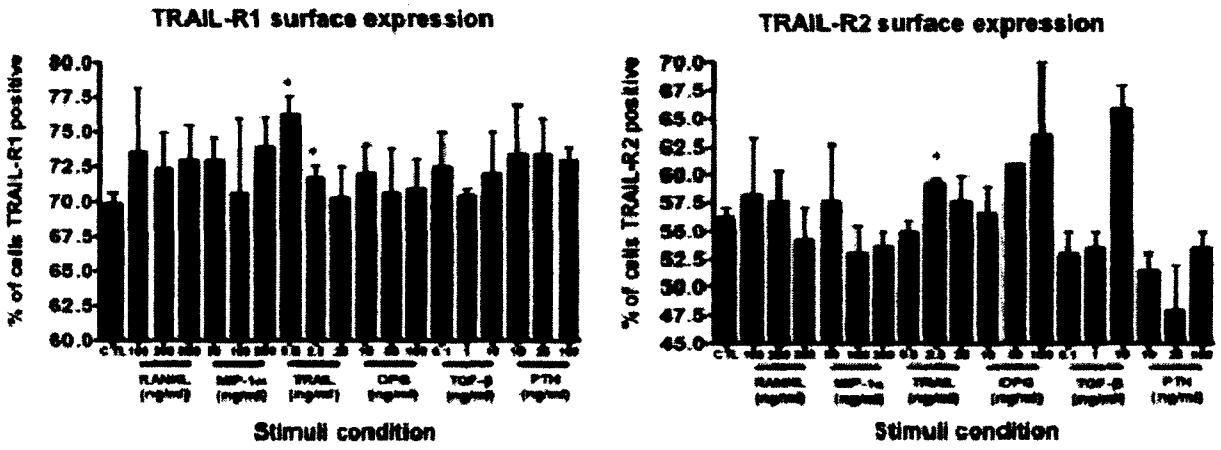

Figure 14: Surface expression of death receptors evaluated by immunocytochemistry:

The cells were treated over a period of six days at various concentrations of the six target cytokines. Staining of the receptors was performed with specific antibodies directed towards TRAIL-R1, -R2, –R3 (results not shown), or non-specific IgG acting as controls (A). The immunocytochemistry was carried out with the LSAB kit from DAKO. Quantification was achieved by counting positive MNCs (those stained red: identified by black arrowheads; non-marking negative cells are identified with white arrowheads), and these results were presented in percentage of positive MNCs for TRAIL-R1 expression (E) and TRAIL-R2 expression (F). N=3 *=$p<0.05$

could blunt or attenuate the observed modulation of the TRAIL-R expression in the specific osteoclast population.

APOPTOSIS:

To determine whether any of the augmentations or diminutions in the TRAIL-R expression induced by the various cytokines tested and observed at a mRNA level (OPG, TGF-β, PTH), or at a protein level (MIP-1α, TRAIL) would have any applicable significance, functional TRAIL-induced apoptosis tests were performed. The human osteoclasts underwent the standard culture period, then were deprived in a solution of OPTI-MEM with 1% FBS for 24 hours. After this period of incubation, the cells were subjected to 10 ng/ml of TRAIL (excluding a control well in each labtek), for another period of 24 hours, and apoptosis was analyzed using TACS blue label. At this point they were fixed, stained, and counted.

TRAIL is a well known apoptosis-inducing factor for osteoclasts, in a dose-dependent manner, and induced a significant increase in the rate of osteoclast apoptosis in control cultures: a ten percent increase with a p-value of 0.0024. Although significant, the modulation of TRAIL-R observed at the osteoclast surface by TRAIL, was likely not very important with a magnitude of few percents, and were only observed at the lowest doses of TRAIL. We have therefore evaluated their relevance by studying their impact on osteoclast apoptosis. However, no significant change in apoptosis occurred with TRAIL or with any of the co-factors and cytokines tested (Figure 15). If TRAIL induced TRAIL-R1 and TRAIL-R2 receptor expression, an increase in TRAIL-induced apoptosis would have been

expected, however we observed a trend towards a decrease in the rate of apoptosis induced by TRAIL.

MIP-1α also increased TRAIL-R expression (TRAIL-R1 and TRAIL-R3) in the whole protein extract analyzed by WB, but no significant modulation of osteoclast apotosis was observed in the functional apoptosis test. However, because of the variability of this test, the number of experiments (n=3) is of yet too low to expect any significant changes. We believe that these factors, particularly TRAIL and MIP-1α could make a difference in the apoptotic rate by modulation of the TRAIL-receptor expression, and further studies are needed and will be performed to complete this study and validate our results.

