Tumor Necrosis Factor-a Regulation of Osteoclast Formation in RAW 264.7 Cells

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ABSTRACT

Bone resorption is dependent on the differentiation of osteoclast progenitor cells into mature osteoclasts. The roles of receptor activator of NF-κB ligand (RANKL), the decoy receptor for RANKL (osteoprotegerin, OPG) and macrophage-colony stimulating factor (M-CSF), which are produced by osteoblasts or stromal cells, in osteoclastogenesis are well established. RANKL and M-CSF are both considered to be essential for osteoclastogenesis, while OPG is an inhibitor of RANKL actions. Tumor necrosis factor (TNF-α) is a potent stimulator of bone resorption, especially under inflammatory conditions. However, the role of TNF-α in osteoclast proliferation, differentiation and activation is still unclear. It has been reported previously that TNF-α stimulates bone resorption indirectly via a primary effect on osteoblasts to increase RANKL or decrease OPG. On the other hand, TNF-α has also been proposed to have a direct action on the proliferation and differentiation of osteoclast progenitors [1].

In our current research, we use RAW 264.7 cells, a cell line of monocyte-macrophage lineage. RAW 264.7 cells make M-CSF but do not express RANKL. They can develop into osteoclasts if given RANKL. Our hypothesis is that TNF-α cannot stimulate osteoclastogenesis without RANKL in RAW 264.7 cells but can enhance the effects of RANKL on osteoclastogenesis via the induction of cyclooxygenase-2 (COX-2) and production of prostaglandins.

The aims of this study are to determine if TNF-α can stimulate osteoclastogenesis in RAW 264.7 cells in the absence of RANKL and if TNF-α enhances the effect of added
RANKL in this system via the TNF-α induction of prostaglandins produced by cyclooxygenase (COX-2).

This project will help determine the role of TNF-α in osteoclast formation. This will give us a better understanding of the processes involved in bone remodeling in orthodontic tooth movement as well as in several diseases such as rheumatoid arthritis and periodontitis.
INTRODUCTION

Objective of Research

Previous research has shown that proinflammatory cytokines, such as TNF-α and interleukin-1 (IL-1), play a role in the biologic processes involved in orthodontic tooth movement. The inhibition of cytokine activity by the addition of soluble receptors reduced the amount of tooth movement by 50% and also reduced the number of osteoclasts [2].

Prostaglandins (PGs) are potent stimulators of bone resorption and are produced largely by the induction of COX-2 in osteoblasts [21]. The effect of prostaglandins on tooth movement has been studied extensively. Yamasaki et al [3] suggested that orthodontic mechanical stress induced synthesis and secretion of prostaglandins (PGs) by localized cells, which stimulated osteoclastic bone resorption. When PGE₁ or PGE₂ were injected in the gingiva near the upper first molar in rats, osteoclasts and alveolar bone resorption were observed. On the other hand, the administration of indomethacin, an inhibitor of PG production by both COX-1 and COX-2, suppressed the appearance of osteoclasts and bone resorption. Davidovitch et al [4] reported the involvement of PGE₂ in bone remodeling in orthodontically treated cats. Guinta et al [5] treated miniature pigs with indomethacin and showed a significant decrease in the extent of resorption surfaces histologically.

Cytokines, such as TNF-α and IL-1, have been shown to induce COX-2 expression and PG production in osteoblasts and in cultured bone marrow cells [21] [6]. These cytokines are potent inducers of bone resorption, and some of their effects on resorption may be mediated via their induction of COX-2 and PGs [21]. Most of the regulation of
osteoclastogenesis by cytokines and PGs is thought to occur via the induction of RANKL in osteoblasts, which then binds to RANK receptor on cells of the hematopoietic lineage. However, it has been proposed that TNF-α can induce osteoclastogenesis by acting directly on cells of the hematopoietic lineage, independently of RANKL. Therefore, the objective of this research is to determine if TNF-α can stimulate osteoclast formation via induction of COX-2 in cells of the hematopoietic lineage.

**Background**

**Osteoclasts**

Osteoclasts are multinucleated giant cells that resorb bone. They develop from the hemopoietic cells of the monocyte-macrophage lineage. Major characteristics of osteoclasts are (1) Tartarate-resistance acid phosphatase (TRAP) activity, (2) expression of calcitonin receptors (CTR), (3) multinucleation, and (4) ability to resorb mineralized bone. TRAP is a resorptive enzyme identified in both the ruffled border of the osteoclast membrane and the secretions in the resorptive space [7].

Osteoblasts/stromal cells are crucially involved in osteoclast development. Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors is necessary for induction of osteoclast differentiation.[8] Osteoclast differentiation involves several major stages outlined in Figure 1.

Osteoclasts arise from hematopoietic stem cells (HSCs). HSCs give rise to colony forming unit-granulocyte/macrophages (CFU-GMs). Macrophage/monocyte-colony stimulating factor (M-CSF) stimulates the proliferation of CFU-GMs (osteoclast precursors), which lack two osteoclast markers: TRAP and CTR. The mononuclear
precursors differentiate into prefusion osteoclasts (positive for both TRAP and CTR) with the stimulation of M-CSF and RANKL. The prefusion osteoclasts will further differentiate by fusion to become multinucleated cells (with continuous stimulation of M-CSF and RANKL). When these multinucleated cells become functional, they have a ruffled border [9]. RANKL continues to play an important role in activating osteoclasts by stimulating formation of the ruffled membrane [10].

