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Endogenous Production of TGF-β Is Essential for Osteoclastogenesis Induced by a Combination of Receptor Activator of NF-κB Ligand and Macrophage-Colony-Stimulating Factor¹

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Osteoclasts are the cells primarily responsible for bone resorption, and are of hemopoietic stem cell origin. Precursors of osteoclasts have been demonstrated to share common properties with those of a monocyte/macrophage (M/Mφ) cell lineage (1, 2). Recent extensive studies have increased our understanding of osteoclast biology, particularly osteoclastogenesis. Many systemic hormones and local cytokines pathophysiologically participate in regulating osteoclast differentiation, including M-CSF, IL-1, IL-6, IL-11, TNF-α, 1,25-dihydroxyvitamin D₃, parathyroid hormone, and PGs (3, 4). Osteoclast differentiation factor/osteoprotegerin ligand/TNF-related activation-induced cytokine/receptor activator of NF-κB (RANK) ligand (RANKL) has recently been identified as the most important and critical molecule for osteoclast development (5, 6). Bone marrow stroma/osteoblasts produce this molecule on the plasma membrane in response to several osteotropic factors, and osteoclast precursors express a receptor of RANKL (RANK). Most recently, mice with a disrupted RANKL gene were found to have severe osteopetrosis (7). Therefore, the RANKL/RANK system is considered to be an essential signal for osteoclast differentiation in the interaction between stromal cells and cells of the osteoclast lineage. Like the interaction of osteoclast precursors/stromal cells, RANKL is expressed on activated T cells and activates mature dendritic cells that express RANK on their plasma membrane, implying a role for T cell-dendritic cell interaction during an immune response (7–9).

Soluble RANKL (sRANKL) lacking transmembrane and intra-cellular regions is now available and has allowed us to elucidate the role of RANKL in osteoclast development and function in more detail. Osteoclasts have been shown to be formed from spleen cells, nonadherent bone marrow cells, or peripheral blood-derived monocytes in the presence of M-CSF and sRANKL in the absence of stromal cells (5, 6, 10, 11). In addition, a macrophage-like cell line has been demonstrated to potentially differentiate into osteoclasts when treated with M-CSF and sRANKL (12). However, the efficiency for osteoclast formation was low in the above cultures, suggesting the requirement for other factors for osteoclastogenesis. Furthermore, due to the lack of a population of osteoclast progenitors that synchronously differentiate into osteoclasts, the molecular mechanisms regulating the process of osteoclastogenesis have remained uncertain.

In this study we developed a new isolation method for obtaining osteoclast progenitors. By this procedure, we isolated M/Mφ-like hemopoietic cells from mouse unfractionated bone cells; these isolated cells are potentially capable of differentiating into osteoclasts in response to M-CSF and sRANKL. Surprisingly, neutralizing Ab against TGF-β completely blocked osteoclast formation from the
precursors induced by sRANKL/M-CSF signaling. In addition, exogenous TGF-β induced the further commitment and maturation of osteoclast progenitors into mature osteoclasts in the absence of stromal cells. In contrast, many studies using in vitro culture systems containing stromal cells have demonstrated that TGF-β inhibited the differentiation and function of osteoclasts (13, 14). Thus, TGF-β possesses multifunctional biological activities. Target cells of TGF-β are heterogeneous in bone, including bone-forming and -resorbing cells, hemopoietic cells, and bone marrow stromal cells (15–17). Therefore, it has been difficult to elucidate the precise and direct action of TGF-β on osteoclast development. Here we report that endogenous production of TGF-β by M/Mβ-like hemopoietic cells and the derived osteoclast precursors is essential for osteoclastogenesis induced by a combination of RANKL and M-CSF. Our findings expand the established roles of TGF-β in osteoclastogenesis and provide a novel insight into bone metabolism.

