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Interaction of Fas Ligand and Fas Expressed on Osteoclast Precursors Increases Osteoclastogenesis

Hyewon Park,* Youn-Kwan Jung, † Ok-Jin Park,* Yeon Ju Lee,* Je-Yong Choi,** and Youngnim Choi,*†

We incidentally found that osteoclast precursors and mature osteoclasts express Fas ligand (FasL) as well as Fas, which was confirmed by flow cytometry, immunofluorescent staining, and RT-PCR. The aim of this study was to determine the role of FasL in differentiation and cell death of osteoclasts. To study the role of FasL in osteoclastogenesis, neutralizing anti-FasL mAb or rFasL was added during receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis using bone marrow-derived macrophages. Neutralization of endogenous FasL by anti-FasL mAb decreased osteoclastogenesis, whereas rFasL enhanced osteoclast differentiation in a dose-dependent manner. In addition, rFasL up-regulated the secretion of osteoclastogenic cytokines, such as IL-1β and TNF-α, and the activation of NF-κB. Functional blocking of IL-1β and TNF-α using IL-1 receptor antagonist and soluble TNFR confirmed that those cytokines mediated the effect of FasL on osteoclastogenesis. The osteoclast precursors were relatively resistant to rFasL-induced apoptosis especially before RANKL treatment, resulting in minimal cell loss by rFasL treatment during osteoclastogenesis. Although rFasL increased the cell death of mature osteoclasts, growth factor withdrawal induced much more cell death. However, anti-FasL mAb did not affect the survival of mature osteoclasts, suggesting that the endogenous FasL does not have a role in the apoptosis of osteoclasts. Finally, in contrast to the effect on apoptosis, rFasL-assisted osteoclastogenesis was not mediated by caspases. In conclusion, FasL has a novel function in bone homeostasis by enhancing the differentiation of osteoclasts, which was not considered previously.

Osteoclast differentiation requires many important determinants such as receptor activator of NF-κB ligand (RANKL), receptor activator of NF-κB ligand (RANKL), receptor activator of NF-κB, M-CSF, PU.1, c-Src, and c-fos, etc., which can be classified as cytokines, signaling molecules, and transcription factors (1). Together with these key molecules, osteoclast differentiation is also regulated at various steps by many other modulators such as TNF-α, IL-1, IFN-β, and NF-ATc1 (2).

Among cytokine determinants, M-CSF was the first molecule found to be essential for osteoclastogenesis, and otopop mice defective in M-CSF gene showed osteopetrosis (3, 4). RANKL, cloned from osteoblasts and T cells almost at the same time, was effective in M-CSF gene showed osteopetrosis (3, 4). RANKL, cloned from osteoblasts and T cells almost at the same time, was sufficient to induce differentiation of osteoclasts from their precursors in the presence of M-CSF in vitro (5). Mice that are deficient of RANKL or its receptor activator of NF-κB exhibited severe osteopetrosis accompanied by the lack of osteoclasts (6, 7).

Although M-CSF and RANKL are the two essential cytokines for osteoclast differentiation, many other cytokines have positive or negative effects on osteoclastogenesis (8, 9). During immune response, macrophages, the potential osteoclast precursors, interact with T cells as APC for naïve T cells or target cells for effector T cells. Because activated T cells express RANKL, T cell–mediated bone destruction was shown in cIa4−/− mice (10). However, T cells could also regulate osteoclastogenesis negatively through IFN-γ (11). In addition, we have shown that activated CD8+ T cells substantially inhibit osteoclastogenesis (12). Fas ligand (FasL) (CD95 ligand, TNFSF6, CD178) is a major death ligand expressed by activated CD8+ T cells. Fas (TNFRSF6, CD95) received great attention as a major death receptor that mediates immune homeostasis and the effector function of CTL and NK cells (13, 14). While exploring the possibility that activated CD8+ T cells may inhibit osteoclastogenesis through FasL, we incidentally found that osteoclast precursors express FasL, as well as Fas, and the FasL increases osteoclastogenesis rather than inhibits it. There are two reports regarding the role of Fas in the apoptosis of osteoclasts (15, 16). In this study, we now report the novel function of FasL/Fas in osteoclast differentiation.

