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Regulation of Osteoclastogenesis and RANK Expression by TGF-β1

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Abstract Transforming growth factor-β (TGF-β) has been shown to both inhibit and to stimulate bone resorption and osteoclastogenesis. This may be due, in part, to differential effects on bone marrow stromal cells that support osteoclastogenesis vs. direct effects on osteoclastic precursor cells. In the present study, we used the murine monocytic cell line, RAW 264.7, to define direct effects of TGF-β on pre-osteoclastic cells. In the presence of macrophage-colony stimulating factor (M-CSF) (20 ng/ml) and receptor activator of NF-κB ligand (RANK-L) (50 ng/ml), TGF-β1 (0.01–5 ng/ml) dose-dependently stimulated (by up to 120-fold) osteoclast formation (assessed by the presence of tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells and expression of calcitonin and vitronectin receptors). In addition, TGF-β1 also increased steady state RANK mRNA levels in a time- (by up to 3.5-fold at 48 h) and dose-dependent manner (by up to 2.2-fold at 10 ng/ml). TGF-β1 induction of RANK mRNA levels was present both in undifferentiated RAW cells as well as in cells that had been induced to differentiate into osteoclasts by a 7-day treatment with M-CSF and RANK-L. Using a fluorescence-labeled RANK-L probe, we also demonstrated by flow cytometry that TGF-β1 resulted in a significant increase in the percentage of RANK+ RAW cells ($P < 0.05$), as well as an increase in the fluorescence intensity per cell ($P < 0.05$), the latter consistent with an increase in RANK protein expression per cell. These data thus indicate that TGF-β directly stimulates osteoclastic differentiation, and this is accompanied by increased RANK mRNA and protein expression. J. Cell. Biochem. 83: 320–325, 2001.

Key words: RANK; TGF-β1; osteoclastogenesis; RAW cells

Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that has significant effects on skeletal metabolism [Bonewald and Dallas, 1994; Centrella et al., 1994]. In vivo studies have generally found that TGF-β increases bone turnover. Thus, local injection of TGF-β into the subcutaneous tissue over the calvariae of mice results in an increase in bone resorption and formation [Marcelli et al., 1990]. Similarly, transgenic mice overexpressing TGF-β2 from the osteocalcin promoter demonstrate progressive bone loss associated with increased bone turnover and a skeletal phenotype similar to hyperparathyroid bone disease [Erlebacher et al., 1996]. In addition, transgenic mice expressing a dominant negative non-functional type II TGF-β receptor driven by the osteocalcin promoter demonstrate an increase in bone mass associated with reductions in osteoclast number and activity [Filvaroff et al., 1994].

In vitro studies attempting to define TGF-β effects on bone resorption and on osteoclastogenesis have found variable results, depending on the model system used. Thus, TGF-β has been shown to induce apoptosis of mature osteoclasts [Hughes et al., 1996] and to stimulate osteoprotegerin (OPG) production in bone marrow stromal and osteoblastic cells [Murakami et al., 1998; Takai et al., 1998]. Since OPG binds to receptor activator of NF-κB ligand (RANK-L), thus preventing it from activating RANK on osteoclastic lineage cells [Lacey et al., 1998], the TGF-β1 induced increase in OPG production by marrow stromal cells was accompanied by a decrease in osteoclastogenesis. However, other studies using systems containing both osteoclast precursor cells and stromal/osteoblastic cells have found an induction of osteoclastogenesis by TGF-β [Fuller et al., 2000]. These variable results of

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TGF-β on osteoclast development could be due, in part, to differing actions of TGF-β on osteoclast precursor cells vs. the bone marrow stromal cells that support osteoclastogenesis.