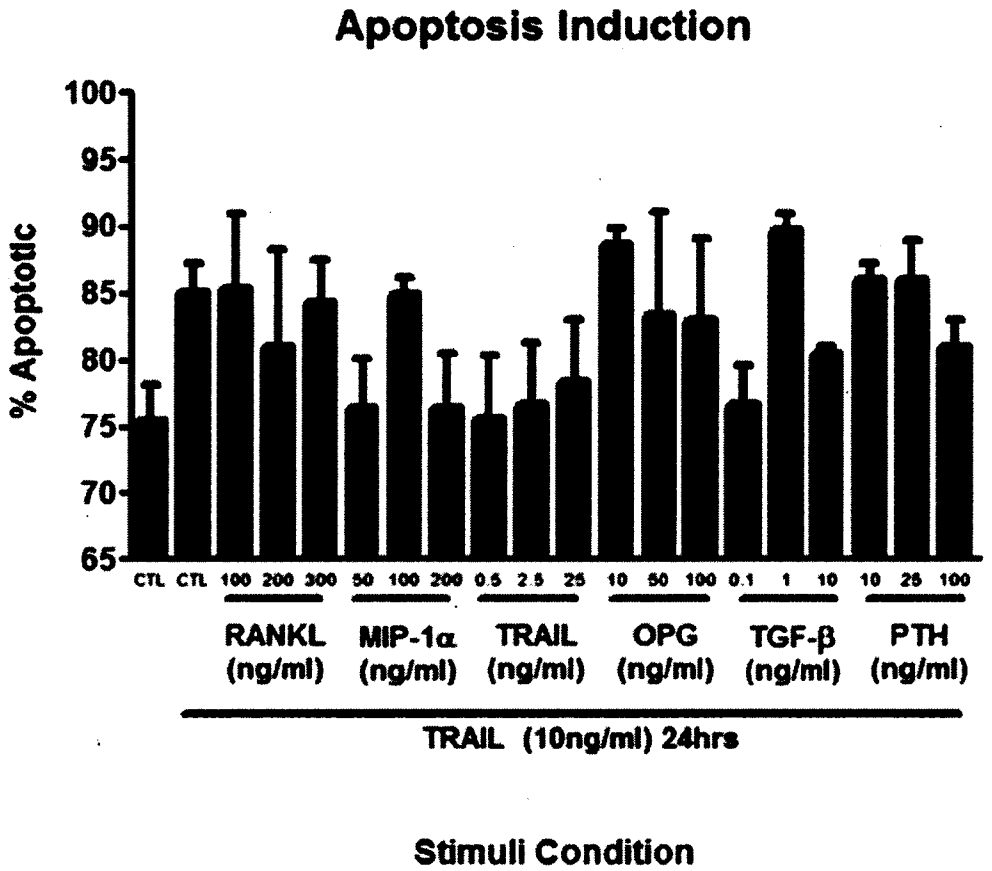

Figure 15: Apoptosis induction in treated human osteoclasts post-TRAIL stimulation

The cells were treated over a period of six days at various concentrations of the six target cytokines. All conditions underwent 24 hours of deprivation at 1% FBS OPTI-MEM, followed by 24 hours of treatment with 10 ng/ml of TRAIL. Staining of nuclei of apoptotic cells was performed with the TACS blue label apoptosis kit. Quantification was achieved by counting positive MNCs (those with blue nuclei; non-marking, non-apoptotic nuclei stain red), and these results were presented in percentage of positive MNCs. N=3

DISCUSSION

The induction of apoptosis by extracellular signals involves ligands related to TNF. To better understand potential pathways of TRAIL or RANKL signaling in osteoclast biology, it is necessary to further discuss pre-established knowledge of TNF family pathways. TNF-α is an important member of the TNF family regarding modulation of osteoclast formation and function. It exerts its function through two receptors; TNFR1 or p55, which contains a death domain, and TNFR2 (p75), which does not (Feng et al., 2005). TNF-α is essentially known as a potent pro-inflammatory factor. By engaging TNFR1, TNF-α activates transcription factors, leading to the induction of pro-inflammatory and immunomodulatory genes. In some circumstances, TNF-α may also induce apoptosis. However, TNF-α rarely triggers apoptosis unless protein synthesis is blocked. This suggests the existence of cellular factors that can suppress the apoptotic stimulus generated by TNF-α (Ashkenazi et al., 1998). Unlike TNF family member RANKL, TNF-α is not essential for osteoclast formation, though it has been shown to promote osteoclastogenesis in vitro, through the production of RANKL or by a mechanism independent of RANKL (Kobayashi et al., 2000, Manolagas et al., 1995). TNF-α signaling through TNFR1 appears to act as a survival factor in osteoclast differentiation by activating NF-κB. TNF-α-enhanced survival of osteoclasts appears to be associated with a reduction in apoptosis and the suppression of caspase activation (Lee et al., 2001). Knockout mice deficient in TNF-α and also TNFR1 have a reduction in bone loss post-ovariectomy (in which estrogen-mediated resorption suppression is stopped) (Roggia et al., 2001). Another study has demonstrated that nude mice (lacking T lymphocytes) have the same protection from bone loss (Cenci et al., 2000). This shows that circulating T cells are the major producers of estrogen-regulated TNF-α in the bone marrow microenvironment. In other inflammatory bone loss conditions, like rheumatoid arthritis, TNF-α is produced by activated macrophages (Romas et al., 2002).

Like other members of the TNF family, TNF-α acts on the osteoclast through the activation of many signaling pathways including NF-κB, JNK, p38, ERK and Akt in osteoclasts and their precursors (Wei et al., 2002). However, the signaling cascades by which it activates these pathways have yet to be delineated. Yet, the promoters for many of

these signaling pathways have been identified in the TRAIL promoter region, highlighting potential for cross-talk or even cross-activation.

Each TNF-α receptor affects osteoclasts differently, with TNFR1 acting as a positive regulator of formation and function, while TNFR2 is inhibitory (Abu-Amer et al., 2000). This same principle rests a theory for TGF-beta action. TGF-beta represents another osteoclast apoptosis inducer with multiple receptors, whose function may be synergistic, antagonistic, or wholly unrelated. While TNFR2 binds TNF-α more tightly, it is in fact not the primary receptor in most cell types, and particularly not in osteoclasts (Dempsey et al., 2003). And while TNFR1 has a DD that initiates its signaling within the cytoplasm, it rarely induces apoptosis through this receptor. In most cases, it simultaneously induces activation of NF-κB and JNK, which prevent the osteoclast from entering apoptosis by blocking the pathway (Dempsey et al., 2003). TNFR1 activates NF-κB through two adaptor proteins; TRAF2 and receptor interacting protein (RIP). TRADD can interact with these proteins in place of FADD, leading to recruitment of IKK and its subsequent activation (Devin et al., 2000). Many of the non-apoptotic pathways associated with TNFR1 signaling in osteoclasts are very similar or identical to those activated by RANK that are necessary for osteoclast formation and activation. It is currently unknown why TNFR2 is inhibitory in osteoclast formation and function, given that it activates NF-κB through similar TRAF2 pathways (Feng, 2005). Since the major difference between the two receptors is the lack of DD in TNFR2, it is possible that some region on the death domain is responsible for the signaling change that activates osteoclasts rather than repressing them. With this information, one might assume a similar mode of activity carried out by the TRAIL receptors: that the TRAIL decoy receptor –R3 (which is GPI anchored) may be truly just a decoy, while TRAIL-R1, -R2 and –R4 are capable of osteoclast activation and probable receptor modulation on top of their canonical functions as either apoptosis inducers or decoys; that this activity is made possible by a portion of the death domain (a reminder that the –R4 possesses a partial death domain, leaving it incapable of signaling apoptosis, but permitting it to activate NF-kB).

TRAIL was the only cytokine of the six tested throughout the study to have a modulatory effect on the surface expression of the TRAIL receptors. In each case, the change occurred with the intermediary dose of TRAIL at 2.5 ng/ml (Figure 14). This

produced a bell-curve effect in the results. One explanation is that at lower doses, when apoptosis was not induced, or at higher doses when apoptosis may be strongly induced, other signaling through the TRAIL receptors were made more evident.