Materials and Methods

Mouse and reagents

Five- to 6-wk-old C57BL/6 mice (Samtako) were used for all experiments. The handling of the animals and the experimental protocol were approved by Seoul National University Animal Care and Use Committee. Murine recombinant M-CSF and RANKL were purchased from PeproTech. Human rFasL was purchased from Alexies or PeproTech. Because the m.w. of rFasL from two companies were different, when rFasL from Peprotech was used, half of described concentration was used to get equivalent effect. Murine recombinant IL-1 receptor antagonist (IL-1ra) and soluble type 1 TNFR (sTNFR) were purchased from R&D Systems. The Abs used in culture were dialyzed for 48 h to remove sodium azide. A general caspase

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inhibitor z-VAD-fmk and a noncaspase cysteine protease inhibitor z-FA-fmk (BD Biosciences) were dissolved in DMSO to make 10 μM stock solutions.

**Osteoclast culture, functional, and tartrate-resistant acid phosphatase (TRAP) staining**

Osteoclast culture was done as described before (17). Briefly, bone marrow cells obtained from the long bones of mice were suspended in α-MEM containing 10% FBS following RBC lysis, and cultured in 100-mm dishes in the presence of 1 ng/ml M-CSF for 24 h. By harvesting nonadherent cells, stroma-free bone marrow cells were plated at 5 × 10^4 cells/200 μl well into 96-well plates, and cultured in the presence of 20 ng/ml M-CSF for 2 days to generate bone marrow-derived macrophages (BMMs). After nonadherent cells were washed away, BMMs were further cultured with new medium containing M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the absence or presence of anti-Fasl mAb (BD Biosciences), rFasL, DMSO, z-VAD-fmk, or z-FA-fmk for 4 days. To block IL-1 and TNF-α during osteoclastogenesis, IL-1ra (100 ng/ml), TNFR (500 ng/ml), or both were added in the absence or presence of rFasL (100 ng/ml) during RANKL-induced osteoclastogenesis. Osteoclastogenesis using RAW264.7 cells (American Type Culture Collection) were performed by plating fresh thawed cells at 2 × 10^4 cells/well into 96-well plates and culture with RANKL (40 ng/ml) for 4 days. Cultures were fed with new medium every 2 days, and culture supernatants were saved sometimes for ELISA analysis. For TRAP staining, cells were fixed with 3% formaldehyde in acetic acid for 30 s after washing with PBS and stained for TRAP using a leukocyte acid phosphatase-staining kit (Sigma-Aldrich). TRAP* multinuclear cells (MCs) with three or more nuclei were regarded as osteoclasts and counted under an inverted-phase contrast microscope.

**Flow cytometric analysis**

Stroma-free bone marrow cells were cultured with M-CSF (20 ng/ml) for 2 days to generate BMMs. BMMs were further cultured with M-CSF (20 ng/ml) and RANKL (40 ng/ml) for another 2 days. BMMs or cells further cultured with M-CSF and RANKL were harvested by a cell scraper and transferred to FACS tubes. Cells were washed with PBS and blocked with mouse IgG (Sigma-Aldrich). Then, cells were double stained with anti-CD11b mAb clone M1/70-PerCP and either anti-Fas mAb clone Jo2-FITC or anti-Fasl mAb clone M3L3, followed by goat anti-hamster Ab FITC. As isotype controls, cells were also stained with anti-CD11b mAb PerCP and either FITC-conjugated hamster isotype control Ab or secondary Ab alone. All primary and secondary Abs were from BD Biosciences. The stained cells were analyzed with FACS Calibur (BD Biosciences).

