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The Fas/Fas Ligand System Inhibits Differentiation of Murine Osteoblasts but Has a Limited Role in Osteoblast and Osteoclast Apoptosis

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Apoptosis through Fas/Fas ligand (FasL) is an important regulator of immune system homeostasis but its role in bone homeostasis is elusive. We systematically analyzed: 1) the expression of Fas/FasL during osteoblastogenesis and osteoclastogenesis in vitro, 2) the effect of FasL on apoptosis and osteoblastic/osteoclastic differentiation, and 3) osteoblastogenesis and osteoclastogenesis in mice deficient in Fas or FasL. The expression of Fas increased with osteoblastic differentiation. Addition of FasL weakly increased the proportion of apoptotic cells in both osteoclastogenic and osteogenic cultures. In a CFU assay, FasL decreased the proportion of osteoclast colonies but did not affect the total number of colonies, indicating specific inhibitory effect of Fas/FasL on osteoblastic differentiation. The effect depended on the activation of caspase 8 and was specific, as addition of FasL to osteoblastogenic cultures significantly decreased gene expression for runt-related transcription factor 2 (Runx2) required for osteoblastic differentiation. Bone marrow from mice without functional Fas or FasL had similar osteoclastogenic potential as bone marrow from wild-type mice, but generated more osteoblast colonies ex vivo. These colonies had increased expression of the osteoblast genes Runx2, osteopontin, alkaline phosphatase, bone sialoprotein, osteocalcin, and osteoprotegerin. Our results indicate that Fas/FasL system primarily controls osteoblastic differentiation by inhibiting progenitor differentiation and not by inducing apoptosis. During osteoclastogenesis, the Fas/FasL system may have a limited effect on osteoclast progenitor apoptosis. The study suggests that Fas/FasL system plays a key role in osteoblastic differentiation and provides novel insight into the interactions between the immune system and bone.


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2 Address correspondence and reprint requests to Dr. Nataša Kovac'č, Department of Anatomy, University of Zagreb School of Medicine, Šalata 11, Zagreb, Croatia. E-mail address: natasa@mef.hr

3 Abbreviations used in this paper: FasL, Fas ligand; AP, alkaline phosphatase; mm, recombinant mouse; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TRAP, tartrate-resistant acid phosphatase; qPCR, quantitative PCR; PI, propidium iodide; NT, nick translation; OC, osteocalcin; DAPI, 4',6-diamidino-2-phenylindole; Runx2, runt-related transcription factor 2; BSP, bone sialoprotein; Ct, cycle threshold; OPG, osteoprotegerin; ΔRn, normalized fluorescence reporter signal.

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Materials and Methods

Mice

Twelve-week-old female C57BL/6j mice, mice homozygous for a mutation in the FasL gene (gld) (4), and mice deficient in the Fas gene (Fas −/−) were used in experiments. Both deficient strains were on the C57BL/6j background. The Fas −/− mice were a gift from Dr. M. Simon (Max Planck Institute for Immunobiology, Freiburg, Germany). Mice homozygous for the gld mutation on a C57BL/6j background were originally obtained from Dr. E. R. Podack (Miami University, Miami, FL). All animal protocols were approved by the Ethics Committee of the University of Zagreb, School of Medicine (Zagreb, Croatia).

Cell culture

Bone marrow was flushed out of the medullar cavity of long bones from at least six animals per group and used for cell culture. For osteoblastic differentiation, cells were seeded in 6-well culture plates at a density of 3 × 10^6 cells/well in 3 ml of α-MEM supplemented with 10% FCS (HyClone) (17). Osteoblastic differentiation was induced by the addition of 50 μg/ml ascorbic acid, 10−3 M dexamethasone, and 8 mM β-glycerophosphate (termed osteoblastogenic cultures). Osteoblast colonies were identified histochemically by the activity of alkaline phosphatase (AP), using a commercially available kit (Sigma-Aldrich). For the induction of apoptosis, cells were cultured in 24-well culture plates (Corning-Costar), at a density of 1 × 10^6 cells/well in 0.7 ml α-MEM with 10% FCS/well. For fluorescence microscopy, cells were plated in 4-well chamber slides at a density of 1 × 10^5 cells/well in 0.5 ml culture medium/well. Osteoblast-like markers and purity of cultures were checked on day 14 of osteoblastogenic culture, when AP-positive colonies comprised minimally 75% of total colonies, compared with <20% of AP-positive colonies in cultures without addition of ascorbic acid, dexamethasone, and β-glycerophosphate. The total number of colonies was determined after staining for AP with ethanol and subsequent staining with methylene blue.