Interestingly, we found that TRAIL upregulated both of its death receptors, DR4 and DR5 (TRAIL-R1 and –R2, respectively). This confirms similar findings regarding DR5 (Brunetti et al., 2007). but is the first to do so with the human osteoclast. High expression of TRAIL-R1 (and TRAIL-R4) was recently shown to correlate to significant cell death in pancreatic ductal adenocarcinoma patients (Sanlioglu et al., 2009). However, the augmentation of TRAIL-R1 and –R2 receptors caused by TRAIL in the human osteoclasts in vitro did not correlate to increased apoptosis in our study. As previously mentioned, the number of samples tested was too small to be conclusive. However, two other possibilities may be considered: that augmentation of TRAIL-R1 and –R2 is extraneous as long as there are already death receptors for TRAIL expressed, or that the augmentation observed was too small on a relative scale to impact the apoptotic toll. Also interesting in this surface expression is that no significant change was reported in the mRNA expression of the same receptors upon TRAIL expression, and only a trend towards increase in protein expression was observed in experiments performed using the whole cell lysates. There again we keep in mind that only 3 samples of mRNA extracts were analyzed, and that osteoclast cultures also contain non-osteoclastic cells that could have modify the osteoclast response to TRAIL. However, this leads one to believe that whatever modification is taking place does so either on the level of receptor transport, protein packaging, or prevention of protease activity.

Despite the global influence of RANK signaling in osteoclasts, in our experimental model, RANKL stimulation did not appear to modulate TRAIL-Rs in any way. Yet, to produce the osteoclasts themselves, we were already stimulating with significant doses of RANKL. Thus it is difficult to decisively remark on effects of further RANK signaling within these tests. And so, since we cannot examine these cells in a vacuum, we must therefore seek the most probable explanations.

There is at least some possibility that RANK already plays a part in rendering the osteoclast less susceptible to TRAIL-induced apoptosis. Conversely, it is entirely possible that RANKL has no effect on TRAIL through its receptors. It already regulates several

pro-survival pathways along with its myriad other pro-osteoclastic functions. In addition, the doses in our model were significantly larger than anything one might observe in vivo. It is possible that the receptors were already saturated, and our addition stimulation was effectively null. Either way, with the information obtained in this study, we can safely say that within the confines of this experimental model, the TRAIL receptors do not modify expression in response to RANKL.

MIP-1α is another factor that has been involved in diseases characterized by a high level of osteoclast activity. MIP-1α is known to stimulate osteoclast formation and bone resorption (Choi et al., 2000). This chemokine increases osteoclast differentiation in human bone-marrow cultures, as the result of a direct effect on osteoclast progenitors. MIP-1α also enhances the effects of IL6, PTHrP and RANKL on osteoclast formation (Han et al., 2001). This chemokine may also directly act on mature osteoclasts, and in a murine model, MIP-1α has been shown to increase osteoclast motility, although it did not alter osteoclast bone resorbing activity, adhesion, or survival (Yu et al., 2004). MIP-1α may also act indirectly on these cells by stimulating RANKL production (Tsubaki et al., 2007).

In our study, MIP-1α was able to induce change in the production of both TRAIL-R1 and –R3 at the protein level in osteoclast cultures. This was achieved without a significant elevation in mRNA and with no obvious change to surface expression of either receptor. MIP-1α, like many other CC chemokines, can activate more than one CC chemokine receptor (CCR) expressed on the osteoclast. In fact, its mechanism in myeloma cells appears to necessitate activation of CCR1 and CCR5 in cooperation to trigger its signaling pathway (Oba et al., 2005). This hints at a complex network of intracellular pathways necessary to induce full expression of the physiological function of MIP-1α (New et al., 2003). In fact, CCR1 has been identified in monocytes but has yet to be studied in osteoclasts, while CCR5 has not been observed to be expressed even in monocytes (Nardelli et al., 1999). This likely means that like many other CCLs, MIP-1α's method of signal transduction and consequently its effects, vary from one cell type to another. Thus to identify the exact pathway by which it operates in osteoclasts, and particularly in our model demands a degree of speculation. CCRs activate a network of signaling pathways that have been shown to play important roles in regulation of

physiological and pathophysiological events throughout the body, and their influence is matched only by their complexity.

Chemokines can exert their effects through at least nineteen known G protein-coupled receptors (GPCRs) (New et al., 2003). One remarkable feature of these receptors is their apparent lack variety of selectivity in regards to ligand binding. For example, MIP-1α is one of eleven identified chemokines associated with CCR1 binding and activation (Chou et al., 2002). Likewise, while a receptor for MIP-1α, CCR5 has received the majority of its publicity through the identification of its role as a co-receptor for HIV (Deng et al., 1997). Another example of the versatility of these receptors is in their GPCR activity. While CCRs are believed to primarily signal through Gi/o-coupled heterotrimeric G proteins to inhibit adenylyl cyclase (AC) and regulate Ca^{2+} flux, research is beginning to show that CCRs can couple to other G proteins, providing a wider influence on intracellular signaling pathways and gene transcription (New et al., 2003).

Since the primary known target of CCRs is adenylyl cyclase, it makes an interesting first case in examination of osteoclasts. AC is an enzyme which catalyses the synthesis of cAMP from ATP. There are 9 known isozymes of AC; all of which are activated by interaction with Gαs subunits. Each AC isozyme, however, exhibits varying sensitivity to these subunits: Gαi, Gαz and Gαo. Some are selectively inhibited, while others are activated, meaning that binding of the CCR and subsequent Gi/o-activation may inhibit or activate AC depending on their tissue-specific G protein and AC isozyme expression. While tests have not been performed in osteoclasts, human embryonic kidney 293 cells (HEK-293) that stably expressed CCR1 inhibited forskolin-stimulated cAMP accumulation upon stimulation with MIP-1α (Myers et al., 1995). Similar stimulation of CCR5 in CHO cells, with MIP-1β inhibited AC activity (Blanpain et al., 2001). More specifically, overexpression of Gαi2 strongly re-enforced this mechanism of action, suggesting that it is a subunit of interest in the case of CCR5 AC inhibition (Zhao et al., 1998). The opposite effect was observed with MIP-1α stimulation in human hepatoma cells, with an inhibition of cAMP formation. MIP-1α's effects on T lymphoblasts (cell polarization and adhesion, signals it also propagates in pre-osteoclasts), were blocked by PKA inhibitors, indicating its activity runs through cAMP (del Pozo et al., 1995). PKA activity may thus be responsible for the specific cascade of genes responsible for the

increase in production of TRAIL receptors. This would also mean that this same activity does not provide for transport to the membrane, or that any such activity would be blocked by concurrent signaling within the cell, given that the increase in protein production was not linked to an increase in receptor expression.