**In situ immunofluorescent staining**

To investigate the expression of Fas and FasL on mature osteoclasts, we conducted in situ immunofluorescent staining, as described previously (18). Briefly, BMMs were seeded at 5 × 10^4 cells/well in 8-chamber slide and cultured with α-MEM containing 10% FBS, M-CSF (20 ng/ml), and RANKL (40 ng/ml) to generate mature osteoclasts. Mature osteoclasts in 8-chamber slide were washed with PBS and fixed with 4% formaldehyde in PBS (pH 7.4) for 10 min. After blocking with 2% BSA/PBS for 1 h, cells were stained with either anti-Fas polyclonal Ab (1:200) (Oncogene Research Products) and anti-Fasl mAb (1:200) (Biolendeg) or rabbit IgG and mouse IgG (1:200) as control in 1% BSA. Subsequently, cells were stained with Alexa Fluor 488 donkey anti-rabbit IgG Ab (1:200) and Alexa Fluor 555 donkey anti-mouse IgG Ab (1:200) (Molecular Probes). To investigate the translocation of NF-κB by Fasl or FasL, BMMs or mature osteoclasts cultured in 8-chamber slide were treated with or without Fasl (250 ng/ml) in serum-free medium for 30 min. Following wash with PBS, cells were fixed with 4% formaldehyde in PBS (pH 7.4) for 5 min, washed, permeabilized with 0.25% Triton X-100 in PBS for 5 min on ice, and washed three times again. After blocking with 2% BSA/PBS for 30 min, the cells were stained with anti-NF-κB Ab (1:200) (Santa Cruz Biotechnol- ogy) in 1% BSA/PBS, followed by FITC-conjugated goat anti-mouse Ab (Molecular Probes) in 1% BSA/PBS. Cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), mounted with anti-fade mounting solution (Molecular Probes), and photographed under a fluorescence microscope.

**RT-PCR**

Total RNA (2 μg) was subjected to reverse transcription with (dT)16 and 1 μl of Superscript II enzyme (Invitrogen Life Technologies) in 20 μl reaction mix at 42°C for 1 h. Two microtiter of reverse-transcription products were subjected to PCR in cycling conditions: 94°C, 1 min; 58°C, 30 s; 72°C, 45 s for 35 cycles, except FasL. For FasL, it was amplified for 40 cycles as first PCR, and then 2 μl of amplified product was further amplified for 25 cycles using nested primers. Primer sequences used are as follows: 5'-GACGTGCAAATGAATGGGGGT-3' and 5'-AGTGTG GGGTGAATTTTC-3' for Fas; 5'-GGGCCCTCCTCAAGCATGTT-3' and 5'-GGAGGGTCTCATAGATGCTTCC-3' for the primary PCR of FasL; 5'-TCAGTTTCCCTGCTTACCT-3' and 5'-TGGGTTGGTC TATTGCTTT-3' for the secondary PCR of FasL; 5'-TCAAGGTCCT TCTCATT-3' and 5'-GAGCCAAGAGGAGTAT-3' for caspase 8, GAPDH gene amplified with primers 5'-AATCTGGTGATGGA AACG-3' and 5'-ACATCTGGGTTAGGAACA-3' for 35 cycles served as control for RNA input. All primers were designed to amplify at least two exons.

**MTT assay**

Mature osteoclasts were differentiated by the culture of BMMs with M-CSF (20 ng/ml) and RANKL (40 ng/ml) for 4 days, to which 20 μl/well MTT reagent (R&D Systems) was added and incubated for 2 h at 37°C. Purple precipitates were solubilized by incubation with 100 μl of detergent reagent overnight at room temperature, and the absorbance was measured with a microplate reader (Bio-Rad).

**The measurement of cell death by SYTOX Green staining**

Mature osteoclasts were differentiated by the culture of BMMs with M-CSF (20 ng/ml) and RANKL (40 ng/ml) for 4 days. To measure the effect of rFasL on overall cell death during osteoclastogenesis, mature osteoclasts were stained with 5 μM SYTOX Green (Molecular Probes) right away, and fluorescence was measured with a fluorometer (BMG Labtech). To measure the effect of rFasL on cell death during the process of osteoclastogenesis, cells were further incubated with new complete α-MEM medium alone or medium containing either FasL (250 ng/ml) or anti-FasL mAb (10 μg/ml) in the presence or absence of M-CSF plus RANKL for 16 h. Cells were stained with SYTOX Green, and fluorescence was measured with a fluorometer. To take photographs, Hoechst 33258 (25 μg/ml) was added to SYTOX Green-stained cells and photographed under an inverted fluorescence microscope.