RAW 264.7 Cells

There is only one known clonal cell line that can give rise to osteoclasts in vitro. The RAW 264.7 cell line is a functional macrophage cell line transformed by the Abelson Leukemia Virus. These cells produce cytokines in response to lipopolysaccharide (LPS) and can make osteoclasts in response to RANKL [11-13]. Since they produce M-CSF, it is not necessary to add M-CSF to cultures.

RANKL, M-CSF and Osteoprotegerin

RANKL is the ligand for the receptor activator of NF-κB. It is also called osteoprotegerin ligand (OPGL) [8]. It is a member of the TNF superfamily. It is a cell surface molecule expressed by marrow stromal cells and osteoblasts, and by activated T lymphocytes [14]. It is involved in bone metabolism by mediating osteoclast differentiation, function and survival [11]. The discovery of RANKL helped establish that osteoblasts/stromal cells support osteoclast differentiation primarily by serving as a source of RANKL as well as M-CSF [10]. Osteoblast/stromal cells express both M-CSF and RANKL (membrane-bound and soluble), which bind to their respective
receptors, c-fms and RANK, expressed on osteoclast precursors to stimulate osteoclast formation. *In vitro*, M-CSF and RANKL have been shown to be sufficient for osteoclastogenesis [15]. Both RANKL knockout and RANK knockout mice show features of osteopetrosis with a complete absence of osteoclasts in bone[16].

Osteoblasts/stromal cells also produce osteoprotegerin (OPG), a soluble decoy receptor for RANKL. OPG inhibits RANKL function by competing with RANK for RANKL [9].

**TNF-α**

TNF-α also modulates osteoclast formation and function [17]. TNF-α exerts its function via two receptors. TNF-α receptor 1 (TNFR1), or p55, contains a death domain (DD), and the binding of TNF-α to TNFR1 triggers programmed cell death [18]. TNFR2, or p75, lacks a DD [9]. TNF-α signal pathways stimulate bone resorption as well as inhibit bone formation. Binding of TNF-α to its receptor activates TNF-α receptor-associated death domain (TRADD), which in turn stimulates two well known pathways: (1) activation of nuclear factor kappa B (NF-κB), Jun kinase (JNK), p38 kinase, protein kinase C (PKC), and (2) activation of Fas activated death domain (FADD), a protein that triggers the pro-apoptotic caspases and cell death. These pathways are not mutually exclusive, which makes the study if TNF-α action difficult [17].

Unlike mice lacking RANKL or RANK, mice lacking TNF-α or its receptor do not exhibit any bone defects [19], indicating that TNF-α mediated signaling is not essential for skeletal development and physiologic bone remodeling. Inflammatory cytokines
like TNF-α and IL-1 are secreted by macrophages and/or T-cells during pathologic bone resorption, for example, in rheumatoid arthritis, periodontitis, and loosening of implants [20].

_prostaglandins (PGs) and cyclooxygenase-2 (COX-2)_

Conversion of arachidonic acid (AA)—a 20 carbon fatty acid derived from the cellular lipid bilayer—by cyclooxygenase (COX or prostaglandin endoperoxide synthase) is the committed step in PG synthesis [21] (Figure 2). PGs are eicosanoids produced by COX and exhibit numerous functions throughout the body.

There are two isoforms of COX, COX-1 and COX-2, which have significant sequence homology and identical catalytic activity, but their expression pattern is markedly different. They have significant differences in mRNA splicing, stability and translational efficiency, and they use different substrate pools [6]. The COX enzymes are associated with two types of receptors: G-protein coupled receptors (transmembrane receptors), and peroxisome proliferators-activated receptors (PPARs), which are members of the nuclear receptor family of transcription factors. The most abundant PG produced by osteoblasts, PGE₂, is associated with four classes of receptors, EP₁-EP₄, which are G-protein coupled receptors [21].

COX-1 and COX-2 are produced throughout the human body. COX-1 is constitutively expressed in nearly all tissues (including osteoblasts) while COX-2 is inducible. It was hypothesized that COX-2 is responsible for acute PG responses associated with inflammation and pain, while COX-1 produces those prostanoids needed for ongoing “housekeeping” functions, including maintenance of renal blood
flow, platelet aggregation, and gastric cytoprotection. COX-2 is produced in response to IL-1, TNF-α, TGF-α, TGF-β, parathyroid hormone, 1,25(OH)₂D₃ and mechanical loading of bone [21]. The induction of COX-2 is transient, with a return to base-line within 24-48 hours [6].

Non-selective, non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, inhibit PG production by competing directly with AA for binding to the cyclooxygenase catalytic site. Therefore, they are used to study the role of endogenous PGs. NS-398 is a selective NSAID that inhibits COX-2 activity at a concentration of 0.01μM, but loses its selectivity at higher doses [21].

PGs appear to have a dual role in bone remodeling by enhancing both osteoclast and osteoblast formation. Although PGs are known to be potent mediators of bone resorption, their actions are complex. Osteoclastogenesis is enhanced because of an increase in RANKL production. Induction of RANKL has been shown to be essential for resorption by PGE₂ [22], but PGE₂ may also have some stimulatory effects on the hematopoietic lineage as well [21]. On the other hand, PGE₂ has also been shown to inhibit the activity of mature osteoclasts [21]. Han et al [23] have shown that RANKL can induce COX-2 expression, which results in production of PGE₂ in RAW 264.7 cells. PGs also seem to increase osteoblast formation by recruiting osteoblast precursors from a population of non-adherent mesenchymal precursors in the bone marrow [24]. Thus, PGs can stimulate both resorption and formation and the balance of these activities in vivo may determine whether bone is gained or lost.
**TNF-α and Osteoclastogenesis**

In bone metabolism, it has been shown that numerous TNF family members including RANKL, TNF-α, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) play pivotal roles in the differentiation, function, survival and/or apoptosis of osteoclasts [9]. TNF-α can induce RANKL production by osteoblasts or marrow stromal cells and promote osteoclastogenesis. There is also the possibility that TNF-α can induce osteoclastogenesis independent of RANKL by acting directly on cells of the hematopoietic lineage.