Materials and Methods

Antibodies
Neutralizing mAb (clone, 1D11) against TGF-β1, -2, and -3 and isotype control mouse IgG1 were obtained from R&D Systems (Minneapolis, MN). Polyclonal rabbit anti-RANK Ab was provided by Snow Brand Milk Products Co., Ltd. (Kobuchisawa, Japan) and anti-human CD11b (clone, 2.4G2), Ab, biotinylated anti-CD11b (Mac-1)–chain (clone M1/70), anti-CD11a (LFA-1 α-chain; clone 2D7), anti-CD44 (clone IM-7) and anti-CD61 (integrin β3; clone C9.G2) Abs and PE-labeled anti-CD14 (clone rmc5-3) Ab were purchased from PharMingen International (San Diego, CA). Biotinylated anti-F4/80 (clone A1), FITC-labeled anti-integrin αv, and unlabeled anti-DEC-205 (clone NLDC-145) were obtained from Srotec (Kilddington, U.K.), Sumitomo Electronic (Osaka, Japan), and BMA Biomedicals (Augst, Switzerland), respectively. Anti-p50 (sc-114X) and anti-p65 (sc-109X) Abs were purchased from Santa Cruz Biotechnology (San Diego, CA). Anti-p50 (sc-114X) and anti-p65 (sc-109X) Abs were purchased from Santa Cruz Biotechnology (San Diego, CA). Neutralizing mAb (clone, 1D11) against TGF-β1, -2, and -3 and isotype control mouse IgG1 were obtained from R&D Systems (Minneapolis, MN). Polyclonal rabbit anti-RANK Ab was provided by Snow Brand Milk Products Co., Ltd. (Kobuchisawa, Japan) and anti-human CD11b (clone, 2.4G2), Ab, biotinylated anti-CD11b (Mac-1)–chain (clone M1/70), anti-CD11a (LFA-1 α-chain; clone 2D7), anti-CD44 (clone IM-7) and anti-CD61 (integrin β3; clone C9.G2) Abs and PE-labeled anti-CD14 (clone rmc5-3) Ab were purchased from PharMingen International (San Diego, CA). Biotinylated anti-F4/80 (clone A1), FITC-labeled anti-integrin αv, and unlabeled anti-DEC-205 (clone NLDC-145) were obtained from Srotec (Kilddington, U.K.), Sumitomo Electronic (Osaka, Japan), and BMA Biomedicals (Augst, Switzerland), respectively. Anti-p50 (sc-114X) and anti-p65 (sc-109X) Abs were purchased from Santa Cruz Biotechnology (San Diego, CA).

Isolation of M/Mβ-like hemopoietic cells from mouse unfractinated bone cells

Mouse unfractinated bone cells were prepared from femora and tibiae of 4- to 5-week-old C57BL/6J mice (Shizuoka Laboratories Animal Center, Shizuoka, Japan). After removal of connective soft tissues, the bones were minced into small pieces in α-MEM (ICN Biomedicals, Aurora, OH) supplemented with 10% FBS (Intergen, Purchase, NY) and 100 U/ml of penicillin. The cells were dissociated from the bone fragments by vortexing and were filtered through a nylon mesh with a 70-μm pore size. The cells obtained in suspension were used as mouse unfractinated bone cells. The unfractinated bone cells (107 cells) were seeded and cultured for 6 days in α-MEM medium containing 10% FBS and PGE2 (10 μg/ml) at a density of 2,000 cells/cm2. Unlabeled anti-integrin αv, and unlabeled anti-DEC-205 (clone NLDC-145) were obtained from Srotec (Kilddington, U.K.), Sumitomo Electronic (Osaka, Japan), and BMA Biomedicals (Augst, Switzerland), respectively. Anti-p50 (sc-114X) and anti-p65 (sc-109X) Abs were purchased from Santa Cruz Biotechnology (San Diego, CA). Mouse unfractinated bone cells were prepared from femora and tibiae of 4- to 5-week-old C57BL/6J mice (Shizuoka Laboratories Animal Center, Shizuoka, Japan). After removal of connective soft tissues, the bones were minced into small pieces in α-MEM (ICN Biomedicals, Aurora, OH) supplemented with 10% FBS (Intergen, Purchase, NY) and 100 U/ml of penicillin. The cells were dissociated from the bone fragments by vortexing and were filtered through a nylon mesh with a 70-μm pore size. The cells obtained in suspension were used as mouse unfractinated bone cells. The unfractinated bone cells (107 cells) were seeded and cultured for 6 days in α-MEM medium containing 10% FBS and PGE2 (10 μg/ml) at a density of 2,000 cells/cm2. Unlabeled anti-integrin αv, and unlabeled anti-DEC-205 (clone NLDC-145) were obtained from Srotec (Kilddington, U.K.), Sumitomo Electronic (Osaka, Japan), and BMA Biomedicals (Augst, Switzerland), respectively. Anti-p50 (sc-114X) and anti-p65 (sc-109X) Abs were purchased from Santa Cruz Biotechnology (San Diego, CA).