**Annexin V staining**

BMMs or cells further cultured with M-CSF (20 ng/ml) and RANKL (40 ng/ml) for 2 days were incubated with various doses of rFasL for 4 h. During the incubation with FasL, M-CSF or M-CSF and RANKL were present in the medium to exclude apoptosis by growth factor withdrawal. Cells were harvested and transferred to FACS tubes. After wash, cells were incubated with annexin V FITC (BD Biosciences) and propidium iodide (PI) (Sigma-Aldrich) in binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2) for 15 min, and immediately analyzed with FACS Calibur.

**Apoptosis assay of T cells**

CTLL-2 cells (2 × 10^6), a murine IL-2-dependent T cell line, were incubated with rFasL (250 ng/ml) for 4 h in the presence of 1 ng/ml murine IL-2 (PeproTech) and subjected to annexin V assay. Otherwise, CTLL-2 cells (2 × 10^6) were incubated with FasL (250 ng/ml) in the presence of 1 ng/ml IL-2 for 16 h, and cell death was analyzed by flow cytometric enumeration following PI staining, as described before (19).

**ELISA**

The amounts of IL-1β and TNF-α secreted into medium during osteoclast differentiation were measured using ELISA kits (R&D Systems), according to the manufacturer’s instruction.

**EMSA**

Murine macrophage cell line RAW 264.7 cells (American Type Culture Collection) were cultured in DMEM containing 10% FBS. To determine Fas–mediated NF-κB activation, 80% confluent cells in a 100-mm culture dish were serum starved overnight and treated with rFasL (250 ng/ml) for 4 h. Nuclear extracts were prepared, as described previously (20). Briefly, collected cells were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, and 10 μg/ml aprotinin) and swelled on ice for 15 min. After swelling, Nonidet P-40 was added to make a final concentration of 0.1%, and the cell lysate was subjected to vigorous vortex. The lysate was cen-trifuged at 13,000 rpm at 4°C for 30 s, the supernatant was discarded, and 50 μl of high salt buffer (20 mM HEPES (pH 7.9), 25% glycerol, 0.4 M KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) was added to remaining nuclear pellets. The pellets in high salt were mixed.
globally at 4°C for 20 min. Nuclear extracts were then cleared by centrifugation at 13,000 rpm for 5 min, and their protein contents were quantified by a Bio-Rad protein assay kit (Bio-Rad). EMSA was performed according to the manufacturer’s protocol (Promega). Briefly, 10 μg of the nuclear extract was incubated with 1× gel shift binding buffer containing 10 mM Tris (pH 7.5), 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 4% glycerol, and 50 μg/ml poly(dI–dC). Competition was performed with a 25-fold molar excess of unlabeled oligonucleotides containing NF-κB consensus sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') at room temperature for 10 min, and then 32P-labeled NF-κB consensus oligonucleotides were added. After 30 min, the reaction product was subjected to electrophoresis through a 6% native polyacrylamide gel in 0.5 TBE buffer (50 mM Tris-HCl (pH 8.5), 50 mM borate, and 1 mM EDTA). Then, the gel was dried in a vacuum for 30 min and exposed to x-ray film.

Statistics

All presented data are the representative of two to three experiments with similar results. The data were expressed as mean ± SEM of three to four experimental samples. An unpaired two-tailed t-test assuming equal variances was performed to compare the means of two groups. For each figure, the error bars indicate SEM.

Results

Expression of Fas and FasL by osteoclast precursors and mature osteoclasts

In our system, cell fusion appeared on the third day of culture with M-CSF and RANKL, and mature osteoclasts were obtained by 4-day culture with M-CSF and RANKL. To determine the expression of Fas and FasL during osteoclast differentiation, three different methods were used, including flow cytometric analysis, immunofluorescent staining, and RT-PCR.

