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Roles of Stromal Cell RANKL, OPG, and M-CSF Expression in Biphasic TGF- β Regulation of Osteoclast Differentiation

MARY KARST¹, GENEVIEVE GORNY¹, RACHELLE J. SELLS GALVIN², and MERRY JO OURSLER^{1,3,*}

¹Department of Biology, University of Minnesota, Duluth, Minnesota

²Lilly Research Labs, Eli Lilly and Company, Indianapolis, Indiana

³Endocrine Research Unit, Mayo Clinic and Foundation Rochester, Minnesota

Abstract

To better understand the complex roles of transforming growth factor-beta (TGF- β) in bone metabolism, we examined the impact of a range of TGF- β concentrations on osteoclast differentiation. In co-cultures of support cells and spleen or marrow osteoclast precursors, low TGF- β concentrations stimulated while high concentrations inhibited differentiation. We investigated the influences of TGF- β on macrophage colony stimulating factor (M-CSF), receptor activator of NF- κ B ligand (RANKL), and osteoprotegerin (OPG) expression and found a dose dependent inhibition of M-CSF expression. RANKL expression was elevated at low TGF- β concentrations with a less dramatic increase in OPG. Addition of OPG blocked differentiation at the stimulatory TGF- β dose. Thus, low TGF- β concentrations elevated the RANKL/OPG ratio while high concentrations did not, supporting that, at low TGF- β concentrations, there is sufficient M-CSF and a high RANKL/OPG ratio to stimulate differentiation. At high TGF- β concentrations, the RANKL/OPG ratio and M-CSF expression were both repressed and there was no differentiation. We examined whether TGF- β -mediated repression of osteoclasts differentiation is due to these changes by adding M-CSF and/or RANKL and did not observe any impact on differentiation repression. We studied direct TGF- β impacts on osteoclast precursors by culturing spleen or marrow cells with M-CSF and RANKL. TGF- β treatment dose-dependently stimulated osteoclast differentiation. These data indicate that low TGF- β levels stimulate osteoclast differentiation by impacting the RANKL/OPG ratio while high TGF- β levels repress osteoclast differentiation by multiple avenues including mechanisms independent of the RANKL/OPG ratio or M-CSF expression regulation.

Transforming growth factor-beta (TGF- β) is a ubiquitous multifunctional cytokine that has a spectrum of influences. The variety of reported responses to TGF- β depends, at least in part, on experimental conditions as well as the cell type under study. Within the bone environment, TGF- β is a key regulator of bone metabolism. Although all TGF- β isoforms bind to the same receptor complex, there have been some reports of different cellular responses to the different isoforms (Jennings et al., 1988; Segarini et al., 1988; ten Dijke et

*Correspondence to: Merry Jo Oursler, Department of Biochemistry and Molecular Biology, Mayo Foundation, 200 First Street SW, Rochester, MN 55905. moursler@d.umn.edu.

al., 1990; Lyons et al., 1991; Liu et al., 2000). In the presence of stromal support cells, TGF- β 1 has a biphasic effect on osteoclast differentiation from marrow precursors, in that TGF- β 1 stimulates differentiation at a low dose while inhibiting differentiation at a higher dose (Shinar and Rodan, 1990; Mundy, 1991; Yamaguchi and Kishi, 1995). Spleen cells, as well as marrow cells, contain osteoclast precursors and the possibility of a biphasic effect of TGF- β on spleen cell precursor differentiation has not yet been studied.

A great deal of information on osteoclast differentiation has been investigated using a co-culture system of osteoclast precursors from either spleen or marrow combined with a support cell line, such as osteoblasts or stromal cells (Udagawa et al., 1990; Takahashi et al., 1995). From these studies, it has been demonstrated that many factors influence osteoclast differentiation through effects on support cells (Khosla, 2001; Suda et al., 2001). It has been well-documented that macrophage colony stimulating factor (M-CSF) is required for osteoclast differentiation (Yoshida et al., 1990; Kodama et al., 1991; Takahashi et al., 1991; Suda et al., 1993). Although it has been documented that TGF- β influences M-CSF stimulated osteoclast differentiation, the impact of TGF- β on M-CSF expression has not been investigated during osteoclastogenesis (Sells Galvin et al., 1999; Fox et al., 2003). It is a goal of the research described here to address this question. There is also overwhelming evidence that interaction with support cell-derived receptor activator of NF- κ B ligand (RANKL) induces osteoclast differentiation (Simonet et al., 1997; Suda et al., 2001). Osteoprotegerin (OPG) is a secreted stromal cell-derived decoy receptor that specifically binds RANKL and inhibits osteoclast differentiation (Simonet et al., 1997; Suda et al., 2001). The balance of RANKL relative to OPG expression modulates the rate of osteoclast differentiation and many factors that influence osteoclast differentiation do so by regulating OPG and RANKL expression in stromal support cells (Khosla, 2001; Theill et al., 2002). TGF- β 1 treatment of stromal cells at relatively high doses (levels that inhibit osteoclast differentiation in co-cultures of marrow precursors with stromal cells) induces OPG and inhibits RANKL expression (Takai et al., 1998; Sells Galvin et al., 1999; Thirunavukkarasu et al., 2001; Quinn et al., 2001). It is, therefore, hypothesized that this modulation is responsible for TGF- β -mediated repression of osteoclast differentiation, but this has not yet been tested. It is a goal of the research described here to address this question.