However, effects of direct and isolated MIP-1α stimulation in human mature osteoclasts are still unknown, and cannot be accurately estimated. Nevertheless, such effects are likely as human osteoclasts express CCR1 and CCR5, and may also expressed MIP-1α (Kim et al., 2006). While we know that different CC receptors, and G proteins have been shown to regulate the AC/cAMP/PKA/CREB pathway, it has also been shown that varying CCLs can impact a system differently as well. While both MIP-1α and RANTES are ligands for CCR1 and CCR5, MIP-1α increases cAMP in one cell line, while RANTES has no effect (Murphy et al., 2000, Mantel et al., 1995).

The activity of chemokine receptors is most frequently determined by measuring changes in intracellular Ca^{2+} levels following stimulation. This can be a more complicated assay with the osteoclasts, however, who traffic heavily in calcium during the resorptive process. The mobilization of calcium from intracellular stores indicates an activation of PLC-β by G protein subunits. Phosphoinositide-specific phospholipase C (PLC) is an enzyme responsible for catalyzing hydrolysis of phosphatidylinositol to inositol-triphosphates (IP3) and diacylglycerol (DAG). CC chemokines have also been shown to be able to promote calcium fluxes through the cADPR/ryanodine receptor pathway, notable in osteoclasts as high Ca^{2+} levels ensure induction of NFATc1, leading to osteoclastogenic signaling (Maghazachi, 2005). While MIP-1α's exact mechanism of osteoclast stimulation is unknown, it is certainly possible that calcium induction plays a role. These transcription promoters may also be responsible for our observed MIP-1α activity in the human osteoclast regarding TRAIL-R production. A western blot for products of NFATc1, PLC, or PKA activity might be useful for determining the exact route of MIP-1α signaling.

In COS-7 cells transfected with CCR1 receptors, IP3 formation was not promoted post- MIP-1α stimulation (Kuang et al., 1996). However, when they were co-transfected with PLC-β, IP3 formation was induced by MIP-1α, suggesting that dependent on the cells G-protein subunit composition, CCR1 and MIP-1α can induce PLC activity. In the same cell line transfected with CCR5, MIP-1β stimulated intracellular Ca^{2+} release, indicating

that both MIP-1α receptors are capable of inducing the same PLC/IP/DAG/PKC pathway (Blanpain et al., 2001)

GPCRs also regulate cell proliferation and differentiation through the MAPKs. The serine/threonine protein kinases can phosphorylate several different transcription factors that may be responsible for changes in receptor expression as well. There are at least three subtypes of MAPK: the extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), and p38 MAPK. These pathways are all regulated in the osteoclast as well, and associated with anti-apoptotic RANKL signaling, as well as pro-apoptotic TGF-β signaling. MAPK activation could very well be linked to our observed effect. While possibly not the primary intended action, minor upregulation of TRAIL-R may take place as a sort of side effect of MIP-1α–induced MAPK signaling in the human osteoclast. MIP-1α's receptors have been shown to induce PLC dependent MAPK activation, and in fact has been linked to both pro-apoptotic and cell proliferation/cell cycle progression pathways, depending on which receptor and associated cytokine (Kim et al., 2003, Misse et al., 2001). Thus, again it is plausible that MIP-1α could induce MAPK signaling altering gene transcription of TRAIL receptors through CCR signaling.

We saw that stimulation with 10 ng/ml of TGF-β resulted in a reduction of TRAIL-R1 mRNA. The best identified signaling pathway for TGF-β within osteoclasts is through Smads. Like other members of the TGF-β family, TGF-β itself transmits signals through Smad-dependent and Smad-independent pathways after receptor activation. The independent pathways include ERK, JNK, and MAPK (Derynck et al., 2001). There are three classes of Smads: receptor-regulated (r-Smad), common partner BMP or TGF-β mediators (co-Smad), and inhibitory Smads (I-Smads). Upon ligand stimulation and activation through the type II receptor, the type I receptor phosphorylates the r-Smad, allowing it to form complexes with Co-Smads (Massague, 2000). This complex translocates to the nucleus, regulating target gene transcription by interacting with transcription factors, co-activators and co-repressors. The I-Smads, of course, can interfere with the co- and r-Smads, negatively regulating signaling.

In our example, the quantity of TGF-β is significant, and likely to induce apoptosis in a large number of the cells (Houde et al., 2009). It is possible that Smad3 activation for apoptosis also has a regulatory effect on TRAIL-R1 production. It is unlikely that the

downregulation is intentionally protective, as the cytokine and dosage in question are lethal to the osteoclast. It is quite possibly a byproduct of an already active signaling pathway, reducing energetic demands of a cell preparing to enter apoptosis.

PTH presents an interesting case as well. Unpublished data from a previous study by Roux et al in 2003 hinted at effects of parathyroid hormone on osteoclasts' TRAIL-R expression. In a hyperparathyroidism lesion, characterized by a high bone turnover and numerous osteoclasts close to resorbed bone areas, TRAIL-R1 and TRAIL-R2 were strongly expressed by resorbing osteoclasts, and osteoclasts present in the stroma, with a reduced decoy receptor expression. Given that the major component of hyperparathyroidism is oversecretion of PTH, this led to the possible conclusion that PTH could be implicated in TRAIL-R modulation.

However, while PTH can stimulate bone resorption and formation through activation of osteoclast signaling, it does so indirectly, as its receptors are expressed on osteoclast precursors, and more importantly, osteoblasts, but the expression of PTH receptors on human osteoclasts themselves are still controversial (Rosen et al., 2004, Dempster et al., 2005). So in the model of multiple myeloma or hyperparathyroidism, the effects of PTH may be great in vivo, and all but knocked out in a near-homogenous in vitro osteoclast study. In our study, PTH stimulation produced almost no effect on TRAIL-receptor expression. While a small increase in TRAIL-R2 mRNA was observed upon PTH treatment, this may be an echo of an extinguished signal within the precursors, or an effect on the few monocytic cells remaining in culture along with the osteoclasts. Or this may rely to an effect of PTH on TRAIL-R2 mRNA expression, to small to be detectable at a protein level or at the surface of osteoclasts in our set of experiments.