In flow cytometric analysis, osteoclast precursors were analyzed before cell fusion. To gate on myeloid cell lineage, cells were stained with CD11b. Following culture with M-CSF for 3 days, 90% of BMMs expressed CD11b, and further culture with M-CSF plus RANKL for 2 days increased the percentage of CD11b+ cells to 97 (Fig. 1A, density plot). Upon the flow cytometric analyses of osteoclast precursors, two distinct populations were observed: small cells with CD11blow (R2) and large cells with CD11bhigh (R3) (Fig. 1A, density plot). Although Fas was expressed ubiquitously at high level, FasL was expressed weakly only on the CD11bhigh cells. The levels of Fas and FasL expression did not change much, but the proportion of CD11bhigh cells was increased by the culture with M-CSF and RANKL (Fig. 1A).

In immunofluorescent staining, Fas- and FasL-positive signals were observed on all mono- and multinuclear cells, although it was difficult to compare the levels of expression (Fig. 1B). We surmised that the small CD11blow cells represented less adherent cells, and those were removed during washing steps in immunofluorescent staining. Indeed, vigorous washing before the collection of cells removed the small CD11blow cell population upon flow cytometric analysis (data not shown).

In RT-PCR analysis, the expression of cathepsin K, a marker of osteoclasts, was detected after 2-day culture with M-CSF and RANKL. Fas expression was slightly increased by 1-day culture with M-CSF and RANKL, and then sustained it through differentiation. The level of FasL expression was very low and required proper detection. FasL was also increased by culture with M-CSF and RANKL, but decreased in mature osteoclasts (Fig. 1C). These semiquantitative RT-PCR data showed fairly good correlation with the observations made by flow cytometry and immunofluorescent staining. In addition, RNA samples from RAW264.7 cells cultured with RANKL were subjected to RT-PCR. The sequence of amplified products was verified to be FasL by sequencing.

These results indicate that Fas and FasL are constitutively expressed during osteoclast differentiation.
Interaction of Fas and FasL enhances RANKL-induced osteoclastogenesis

Formation of mature osteoclasts needs vigorous cell fusion among osteoclast precursors. It suggests that the interaction of FasL and Fas on osteoclast precursors may play an important role during osteoclast differentiation. To know the functional consequence of Fas and FasL interaction, two different approaches were used. First, functional blocking of endogenous FasL to Fas signaling by neutralizing anti-FasL mAb inhibited RANKL-induced osteoclastogenesis in a dose-dependent manner, which was quite unexpected finding considering the well-known function of Fas as a death receptor (Fig. 2). Second, direct addition of rFasL to BMM culture substantially increased RANKL-induced osteoclast formation (Fig. 3). In the absence of RANKL, however, rFasL could not induce osteoclast differentiation, although weak TRAP-positive cells were occasionally observed (Fig. 3A). Similar enhancement effect on osteoclast formation by rFasL was observed using RAW264.7 cells, too, when the cells were exposed to rFasL for the first 24 h during 4-day culture with RANKL (data not shown). These results clearly indicate that interaction of FasL and Fas on osteoclast precursors stimulates osteoclast differentiation.