Osteoclastic differentiation of bone marrow cells was stimulated by the addition of 10 ng/ml of both recombinant mouse (rm) FasL and 5 μg/ml anti-Fas mAb. FasL treatment induced apoptosis in 50 – 60% of Con A-stimulated Jurkat cells after treatment with anti-6x-histidine mAb only. Furthermore, 1.0 μg/ml rmFasL and 5 μg/ml mitogen Con A (Sigma-Aldrich) for 48 h in RPMI 1640/10% FCS at 37°C with 5% CO2. After that lymphocytes were treated with 0.5 μg/ml rmFasL and 5 μg/ml anti-6x-histidine mAb. FasL treatment induced apoptosis in 50 – 60% of Con A-stimulated T cells, whereas there were <15% of apoptotic cells in cultures treated only with anti-fx-histidine mAb. Furthermore, 1.0 μg/ml rmFasL-induced apoptosis of minimally 65% of Jurkat cells. There were <5% of apoptotic Jurkat cells after treatment with anti-6x-histidine mAb only.

Induction of apoptosis

For the induction of apoptosis, 0.5 μg/ml rmFasL and 5 μg/ml mAb to polyhistidine tag (anti-6x-histidine; R&D Systems) required for FasL cross-linking were added to osteoblastogenic and osteoclastogenic cultures (termed osteoclastogenic cultures). After 6 days of culture, cells with three or more nuclei per cell, stained positively for tartrate-resistant acid phosphatase (TRAP), were considered osteoclasts and counted per well. Osteoclastic phenotype was further confirmed by quantitative PCR (qPCR) for cDNA synthesized from cultured cells using a commercial kit (Sigma-Aldrich). For the analysis of the morphology of cell nuclei stained with DAPI, membrane binding of annexin V, and staining of nuclei by the NT method (19). In osteoclastogenic cultures, only cells with three or more nuclei were analyzed.

Flow cytometry

Cells (1 × 10^6) were suspended in 100 μl of PBS with 0.1% NaN3, and incubated with FITC-conjugated anti-Fas mAb (BD Pharmingen), or a PE-conjugated anti-FasL mAb (BD Pharmingen), as well as appropriate isotype control mAb (Sigma- Aldrich). Cells were analyzed using the CellQuest software (BD Biosciences), 2 × 10^4 events were collected from each sample. Dead and fragmented cells were excluded from the analysis on the basis of their light scatter properties and labeling with propidium iodide (PI). Positively stained populations were delineated using the signal of the isotype control. Annexin V (BD Pharmingen) and PI staining were performed according to the manufacturer’s instructions. Single DNA-strand breaks in apoptotic nuclei were detected using a nick translation (NT) assay (19). The acquired data from 2 × 10^4 events per sample were analyzed using the CellQuest software (BD Biosciences).

Immunofluorescence

For in situ immunofluorescence analysis of Fas or FasL, expression and apoptosis detection, bone marrow cells were cultured in chamber slides. Cells were washed with 0.5 ml of PBS, fixed in 4% formaldehyde in PBS for 15 min, washed again with PBS, and then nonspecific binding was blocked by incubation for 15 min with a 3% solution of BSA in PBS. Cells were then incubated with anti-Fas or anti-FasL mAb diluted 1/100 in 3% BSA in PBS for 30 min at room temperature (RT). After washes with PBS, cell nuclei were stained with 2 μg/ml 4, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 15 min. Apoptosis was detected by examining the morphology of cell nuclei stained with DAPI, membrane binding of annexin V, and staining of nuclei by the NT method (19). In osteoclastogenic cultures, only cells with three or more nuclei were analyzed.