The above observations have led us to examine the responses of osteoclast precursors resident in both marrow and spleen to a broad range of TGF- β 1 and TGF- β 2 concentrations, the impact of stimulatory and inhibitory TGF- β 1 doses on M-CSF, RANKL, and OPG expression, and how these changes influence osteoclast differentiation. We have used as our model system the co-culture of osteoclast precursors from mouse spleen and marrow with ST2 stromal cells.

MATERIALS AND METHODS

Material

Unless otherwise noted, all chemicals were from Sigma Chemical Co., St. Louis, MO.

In vitro osteoclast differentiation with stromal support cells

Mouse marrow and spleen containing osteoclast precursors were obtained from female BalB/c mice (Taconic, Germantown, NY). Four to six-week-old mice were sacrificed and long bones of the hind limbs and spleen were aseptically removed. The distal ends of bones were clipped and the marrow flushed out by injecting sterile Mosconas buffer (8% NaCl, 0.2% KCl, 0.06% $\text{NaH}_2\text{PO}_4 + \text{H}_2\text{O}$, 2% glucose, 0.02% bicarbonate) into the marrow cavity with a 27-gauge needle. Marrow cells were counted and stored at 2.4×10^6 cells/tube in liquid nitrogen until used. Freezing media consisted of 12% dimethylsulfoxide (DMSO) in FBS as has been previously reported (Wesolowski et al., 1995). To generate marrow-derived osteoclasts, precursors were cultured with ST2 stromal cells (Riken Cell Bank, Tsukuba, Japan) during differentiation. ST2 cells (passage 10–13) were plated (4×10^4 cells/well) in a 48-well plate (Fisher, Pittsburgh, PA) 24 h prior to the addition of osteoclast precursors (1.5×10^6 marrow mononuclear cells or 4.8×10^7 spleen cells per plate) as previously reported (Gingery et al., 2003). Recombinant human TGF- β 1 or TGF- β 2 (R&D Systems, Minneapolis, MN) were added to four replicate wells for each dose. In some experiments, 1 ng/ml OPG, 25 ng/ml M-CSF (R&D), or 60 ng/ml RANKL (Calbiochem, La Jolla, CA) were added as indicated in the figure legends. The media was changed every 3 days. On day 6 for spleen cultures and either day 9 or 10 for marrow cultures, the cell co-cultures were washed three-times with phosphate buffered saline ($1 \times \text{PBS}$: 1.7 mM KH_2PO_4 , 5 mM Na_2HPO_4 , 150 mM NaCl, pH to 7.4) and fixed with 1% paraformaldehyde in PBS. After incubating for 30 min in the fixative, the cells were rinsed with water three-times and stored in water at 4°C until they were evaluated for differentiation.

Determination of differentiation

Tartrate resistant acid phosphatase (TRAP) staining was used to visualize differentiated cells according to manufacturer's directions (Sigma Chemical Co.). The number of multinucleated TRAP positive cells was counted using an Olympus Takyo inverted microscope at 200 $\times$ magnification.