Effect of rFasL on the apoptosis of osteoclast precursors and mature osteoclasts

One potential mechanism for the stimulatory effect of FasL on osteoclastogenesis may be the increase of precursor cells. We determined the effect of rFasL on the overall cell survival and death during osteoclastogenesis by 4-day culture with M-CSF and...
RANKL. Cell death was quantified by a fluorometer after staining with SYTOX Green that penetrates only damaged membrane and becomes fluorescent upon binding to DNA (Fig. 4A). Significant change in cell death was observed at 250 ng/ml rFasL compared with control; however, it resulted in only ~10% reduction in total viable cells (Fig. 4B). As a control experiment for the effectiveness of rFasL, CTLL-2 T cells were incubated with 250 ng/ml rFasL for 16 h, which induced 91% reduction in cell viability. This result clearly indicates that the cells of osteoclast lineage are resistant to Fas-mediated apoptosis. We further studied the effect of rFasL on cell death at different differentiation status. Annexin V assay revealed that osteoclast precursors were quite resistant to FasL-induced apoptosis especially before RANKL treatment. Following culture with M-CSF and RANKL for 2 days, cells showed a small, but significant increase (1.5-fold compared with nontreated control) in cell death at 250 ng/ml rFasL (Fig. 4C), which is comparable to that observed by 4-day treatment (Fig. 4B). The same concentration of rFasL induced the apoptosis of CTLL-2 T cells much more efficiently (from 9.8 to 58.5%) (data not shown). Wu et al. (15) reported Fas-mediated apoptosis in mature osteoclasts. To confirm this finding, we incubated mature osteoclasts with rFasL or anti-FasL mAb in the absence or presence of M-CSF and RANKL for 16 h and measured cell death by SYTOX Green stain. Regardless of the presence of M-CSF and RANKL, rFasL increased cell death compared with nontreated control, confirming the previous report (Fig. 4D). However, survival factor withdrawal induced twice more cell death than rFasL (Fig. 4D). In the meantime, the anti-FasL mAb had no significant effect on overall cell death, suggesting that FasL expressed on osteoclasts has more of a role in osteoclastogenesis than cell death (Fig. 4D). Collectively, these results indicated that the enhancement of osteoclastogenesis by rFasL is due to the facilitation of differentiation and not the increase of precursor cells. In addition, although FasL-mediated apoptosis is increased along the differentiation of osteoclasts, the cells of osteoclast lineage are very resistant to FasL-induced apoptosis, resulting in the minimal loss of viable cells.

**FasL increased the production of IL-1β and TNF-α with activation of NF-κB**

Because Fas-mediated induction of inflammatory cytokines through NF-κB activation has been reported (21, 22), we postulated that the FasL-induced up-regulation of osteoclastogenesis may be mediated by production of osteoclastogenic cytokines such as IL-1β and TNF-α.

![Image](http://www.jimmunol.org/)
as IL-1β and TNF-α. Supernatants were collected after 2-day culture with M-CSF and RANKL when mature osteoclasts were not formed yet. As shown in Fig. 5A, rFasL increased IL-1β and TNF-α productions. However, this up-regulation of IL-1β and TNF-α productions was not significant in the absence of M-CSF and RANKL (data not shown). Production of proinflammatory cytokines is often regulated by NF-κB. To see whether rFasL induces NF-κB activation in osteoclast precursors, translocation of NF-κB into nucleus was examined by immunofluorescent staining. Indeed, rFasL induced the translocation of NF-κB into nucleus, which was more evident in mononuclear precursors than in mature osteoclasts (Fig. 5B). EMSA also confirmed NF-κB activation by rFasL (Fig. 5C). To confirm the contribution of up-regulated IL-1β and TNF-α to the rFasL-assisted osteoclastogenesis, functional blocking experiments were performed using IL-1ra and TNFR.

Although the blocking of either IL-1β or TNF-α alone was not enough to inhibit the effect of rFasL, the blocking of both IL-1 and TNF-α inhibited it (p < 0.005) (Fig. 6). These results indicate that the stimulatory effect of FasL on osteoclastogenesis is mediated by production of IL-1β and TNF-α.

The role of caspase in osteoclastogenesis and apoptosis

Most FasL-induced responses are mediated by the activation of caspase cascades. We questioned whether the rFasL-induced enhancement of osteoclastogenesis or apoptosis is dependent on the caspase activation. In the absence of rFasL, a general caspase inhibitor z-VAD-fmk decreased osteoclast formation marginally with no statistical significance, and the vehicle for peptide inhibitors DMSO had no significant effect on osteoclast differentiation either. The rFasL-assisted osteoclastogenesis was inhibited by neither z-VAD-fmk nor DMSO. Interestingly, a noncaspase cysteine protease inhibitor z-FA-fmk, a negative control for z-VAD-fmk, inhibited RANKL-induced osteoclastogenesis completely (p < 0.005).