Fas and FasL ELISA

Cultured cells were lysed for 1 h on ice in buffer (300 mM NaCl, 50 mM Tris-Cl, 0.5% Triton-X 100 (pH 7.6)) containing protease inhibitors (Protease Inhibitor Cocktail Tablets; Roche). After centrifugation at 1,500 × g for 15 min at 4°C, the supernatant was collected and centrifuged again for 15 min at 14,000 × g at 4°C. Protein concentration in the samples was determined using a commercial kit (BCA protein assay; Pierce Biotechnologies). The concentration of Fas and FasL proteins was determined using commercial kits (Quantikine, Mouse Fas and Fas Ligand Immunoassays; R&D Systems). Briefly, samples were added to Fas- or FasL-specific mAb-precoated plates and incubated for 2 h at RT on a horizontal orbital microplate shaker, washed five times, and incubated for the next 2 h with HRP-conjugated Fas or FasL-specific Ab. After further washing, the reaction was visualized with tetramethylbenzidine and arrested with hydrochloric acid. OD was determined within 15 min, on a microplate reader (Bio-Rad) set to 450 nm excitation wavelength.

Gene expression analysis

Total RNA was extracted from cultured cells using a commercial kit (TriPure; Roche). For PCR amplification, 2 μg of total RNA was converted to cDNA by reverse transcriptase (Applied Biosystems). The amount of cDNA corresponding to 20 ng of reversely transcribed RNA was amplified by qPCR, using specific amplimer sets designed by Primer Express software (Applied Biosystems) for β-actin (sense, 5′-CATTGTCGACAGGATGCAAA-3′, antisense, 5′-GCTGATCCACTCCTGTGGA-3′), RANK (sense, 5′-GACA CTGGAGGACACCCAAA-3′, antisense, 5′-ACAACGTCCCTTGGAG CT-3′), RANKL (sense, 5′-TGACGACATGGCTGTGTTCC-3′, antisense, 5′-CCCAATGT TTCGTTGACCTTC-3′); M-CSF receptor (c-fms) (sense, 5′-AGTCCACGGCTATGCTGAT-3′, antisense, 5′-TAGTCGGAGAGTCTCC-3′); and osteopontin (OP) (sense, 5′-CAAGCAGAGGGCAATAA GGT-3′, antisense, 5′-AGGCCGCTTCATCAAGGACT-3′), with SYBR Green chemistry (SYBR Green Master Mix; Applied Biosystems). Expression of Fas, Fasl, and runt-related transcription factor 2 (Runx2), AP, osteopontin, bone sialoprotein (BSP), osteoprotegerin (OPG), and calcitonin receptor was analyzed using commercially available TaqMan Assays (two primers and Fam/Mgb-labeled probe; Applied Biosystems) and TaqMan chemistry. Quantitative PCR was conducted using an ABI Prism 7000.
FIGURE 1. Expression of Fas and FasL during osteoblastic and osteoclastic differentiation in vitro. A, Representative histograms of cells from osteoblastogenic and osteoclastogenic cultures, and Con A-stimulated lymph node lymphocytes (T ly), labeled with mAbs to Fas (anti-Fas-FITC) or FasL (anti-FasL-PE). B, Proportion of cells in osteoblastogenic cultures labeled with mAbs to Fas or FasL. Labeled cells were delineated according to the signal from isotype control mAbs. Day 0 represents Fas/FasL expression on freshly isolated bone marrow cells. Statistics: \( p < 0.05 \) (ANOVA and Student-Newman-Keuls post hoc test); *, FasL expression vs days 4, 7, 9, 14, and 17; **, FasL expression vs days 0, 9, 11, and 14; ***, Fas expression vs days 0, 9, 11, 14, and 17. C, Fas/FasL protein concentration in osteoblastogenic cultures, determined by ELISA and normalized to total protein content. Statistics: \( p < 0.05 \) (ANOVA and Student-Newman-Keuls post hoc test); *, FasL expression vs all other time points; **, Fas expression vs days 0, 4, 7, 9, and 11. D, Expression of Fas/Fasl mRNA in osteoblastogenic cultures. Expression was calculated according to the standard curve for Fas/Fasl expression in the calibrator sample (cDNA from osteoblastogenic culture) and normalized to the mRNA quantity for \( \beta\)-actin (“endogenous” control). Statistics: \( p < 0.001 \) (t test); *, Fasl expression vs all other time points; **, Fas expression vs days 0, 9, 11, 14, and 17; ***, Fas expression vs all other time points. E, Proportion of cells in osteoclastogenic cultures labeled with mAb to Fas or FasL. Labeled cells were delineated according to the signal from isotype control mAbs. Day 0 represents Fas/Fasl expression on freshly isolated bone marrow cells. Statistics: \( p < 0.05 \) (ANOVA and Student-Newman-Keuls post hoc test); *, Fas and FasL expression vs all other time points; **, Fas expression vs days 0, 2, and 4. F, Fas/Fasl protein concentration in osteoclastogenic cultures, determined by ELISA and normalized to total protein content. Statistics: \( p < 0.05 \) (ANOVA and Student-Newman-Keuls post-hoc test); *, Fas expression vs all other time points; **, Fas expression vs days 0, 2, 4, 7, and 9; ***, Fas expression vs all other time points.
Sequence Detection System (Applied Biosystems). Each reaction was performed in duplicate or triplicate in a 25-μl reaction volume (8). The generated data were analyzed by plotting the normalized fluorescence receptor signal (ΔRn) vs cycle number. An arbitrary threshold was set on the linear phase midpoint of the log ΔRn vs cycle number plot. The cycle threshold (Ct) value was defined as the cycle number at which ΔRn crossed this threshold. The expression of specific genes was calculated according to the standard curve of gene expression in the calibrator sample (cDNA from osteoclastogenic culture) and normalized to the expression of the gene for β-actin (“endogenous” control) (8).