RT-PCR
Total RNA (1 μg) extracted from cells in the culture was used as a template for cDNA synthesis. cDNA was prepared by use of a Superscript II reverse transcription system (Life Technologies, Gaithersburg, MD). Primers were synthesized on the basis of the reported mouse cDNA sequences for TRAP, integrin αv, integrin β3, calcitonin receptor, cathepsin K, RANK, CD14, TGF-β1, TGF-β2, TGF-β3, TGF-β receptor I (TGF-RI), and TGF-RII. Sequences of the primers used for PCR were as follows: TRAP forward, 5′-GGCATCGCTCATCTCCACAGT-3′; TRAP reverse, 5′-CATCTA-3′; integrin αv forward, 5′-GAGTACTTGCGCTCA-3′; and integrin αv reverse, 5′-GTTGCCGCTTCCCCAAGCAG-3′; integrin β3 forward, 5′-TTACCCCGTGAGCCTCATACTA-3′; and integrin β3 reverse, 5′-CTGTCTCTTCTTCTTCTTCTTCTTCT-3′. CD14 forward, 5′-GAGAAGAGACTCACCAGAAGC-3′; CD14 reverse, 5′-GAAGAGATTGCTAAC-3′; integrin αv forward, 5′-GCGGAGCGAATTGATTGTAATT-3′; and integrin αv reverse, 5′-GCTTACAGGAGACCCGACAG-3′. Amplification was conducted for 22–32 cycles, each of which consisted of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min with biotinylated anti-CD11b, anti-CD11a, anti-CD44, anti-F4/80, or anti-integrin β3 Abs; with FITC-labeled anti-integrin αv; with PE-labeled anti-DEC-205; or with unlabeled anti-DEC-205 Abs. For staining with the biotinylated and unlabeled Abs, the stained cells were incubated with avidin-FITC (PharMingen) and FITC-conjugated anti-rat IgG (PharMingen), respectively. After staining with an excess of mouse IgG, the cells were analyzed without gating on a FACStar (Becton Dickinson, San Jose, CA).

Flow cytometry
After isolation of M/Mβ-like hemopoietic cells, the cells were suspended in ice-cold PBS containing 0.5% BSA, 0.1% sodium azide, and 1 mM glucose. Before being stained for cell surface Ags, the progenitors were preincubated with anti-CD16/122 Ab or an excess of mouse IgG (Sigma) to reduce nonspecific binding of Abs. The pretreated cells were stained for 30 min with biotinylated anti-CD11b, anti-CD11a, anti-CD44, anti-F4/80, or anti-integrin β3 Abs; with FITC-labeled anti-integrin αv; with PE-labeled anti-CD44; or with unlabeled anti-DEC-205 Abs. For staining with the biotinylated and unlabeled Abs, the stained cells were secondarily incubated with avidin-FITC (PharMingen) and FITC-conjugated anti-rat IgG (PharMingen), respectively. After staining with anti-CD16/122 Ab or an excess of mouse IgG, the cells were analyzed without gating on a FACStar (Becton Dickinson, San Jose, CA).

Estimation of osteoclastogenesis from M/Mβ-like hemopoietic cells

Isolated M/Mβ-like hemopoietic cells were seeded at an initial density of 1 × 105 cells/cm2 and cultured in α-MEM/10% FBS with or without several cytokines and/or other agents. The culture medium was exchanged every 3 days. After a culture period of the desired length, the cells were fixed in 10% formalin and stained for TRAP activity with a leukocyte acid phosphatase kit (Sigma). The numbers of total cells, TRAP-positive mononuclear cells, and TRAP-positive MNCs were counted under a microscope. TRAP-positive mononuclear cells and MNCs were considered to be precursors and osteoclasts, respectively. The percentage of M/Mβ-like hemopoietic cells of TGF-β-mediated osteoclastogenesis is essential for osteoclastogenesis induced by a combination of RANKL and M-CSF. Our findings expand the established roles of TGF-β in osteoclastogenesis and provide a novel insight into bone metabolism.
agarose gel electrophoresis, and the bands were then visualized by ethidium bromide staining.

**Western blot analysis**

After the isolated M/Mfo-like hemopoietic cells had been treated with M-CSF and/or TGF-β for 2 days, the cells were washed with PBS; scraped into a solution consisting of 10 mM sodium phosphate (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM aminoethylbenzenesulfonfyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin; and sonicated for 15 s. The protein concentration in the cell lysate was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Each sample containing equal amounts of protein was subjected to 10% SDS-PAGE and, after being blocked with 5% skim milk, the membrane was incubated with anti-RANK Abs or nonimmune rabbit IgG and subsequently with peroxidase-conjugated anti-rabbit IgG Ab. Immunoreactive proteins were visualized with Western blot chemiluminescence reagents (DuPont-New England Nuclear Products, Boston, MA) following the manufacturer’s instructions.