Since primary marrow cultures contain both pre-osteoclastic as well as osteoblast/stromal cells, it is not possible using this system to dissociate the effects of TGF-β on these two populations. Thus, in the present study, we used the murine monocytic cell line, RAW 264.7, which can be induced to differentiate into mature osteoclasts in the presence of RANK-L and macrophage-colony stimulating factor (M-CSF), to examine the direct effects of TGF-β on osteoclast precursor cells in the absence of osteoblastic/stromal cells. RAW 264.7 cells that are induced to differentiate into osteoclasts in the presence of RANK-L express genes typical of osteoclast lineage cells, including TRAP, cathepsin K, integrin $\alpha_v\beta_3$, c-src, and calcitonin receptor (CTR) [Hsu et al., 1999]. In addition, we also tested whether TGF-β1 effects on osteoclast differentiation were accompanied by changes in RANK mRNA and protein levels. Our findings indicate that, in the presence of RANK-L and M-CSF, TGF-β1 dramatically increases osteoclast differentiation of RAW 264.7 cells accompanied by a dose dependent increase in the expression of CTR and vitronectin receptor (VTR) mRNA levels. RANK mRNA and protein levels, as well as the percentage of RAW 264.7 cells expressing RANK on the cell surface, were also significantly increased by TGF-β1.

MATERIALS AND METHODS

Osteoclastogenesis Assay

RAW 264.7 cells were plated in 24 well plates at a seeding density of 3,000 cells/well in DMEM/10% calf serum at 37°C in a humidified atmosphere containing 5% CO_2. The treatment of cells started after the cells had been incubated for 24 to 48 h at 37°C. Cells were treated with different concentrations of TGF-β1 (ranging from 0.1 to 20 ng/ml, R & D Systems, Minneapolis, MN) in the presence of RANK-L (50 ng/ml, kindly provided by Amgen, Inc., Thousand Oaks, CA) and M-CSF (20 ng/ml, R & D System, Minneapolis, MN) for 7 days, and the medium was changed every three days. Osteoclast formation was measured by quantitating the presence of TRAP multinucleated positive cells (more than three nuclei) using cytochemical staining.

Cell Cultures for Extracting Total RNA and Fluoresence Activated Cells Sorting (FACS) Staining

RAW cells were plated at $2–3 \times 10^5$ cells/well in six well plates in DMEM/10% calf serum. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h to allow attachment. The medium was then changed to DMEM/0.1% BSA for 24 h before the addition of different concentrations of TGF-β1 ranging from 0.1 to 20 ng/ml dissolved in DMEM/0.1% BSA. For dose response studies, the cells were treated for 48 h and total RNA was extracted. For time course studies, cells were treated for 12, 24, 36, 48, and 72 h and total RNA extracted.

Semi-Quantitative Real Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using the Qiashredder and RNeasy kit from Qiagen (Hilden, Germany). One to two microgram of total RNA was used for cDNA synthesis. The reaction was carried out at 42°C for 2 h in a 20 μl mix of 1× AMV-RT incubation buffer, 2.5 μM poly-dT primer, 1 mM each of dATP, dCTP, dGTP, and dTTP, 20 U of RNase inhibitor, and 20 U of AMV-RT. After stopping the reaction by heating to 95°C, the volume was diluted to 200 μl, and 5 μl aliquots of this cDNA were used for PCR using a Light-Cycler (Roche Diagnostics) or conventional semi-quantitative PCR for some reactions

For the Light-Cycler, the PCR reaction was carried out in a 20 μl mix of 1× LightCycler FastStart reaction mix (containing a 200 μM mix of dATP, dCTP, dGTP, and dUTP, FastStart Taq DNA polymerase, and SYBR green I dye), 3 mM $MgCl_2$ (unless otherwise stated) and the appropriate primers at 0.375 μM.

Conventional semi-quantitative PCR reactions were carried out in a 25 μl mix of a 10 μl aliquot of diluted cDNA, 1× PCR buffer, 10 mM dNTP's, oligo primers (sense and anti-sense) at 10 μM, 0.1 μl Taq polymerase, and 0.25 μl ^{32}P.