ST2 cell treatment, RNA isolation, real time polymerase chain reaction

ST2 cells were plated in 100 mM dishes and maintained until confluent. One plate was harvested as a time zero. The remaining cultures were maintained in base medium or base medium supplemented with 7×10^{-3} M ascorbic acid, 1×10^{-7} M dexamethasone, and 1×10^{-5} M vitamin D₃ as above and treated with either vehicle or a range of TGF- β 1 concentrations for 3 days. RNA was isolated using Trizol Reagent according to manufacturer's directions (Gibco BRL, Grand Island, NY). The RNA was stored at -70°C until analyzed. Following LiCl precipitation to remove DNA, cDNA was synthesized by standard protocol: 4 μg total RNA was heat denatured at 68°C for 15 min in reverse transcription reaction buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl_2 , 50 mM DTT, 1 μM dNTPs, 500 ng oligo-dT primer). Following heat denaturation, 1 U of MMLV-RT (Invitrogen, Carlsbad, CA) was added and the mixture incubated at 37°C for 45 min followed by a 68°C incubation for an additional 15 min. The resultant cDNA was diluted 10-fold prior to analysis and 2 μl used for each reaction as follows: PCR buffer (20 mM Tris-HCl, 50 mM KCl, 3 mM MgCl_2 , 300 nM of both the upstream and downstream primers

(see Table below), and 1 U of Taq Polymerase (Promega, Madison, WI)). As control, tubulin was amplified simultaneously in separate reactions. Message levels are examined using the BioRad iCycler according to the specifics recommended by the manufacturer. The amount of target cDNA in the sample, relative to tubulin, was calculated using the formula 2^{-Ct} , where Ct is the difference between the target and tubulin levels. The results were graphed as relative quantification of the target gene compared to a control (vehicle) treatment. The Table below was the primer sequences used for amplification. All results were standardized to a corresponding tubulin reaction that is carried out simultaneously.

Target mRNA	5' Primer	3' Primer
M-CSF	CTCTGGCTGGCTTGGCTTGG	GCAGAAGGATGAGGTTGTG
OPG	ACGGACAGCTGGCACACCAG	CTCACACACTCGGTTGTGGG
RANKL	CCAGTGAAGCAGCAGCCAGC	CCCTCTCATCAGCCCTGTCC
Tubulin	CTGCTCATCAGCAAGATCAGAG	GCATTATAGGGXTCCACCACAG

Spleen and marrow precursors cultured without support cells

Marrow and spleen cells were harvested as outlined above and plated at 1×10^6 marrow cells per well and 2×10^6 spleen cells per well in a 48-well plate as we have detailed (Sells Galvin et al., 1999). Base medium was supplemented with 7×10^{-3} M ascorbic acid prior to plating the cells and cultures were supplemented with 30 ng/ml of RANKL and 25 ng/ml M-CSF with either vehicle, 2×10^{-4} ng/ml or 1 ng/ml TGF- β 1. The culture media was changed every 3 days and cells were fixed in 1% paraformaldehyde in PBS to terminate culture. The cells were TRAP stained and evaluated as outlined above.

Statistical analysis

The effects of treatment are compared with the control values by one-way analysis of variance (ANOVA). Significant treatment effects are further evaluated by the Student's *t*-test. All analyses are performed with JMP version 4.04.

RESULTS

TGF- β dose responses

Dose response studies were performed to establish the impact of a broad range of TGF- β concentrations on osteoclast differentiation from both spleen and marrow precursors. Both TGF- β 1 (Fig. 1A,B) and TGF- β 2 (Fig. 1C,D) had biphasic effects on differentiation that were nearly indistinguishable. TGF- β stimulation of osteoclast differentiation at 2×10^{-4} ng/ml was significant and, at higher concentrations, the number of osteoclasts significantly decreased below control levels, with complete inhibition of osteoclast differentiation occurred at a dose of 2 ng/ml TGF- β . We focused additional studies on the low dose stimulation of differentiation and observed significant stimulation at both 1×10^{-4} and 2×10^{-4} ng/ml TGF- β 1 with higher, but not statistically significant levels at 3×10^{-4} ng/ml (Fig. 1E).