**EMSA**

Nuclear extracts were prepared from M/Mfo-like hemopoietic cells pretreated for 2 days with M-CSF alone or with M-CSF and TGF-β as previously described (19). Double-stranded oligonucleotides containing an NF-kB binding site (5′-AGTTGAGGGACTTCCAGGC-3′) were radiolabeled with [32P]ATP and combined with 1 μg of nuclear extracts for 20 min at room temperature using a gel shift assay system (Promega, Madison, WI). The specificity of the reaction was confirmed by competition with a 50-fold molar excess of nonlabeled oligonucleotides. The protein-DNA complexes were resolved by 7.2% PAGE in 0.5 M urea and visualized by autoradiography. In the supershift experiment, the nuclear extracts were incubated with anti-p50 or anti-p65 Ab for 30 min on ice after binding to the oligonucleotides, and then were subjected to PAGE.

**Statistical analysis**

Significant differences between means of group were analyzed by one-way ANOVA and Dunnett’s test.

**Results**

**Characterization of M/Mfo-like hemopoietic cells isolated from a culture of mouse unfractionated bone cells**

When unfractionated bone cells prepared from 4- to 5-wk-old mice were cultured in the presence of a low dose of PGE2 (10^-8 M) for 6 days, the stromal cells proliferated to overconfluence in the culture and formed a sheet of cells. Morphologically appearing macrophage-like cells adhered to the substratum under the stromal cell layer. After the stromal cell sheet was detached, the remaining cells were isolated. As shown in Fig. 1, A and B, these cells revealed a mononuclear macrophage-like shape with a relatively large cytoplasm, and all the cells were capable of phagocytosing latex beads. These cells required M-CSF for their survival (data not shown). In addition, they were TRAP negative. Cell surface molecules expressed on the isolated cells were analyzed by flow cytometry using various Abs (Fig. 1C). The cells were positive for CD11b (Mac-1 α-chain), CD44, F4/80, and CD11a (LFA-1) and weakly positive for CD14 and integrin αv, but negative for integrin β3, which is expressed on mature osteoclasts, and DEC205, which is expressed on dendritic cells. Taken together, these findings indicate that these cells belonged to the M/Mfo lineage.

In vivo and in vitro studies have demonstrated that RANKL in cooperation with M-CSF is essential for generation of osteoclasts from hemopoietic cells (5, 6). When the cells isolated by the above procedure were incubated for 6 days in the presence of M-CSF and sRANKL, TRAP-positive MNCs were formed in the culture in a dose-dependent manner, as shown in Fig. 2. The total number of nuclei in the cultures was increased by the addition of M-CSF, indicating that the proliferation of the cells was dependent on M-CSF. M-CSF also increased the percentage of nuclei in TRAP-positive cells as an indicator of commitment to the osteoclast lineage and the fusion index of TRAP-positive MNCs, defined as the percentage of cells participating in the fusion. These values at 20 ng/ml of M-CSF were 28 and 17%, respectively, producing a maximal effect; and no further stimulation was observed at higher concentrations. On the other hand, although the total nuclear number was not changed by the addition of sRANKL, the percentage of nuclei in TRAP-positive cells and the fusion index of TRAP-positive MNCs were increased in a concentration-dependent fashion, with a maximal stimulation at 40 ng/ml. When TRAP-positive MNCs generated in the presence of M-CSF and sRANKL were replated on dentine slices, these cells resorbed dentine and formed pits on the surface (Fig. 2D). In addition, semiquantitative PCR analysis revealed that treatment with M-CSF and sRANKL for 6 days induced an increase in the levels of mRNA for TRAP, cathepsin K, calcitonin receptor, and integrin β3, all of which are abundantly expressed in osteoclasts (Fig. 3). On the other hand, the addition of sRANKL decreased the level of CD14 mRNA. These effects of M-CSF and sRANKL indicate that the isolated hemopoietic cells were potentially capable of differentiating into mature osteoclasts. In addition, these results demonstrate that M-CSF and RANKL play crucial roles in survival, proliferation, and differentiation of osteoclast progenitors, consistent with the conclusion of previous studies (5, 6). However, the percentages of nuclei in TRAP-positive cells and the fusion index were not very high, indicating that all the cells did not differentiate into the osteoclast lineage.