Lam et al [14] showed that TNF-α targets both marrow stromal cells and hematopoietic osteoclast precursors, but directly impacts the latter only in the presence of permissive levels of RANKL. A pure population of murine osteoclast precursors failed to undergo osteoclastogenesis when treated with TNF-α. However, TNF-α dramatically stimulated differentiation in macrophages primed by less than 1% of the amount of RANKL required to induce osteoclast formation. Administration of TNF-α to RANK deficient animals failed to induce osteoclastogenesis, indicating that TNF-α could not substitute for RANKL in physiological conditions. Macrophages isolated from marrow cultures when treated with OPG were incapable of TNF-α induced osteoclastogenesis. TNF-α potentiation of RANKL-primed osteoclastogenesis was time-sensitive. TNF-α added concomitantly with the priming dose of RANKL failed to induce osteoclast formation. In contrast, when TNF-α was added 2-4 days after RANKL priming, TNF-α induced osteoclastogenesis was maximal. Outside of this temporal window, TNF-α appeared to drive macrophage development along a nonosteoclastogenic pathway.
Zou et al [25] showed that TNF-α expression can be transcriptionally regulated by RANKL. RANKL increased TNF-α expression by 8-fold and 4.5-fold in RAW 264.7 and bone marrow macrophages (BMMs), respectively. RANKL increased TNF-α transcription rate by 2.9-fold in RAW 264.7 cells. This transcriptional mechanism was dependent on the NF-κB sites in the TNF-α promoter. Hence, it is possible that some of the effects of RANKL might be mediated by RANKL-induced TNF-α.

Wei et al [26] found that TNF-α exerts its osteoclastogenic effect via IL-1-dependent and -independent signaling pathways and that the IL-1-mediated effect involves, at least in part, RANKL-producing stromal cells. IL-1 alone was incapable of promoting osteoclast formation in macrophage/stromal cell coculture. However, IL-1 also directly targeted osteoclast precursors and promoted the osteoclast phenotype in a TNF-independent manner in the presence of permissive levels of RANKL (Figure 3).

On the other hand, Kobayashi et al [18] demonstrated that TNF-α can stimulate osteoclast differentiation in the presence of M-CSF through a mechanism independent of the RANKL-RANK interaction. They used bone marrow cells cultured for 3 days, after which M-CSF dependent bone marrow macrophages (M-BMMs) were isolated and treated with TNF-α. Osteoclast formation induced by TNF-α was inhibited by the addition of antibodies against TNFR1 (no TRAP-positive cells) or TNFR2 (markedly reduced TRAP positive cells), but not by osteoprotegerin (OPG, a decoy receptor for RANKL), nor the Fab fragment of anti-RANK antibody. Real time-polymerase chain reaction (RT-PCR) showed that M-BMM expressed RANKL mRNA, but its levels were low and not upregulated by adding TNF-α.
Azuma et al [1] using a cell culture similar to that used by Kobayashi et al [18], found that TNF-α was a crucial differentiation factor for osteoclasts in the presence of M-CSF, via the p55 TNF receptor. TNF-α directly induced the formation of TRAP-positive multinucleated cells (MNCs) in a dose-dependent manner at TNF-α concentrations of 10 ng/ml and above. The number of TRAP-positive MNCs in TNF-α-treated groups was about 50% of that in the soluble RANKL-treated groups. The bone resorption activity of TNF-α induced MNCs was also lower than that of the RANKL-induced MNCs.

Zou et al [27] used cell cultures derived from three different mouse strains, Balb/c and C57BL/6 (strains known to differ in inflammatory responses and cytokine modulation) as well as RAW 264.7 cells. In the Balb/c cells, the following results were seen:

1. TNF-α enhanced osteoclastogenesis in co-culture and in RANKL treated bone marrow cells. Antibodies to TNF-α and TNFR1 inhibited RANKL induction of osteoclastogenesis (also seen in RAW 264.7 cells).

2. TNF-α alone promoted osteoclastogenesis in the presence of M-CSF (but was less efficient than RANKL).

3. RANKL increased abundance of TNF-α mRNA and induced secretion of the TNF-α (also seen in RAW 264.7 cells).

4. OPG inhibited RANKL induced osteoclastogenesis and TNF-α expression, but not TNF-α activity.
In the C57BL/6 cells, TNFR-1 antibodies did not affect RANKL-induced osteoclastogenesis. Therefore, the authors concluded that there were different modes of action of RANKL in the two strains.

Hsu et al [11] showed that the RAW 264.7 cell line expressed high levels of RANK mRNA. Treatment of RAW 264.7 cells with murine RANKL readily stimulated cell differentiation into osteoclast-like TRAP-positive cells. They also showed that RANKL induced JNK activation in RAW 264.7 cells. Activation of JNK was readily detectable after 5 minutes of RANKL exposure. NF-κB activation was not detectable in RANKL-treated RAW 264.7 cells. These data, strongly suggest Jun kinase as a potentially important osteoclastogenic signal transducer.
RESEARCH PLAN

Rationale

For tooth movement to occur, bone resorption has to occur on the compression side and bone deposition on the tension side. Therefore, osteoclasts and osteoblasts play an active role in tooth movement. Under physiologic conditions, osteoblasts secrete RANKL, which helps maintain function, but they do not express IL-1 or TNF-α. However, under pathologic conditions or under the loading conditions necessary for orthodontic tooth movement, these cytokines can be expressed. Since they are potent stimulators of PG formation and bone resorption, they may contribute to the resorption that permits tooth movement [28]. This goal of this project is to examine the role of TNF-α in the formation of bone resorbing cells.