Roles of M-CSF, OPG, and RANKL expression

As shown in Figure 1, TGF- β stimulated differentiation at a low dose while inhibiting differentiation at higher doses. To study this, we examined the impact of these TGF- β concentrations on expression of M-CSF, OPG, and RANKL by real time reverse transcriptase polymerase chain reaction (Fig. 2). We document here that there was a dose dependent inhibition of M-CSF expression such that the levels are repressed although some expression is observed at low TGF- β concentrations (Fig. 2A). Interestingly, RANKL expression was elevated at low TGF- β concentrations only while there is a less dramatic increase in OPG (Fig. 2B,C). The RANKL/OPG ratio is the determining factor in whether there is sufficient RANKL to interact with its signaling receptor in the presence of OPG and our data support that low TGF- β concentrations elevate the RANKL/OPG ratio while high concentrations repressed the ratio (Fig. 2D). Thus, at low TGF- β concentrations, there is sufficient M-CSF and a high RANKL/OPG ratio and differentiation is elevated. We, therefore, added OPG to cultures to determine if reducing the RANKL/OPG ratio would block the low dose stimulation of osteoclast differentiation (Fig. 3). OPG blocked differentiation stimulation in the presence of either 1×10^{-4} or 2×10^{-4} ng/ml TGF- β . As documented above, at high TGF- β concentrations, the RANKL/OPG ratio and M-CSF expression are both repressed and there is no differentiation. These data suggested that the loss of M-CSF and/or the reduction in the ratio of RANKL to OPG was causing the high dose differentiation repression. We, therefore, examined differentiation in the presence of added M-CSF and/or RANKL in the presence of 2 ng/ml TGF- β (Fig. 4). Surprisingly, restoring M-CSF and/or elevating the RANKL/OPG ratio by adding RANKL had no impact on TGF- β -mediated repression of osteoclast differentiation at this suppressive dose. M-CSF addition in the absence of TGF- β stimulated differentiation while RANKL alone or in combination with M-CSF repressed differentiation compared to control levels in the absence of TGF- β . Given reports that high RANKL levels stimulate INF- β production to repress osteoclast differentiation, these latter finding are not surprising (Hayashi et al., 2002).

Spleen and marrow precursors cultured without stromal support cells

The above data supported that high TGF- β doses may have direct impacts on osteoclast precursors that could not be overcome by stromal cell-derived M-CSF and/or RANKL. To examine direct TGF- β influences on osteoclast differentiation, we used mouse model systems where osteoclasts are generated from marrow or spleen precursors from 8–10-week-old mice supplemented with RANKL and M-CSF (Fig. 5). In marrow precursor cultures, there was a dose-dependent increase in osteoclast differentiation with increasing TGF- β concentrations. Spleen cell precursor differentiation was only detected at the higher TGF- β concentration with no TRAP-positive cells present either in the absence of TGF- β or at the lower concentration.

DISCUSSION

As noted above, it has been established that TGF- β has a biphasic effect on osteoclast differentiation when osteoclast precursors originate from the marrow environment and are cultured in the presence of stromal support cells (Shinar and Rodan, 1990; Mundy, 1991; Yamaguchi and Kishi, 1995). Here we document that precursors from both spleen and

marrow tissues respond similarly to TGF- β in a biphasic pattern under these conditions. Given that TGF- β 2 has different influences on some cell types compared with TGF- β 1 (Jennings et al., 1988; Segarini et al., 1988; ten Dijke et al., 1990; Lyons et al., 1991; Liu et al., 2000), we explored whether there were differences between these isoforms in influencing either stimulation or repression of osteoclast differentiation. We document that both isoforms have similar impacts on stimulation and repression of osteoclast differentiation.

Our observations raised the question of the mechanism by which low TGF- β doses stimulate differentiation. We explored this by examining the impact of a range of TGF- β concentrations on M-CSF, OPG, and RANKL expression and document that low TGF- β doses elevated the RANKL/OPG ratio while not completely repressing M-CSF expression in stromal support cells. To examine whether the increased ratio of RANKL to OPG was responsible for increased differentiation, we added OPG during differentiation and found that suppression of the RANKL/OPG ratio in this manner repressed differentiation. Thus, the low dose TGF- β stimulation of spleen and marrow precursors cultured with stromal support cells may be accounted for by regulation of the RANKL/OPG expression ratio in the stromal cells. To our knowledge, this is the first study that TGF- β is capable of stimulating RANKL expression at any concentration. This interesting observation supports the cumulative observations that the TGF- β effects on stromal support cells are complex and may have multiple implications on the roles of TGF- β in bone metabolism.

Studies of inhibitory TGF- β doses were more complex, with repression of the RANKL/OPG ratio and some repression of M-CSF expression. These impacts seems likely to be the cause of repressed differentiation, yet adding M-CSF and/or RANKL to the cultures during differentiation did not alleviate the repression. These data support a here-to-fore undocumented TGF- β impact on osteoclast differentiation independent of these key stimulators of differentiation. Since one possible explanation for differentiation repression independent of stromal cell M-CSF and RANKL expression would be direct repression of precursors, we examined this using a stromal cell-independent culture system. We found that marrow cells cultured in the absence of support cells responded to TGF- β treatment with a dose-dependent stimulation of differentiation. Thus, at a dose that inhibited osteoclast differentiation in co-cultures of stromal cells and precursor cells, there was a marked stimulation of differentiation of the precursor cells cultured without support cells. These data support that the TGF- β -mediated repression observed in the co-cultures is by regulating stromal cell expression of factor(s) in addition to M-CSF, RANKL, or OPG. Thus, our data support that TGF- β targets both support cells and osteoclast precursors during differentiation by influencing multiple targets in osteoclast precursors and support cells. When spleen precursors were evaluated in the absence of support cells, only the higher dose of TGF- β stimulated differentiation. This was an unexpected observation since the lower TGF- β concentration stimulated differentiation in the presence of support cells. This is in conflict with reports using spleen cells from younger mice including our study (Sells Galvin et al., 1999). Whether this is due to the age of the mouse from which the spleens were harvested is currently under investigation.