Amplification reactions specific for RANK and glyceraldehyde phosphate dehydrogenase (GAPDH) were carried out using the Light-Cycler. Reaction products was quantified using a simultaneously amplified series of dilutions of a sequence of known concentration (in this case a vector containing the RANKL gene) to generate a standard curve for each run.

TABLE I. Primer Sequences Used in Quantitative RT-PCR

Murine RANK primers	5′ AAGATG GTTCCAGAAGACGGT
	3′ CATAGAGTCAGTTCTGCTCGGA
GAPDH	5′ ACCACAGTCCATGCCATCAC
	3′ TCCACCACCCTG TTGCTG TA
Murine β5 integrin (VTR)	5′ GCGAAAAGATGCTCTGCA
	3′ GCCGCATGTGCAATTGTA
Mouse calcitonin receptor (CTR)	5′ TTTCAAGAACCTTAGCTGCCAGAG
	3′ CAAGGCACGGACAATGTTGAGAG

Amplification reactions for CTR and VTR were carried out using a conventional thermal cycler. Primer sequences are as shown in Table I.

For the Light-Cycler reactions, all reactions were performed using 3 mM $MgCl_2$. Amplification profiles were as follows: murine RANK, denaturation at 95°C for 0 sec; annealing at 58°C for 7 sec; extension at 72°C for 14 sec; 40 cycles; GAPDH, 95°C for 0 sec; 55°C for 7 sec; 72°C for 20 sec; 35 cycles.

Conventional PCR reaction profiles for CTR: denaturation at 94°C for 1 min; annealing at 60°C for 1 min; extension at 72°C for 1 min; 35 cycles. VTR: denaturation at 94°C for 30 sec; annealing at 50°C for 30 sec; extension at 72°C for 30 sec; 35 cycles.

Quantitation of RANK+ RAW 264.7 Cells and RANK Protein Expression Using FACS

RAW cells were either cultured in the presence of TGF-β1 (5 ng/ml) or vehicle for 48 h. The cells were then trypsinized from the plates and resuspended and washed twice in 1× PBS containing 1% bovine serum albumin (BSA). Cells were incubated in PBS containing 1% BSA for blocking non-specific binding followed by incubation on ice for 45 min with fluorescein isothiocyanate (FITC)-conjugated RANK-L (provided by Amgen, Thousand Oaks, CA). Cells incubated with buffer without FITC-conjugated RANK-L were used as a negative control. The cells were washed three times after incubation and were subsequently resuspended in PBS containing 1% BSA. RANK(+) RAW cells and RANK protein expression were analyzed with a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA).

Statistical Analysis

All values are expressed as mean±SEM. Student's paired *t*-tests were used to evaluate the differences between the samples of interest and the respective controls. A P value of less than 0.05 was considered statistically significant.

RESULTS

TGF-β1 Regulation of Osteoclast Differentiation of RAW 264.7 Cells

RAW 264.7 cells were treated with TGF-β1 for 7 days and TRAP (+) multinucleated cells quantified. As shown in Figure 1, a dose dependent increase in TRAP (+) multinucleated cell formation was observed with increasing concentrations of TGF-β1 in the presence of RANK-L (50 ng/ml) and M-CSF (20 ng/ml), with a 120-fold increase present at the 5 ng/ml dose. Figure 2 is a photomicrograph demonstrating the dramatic increase in the number of TRAP (+) multinucleated cells present after TGF-β1 treatment (A) as compared to control treatment (B). Osteoclast differentiation of RAW 264.7 cells was also accompanied by a dose dependent increase in CTR (Fig. 3) and VTR (Fig. 4) mRNA levels.