Preliminary Data

Preliminary experiments were done in our lab on RAW 264.7 cells. The effects of TNF-α and PGE₂ on RAW 264.7 cells treated with RANKL (30 ng/ml) were observed. The number of osteoclasts increased significantly when treated with TNF-α or PGE₂. The effect of TNF-α was dose-dependent (Figure 4).

A second experiment was conducted to see the effect of a COX-2 inhibitor (NS-398) on RAW 264.7 cells treated with PGE₂ + RANKL and TNF-α + RANKL. There was a significant reduction in the number of TRAP-positive cells with the addition of NS-398 (Figure 5).

To take this one step further, we decided to determine if TNF-α alone can stimulate osteoclast formation. Previous literature has shown that it may [1, 18, 27]. However, these experiments were done in bone marrow macrophages cultured with whole marrow
cells for three days before they were isolated. Therefore these cells had been exposed to RANKL prior to treatment with TNF-α. According to Lam et al [14], this priming with RANKL is crucial for osteoclastogenesis with TNF-α. We propose the use of the RAW 264.7 cell line, which is a macrophage cell line that does produce RANKL and therefore has no exposure to RANKL unless it is added.

This preliminary data and a review of the current literature led us to our current hypothesis.

**Hypothesis**

1. TNF-α cannot stimulate osteoclastogenesis by itself in RAW 264.7 cells but can stimulate osteoclastogenesis in the presence of RANKL.

2. TNF-α can enhance the RANKL stimulated osteoclastogenesis by increasing COX-2 expression and PG production in RAW 264.7 cells.

**Specific Aims**

**Aim 1.** To determine if TNF-α alone can stimulate osteoclastogenesis in RAW 264.7 cells.

**Aim 2.** To determine if TNF-α enhances RANKL induced osteoclastogenesis

**Aim 3.** To determine if TNF-α induces COX-2 expression and PG production in RAW 264.7 cells.

**Aim 4.** To determine the effect of inhibiting PG production by indomethacin (a general inhibitor of both COX-1 and COX-2 activity) and NS-398 (a selective inhibitor of COX-2 activity) on TNF-α induced osteoclastogenesis.
Materials and Methods

Materials

TNF-α was obtained from Roche Diagnostics (Indianapolis, IN). RANKL was from R&D Biosystems (Minneapolis, MN). NS-398 was from Cayman Chemical Company (Ann Arbor, MI). Culture media were purchased from Gibco-BRL (Grand Island, NY). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Cell Culture

We used the RAW 264.7 murine macrophage cell line. Cells were plated at concentrations of 1.2x10^4 cells/well or 6x10^4 cells/well in 12 well dishes in α-MEM with 10% heat activated fetal calf serum (HIFCS), 100 U/ml of penicillin and 50 μg/ml of streptomycin and cultured in a humidified atmosphere of 5% CO₂ at 37°C. We started with an initial cell concentration of 6x10^4 cells/well, but due to the large number of osteoclasts produced, quantification was difficult and we reduced the concentration to 1.2x10^4 cells/well. Cells were treated with RANKL (30 ng/ml), TNF-α (10 ng/ml) and RANKL + TNF-α. The vehicle was phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA). Media were changed on day 2.

To inhibit COX-2 activity, cells were treated with indomethacin (1 μM) or NS-398 (0.1 μM) and the vehicle, 0.1% ethanol, was added to control cultures.

Tatrate Resistance Acid Phosphatase Staining

Cells were fixed at the end of culture with 2.5% glutaraldehyde. A Leukocyte Acid Phosphatase Kit (Sigma) was used to stain for tartrate resistant acid phosphatase
(TRAP, a marker of osteoclasts) following the manufacturer’s instructions. The TRAP positive multinucleated cells (MNCs), with 3 or more nuclei, were counted under the microscope at 10X magnification. Media were collected from each well, placed in dishes covered with parafilm securely and stored at -20°C for PGE2 assays.

**PGE2 Assay**

Medium was removed from cultured cells and PGE2 accumulation was measured on days 2, 3 and 4 using enzyme immunoassay kits. The experiment was repeated three times with three different enzyme immunoassay kits following the manufacturer’s instructions: (1) EIA Cayman (monoclonal), Ann Arbor, MI. The detection range was 7.8-1000 pg/ml. (2) Cayman Express, Ann Arbor, MI. The detection range was 15.6 pg/ml to 2000 pg/ml. (3) Assay Designs, Ann Arbor, MI. The detection range was 38.7 pg/ml to 2600 pg/ml (0.11 to 7.4 nM).