OPG also provided a unique challenge in terms of evaluation. Any change made in the total protein or RNA levels post-exposure to OPG is difficult to assess accurately. While surface expression gives us an accurate depiction of the effects on osteoclasts, we are examining a heterogeneous population and selecting exclusively the osteoclasts, something we are unable to do with total lysate analyses like western blots or PCRs. Ordinarily this is not an issue as we can expect proportionally larger numbers of osteoclasts in culture, but osteoprotegerin is, of course, an anti-osteoclastogenic factor. A notable relative decrease in osteoclast numbers can be seen under the microscope, and at

times, a relative reduction of nuclei in those osteoclasts that do form in vitro. So the risk is run of analyzing osteoclastic changes (or lack thereof) that are represented at much lower amplitude. That being said, OPG still provides an intriguing target in osteoclasts and TRAIL-induced apoptosis, as well as a candidate for modification of TRAIL response in time of disease. One recent study highlighting its relevance to TRAIL was published by the laboratory of Dr. Roux in 2008. They demonstrated that OPG, a known decoy receptor for TRAIL, was able to decrease human osteoclast apoptosis by inhibiting the TRAIL pathway. In serum-deprived states, OPG reduced apoptosis by binding TRAIL, but not by actually reducing mRNA production of the death ligand in the osteoclasts themselves.

The effect of OPG can be expected to vary depending on the concentrations of RANKL and TRAIL in the bone micro-environment. If there is indeed a cellular ligand for OPG, these two soluble ligands would interfere before it could enact any effect. The effect of OPG may also be partially dependent on cellular state and the responsiveness of the osteoclast; whether it has been primed either through positive or negative stimuli to respond to OPG in a manner that may affect apoptosis. For these reasons, it is possible that our experimental model was flawed in regards to the introduction of OPG to our osteoclast-rich environment. Before each stimulation, existing cell culture medium potentially containing competitors or augmenters of OPG signaling was aspirated, and replaced with fresh cell culture medium. This new medium also contained the ligand RANKL, so as to continue promoting osteoclast growth, allowing us to analyze the adult cell line. A possible perspective may be to engineer a ligand for the OPG receptor that would allow us to observe the effect of the ligand itself without being concerned with loss of function from RANKL binding or its signal being washed out in a cytokine rich culture environment.

There is of course the question of our experimental model and its potential effect on the results. It has been established that OCs can be acquired from monocyte/ macrophage precursors obtained from bone marrow, peripheral blood (PBMCs), and umbilical cord blood (CBMs) (Atkins, GJ., et al. 2006). The model for this study uses CBMs in place of PBMCs primarily because they can be isolated in large numbers, and can be more easily directed towards differentiation, thanks to their more uniformly naïve phenotype. To date, there is a limited amount of information regarding any functional

differences between CBM derived OCs and those originating from PBMCs. It has not yet been demonstrated if these cells respond differently to other cytokine signaling pathways than those derived from PBMCs, but a study performed in 2008 by Penolazzi et al. analyzed specific marker expression and apoptosis sensitivity differences between the two. They showed that NF-kB or NFATc1 knockout in OC/PBMCs induced a strong apoptotic response that did not appear in the OC/CBMs. While CBM-derived OC expressed lower apoptotic responses, it has been demonstrated in publication that they are still susceptible to TRAIL-induced apoptosis under proper conditions (Roux, S. et al 2005). And while this study found no change in activation of ERK and Akt proteins between the two OC types, they observed that survival signals Bcl-2 and Bcl-XL were elevated in CBM derived OCs. Interestingly, levels of Bcl-2 and Bcl-XL have been shown to be elevated in osteoclasts in bone disease like MM and Paget's Disease of Bone, helping lead to their characteristic increased OC numbers, size and activity (Barille 2003 and Brandwood, CP., et al. 2003). This same modified gene expression can lead to increased OC resistance to drug-induced cell death (Zhang, Q., et al. 2005). While not the intention of the experimental model, it is possible that these characteristic differences may even contribute to a more accurate representation of the osteoclast environment in such resorptive diseases (particularly MM) that this study is intended to emulate.

OTHER EXPLANATIONS FOR RESISTANCE TO APOPTOSIS: MYELOMA INTERACTION

It is not only TRAIL and its receptors that regulate apoptosis in the bone microenvironment, particularly in cases of disease. Different studies have established that MM patients have an imbalance in OPG/RANKL ratio in the BM environment, and this could affect osteoclast apoptosis, as RANK is a potent osteoclast survival factor. In 2002, Roux et al tested RANK and RANKL expression by immunohistochemistry in MM patients, finding that endeosteal bone surface, vessels, and stromal cells, but not plasma cells express RANKL. They also identified that stromal cells of MM patients express significantly higher levels of RANKL expression than normal subjects (Roux et al., 2002). Independently, it has also been shown that OPG expression in trabecular osteoblasts of

those MM patients who exhibit osteolytic lesions is reduced (Giuliani et al., 2001). In MM patients, RANKL serum levels and RANKL/OPG ratio were shown to be elevated, and carried a direct correlation to bone disease and other markers of bone resorption (Terpos et al., 2003). In fact, this study showed that RANKL/OPG ratio, along with β2-microglobulin (a protein elevated in myeloma and amyloidosis), and c-reactive protein (CRP, a pro-inflammatory, complement-activating molecule) were each prognostic factors regarding survival time and probability in multiple myeloma patients.

Another way that MM works towards bone destruction is through manipulation of the immune system. In other physiopathological conditions, like arthritis, activated T cells regulate bone loss via the expression of RANKL (Kong et al., 1999, Kotake et al., 2001). This is not the only RANKL related mediation by T lymphocytes, of course, as it is known to signal for gene transcription in the T-cells themselves, and has been identified as crucial for the mechanism of hypercalcaemia in some T-cell leukemia (Nosaka et al., 2002). These observations led to a study showing that myeloma plasma cells upregulate RANKL expression and secretion in both activated and autologous T cells (Giuliani et al., 2002). In this same study, a down-regulation of IFN-γ was also observed. This is equally interesting, given that recent evidence has shown that T cells may regulate bone resorption and bone homeostasis through RANKL and IFN-γ cross-talk, as IFN-γ is known to be an osteoclastogenic inhibitor (Takayanagi et al., 2000).