TGF-β1 Effects on RANK mRNA and Protein Levels

As shown in Figure 5, TGF-β1 dose-dependently increased RANK mRNA levels in RAW 264.7 cells. Time-course studies (Fig. 6) showed a time-dependent increase in RANK mRNA levels. To test whether the effects of TGF-β on RANK mRNA levels were present only in undifferentiated RAW cells as in the above experiments or also in cells that had been made to differentiate into osteoclastic cells, RAW cells were first treated with RANK-L and M-CSF for 7 days to induce osteoclastic differentiation and the above studies repeated. As shown in

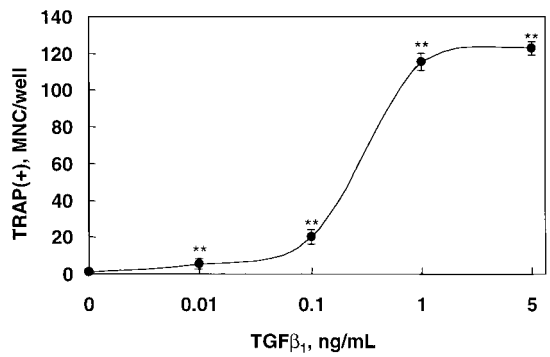

Fig. 1. Effects of TGF-β1 on osteoclastic differentiation of RAW 264.7 cells. RAW cells were stimulated with RANK-L (50 ng/ml), M-CSF (20 ng/ml) and varying concentrations of TGF-β1 for 7 days. The medium was changed every 3 to 4 days and fresh growth factors were added. The cultures were fixed with formalin and the number of TRAP (+) cells containing three or more nuclei was quantitated. Each point represents the mean and the standard error of three replicates. (**$P < 0.001$, n = 3).

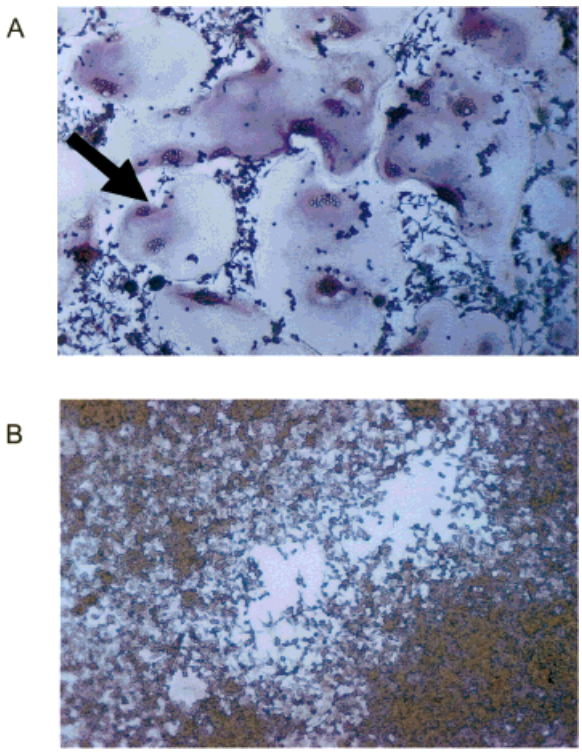

Fig. 2. Photomicrographs demonstrating TGF-β1 effects on TRAP (+) multinucleated cell formation of RAW 264.7 cells (arrow). RAW cells were stimulated with TGF-β1 (5 ng/ml) in the presence of RANK-L (50 ng/ml) and M-CSF (20 ng/ml) for 7 days (**A**) or treated with only RANK-L (50 ng/ml) and M-CSF (20 ng/ml) (**B**). The cultures were then fixed with formalin and stained for TRAP. (20× magnification).

Figure 7, TGF-β1 increased steady-state RANK mRNA levels even in differentiated RAW cells.