Data analysis and interpretation

All experiments were repeated three times and the results from a representative experiment are presented. Results were expressed as the mean ± SD of TRAP-positive osteoclast number in 6 wells of a 48-well plate and osteoblast or total colony number in three wells of a 6-well plate. Differences in the number of osteoclasts or osteoblast colonies between B6, Fasl−/−, and gld mice were analyzed by ANOVA or t test.

Results

Moderate expression of Fas and weak expression of FasL during osteoblastic differentiation

Expression of Fas and FasL was first analyzed by flow cytometry (Fig. 1, A and B). Anti-Fas mAb-labeled ~30% of freshly isolated bone marrow cells. During the first few days of osteoblastogenic culture, the proportion of cells expressing Fas decreased below 5%, and then increased to 30% after day 7, remaining unchanged until the end of culture (day 17). Anti-FasL mAb also labeled ~30% of freshly isolated bone marrow cells and after that <5% of cells up to culture day 9. FasL membrane expression peaked on day 11 of osteoblastogenic culture, when it was found on 30% cells, and then decreased with osteoblastic differentiation to <10% of cells. The signal obtained by both anti-Fas and anti-FasL mAbs was weak, suggesting low expression of Fas and FasL per cell (Fig. 1A, two left panels). Weak signal was apparent in comparison with the clear signal obtained on activated T lymphocytes used as a positive control (Fig. 1A, two right panels). Total Fas protein content was low during the early stages of osteoblastic differentiation, and increased in mature cells of osteoblastogenic culture (days 14 and 17, Fig. 1C). Both Fas and FasL were present in freshly isolated bone marrow (day 0), but FasL level decreased after day 0 and remained low until the end of culture (Fig. 1C). The expression of mRNA for Fas and Fasl, by qPCR, was in accordance with protein expression by ELISA and revealed low Fas and Fasl gene expression in osteoblastogenic cultures (Fig. 1D). The difference in the Ct between Fas/Fasl and β-actin was ~15 cycles for Fas and 20 cycles for Fasl, vs days 0, 4, and 9; **, FasL expression vs all other time points. G, Fasl/Fasl mRNA expression in osteoblastogenic cultures. Expression was calculated according to the standard curve for Fasl/Fasl expression in the calibrator sample (cDNA from osteoblastogenic culture) and normalized to the mRNA quantity of β-actin (“endogenous” control). Statistics: p < 0.001 (t test); *, Fasl/Fasl expression vs all other time points; **, Fasl/Fasl expression vs days 0, 4, and 7. H, Representative micrographs of mature osteoclasts on day 6 of culture (original magnification, ×200), labeled with mAbs to Fas or Fasl; PH, phase contrast image of the same field. Arrows indicate osteoclasts. Results represent arithmetic means (±SD) of percentages of mAb-labeled cells from three experiments (B and E); arithmetic means (±SD) of duplicate samples analyzed by ELISA (C and F); or arithmetic means (±SD) of qPCR triplicates prepared from the same sample (D and G).
indicating a 215- and 220-fold lower expression, respectively, compared with the expression of β-actin. Early in osteoblastogenic cultures, the expression of Fas mRNA was low but it increased later in the culture course, whereas expression of Fasl mRNA was continuously low throughout osteoblastic differentiation (Fig. 1D).