The up-regulation of RANKL in T cells by the MM cells appears to be mediated by release of soluble factors. One of these target cytokines is interleukin-7 (IL-7), known to stimulate RANKL production by T cells and thus induce bone loss (Toraldo et al., 2003). Additionally, higher than normal levels of IL-7 in BM plasma of MM patients have been found in vivo. It was shown as well that MM cells are capable of IL-7 secretion, and that use of anti-IL-7 in co-cultures inhibited MM-induced osteoclastogenesis (Giuliani et al., 2002). Anti-IL-6 treatment also produced similar results, though likely through an alternate pathway. This is because while myeloma cells can produce IL-6, its activity is believed to be a reciprocal one, as stimulation with IL-6 induces IL-7 secretion, and likewise the contrary (Iwata et al., 2002). Also, while it works directly on IL-7, IL-6 has not been shown to directly stimulate RANKL in T lymphocytes, or other cell systems (Hofbauer et al., 1999). Thus, high levels of IL-6 produced by T cells in the BM environment could

induce IL-7 production by MM cells, leading to continued IL-6 production and RANKL stimulation in T cells.

Of course, RANKL, OPG, and the few interleukins described above are not the only cytokines modified in the bone microenvironment in multiple myeloma. One chemokine already discussed that is strongly associated with MM-induced bone destruction is MIP-1α. This protein, also known as macrophage inflammatory protein- 1α, or c-c motif chemokine 3 (CCL3), is best known for its involvement in the acute inflammatory state in the recruitment and activation of polymorphonuclear lymphocytes. In the bone marrow environment, however, MIP-1α is also a chemoattractant for human osteoclasts, and can even induce osteoclast formation in vitro (Kukita et al., 1997, Scheven et al., 1999). Direct production of MIP-1α has been observed in several human myeloma cell lines and MM cells from most patients tested (Uneda et al., 2003). Not only that, higher levels of the MIP-1α protein and mRNA are seen in the BM plasma and isolated BM cells of MM patients than in those of normal subjects (Choi et al., 2000). Alone these facts could lead to the conclusion that in MM patients, MIP-1α induced resorption would be more common. This is the case, as shown in 2001 by Choi et al.; by blocking MIP-1α or its receptor CCR5, MM-induced osteoclast formation was reduced (Choi et al., 2001). As well, a strong correlation between MIP-1α mRNA expression in MM cells and active bone disease has been demonstrated (Magrangeas et al., 2003). One potential weakness of the argument of MIP-1α as an osteoclast activator is that it has been demonstrated to require the RANKL signaling pathway for its effect in osteoclasts. In RANKL knock-out mice, MIP-1α was rendered ineffective (Oyajobi et al., 2003). Perhaps not coincidentally, it has also been shown to induce RANKL in stromal cells (Abe et al., 2002). Another paper published has observed the contrary, showing that MIP-1α could directly stimulate osteoclast progenitors without increasing RANKL expression (Han et al., 2001). This leads to the contrary hypothesis that MIP-1α is capable of activating osteoclasts independently, meaning that while the end result of MIP-1α is undisputed, the pathway (or pathways) that it takes to produce a response in the bone microenvironment require further study. In short, while it may not be as critical as RANKL, MIP-1α has been shown in these studies to be an additional OAF in MM patients, playing a role in the acceleration of lytic bone destruction. Additionally, while it may not work directly on the human osteoclast in vitro,

it is entirely possible that it may contribute to TRAIL or apoptosis resistance in MM through its actions on osteoblasts and the surrounding bone microenvironment.

Another mechanism through which MM acts is through the inhibition of the transcription factor Runx2/Cbfa1. This transcription factor is required for osteoblast and bone formation, as Runx2-/- mice demonstrate total deficiency in these two areas (Komori et al., 1997). The co culture of MM cells with osteoprogenitor cells has been shown to reduce numbers of osteoblast precursors, osteoblast differentiation markers like alkaline phosphatase, and the osteoblasts themselves, performed via blocking Run2x/Cbfa1 activity (Giuliani et al., 2005). This appears to be mediated primarily through cell-to-cell contact with MM and osteoprogenitor cells, involving interactions between VLA-4 on the MM cells and VCAM-1 on the osteoblast progenitors (Giuliani et al., 2005). This is not the only cell contact interaction appearing to inhibit osteoblastogenesis, but the best characterized. For example, NCAM-NCAM interactions between the MM and osteoblastic cells decreases bone matrix production (Barille et al., 1995). What's more, MM cells inhibit Runx2/Cbfa1 promoter activity by production of IL-7. This cytokine is known to inhibit bone formation through this pathway, and is found in elevated levels in MM patient serum (Weitzmann et al., 2002, Giuliani et al., 2002). The resulting decrease in osteoblast activity helps lead to MM bone lesions. Another consideration with the blocking of Runx2/Cbfa1 is that it stimulates the secretion of the osteoclast activity inhibitor OPG in osteoprogenitor cells (Thirunavukkarasu et al., 2000). This is another method by which the MM cells can influence the OPG/RANKL balance, leading to further osteoclastogenesis and resorption. Blocking of the Runx2/Cbfa1 promoter activity may also have indirect effects on the osteoclast, possibly providing another method of apoptosis evasion not accounted for in our model.

Multiple myeloma has also recently been suggested to be a regulator of osteoclast formation through activity in the Wnt signaling pathways. MM cells have been shown to over-express Dickkopf-1 (DKK-1), member of a family of negative regulators of canonical Wnt signaling (Tian et al., 2003). This study also showed a direct relationship between DKK-1 expression in MM cells and bone lesions in patients, indicating a mediation of bone destruction. Yet, the mechanism by which the DKK-1 production is related to bone destruction is unclear, as neutralizing DKK-1 in MM patients aids in restoration of

osteoblastogenesis in mice, but not humans, indicating there are likely other mechanisms at work in conjunction with DKK-1 Wnt inhibition (Giuliani et al., 2005). One hypothesis is that DKK-1 aids in adhesion of stromal cells to MM cells, inhibiting Runx2 and activating osteoclasts (Giuliani et al., 2004).