We have documented that mature osteoclasts secrete and activate TGF- β (Oursler, 1994). Moreover, there is evidence including the studies presented here that osteoclast precursors and mature cells respond directly to TGF- β (Fiorelli et al., 1994; Zheng et al., 1994). Thus, direct TGF- β effects on osteoclast precursors appear to be complex, including dose-dependent stimulation of osteoclast differentiation of precursors from both marrow and spleen tissues. These observations are consistent with TGF- β influences on other cell types in that TGF- β has been reported to either promote or repress either proliferation or differentiation in many cell systems (Massague, 1987; Roberts et al., 1990; Laiho and Keski-Oja, 1992). Taken together, our observations support the concept that multifunctional TGF- β has complex influences on osteoclast differentiation. In conjunction with reports that TGF- β regulates osteoclast activity and survival, it appears that TGF- β impacts on bone resorption are multifaceted and a complete understanding of TGF- β roles in bone metabolism require integration of all of the components of these impacts (Hughes et al., 1994; Pederson et al., 1999).

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Fig. 1.

Transforming growth factor-beta 1 (TGF- β 1) and TGF- β 2 influences on osteoclast differentiation in the presence of stromal support cells. Differentiation of osteoclasts from marrow (**A**, **C**, **E**) and spleen (**B**, **D**) were assessed as detailed in the absence (control) or presence of the indicated TGF- β 1 (**A**, **B**, **E**) or TGF- β 2 (**C**, **D**) concentration. Tartrate resistant acid phosphatase (TRAP) positive multinucleated cells were counted and reported as treated/control. The values are an average of 3–6 replicate experiments with at least four wells per dose in each experiment. * $P < 0.05$ compared to control.

Fig. 2.

TGF- β 1 regulation of macrophage colony stimulating factor (M-CSF), receptor activator of NF- κ B ligand (RANKL), and osteoprotegerin (OPG) mRNAs. ST2 cells were untreated (NONE) or treated with 10^{-5} M vitamin D and 10^{-7} M Dexamethazone (vit D + Dex), with or without the indicated TGF- β 1 concentration for 3 days and RNA was isolated and analyzed as described. The values are representative of two replicate experiments. * $P < 0.05$ compared to no treatment (NONE); ** $P < 0.05$ compared to vit D + Dex.

OPG EFFECTS ON STIMULATION

Fig. 3. OPG effects on differentiation stimulation. Osteoclasts were differentiated in co-cultures of bone marrow and ST2 cells with the addition of the indicated TGF-β1 concentration with or without 1 ng/ml OPG as indicated. TRAP positive multinucleated cells were counted and reported as treated/control. The values are an average of three replicate experiments with four wells per treatment in each experiment. * $P < 0.05$ compared to no TGF-β; ** $P < 0.05$ compared to TGF-β treatment in the absence of OPG.

M-CSF AND RANKL EFFECTS ON REPRESSION

Fig. 4.

M-CSF and RANKL effects on differentiation repression. Osteoclasts were differentiated in co-cultures of bone marrow and ST2 cells in the absence or presence of 2 ng/ml TGF- β 1 with or without 25 ng/ml M-CSF and/or 60 ng/ml RANKL as indicated. TRAP positive multinucleated cells were counted and reported as treated/control. The values are an average of two replicate experiments with three wells per treatment in each experiment. * $P < 0.05$ compared to no treatment.

STROMAL CELL-FREE CULTURES

Fig. 5.

Differentiation in the absence of stromal support cells. Precursors from marrow and spleen were cultured with M-CSF (25 ng/ml) and RANKL (30 ng/ml) in the presence or absence of TGF-β1 as described for 9 days and TRAP stained. Differentiation of cells from spleen and marrow were assessed as detailed. TRAP positive multinucleated cells were counted as outlined. The values are an average of three replicate experiments with at least four wells per dose in each experiment. * $P < 0.05$ compared to no TGF-β treatment.