**FIGURE 5.** Induction of proinflammatory cytokines and activation of NF-κB by rFasL (A) ELISA measurement of IL-1β and TNF-α. BMMs were further cultured with M-CSF (20 ng/ml), RANKL (40 ng/ml), and various doses of rFasL for 2 days, and supernatants were saved for ELISA analysis. Each column represents the mean ± SEM of three cultures, *p < 0.005, vs control. B, Nuclear translocation of NF-κB. BMMs were cultured with M-CSF + RANKL for 4 days and treated with rFasL (250 ng/ml) for 30 min. Cells were fixed, permeabilized, and stained with anti-NF-κB mAb, followed by goat anti-mouse Ab FITC and DAPI. Arrows indicate colocalization of NF-κB and nucleus. C, Activation of NF-κB by EMSA. Nuclear extracts obtained from nontreated or rFasL (250 ng/ml)-treated RAW264.7 cells were incubated with a labeled probe with or without specific competitor and separated through a 6% native polyacrylamide gel. An oligonucleotide containing the NF-κB consensus sequence was used as a labeled probe. NF-κB and oligonucleotide complex (●) and free oligonucleotides (○) were indicated. Competitor lanes (+) containing 25-fold molar excess of unlabeled oligonucleotides are compared with controls without specific competitor (−). Comp and NE represent competitor and nuclear extract, respectively.

**FIGURE 6.** Inhibition of rFasL-mediated osteoclastogenesis by functional blocking of IL-1β and TNF-α. BMMs were further cultured with M-CSF (20 ng/ml) and RANKL (40 ng/ml) in the absence or presence of rFasL (100 ng/ml) for 4 days (Control). As experimental groups, IL-1ra (100 ng/ml), sTNFR (500 ng/ml), or both were added during the 4-day culture with M-CSF and RANKL in the absence or presence of FasL. *p < 0.05, vs no FasL; †, p < 0.005, vs control group (x).
In this study, we report the expression of FasL together with Fas on the cells of osteoclast lineage. Thus, it can be speculated that the interaction of FasL and Fas via cell-to-cell contact during osteoclast differentiation can modulate osteoclastogenesis and bone homeostasis.

Surprisingly, FasL enhanced RANKL-dependent osteoclast differentiation. Direct addition of rFasL increased RANKL-mediated osteoclastogenesis. In addition, disruption of endogenous FasL/Fas interaction in osteoclast precursors using an anti-FasL mAb decreased osteoclastogenesis. These results indicate that FasL plays a role as a positive modulator of RANKL-mediated osteoclastogenesis.

FasL expressed on cell membrane acts as a trimer as most other TNF family members. Although soluble rFasL is less efficient than the membrane form, it behaves as an agonist to receptor rather than an antagonist (25) as we used soluble rRANKL to induce signaling through RANK. Therefore, we believe that signaling through Fas by FasL enhanced osteoclastogenesis. Because reverse signaling through FasL has been proposed before (26), the possibility that reverse signaling induced by anti-FasL Ab inhibited osteoclastogenesis and rFasL blocked the reverse signaling from endogenous Fas to FasL cannot be excluded. However, it is less likely. Yang et al. (27) showed that decoy receptor 3 induced osteoclast differentiation in the absence of M-CSF and RANKL through reverse signaling. Although the ligand for decoy receptor 3 was not clarified in their study, it can bind to FasL, herpes virus entrance mediator (LIGHT), and TL1A. Therefore, the reverse signaling through FasL is not likely to inhibit osteoclastogenesis.