**Exogenous TGF-β in combination with RANKL/M-CSF induces osteoclast formation by all isolated hemopoietic cells**

Cells of the M/Mfo lineage are known to produce several cytokines and growth factors, and their proliferation and differentiation are regulated in an autocrine and paracrine manner (20, 21). Of these factors, TGF-β is expressed not only by monocyte/macrophages but also by MNCs (22). The expression of two types (TGF-RI and TGF-RII) of TGF-β receptor on isolated hemopoietic cells was confirmed by RT-PCR analysis (Fig. 4). These results indicate that the isolated hemopoietic cells are potentially responsive to TGF-β.

Simultaneous addition of TGF-β with sRANKL (40 ng/ml) and M-CSF (10 ng/ml) dose dependently increased the number of TRAP-positive MNCs among the cells cultured for 6 days, with a maximal effect of 12-fold at 1.25-20 ng/ml. At 20 ng/ml of TGF-β, the fusion index was 60%, i.e., 12-fold greater than that in the absence of TGF-β. Besides the TRAP-positive MNCs, almost all the mononuclear cells were TRAP positive (Fig. 5, A and B). However, since the cells died in the presence of TGF-β and sRANKL without M-CSF, TGF-β could not replace M-CSF for the survival of the osteoclast progenitors (data not shown). In addition, the combination of TGF-β and M-CSF without sRANKL supported the survival of the cells, but did not induce the formation of osteoclastic TRAP-positive MNCs. Associated with the enhancement of differentiation into osteoclasts, TGF-β further increased the mRNA levels of TRAP, cathepsin K, calcitonin receptor, and integrins αv and β3, and further decreased the CD14 mRNA level (Fig. 3). The number of pits excavated by the cells cultured with TGF-β, M-CSF, and sRANKL was much greater than that with M-CSF and sRANKL (Fig. 5C). As shown in Fig. 6, the stimulatory effect of TGF-β was dose dependent, consistent with the increase in osteoclastic cell formation. Taken together, the data show exogenous TGF-β to be a potent, but additive, inducer of osteoclastogenesis.
Endogenous TGF-β as well as M-CSF and RANKL participate in osteoclastogenesis

As shown in Fig. 4, the isolated hemopoietic cells expressed TGF-β1 and -β2 as well as their receptors. Therefore, we next examined whether endogenous TGF-β is involved in osteoclast generation in an autocrine fashion. Addition of neutralizing Ab against TGF-β abrogated the stimulation of osteoclast-like cell formation induced by M-CSF and sRANKL, whereas the nonimmune IgG had no effect (Fig. 7). This result shows that osteoclastogenesis induced by M-CSF and RANKL requires the endogenous production of TGF-β by the osteoclast progenitors. Next, to ascertain the action point of endogenous TGF-β in osteoclast development, we examined the effects of different treatment periods with anti-TGF-β on the osteoclast-like cell formation from the isolated hemopoietic cells pretreated with M-CSF and/or TGF-β. When the isolated hemopoietic cells were cultured for 6 days in the presence of M-CSF and sRANKL without TGF-β after pretreatment with TGF-β and M-CSF for the first 2 days, the fusion index of TRAP-positive osteoclastic MNCs formed in the cultures was equivalent to that in the cultures treated for the last 6 days with a combination of M-CSF and TGF-β.
of TGF-β, sRANKL, and M-CSF (Fig. 8B; lanes 2 and 3 from left). The cultures pretreated only with M-CSF required the continuous presence of TGF-β for the high efficiency of osteoclastic cell formation (Fig. 8A; lanes 2 and 3 from left). These data suggest that 2-day pretreatment with TGF-β allows osteoclast progenitors to prime to commit to an osteoclast lineage. However, the expression of the RANK receptor (RANK) in the isolated hemo- poietic cells was not up-regulated by 2-day pretreatment with TGF-β at mRNA and protein levels, whereas the expression was enhanced by M-CSF (Fig. 9, A and B). Instead, the TGF-β pretreatment synergistically stimulated activation of NF-κB evoked by sRANKL as determined by direct EMSA (Fig. 9C). Following the TGF-β pretreatment, treatment for 6 days with anti-TGF-β greatly reduced the osteoclast generation induced by the combination of sRANKL and M-CSF. Likewise, the inhibition was seen in the cultures treated with anti-TGF-β for the last 5 and 3 days, implying that endogenous production of TGF-β is involved in the processes of osteoclast differentiation, including priming and maturation (Fig. 8, A and B; lanes 4–6 from left). Finally, as in the

FIGURE 3. Semiquantitative RT-PCR analysis for the expression of various mRNAs. Total RNA of the M/MΦ-like hemopoietic cells was immediately extracted after isolation of the cells (lanes 1, 5, and 9), or the isolated M/MΦ-like hemopoietic cells were treated with M-CSF (10 ng/ml) alone (lanes 2, 6, and 10), M-CSF plus sRANKL (40 ng/ml; lanes 3, 7, and 11), or M-CSF, sRANKL, and TGF-β1 (10 ng/ml; lanes 4, 8, and 12) for 6 days, and then total RNA was extracted from each culture. The primers used were designed for mouse genes of TRAP, cathepsin K, CD14, integrins α and β, calcitonin receptor, and β-actin. Each cDNA was amplified for the indicated number of PCR cycles.