Most myeloma patients also have elevated levels of IL-3, a cytokine associated with both osteoblast inhibition and osteoclast stimulation (Lee et al., 2004). IL-3 has been shown to block differentiation of preosteoblasts to mature osteoblasts at concentrations comparable to those observed in the bone marrow plasma of these MM patients (Lee et al., 2004). Contributing to the MM cells activity in the bone microenvironment is the presence of TNF-α, a cytokine produced by the MM cells associated with osteoclastogenesis that also increases the inhibitory effect of IL-3 (Ehrlich et al., 2005). This makes IL-3 production in myeloma a double contributor to the appearance of the characteristic lytic lesions; stimulating and activating osteoclasts while indirectly inhibiting osteoblastogenesis. It is currently unknown whether IL-3 modulation is effective in treatment of MM bone disease.

Studies have also indicated that MM cells act directly on mature osteoblasts, inhibiting proliferation and up-regulating apoptosis (Evans et al., 1992, Silvestris et al., 2003). In fact, it has recently been shown that MM cells sensitize osteoblastic cells to cell death mediated by the apoptosis inducing ligand TRAIL (Tinhofer et al., 2006). Yet it is not just MM cells that act on osteoblasts: it has been established that the relationship is two-sided, with activity affecting the myeloma cells as well. Studies have shown that osteoblasts help regulate MM cell growth and survival by production of IL-6 when in co culture with these cells (Karadag et al., 2000). High IL-6 levels in the bone microenvironment have long been known to contribute to MM cell growth. And while MM cells are sensitizing the osteoblasts to TRAIL, osteoblasts in contact with them are secreting OPG, a decoy receptor for both RANKL and TRAIL, helping protect the MM cells from apoptosis. Yet even with the positive effects named, recent studies have shown that osteoblasts may reduce the stimulatory effect that osteoclasts have on MM cell survival and proliferation, indicating that by some other as yet undefined mechanism, the osteoblasts are concurrently working both for and against myeloma cells (Yaccoby et al., 2006). However, since the reciprocal activity from osteoclast activity is so great, this study

suggests that an increase in bone formation in MM patients could mark a reduction in myeloma tumor burden. This might also aid in restoring normal osteoclast activity through the addition of osteoblasts to help regulate them.

MM cells develop almost exclusively in the bone marrow microenvironment, and while a great deal of this is attributable to their cell-cell interactions with stromal cells and the IL-6 produced by the stromal cells, the osteoclasts play a major part in this nurturing environment for myeloma growth. When osteoclast activity is inhibited, either by inducing apoptosis or preventing RANKL signaling, not only is bone destruction stopped, but tumor progression is slowed and tumor burden reduced (Yaccoby et al., 2002, Dhodapkar et al., 1998). Osteoclasts have been shown to have potent growth-promoting and antiapoptotic effects on MM cells (Abe et al., 2004). Some of this MM cell growth and survival is increased by the act of resorption itself, as the degradation of the bone matrix results in the release of growth factors and cytokines that help stimulate the MM cells. Another important mechanism is seen in a cell-to-cell contact manner involving OPN and IL-6 production by osteoclasts. Contact between the two cell types has been demonstrated to lead to increased IL-6 production by osteoclasts (Abe et al., 2004). Furthermore, in the presence of MM cells, osteoclasts produce OPN, an angiogenic and immunoregulatory factor regarded as a metastasis gene (Asou et al., 2001, Ashkar et al., 2000). OPN has also been associated as a resorption stimulating factor, and a bone mineralization inhibitor, making its elevated production an even more serious factor in cases of bone lesions (Shapses et al., 2003, Steitz et al., 2002). Finally, contact itself between the osteoclasts and myeloma cells is also responsible for MM cell growth, mediated by VLA-4 and $\alpha v\beta 3$-integrin adhesion (Abe et al., 2004).

As discussed, many routes have been identified as modulators of TRAIL-induced apoptosis. For more than ten years it has been suggested that the modulation of decoy receptor expression could affect the sensitivity of cells to TRAIL (Sheridan et al., 1997). In the past five years it has been suggested that over-expression of the decoy receptors could induce resistance to TRAIL-induced apoptosis, contributing to increased osteoclast activity in myeloma and other bone diseases (Colucci et al., 2005, Roux et al., 2005). The modulation of TRAIL's receptors at the mRNA level most likely requires an influence at the level of their transcription promoters. To this end, numerous transcription factor

binding sites, including NFAT, AP-1 and Sp1, have been identified in the TRAIL promoter region (Wang et al., 2000). These binding sites become increasingly relevant when it is considered that pathways related to these sites (such as NF-kB, NFAT, and Akt) are strongly implicated in osteoclast survival outside of TRAIL involvement (Gingery et al., 2003, Harper et al., 2003, Golden & Insogna, 2004, Igarashi et al., 2004). Further linking of TRAIL to Akt is evidenced by a study by Nam et al. in 2003 linking Akt to increased FLIP production (Nam et al., 2003). One possible mechanism for TRAIL-induced Akt reduction would be to follow closely that of TNF-α, modulating PKC activity via the TRAIL receptor (Martin et al., 2010). If the mechanism is similar to that of TNF-α, this as yet un-established signaling pathway might work by binding the TRADD complex at the intracellular DD of the receptor, recruiting TRAF and subsequently p62, leading to a modulation of PKC activity, thereby decreasing Akt activation. This would lead to an increase in levels of TRAIL-R2 (and possibly –R1) expressed, while decreasing levels of antiapoptotic proteins like surviving and Bcl-2 (Figure 16) However, as of yet, TRAIL-Rs are not known to interact with p62 or any of its subunits, and neither is TRAIL known to interact with TNF-RI, the receptor shown to be capable of inducing this pathway.