We next assessed whether TGF-β1 treatment of RAW 264.7 cells was accompanied by an increase in the percentage of cells expressing

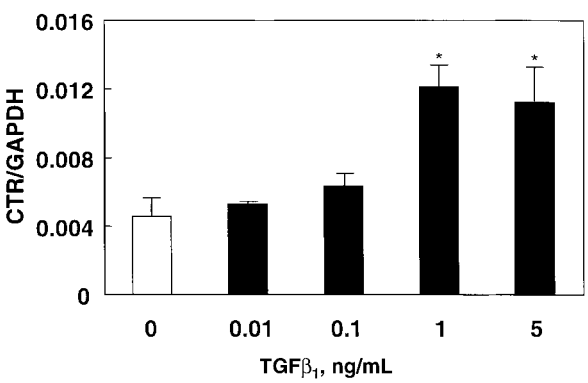

Fig. 3. Effects of TGF-β1 on CTR mRNA levels in RAW 264.7 cells. Cells were stimulated with RANK-L (50 ng/ml) and M-CSF (20 ng/ml) and TGF-β1 at varying concentrations for 7 days. CTR and GAPDH mRNA levels were analyzed using semi-quantitative RT-PCR. (*$P < 0.05$, n = 3).

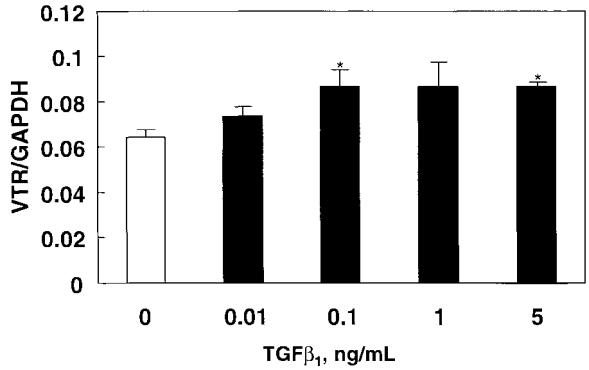

Fig. 4. Effects of TGF-β1 on VTR mRNA levels in RAW 264.7 cells. Cells were stimulated with RANK-L (50 ng/ml) and M-CSF (20 ng/ml) and TGF-β1 at varying concentrations for 7 days. VTR and GAPDH mRNA levels were analyzed using semi-quantitative RT-PCR. (*$P < 0.05$, n = 3).

RANK on the cell surface and/or in the amount of RANK protein expressed per cell. As shown in Figure 8A, FACS analysis of RAW cells treated either with vehicle or TGF-β1 demonstrated a significant increase in the percentage of RANK (+) RAW cells following TGF-β1 treatment. In addition, TGF-β1 treatment also resulted in a significant increase in the fluorescent intensity per cell, consistent with an increase in RANK protein expression per cell (Fig. 8B).

DISCUSSION

TGF-β1 effects on bone resorption and on osteoclastogenesis have been variable, depending on the model system used. In the present study we used a homogenous, clonal population of murine monocytic cells (RAW 264.7) to define

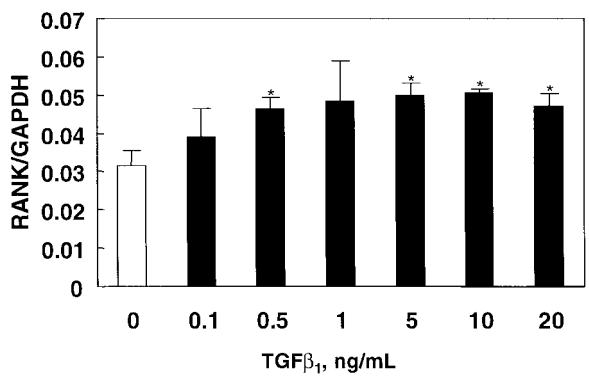

Fig. 5. TGF-β1 effects on RANK mRNA levels in RAW 264.7 cells. Cells were treated with different concentrations of TGF-β1 (0, 0.1, 0.5, 1, 5, 10, and 20 ng/ml) for 48 h in DMEM/0.1% BSA and RANK and GAPDH mRNA levels were analyzed using semiquantitative real time RT-PCR as described in the Methods. (*$P < 0.05$, n = 3).