**RNA Extraction, Real Time-PCR**

Cells were plated in 12-well dishes at a concentration of 1.2x10^4 cells/well and treated with vehicle, RANKL (30 ng/ml), TNF-α (10 ng/ml) or RANKL + TNF-α. Three wells of cells were pooled for one sample. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, California) in accordance with the manufacturer’s directions. 2-5 μg of total RNA was converted to cDNA by the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following manufacturer’s instructions. Quantitative PCR for gene expression was performed in 96-well plates using Assays-on-Demand Gene Expression system (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the endogenous control. Each sample
was amplified in duplicate. Primers were tested for equal efficiency over a range of
target gene concentrations. The PCR reaction mixture (20 μL/well including 2X TaqMan Universal PCR Master Mix, 20X Assays-on-Demand Gene Expression Assay Mix and 40 ng of cDNA) was run in Applied Biosystems ABI Prism 7300 Sequence Detection System instrument utilizing universal thermal cycling parameters. A pool of reversed transcribed samples was the calibrator. We used the relative standard curve method, which sets standard curves for the target gene and endogenous control (GAPDH) by serially diluting total RNA, normalizes the amount of the target gene calculated from the standard curve to the amount of the control gene calculated from the standard curve, and compares the fold induction to the calibrator sample.

Statistical Analysis

All values are depicted as the mean ± standard error of the mean (SEM). Statistical analysis was performed using SigmaStat® for Microsoft Windows®, version 2.03 (San Rafael, CA). To compare multiple treatment groups, differences were examined by one-way analysis of variance (ANOVA) followed by the post hoc Bonferroni’s test.
RESULTS

SPECIFIC AIM #1: To determine if TNF-α alone can stimulate osteoclastogenesis in RAW 264.7 cells

No osteoclasts were observed in the control cultures. In the wells treated with TNF-α alone, there appeared to be an increase in the number of TRAP stained mononuclear cells on day 3 of the culture as compared to the control. The mononuclear cells also appeared to aggregate to form clusters. Osteoclast formation, defined as TRAP positive MNCs, began on day 4. The microscopic images of the wells showed large, MNCs (≥ 3 nuclei) that were stained purple due to TRAP activity (Figures 6 and 7). This experiment was repeated five times. In three of the experiments, the peak in TNF-α induced osteoclast formation was on day 4 (Figures 8 and 9). In the remaining two experiments, the peak in the number of osteoclasts was observed on day 5 (Figure 10). There was a large variation in the number of TNF-α induced osteoclasts in the different experiments. For example, in one experiment, there were 189 ± 5 osteoclasts on day 4 and 462 ± 33 osteoclasts on day 5 (Figure 10, expt 5). In another experiment, there were 7 osteoclasts on day 4 and 1.5 ± 0.5 on day 5 (Figure 9). The reason for this variability is unclear. There are several possibilities: 1) changes in the phenotype with the passage of the cells, 2) unintended differences in the plating densities, and 3) decreased efficacy of the TNF-α stock over time.

SPECIFIC AIM #2: To determine if TNF-α can enhance RANKL induced osteoclastogenesis

When the RAW 264.7 cells were treated with RANKL + TNF-α, there was a statistically significant increase in the number of osteoclasts on day 3 (p<0.05) and on day 4 (p<0.01) as compared to RANKL alone (Figures 7 and 11). This experiment was
repeated 4 times with similar results. On day 5, there was a decrease in the number of osteoclasts with RANKL + TNF-α as compared to RANKL alone. However, this difference was not statistically significant. Examination of the wells on day 5 showed the appearance of ghost cells, which lacked the TRAP positive staining as well as an intact cell membrane (Figure 7).

**SPECIFIC AIM #3: To determine if TNF-α induces COX-2 expression and prostaglandin production in RAW 264.7 cells**

Media from the cell culture experiments were analyzed for PGE₂ production via Enzyme Immunoassay (EIA). The experiment was repeated three times with three different assay kits. There was an n=1 in each treatment group.

1. Cayman EIA (7.8 – 1000 pg/ml): The PGE₂ levels were below the detection range in the control cultures. Treatment with TNF-α increased PGE₂ accumulation in the media (the values ranged from 17.8 to 78.6 pg/ml). (Table 1).

2. Cayman Express (15.6 – 2000 pg/ml): The PGE₂ levels were below the detection range on days 2 and 4 of the control cultures. On day 3, PGE₂ level was measured at 20.9 pg/ml, which is barely detectable. Treatment with TNF-α increased PGE₂ accumulation in the media by 5-fold (105.7 pg/ml) or more as compared to the control (Table 2).

3. Assay Design EIA kit (detection range 38.7 – 2600 pg/ml): The PGE₂ levels were below the detection range in the control cultures. Treatment with TNF-α increased PGE₂ accumulation in the media and the values ranged from 77.85 to
439.6 pg/ml (the assay was repeated twice on each sample and the given values are a mean of the two readings) (Table 3).

There was a consistent trend in all three assays of increasing PGE$_2$ levels from day 2 through day 4 when treated with TNF-$\alpha$. These assays were also performed on media from cells treated with TNF-$\alpha$ + indomethacin. Levels of PGE$_2$ in the media from indomethacin treated cells were below the detectable range.

Real time PCR conducted on day 3 showed a small induction of COX-2 mRNA when RAW 264.7 cells were treated with TNF-$\alpha$ (10 ng/ml). This increase in COX-2 expression was statistically significant ($p<0.01$) (Figure 12). However, since COX-2 is a transiently expressed gene, we may have missed a larger induction earlier in culture.