We also studied the role of FasL in the death of osteoclasts at different time points from early osteoclast precursors (Fig. 4D). Our results suggested that the cells of osteoclast lineage are very resistant to FasL-induced apoptosis, although the susceptibility increases with differentiation (Fig. 4C and D). Even in the mature osteoclasts, survival factor withdrawal induced much more cell death than rFasL (Fig. 4D). Although we did not count the percentage of dead cells, we can deduce that cell death by rFasL is far less than 50% in the presence of M-CSF plus RANKL. It is quite low compared with T cells that show 91% decrease in viable cells in the presence of IL-2 and the same concentration of rFasL. Therefore, treatment with rFasL during osteoclast differentiation did not change the amounts of total viable cells as much as expected, resulting in just 10% loss at 250 ng/ml (Fig. 4B). Higher concentration (500 ng/ml) caused 22% reduction in viable cells, and the effect on osteoclastogenesis enhancement decreased compared with 250 ng/ml (data not shown). These suggest that the enhancement of osteoclastogenesis by rFasL is not due to the increase of precursor cells and increase in osteoclast formation is greater than cell death by rFasL during osteoclastogenesis. Considering the low level of FasL and resistance to Fas-mediated apoptosis in osteoclasts, it is not surprising that the endogenous FasL does not have a major role in apoptosis (Fig. 4D).

Now it is known that almost all members of the TNFR family are able to activate NF-κB and Fas is drawing new attention as a mediator of inflammation (28, 29). Actually, Fas-mediated induction of inflammatory cytokines has been observed in astrocytes (30), dendritic cells (31), macrophages (21, 32), synoviocytes (33), neutrophils (34), bronchial epithelial cells (35), and vascular smooth muscle cells (36). Furthermore, it was shown that the NF-κB activation is essential for the FasL-induced IL-8 production (22). In response to rFasL, the cells of osteoclast lineage also activated NF-κB and produced inflammatory cytokines IL-1β and TNF-α (Fig. 5). IL-1β and TNF-α have been known as osteolytic factors for a long time, and their molecular mechanisms were recently elucidated (37, 38). In addition, the effect of rFasL was
inhibited completely by blocking both IL-1β and TNF-α (Fig. 6). Therefore, we propose that the up-regulated production of IL-1β and TNF-α with NF-kB activation causes the effect of FasL on osteoclast differentiation.

What determines the response of cells to Fas signaling between death and NF-kB activation is not clear. Both pathways are dependent on Fas-associated death domain protein and caspase-8, but caspase-inactive point mutant activated NF-kB as potent as wild-type caspase-8, suggesting that the two pathways are uncoupled (22, 39). Jurkat cells, which are highly sensitive to FasL-induced apoptosis, showed NF-kB activation when the apoptosis was blocked by general caspase inhibitor Z-VAD-fmk (22). It suggested that delayed or inefficient induction of apoptosis via Fas should be sufficient to allow the activation of NF-kB pathway. In contrast, inhibition of NF-kB activation completely abrogated the resistance of leukemic eosinophils to Fas-mediated killing (40). The cells of osteoclast lineage increased the sensitivity to Fas-mediated killing (Fig. 4, C and D), but reduced the translocation of NF-kB into nucleus (Fig. 5B) along the differentiation.

Interestingly, the noncaspase cytotoxic protein inhibitor z-FA-fmk had a potent inhibitory effect on osteoclastogenesis (Fig. 7A). Cathepsin K is abundant cysteine protease found in osteoclasts, and the inhibition of cathepsin K by antisense oligonucleotides inhibited osteoclast differentiation as well as bone resorption activity (41). Therefore, z-FA-fmk might have blocked RANKL-induced osteoclast differentiation by inhibiting cathepsin K, although other cytotoxic proteases might be involved too.

Recently, Ozeki et al. (42) reported the constitutive expression of FasL, but induced expression of Fas by inflammatory cytokines on MC3T3-E1 cells. If it is true in the primary osteoblasts, FasL on osteoblasts may have an important role in osteoclastic differentiation and apoptosis. Oppositely, FasL and Fas on osteoclasts can affect various functions of osteoblasts. There are two interesting reports regarding the role of Fas in bone homeostasis in vivo. Katavic et al. (43) showed the increased bone mass and decreased osteoclasts in gld mice along the differentiation. Wu et al. (15) reported decreased bone mineral density and insufficient to allow the activation of NF-kB by general caspase inhibitor Z-VAD-fmk (22). It suggested that death of osteoclasts, which was not considered previously.

Our study widens the boundary of thought: FasL/Fas has a role in differentiation as well as death of osteoclasts, which was not considered previously.

Disclosures

The authors have no financial conflict of interest.

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