FIGURE 2. Isolated M/MΦ-like hemopoietic cells potentially differentiate into mature osteoclasts. The isolated cells (2 × 10⁴ cells) were cultured with various concentrations of M-CSF (A) in the presence of sRANKL (40 ng/ml), or the cells (5 × 10⁴ cells) were cultured with various doses of sRANKL (B) in the presence of M-CSF (10 ng/ml) for 6 days. At the end of the culture period, the cells were stained for nuclei and TRAP activity. The numbers of total nuclei and of TRAP-positive mononuclear cells and MNCs were counted. The total number of nuclei (□) represents the proliferation of the cells in the culture. The percentages (●) of nuclei in TRAP-positive mononuclear cells and of MNCs per total nuclear number show the rate of commitment of the M/MΦ-like hemopoietic cells into cells of the osteoclast lineage. Fusion index (○) indicates the rate of TRAP-positive osteoclast precursors that participate in cell fusion resulting in mature osteoclasts. Values are the mean ± SE for three cultures in a representative experiment. C, Photomicrograph of the TRAP-stained cells cultured for 6 days with sRANKL (40 ng/ml) and M-CSF (10 ng/ml). D, The cells treated for 5 days with sRANKL and M-CSF were replated on dentine slices and incubated for 1 day. Resorption pits formed by the cells were stained with acid hematoxylin. The bar in each photograph indicates 100 μm.

FIGURE 4. Expression of TGF-β1 and TGF-β2 receptors. Total RNA of the M/MΦ-like hemopoietic cells was immediately extracted after isolation of the cells (lane 1), or the isolated M/MΦ-like hemopoietic cells were treated with M-CSF (10 ng/ml) alone (lane 2), M-CSF plus sRANKL (40 ng/ml; lane 3), or M-CSF, sRANKL, and TGF-β1 (10 ng/ml; lane 4) for 6 days, and then total RNA was extracted from each culture. The primers used were designed for mouse genes of TGF-β1, TGF-β2, TGF-β3, and types I and II of TGFR. The numbers in parentheses indicate the numbers of PCR cycles.
cultures of osteoclast progenitors, anti-TGF-β Ab abolished the formation of TRAP-positive osteoclastic MNCs induced by M-CSF plus sRANKL or 1,25(OH)₂D₃ in cultures of unfractionated bone cells (Fig. 10), indicating that the requirement of endogenous TGF-β for osteoclastogenesis is not restricted to cultures of isolated hemopoietic cells.

Discussion
In this study we succeeded in developing a new isolation method for obtaining a homogenous population of osteoclast progenitors. The progenitors possessed common phenotypes of monocyte/macrophages, representing M/Mφ-like hemopoietic cells. Using the stroma-free culture system of isolated M/Mφ-like hemopoietic
cells, we demonstrated that TGF-β directly acts on the hemopoietic cells to enhance the osteoclast formation elicited by a combination of sRANKL and M-CSF. The isolated hemopoietic cells expressed mRNAs of TGF-β and -β2 and TGF-RI and -II throughout the culture period, suggesting that osteoclast progenitors are both the TGF-β-producing cells and cells responsive to TGF-β. Because various hemopoietic cells express TGF-β and TGF-β receptors and their proliferation and differentiation are widely regulated by TGF-β (23, 24), and because osteoclasts are of hemopoietic origin (25), these expressions in the isolated cells are not surprising. In fact, the production of TGF-β and the expression of TGF-β receptors have been previously reported in chick osteoclasts (22) and in osteoclastic MNCs derived from human giant cell tumors of bone (26). In addition, since anti-TGF-β Ab greatly suppressed the osteoclast formation from isolated cells, endogenous production of autocrine-acting TGF-β by hemopoietic cells appears to be required for osteoclast differentiation. Furthermore, TGF-β induced both the priming of hemopoietic cells to differentiate into the cells of osteoclast lineage and the maturation of these cells.