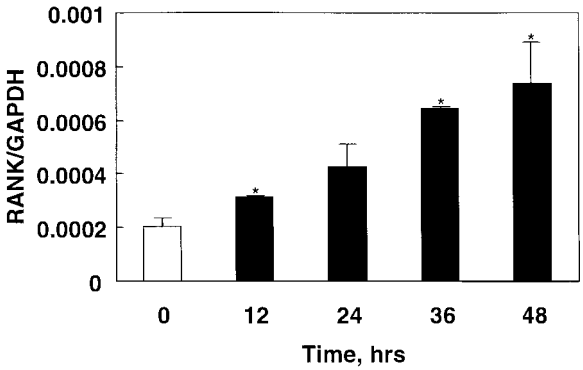

Fig. 6. Time-course of TGF-β1 effects on RAND mRNA levels in RAW 264.7 cells. Cells were treated with 5 ng/ml of TGF-β1 for 0, 12, 24, 26, or 48 h in DMEM/0.1% BSA. RANK and GAPDH mRNA levels were analyzed using semiquantitative real time RT-PCR as described in the Methods. (*$P < 0.05$, n = 3).

the direct effects of TGF-β1 on osteoclast development and on RANK mRNA and protein expression. The main advantage of this system is that it does not contain any osteoblastic/bone marrow stromal cells, which are also targets for TGF-β action [Murakami et al., 1998]. Thus, since TGF-β may have opposing actions on pre-osteoclastic vs. marrow stromal cells in terms of effects on osteoclast development, the RAW cell system allowed us to focus just on the pre-osteoclastic cells.

We found that in the presence of RANK-L and M-CSF, TGF-β1 markedly (by up to 120-fold) increased osteoclast development of the monocytic/pre-osteoclastic cells, as assessed by the presence of TRAP staining in multinucleated

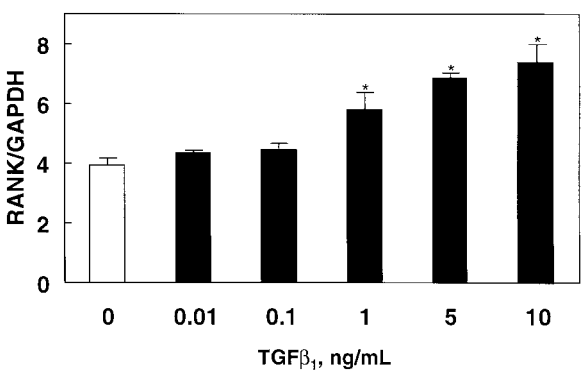

Fig. 7. TGF-β1 effects on differentiated RAW 264.7 cells. Cells were treated with RANK-L (50 ng/ml) and M-CSF (20 ng/ml) for 7 days and were then treated with different concentrations of TGF-β1 (0, 0.1, 0.5, 1, 5, and 10 ng/ml) for 48 h in DMEM/0.1% BSA. RANK and GAPDH mRNA levels were analyzed using semiquantitative real time RT-PCR as described in the Methods. (*$P < 0.05$, n = 4).