**SPECIFIC AIM #4: To determine the effect of inhibiting PG production with indomethacin and NS-398 on TNF-$\alpha$ induced osteoclastogenesis**

The results of adding either a nonselective COX inhibitor (indomethacin) or a selective COX-2 inhibitor (NS-398) to the RAW 264.7 cell cultures were very similar. When either indomethacin (Indo, 1 $\mu$M) or NS-398 (0.1 $\mu$M) were added to the cells, there was either an increase or no change in osteoclast number. On day 3, RANKL + Indo (820±25 cells/well) showed a significant increase compared to RANKL alone (507±18 cells/well) (Table 4 and Figure 13). Similarly, on day 3, TNF-$\alpha$ + NS-398 (4.3±0.3 cells/well) was significantly greater than TNF-$\alpha$ alone (1.3±0.9 cells/well) as was RANKL + TNF-$\alpha$ + NS-398 (343.3±13 cells/well) compared to RANKL + TNF-$\alpha$ (255±21 cells/well) (Table 5 and Figure 14). Neither of these inhibitors of PG production caused a decrease in the number of MNCs formed, except on day 5. On day 5, when treated with TNF-$\alpha$ + Indo as compared to TNF-$\alpha$, the osteoclast number
decreased by a mean of 168 cells. Similarly, in the TNF-\(\alpha\) + NS398 treatment group, there was a mean decrease of 63 in the number of osteoclasts as compared to TNF-\(\alpha\) alone. With RANKL+TNF-\(\alpha\) + NS 398, the osteoclast number decreased by 111 as compared to RANKL + TNF-\(\alpha\). However, none of these decreases were statistically significant. All other treatments, showed either a significant stimulatory effect or no change in osteoclast number when either indomethacin or NS-398 were added to the different treatments (Tables 4 and 5, Figures 13 and 14).
DISCUSSION

Since RAW 264.7 cells do not make RANKL, the presence of TRAP stained MNCs in RAW 264.7 cells treated with TNF-α indicates that TNF-α alone can induce osteoclastogenesis by acting on the hematopoietic lineage. There was an increase in the number of TRAP stained mononuclear cells on day 3, and they appeared to aggregate into clusters of cells. This could suggest the beginning of the transformation into osteoclasts, as multiple mononuclear cells fuse together to form MNCs (a key characteristic of osteoclasts). This observation is consistent with the recent studies of Li et al [29] and Yao et al [30] that concluded TNF-α could increase the proliferation of osteoclast precursors in bone marrow by enhancing c-Fms (the receptor for M-CSF) expression in the osteoclast progenitor pool. These progenitors would then have a greater proliferative response to M-CSF. Hence, it would be interesting to quantify the cell numbers and examine the mRNA and protein expression of c-Fms in RAW 264.7 cells treated with/without TNF-α. It would also be interesting to examine the expression of M-CSF and the response to added M-CSF in cells treated with/without TNF-α.

These authors (Li; Yao) found that RANKL was necessary to produce mature multinucleated osteoclasts in their bone marrow culture system. On the other hand, consistent with our data, Zou et al [27] reported that TNF-α alone can induce the formation of TRAP positive MNCs in RAW 264.7 cells. However, no RAW 264.7 data were actually shown in their paper, and there was no report of whether or not the TRAP positive MNCs formed in response to TNF-α in RAW 264.7 cells could resorb bone. A recent article by Hotokezaka et al [31] suggests that TNF-α alone can induce the fusion
of TRAP positive mononuclear cells (derived from RAW 264.7 cells) into TRAP positive MNCs through the TNF-α receptors. Subsequent activation of signaling pathways involving PI3K, Src, ERK and JNK molecules was required for the cell fusion. In the future we will need to demonstrate that our TRAP positive MNCs are truly mature osteoclasts by placing them on bone slices and showing that they can form pits.

TNF-α was not as effective as RANKL in our model. The total number of TRAP positive MNCs formed after treatment with TNF-α was always lower than the number formed with RANKL alone in the same experiment. Similar results were found in the study by Azuma et al [1] where the number of TRAP-positive MNCs in TNF-α-treated groups was about 50% of that in the soluble RANKL-treated groups. Thus, it seems likely that under normal physiologic conditions, the RANKL pathway for osteoclastogenesis would predominate. Although we did demonstrate that a significant increase in the number of osteoclasts with RANKL + TNF-α as compared to RANKL alone, we must do more studies to determine whether this effect is additive or synergistic. Our previous experiments indicated 30 ng/ml of RANKL produces maximal osteoclastogenesis in RAW 264.7 cells. Hence, the observation that TNF-α can increase the effects of 30 ng/ml RANKL on osteoclastogenesis suggests that the two agonists work in part by independent pathways. The decrease in osteoclast number seen on day 5 with RANKL + TNF-α as compared to RANKL alone is possibly due to an acceleration of osteoclast formation, resulting in an earlier peak of osteoclast formation in the combination treatment, followed by normally occurring cell death, or perhaps by
increased apoptosis/cell death with the joint treatment. This could be studied by doing more precise time courses and using stains to demonstrate the apoptotic cells.

One of the ways that TNF-α may enhance the effect of RANKL is to upregulate the RANK receptor. The combination of TNF-α and RANKL was found to synergistically upregulate RANK expression in osteoclast precursor cells isolated from marrow cultures by Zhang et al [32]. We can use leftover RNA extracted from our cultures to investigate this possibility in RAW 264.7 cells. These authors also found that that TNF-α enhanced RANKL induced osteoclastogenesis by interaction of the signaling pathways downstream from the RANK and TNF-α receptor 1 and that these coupling effects were dependent on the TNF-α receptor 1.

Although RAW 264.7 cells are not supposed to express RANKL, we considered the possibility that TNF-α could induce RANKL in these cells. We tried to measure RANKL mRNA by real time PCR but it was undetectable (data not shown). Other possible experiments to confirm that RANKL was not involved in the effects of TNF-α would be to see what happened to the effects of TNF-α after treating cultures with OPG, knocking down receptors for TNF-α with small interfering RNAs, or using specific blocking antibodies for TNF-α.