We isolated M/Mφ-like hemopoietic cells from cultures of unfractionated bone cells treated with PGE2. These cells required M-CSF for their survival and growth. In addition, the isolated cells expressed various monocyte/macrophage-phenotypic surface Ags, and showed phagocytic activity. GM-CSF also supported the survival, but neither stimulated the proliferation nor induced osteoclast generation even in the presence of sRANKL (data not shown). It has been recently demonstrated that human cells sharing monocyte/macrophage phenotypes were capable of differentiating into dendritic cells and osteoclasts dependent on GM-CSF and M-CSF, respectively (27). In addition, when the isolated M/Mφ-like hemopoietic cells were cultured with LPS (28), these cells did not differentiate into an osteoclastic cell lineage even with sRANKL, M-CSF, and TGF-β (data not shown). Taken together, the available data indicate that the isolated cells represent bipotential immature monocytes/macrophages.

TGF-βs are multifunctional cytokines that widely regulate the proliferation and differentiation of a variety of cell types, including epithelial and mesenchymal cells (29, 30). Numerous studies on bone cells have indicated that TGF-β stimulates the growth and differentiation of osteoprogenitors to become bone matrix-producing cells (31, 32). Thus, TGF-βs are positive regulators of bone formation. However, the effect of TGF-β on bone resorption is controversial. A stimulatory effect of TGF-β on bone resorption was observed in organ cultures of mouse calvariae (33). In contrast, TGF-β inhibited the osteoclastic bone resorption in fetal rat long bones (34). Furthermore, Hughes et al. (35) showed that TGF-β promoted the apoptosis of osteoclasts in culture of bone

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**FIGURE 8.** Effect of neutralizing anti-TGF-β Ab on priming and maturation of osteoclast differentiation. The isolated M/Mφ-like hemopoietic cells were pretreated for 2 days with M-CSF (10 ng/ml) alone (A) or with TGF-β1 (10 ng/ml) plus M-CSF (B). Then, after the factors had been removed by washings, the pretreated cells were further treated for 6 days with various combinations of M-CSF, TGF-β, and/or sRANKL (40 ng/ml). Neutralizing anti-TGF-β Ab (20 μg/ml) or isotype control IgG1 (20 μg/ml) was added to the cultures at day 0 (D-0), day 1 (D-1), or day 3 (D-3) after the start of treatment. Values are the mean ± SE for three cultures in a representative experiment. *p < 0.01 vs culture treated with M-CSF and sRANKL, by Dunnett’s analysis.

**FIGURE 9.** Expression of RANKL receptor (RANK) and activation of NF-κB in M/Mφ-like hemopoietic cells. A and B, Total RNA and membrane proteins were immediately extracted from the isolated untreated M/Mφ-like cells (A, lanes 1 and 4; B, lanes 1, 4, and 7) and from the cells treated for 2 days with M-CSF at 10 ng/ml (A, lanes 2 and 5; B, lanes 2, 5, and 8), or with M-CSF and TGF-β1 at 10 ng/ml (A, lanes 3 and 6; B, lanes 3, 6, and 9). Western blotting (A) and RT-PCR (B) analyses for RANK expression were performed. Each cDNA was amplified for the indicated number of PCR cycles. C, EMSA. The isolated M/Mφ-like cells were precultured in the absence (lanes 1–5) or presence (lanes 6–12) of TGF-β1 (10 ng/ml) with M-CSF (10 ng/ml) for 2 days. After preculture, the cells were treated with sRANKL (40 ng/ml) for 0 h (lanes 1 and 6), 0.5 h (lanes 2 and 7), 1 h (lanes 3 and 8), 2 h (lanes 4 and 9), and 4 h (lanes 5 and 10). Then, nuclear proteins in the cells were extracted and subjected to EMSA. Lanes 11 and 12, EMSA using a sample from the cells treated with sRANKL for 1 h after TGF-β pretreatment (lane 8) were performed in the presence of anti-p65 and anti-p50 Abs, respectively.
marrow cells consisting of a heterogeneous population. Therefore, the inhibition of bone resorption by TGF-β may in part be attributed to the induced osteoclast apoptosis, although we did not observe such a stimulatory effect of TGF-β on osteoclast apoptosis in our culture system. Regarding osteoclastogenesis, the inhibition by TGF-β was demonstrated in cultures of bone marrow cells, which contained stromal cells, and in cocultures of bone marrow cells or spleen cells and stromal cells (13). On the other hand, TGF-β was reported to stimulate the formation of osteoclast-like cells in cultures of a human leukemia cell line, FLG 29.1, in an autocrine manner (36). Transgenic mice overexpressing TGF-β2 exhibited an osteoporosis-like phenotype due to the increased osteoclastic function (37, 38), and transgenic mice expressing dominant-negative type II TGF-β receptor decreased osteoclastic bone resorption (39), suggesting a locally positive participation of TGF-β in osteoclast development. Sells et al. (40) recently demonstrated that TGF-β in combination with RANKL and M-CSF enhanced osteoclast-like formation in cultures of bone marrow cells and spleen cells containing few osteoblastic/stromal cells. In addition, TGF-β was demonstrated to stimulate osteoclast formation in cocultures of spleen cells and T lymphocytes expressing RANKL (41). Our findings are consistent with those results, although target cells of TGF-β were not defined due to the heterogeneity of hematopoietic cells in those culture systems (40, 41). The isolated cells examined in this study consisted of a homogeneous population with monocyte/macrophage phenotypes, and all of them differentiated into cells of osteoclast lineage by TGF-β treatment in the presence of sRANKL and M-CSF. Therefore, TGF-β directly acts on osteoclast progenitors to stimulate their differentiation into osteoclasts. Taken together, the overall effects of TGF-β on osteoclastogenesis are dependent on the cell population.