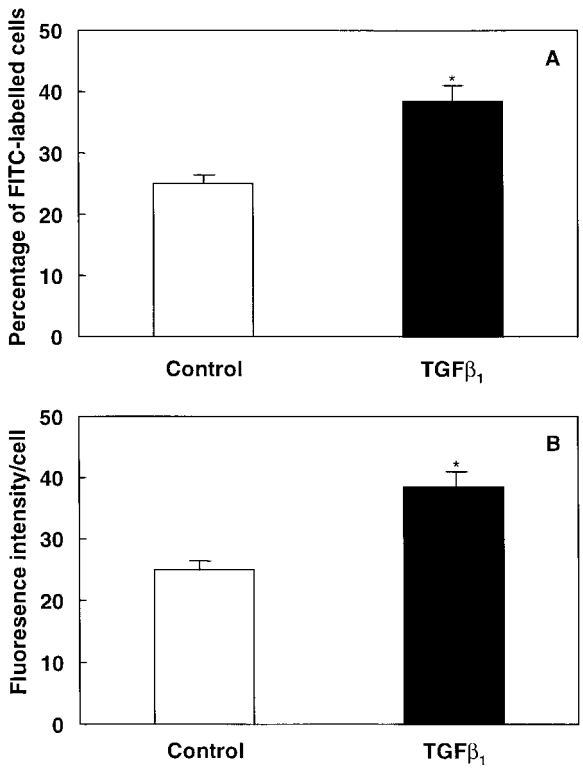

Fig. 8. FACS analysis of RANK protein expression on RAW cells. Cells were treated with either 5 ng/ml of TGF-β1 or vehicle for 72 h and then labeled with a FITC-conjugated RANK-L probe. The cells were then subjected to FACS analysis for expression of RANK on the cell membrane. (**A**) Percentage of labeled cells (*$P < 0.05$, n = 3) and (**B**) Fluoresence intensity per cell (*$P < 0.05$, n = 3).

cells and expression of the osteoclastic markers, CTR and VTR. This was associated with an induction of RANK mRNA and protein levels, as well as an expansion of the number of cells expressing RANK on the cell surface. These findings thus establish that TGF-β acts on pre-osteoclastic cells to promote their differentiation into osteoclasts and that this effect is mediated, at least in part, by increased RANK production.

Previous studies using primary marrow cultures have also found an induction of osteoclastogenesis by TGF-β [Yamaguchi et al., 1995; Fuller et al., 2000]. As noted above, however, while these data are consistent with the findings of the present study, our use of a clonal population of monocytic cells eliminates the possibility that the observed stimulatory effects of TGF-β on osteoclastogenesis are mediated indirectly via effects on bone marrow stromal cells. Our data demonstrating an increase in RANK production in monocytic cells by TGF-β is

also consistent with previous reports that found a similar effect of TGF-β on RANK in T cells [Anderson et al., 1997] and in primary bone marrow cultures [Fuller et al., 2000]. Moreover, we found that RANK mRNA levels were increased as early as 12 h following TGF-β treatment, followed soon thereafter by an increase in RANK protein at the cell membrane. This would suggest that the increase in RANK expression was responsible, at least in part, for the increase in osteoclast differentiation, rather than simply a result of increased osteoclast differentiation. Consistent with this, TGF-β treatment resulted in an increase in RANK mRNA levels both in undifferentiated RAW 264.7 cells and in RAW cells that had been induced to differentiate into osteoclasts by a 7-day treatment with RANK-L and M-CSF.

Binding of RANK-L to RANK results in a cascade of intracellular events, including the activation of the intracellular adaptor protein family, TNFR-associated factor (TRAF), which, in turn, is involved in activation of the NF-κB and Jun N-terminal kinase (JNK) pathways in pre-osteoclastic cells [Anderson et al., 1997; Darnay et al., 1998; Wong et al., 1998; Hsu et al., 1999]. It is possible that, in addition to increasing RANK production, TGF-β results in changes in these or other intracellular mediators of RANK action, with an ultimate increase in osteoclast development. Clearly, further studies are needed to address this issue.

In summary, our data indicate direct effects of TGF-β on monocytic/pre-osteoclastic cells that result in an increase in osteoclast development. These effects are likely mediated, at least in part, by increased RANK production in these cells. The direct stimulatory effects of TGF-β on pre-osteoclastic cells appear to be opposed by effects on bone marrow stromal cells that would lead to an inhibition of osteoclastogenesis (i.e., an increase in OPG production) [Murakami et al., 1998; Takai et al., 1998]. Thus, the net effect of TGF-β on bone resorption and on osteoclastogenesis in vivo and in vitro likely depends on the balance between these competing actions.

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