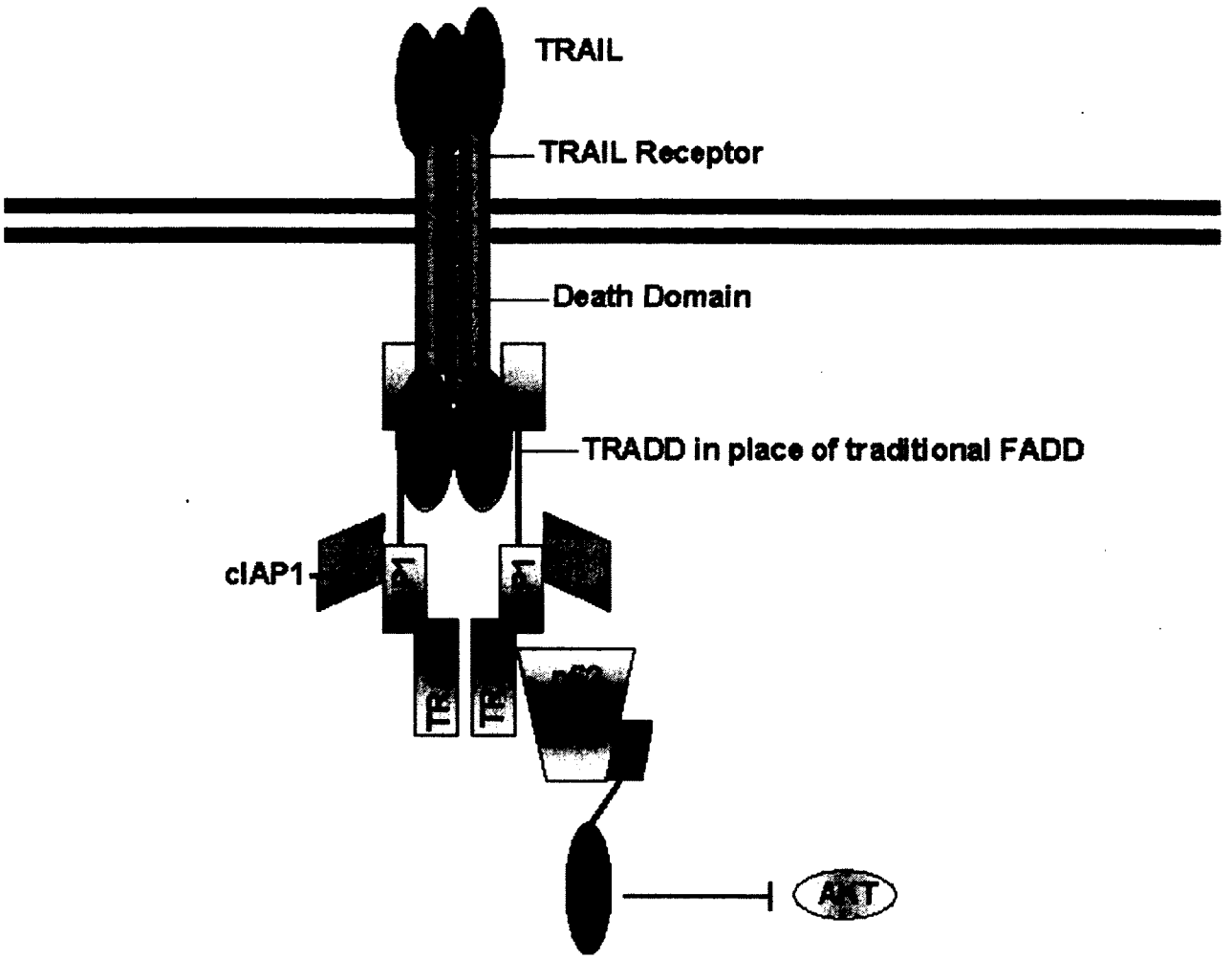

Figure 16: Potential mechanism of TRAIL-induced Akt activity modulation

The effect could to be induced by a modulation of PKC activity (similar to that in TNF alpha) which in turn decreases Akt activation leading to an increase in TRAIL-R2 (DR5) levels and a decrease in the antiapoptotic proteins survivin and bcl-2 levels. (Adapted in part from Martin V et al 2010)

Modulation of its receptors by TRAIL could represent an autocrine positive-feedback control, carried out between protein production and surface transport and expression. These results suggest that with repeated or prolonged TRAIL stimulation, the osteoclast has a built in control in the case that it proves initially incapable to undergo apoptosis. The cell naturally upregulates surface production of the death receptor in response to TRAIL, allowing for an increase in TRAIL activity/signaling.

We observed also on several occasions that an upregulation of mRNA or even protein levels did not equate to a change in surface expression of TRAIL receptors. This is not entirely a surprise, as there are many steps between gene transcription and surface expression. There could be repressor genes, or regulatory proteins standing in the way. Proteases, both intercellular and membrane-bound, represent a threat to newly synthesized TRAIL-R. This becomes a larger issue if the mechanism modulating receptor production or transcription is not fully developed to accommodate these obstacles.

There was also a potential flaw in the experimental method regarding mRNA analysis. Often, particularly in the example of MIP-1α, real-time PCR evaluation of mRNA nearly matched the same augmentation seen in protein at the same doses, but fell just short of significance. A possible explanation for this is a weakening of the signal, so to speak. The lack of consistent mRNA results may have been an effect of the time course used; on average the extractions were performed at least 24 hours from the last stimulation. Recent studies have shown that some modification of TRAIL mRNA occurs over a relatively short timeline, and perhaps a similar period of time is applicable in osteoclasts as well (Aguilera et al., 2009). A proposed method would be stimulation followed by mRNA extraction at 6, 12, and 24 hour periods, helping us evaluate mRNA that otherwise degrade before the extraction is performed.

Finally, we did not find any correlation between protein expression of TRAIL-R and the rate of TRAIL-induced apoptosis in the presence of any of the test conditions. As mentioned earlier, we believe that TRAIL and MIP-1α might have had an effect in experiments using larger numbers of experiments, to allow adequate statistical analysis. In fact it can be noted that the positive control did not register as significantly more apoptotic than the negative control (Figure 15). This is likely attributable to the small number of experiments completed to date (3), further reinforcing the notion that completing further

testing may reveal other significant data, in the instance that significance is established with the control. This is of course expected, as the apoptosis-inducing methods follow protocols established as functional in a previous publication (Roux S. et al., 2005). We intend to complete this work by additional experiments to confirm or invalidate our preliminary data, concerning TRAIL and MIP-1α. Another possibility for this absence of correlation, is that, as suggested in myeloma plasma cells, the status of the TRAIL receptors might not predict TRAIL sensitivity (Mitsiades et al., Blood 2001). Furthermore, even when receptor modulation was observed at any of the three levels evaluated, change from basal expression was small. Therefore there lies the simple possibility that the levels and combinations of cytokines tested do not induce biologically significant change in human umbilical cord blood-derived osteoclasts (from an apoptotic standpoint).

In short, osteoclast apoptosis is the most widely used method in control of bone resorption. Any further understanding of these mechanisms involved in human osteoclast apoptosis may contribute to the application of factors modulating bone resorption. More efficient regulation of osteoclast death and ultimately their function may contribute to improving treatment of bone diseases like osteoporosis, Paget's disease of bone, and multiple myeloma.

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