We also indicated that TGF-β in combination with sRANKL and M-CSF stimulated osteoclast formation in the cultures of unfractionated bone cells. These cultures contained stromal cells, but the stromal cells somehow did not expansively proliferate in the cultures. The inhibitory effect of TGF-β seems to be observed when a large number of osteoblastic/stromal cells are present. It was recently demonstrated that TGF-β increased the expression of osteoprotegerin (identical with osteoclastogenesis-inhibitory factor), which strongly inhibits osteoclastogenesis as a decoy receptor of RANKL (42). Therefore, osteoprotegerin may be at least in part a mediator of the TGF-β inhibitory effect via stromal cells. However, endogenous TGF-β is intrinsically essential for osteoclast development, and the stimulatory effect of exogenous TGF-β is seen under the condition of a minimal number of stromal cells.

PGE₂ has recently been reported to cooperate with RANKL and M-CSF in the promotion of osteoclast formation from hematopoietic cells (43). In a variety of cell types, TGF-β induces the production of PGs mediated by up-regulation of prostaglandin H₂ synthase-2 (44, 45). Those studies suggest that the enhancement of osteoclast formation by TGF-β presented in this study is mediated by endogenous synthesis of PGs. However, since NS-398, a selective inhibitor of PGG/H synthase-2, did not affect the stimulation of osteoclast generation by TGF-β (data not shown), the stimulatory effect of TGF-β is not endogenous PG dependent.

RANKL has been demonstrated to activate NF-κB and c-Jun N-terminal protein kinase (JNK) through RANK in osteoclastic cells as well as in dendritic cells (12, 46, 47). Recent studies indicate that binding of RANKL to RANK caused association of the receptor with several TNF receptor-associated factors (TRAFs), resulting in the activation of NF-κB (46–48). Knockout mice of both NF-κB1 and NF-κB2, and of TRAF6, exhibited severe osteopetrosis due to impaired osteoclast differentiation (49, 50). Therefore, TRAF6 and NF-κB seem to be involved in osteoclastogenesis. In this study we demonstrated that TGF-β synergistically increased the translocation of NF-κB into nuclei induced by RANKL in M/Mδ-like osteoclast progenitors, although TGF-β did not affect the expression of RANK, suggesting an intracellular cross-talk in signalings of TGF-β and RANKL. At present, the detailed molecular signalings of TGF-β that strongly promote osteoclast formation are not known. It was recently reported that TGF-β-activated kinase 1 functionally interacted with IκB kinase to stimulate NF-κB (51). Such an interaction of TGF-β receptor downstream signaling molecules with RANK-associated molecules may at least in part account for the synergistic induction of osteoclastogenesis by TGF-β and RANKL.

Involvement of TGF-β in the pathogenesis of osteopenic disorders has been suggested. It was demonstrated in rheumatoid arthritis that the synovium contained a large number of macrophage-like cells that have a strong ability to produce TGF-β as well as other inflammatory cytokines, such as IL-1 and TNF-α (52, 53), and a high level of endogenous TGF-β was also detectable in other
types of arthritides, including osteoarthritis (54). In addition, the TGF-β concentration in serum was shown to be elevated in osteoporotic women, with good correlation with the bone loss (55). Taken together with our findings, TGF-β may contribute to destruction of bone as well as bone formation in vivo.

In conclusion, TGF-β is intrinsically required for osteoclastogenesis in combination with RANKL and M-CSF. The results presented here expand our knowledge about the multiple roles of TGF-β in bone metabolism.

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