It is also possible that RANKL acts in part to increase osteoclastogenesis via induction of TNF-α. For example, Nakao et al [33] have shown that RANKL stimulates TNF-α expression and that formation of TRAP positive MNCs in RAW 264.7 cultures were reduced by treatment with specific blocking antibodies to TNF-α or to TNF-α receptor 1. We address this issue by using RNA extracted from our cultures to measure TNF-α mRNA levels after treatment with/without RANKL +/− TNF-α.
We found increased PGE$_2$ in the media collected from the wells treated with TNF-$\alpha$. This suggests that TNF-$\alpha$ stimulates PGE$_2$ production and is consistent with studies from Han et al [23]. These authors showed that RANKL transcriptionally upregulated COX-2 expression in RAW 264.7 cells but did not show effects of inhibiting COX-2 activity on osteoclast formation in these cells (only in bone marrow derived osteoclasts). In our studies, the medium PGE$_2$ increased from day 2 to day 4, suggesting that the peak in PGE$_2$ production coincided with the peak in osteoclast formation. However, in contrast to our original hypothesis, we found that inhibiting PG production with NSAIDs, NS-398 or indomethacin, did not decrease osteoclast formation in any of the treatment groups: RANKL, TNF-$\alpha$, or the combination. In fact, in many cases, addition of NSAID significantly stimulated the formation of TRAP positive MNCs.

We had expected that inhibiting PG production would decrease the effect of RANKL since it has been reported that RANKL-induced COX-2 expression and PG production are required for maximal effects of RANKL stimulated osteoclastic differentiation by Han et al [23]. However, these investigators only showed the effects of inhibiting COX-2 activity on RANKL-induced osteoclastogenesis in bone marrow derived osteoclast cultures and not in RAW 264.7 cells. It is likely that the bone marrow derived osteoclast cultures also contained some osteoblasts or stromal cells and perhaps the presence of these cells had some influence on the effects of the NSAIDs. PGs are known to increase osteoblast formation by recruiting osteoblast precursors from mesenchymal precursors in the bone marrow stromal cultures [24].
There are other studies showing that PGs can inhibit osteoclast formation in cultures containing only cells of the hematopoietic lineage. For example, a recent article by Akaogi et al [34] showed that PGE$_2$ could suppress osteoclastogenesis by inhibiting IL-17-induced TNF-$\alpha$ expression in macrophages. Ono et al [35] showed that PGE$_2$ had biphasic effects on spleen cell cultures from mice. Spleen contains hematopoietic lineage cells but no osteoblast lineage cells and spleen cells can differentiate into osteoclasts if given RANKL and M-CSF. In these cells, PGE$_2$ had an initial inhibitory effect on osteoclastogenesis, followed by a stimulatory effect. The stimulatory effect was postulated to be due to factors secreted by T cells in the spleen cultures. Take et al [36] have also reported that PGE$_2$ inhibits osteoclast formation in cultures of cells prepared from human peripheral blood mononuclear cells (PBMCs). These cultures do not contain any osteoblasts or stromal cells and they were selected to contain no T cells as well. They also found that NS-398 enhanced osteoclast formation when these PBMCs were co-cultured with a clonal osteoblast cell line isolated from an osteosarcoma. It is possible that PGs have different effects on different stages of the hematopoietic lineage or that effects will vary depending on other cells in the environment.
SIGNIFICANCE OF THE RESULTS

This study will give us a better understanding of the role of TNF-α in osteoclastogenesis and bone remodeling and its mechanism of action via COX-2 and prostaglandin production. This is turn will help us better understand the therapeutic ability of nonselective NSAIDS and selective COX-2 inhibitors. It will shed some light on the causes of several metabolic bone diseases caused by abnormal osteoclast recruitment and functions such as osteopetrosis, osteoporosis, metastatic bone disease, Paget’s disease, rheumatoid arthritis and periodontal disease [10]. In addition, it will give orthodontists greater insight into the mechanisms involved in stimulation and inhibition of tooth movement. This in turn could improve the quality and efficiency of treatment.
TABLES
<table>
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<th>TREATMENTS</th>
<th>DAY 2</th>
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<th>DAY 4</th>
</tr>
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<tbody>
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<td>ND</td>
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</tr>
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Table 1: PGE₂ production (pg/ml) on days 2, 3 and 4 measured by Cayman EIA. RAW 264.7 cells were treated with vehicle (Control) and TNF-α (10 ng/ml). Cultures were also treated with / without indomethacin (1 μM), a nonselective inhibitor of both COX-1 and COX-2 activity. Units: pg/ml, Detection Range: 7.8 to 1000 pg/ml, ND – non detectable
Table 2: PGE$_2$ production (pg/ml) on days 2, 3 and 4 measured by Cayman Express. RAW 264.7 cells were treated with vehicle (Control) and TNF-α (10 ng/ml). Cultures were also treated with / without indomethacin (1 μM), a nonselective inhibitor of both COX-1 and COX-2 activity.

Units: pg/ml, Detection Range: 15.6 to 2000 pg/ml, ND – non detectable

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Table 3: PGE₂ production (pg/ml) on days 2, 3 and 4 measured by Assay Design EIA Kit. RAW 264.7 cells were treated with vehicle (Control) and TNF-α (10 ng/ml). Cultures were also treated with/ without indomethacin (1 μM), a nonselective inhibitor of both COX-1 and COX-2 activity.

Units: pg/ml, Detection Range: 38.7 to 2600 pg/ml, ND – non detectable

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Table 4: Effect of Indomethacin on osteoclast formation. RAW 264.7 cells were treated with vehicle (Control), TNF-α (10 ng/ml), RANKL (30 ng/ml), or the combination of RANKL + TNF-α. Cultures were also treated with / without indomethacin (1 μM), a nonselective inhibitor of both COX-1 and COX-2 activity. Numbers are means ± SEM for n=3 wells of cells.

*Significant stimulatory effect of indomethacin, p <0.01.

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<td>DAY 4</td>
<td>DAY 5</td>
</tr>
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<td>-----------</td>
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</tr>
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<td>TNF-α</td>
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<td>252.3 ± 18.6</td>
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<tr>
<td>+ NS398</td>
<td>4.3 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.3 ± 3.2</td>
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<td>1420.0 ± 124.2</td>
<td>525.7 ± 39.5</td>
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Table 5: Effect of NS398 on osteoclast formation. RAW 264.7 cells were treated with vehicle (Control), TNF-α (10 ng/ml), RANKL (30 ng/ml), or the combination of RANKL + TNF-α. Cultures were also treated with / without NS398 (0.1 μM), a selective COX-2 inhibitor. Numbers are means ± SEM for n=3 wells of cells.

<sup>a</sup>Significant stimulatory effect of indomethacin, p < 0.05.
FIGURES
Figure 1: Osteoclast differentiation pathway (taken from Feng, X., 2005 [9]).
**Figure 2.** Arachidonic acid metabolic pathway leading to the production of prostaglandins (*taken from Chapter 54 in Principles of Bone Biology by Pilbeam CC et al. [21]).
Figure 3: Mechanisms of TNF-α induced osteoclastogenesis (taken from Wei et al, 2005 [26]).
Figure 4: Effects of TNF-α and PGE₂ on osteoclastogenesis in RAW 264.7 cells treated with 30 ng/ml RANKL.

Cells were cultured for 5 days in the presence of RANKL plus treatments as indicated. There was a significant increase in TRAP positive multinucleated cells (MNCs) when the cells were treated with PGE₂ (1 μM) or with TNF-α (1 and 10 ng/ml).

aSignificantly different from control, P< 0.01;
bSignificantly different from control, P< 0.05
Figure 5: Effects of inhibiting PGE$_2$ production by NS-398 (0.1 μM) on TNF-α induced osteoclastogenesis in RAW 264.7 cells treated with 30 ng/ml RANKL. Cells were treated for 5 days. NS-398, a selective COX-2 inhibitor, inhibited the induction of TRAP positive multinucleated cells (MNCs) stimulated by PGE$_2$ and TNF-α.

*aSignificantly different from control, P< 0.01
Figure 6: TRAP staining following treatment with RANKL (30 ng/ml), TNF-α (10 ng/ml) or RANKL + TNF-α. Cells were treated for the number of days indicated and stained with TRAP. Dishes were scanned into Adobe Photoshop. TRAP positive cells appear pink/purple. With both RANKL and RANKL + TNF-α, there was an increase in TRAP positive cells on days 3 and 4. Although not apparent in this picture, “ghost cells” (structures that appear to be dead TRAP positive cells) appeared on day 5. With TNF-α alone, TRAP positive cells are visible on day 5.
Figure 7: Microscopic images of osteoclast formation following treatment with TNF-α (10 ng/ml), RANKL (30 ng/ml) or RANKL + TNF-α. Mononuclear cells increased in number in cultures treated with TNF-α alone on day 3 and TRAP positive MNCs appeared on days 4 and 5. The combination of RANKL + TNF-α increased the number of TRAP positive MNCs, compared to RANKL alone, on days 3 and 4. Ghost cells appeared on day 5 in RANKL treatment groups.
Figure 8: Quantification of TRAP positive MNCs on days 3, 4 and 5 in RAW 264.7 cell cultures treated with TNF-α (10 ng/ml) alone.

*Significantly different from control, p<0.01.
Figure 9: Quantification of TRAP positive MNCs on days 3, 4 and 5 in RAW 264.7 cell cultures treated with TNF-α (10 ng/ml) alone.

*Significantly different from control, p<0.01.*
Figure 10: Quantification of TRAP positive MNCs on days 3, 4 and 5 in RAW 264.7 cell cultures treated with TNF-α (10 ng/ml) alone. In some experiments TRAP positive MNC number peaked later than day 4.

*aSignificantly different from control, p<0.01.
Figure 11: Effect of TNF-α (10 ng/ml) on RANKL-induced osteoclastogenesis in RAW 264.7 cells on days 3, 4 and 5. On days 3 and 4 of cell culture, there were a significantly greater number of TRAP positive MNCs formed with RANKL+ TNF-α compared to RANKL alone.

*a*Significantly different from control, *P*< 0.01

*b*Significantly different from control, *P*< 0.05
Figure 12: Effect of TNF-α (10 ng/ml) on the induction of COX-2 mRNA expression on day 3 of cell culture.
Bars are means ± SEM for 3 samples.
*aSignificantly different from control, p < 0.01
Figure 13: Effect of Indomethacin on osteoclastogenesis in RAW 264.7 cells
Bars are means ± SEM for 3 samples.

aSignificant stimulatory effect of indomethacin, p <0.01
bSignificant stimulatory effect of indomethacin, p <0.05
Figure 14: Effect of NS 398 on osteoclastogenesis in RAW 264.7 cells. Bars are means ± SEM for 3 samples.

a Significant stimulatory effect of NS 398, $p < 0.01$

b Significant stimulatory effect of NS 398, $p < 0.05$
REFERENCES