Weak expression of Fas and Fasl during osteoclastic differentiation

During osteoclastic differentiation, membrane expression of Fas was continuously weak (Fig. 1A, two middle panels), compared with the signal of corresponding isotype controls and activated T lymphocytes (Fig. 1A, two right panels). Anti-Fas mAb stained 30% of freshly isolated bone marrow cells (day 0) and 20% of cells on culture day 2, whereas anti-Fasl mAb labeled 30% of freshly isolated bone marrow cells and up to 15% of cells from mature osteoclastogenic cultures (Fig. 1E). ELISA revealed varying levels of Fas and low levels of Fasl during the culture (Fig. 1F). Fas and Fasl mRNA expression was also low, ~20-fold and 219-fold lower than that of β-actin and corresponded to the expression levels observed by ELISA (Fig. 1G). Immunofluorescence staining of osteoclastogenic cultures grown in chamber

Table I. Induction of apoptosis by rmFasl during osteoblastic and osteoclastic differentiation in vitro

<table>
<thead>
<tr>
<th>Day of Cell Culture</th>
<th>Treatment</th>
<th>Apoptotic Cells (% DAPI)</th>
<th>Apoptotic Cells/20 Fields (annexin V)</th>
<th>Apoptotic Cells/20 Fields (NT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoblastogenic cultures</td>
<td>7</td>
<td>BSA 3.4 ± 0.5</td>
<td>25 ± 3</td>
<td>9 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-6x-his 3.0 ± 0.8</td>
<td>28 ± 3</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fasl 3.8 ± 1.5</td>
<td>27 ± 4</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>BSA 1.4 ± 0.5</td>
<td>13 ± 3</td>
<td>6 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-6x-his 1.2 ± 0.8</td>
<td>11 ± 3</td>
<td>8 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fasl 2.8 ± 0.8</td>
<td>18 ± 2</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>Osteoclastogenic cultures</td>
<td>3</td>
<td>BSA 4.4 ± 1.8</td>
<td>20 ± 6</td>
<td>16 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-6x-his 3.4 ± 2.8</td>
<td>21 ± 6</td>
<td>15 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fasl 8.8 ± 1.6</td>
<td>25 ± 5</td>
<td>20 ± 3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>BSA 5.6 ± 2.5</td>
<td>9 ± 2</td>
<td>13 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-6x-his 6.1 ± 2.5</td>
<td>10 ± 3</td>
<td>10 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fasl 6.2 ± 1.3</td>
<td>13 ± 3</td>
<td>17 ± 4</td>
</tr>
</tbody>
</table>

*a* Bone marrow cells were cultured in chamber slides and treated overnight with 0.5 μg/ml rmFasl and 5 μg/ml anti-polyhistidine mAb (anti-6x-his) on days 6 and 13 and days 2 and 6, respectively. Control cells were treated with 5 μg/ml anti-6x-his or 0.5 μg/ml BSA. Cell treatment was performed in duplicates, which were counted separately. Apoptosis was detected by examining the morphology of cell nuclei stained with DAPI, membrane binding of annexin V, and staining of nuclei by the NT method. In osteoclastogenic cultures, only cells with three or more nuclei were analyzed.
slides revealed no expression of Fas and FasL on mature osteoclasts but rather on surrounding nonosteoclastic cells (Fig. 1H).

Activation of Fas induces apoptosis of minor proportion of cells from osteoblastogenic and osteoclastogenic cultures

After confirming the presence of Fas in bone cell cultures, we tested the ability of expressed Fas to mediate apoptosis. Spontaneous apoptosis occurred during osteoblastic differentiation in vitro, with ~8% apoptotic cells on day 7 and no increase after the addition of FasL (Fig. 2). On day 14, the proportion of spontaneously apoptotic cells was 20–25%, and the addition of FasL increased this fraction of apoptotic cells by additional 10–15% compared with control cells. The proportion of dead (i.e., necrotic) cells also increased by 5–10% upon addition of FasL (Fig. 2B).

During osteoclastic differentiation in vitro, 5–15% of control cells were labeled with annexin V on flow cytometry (Fig. 3). The proportion of annexin V-labeled cells increased 2- to 3-fold on day 3 of osteoclastogenic culture after treatment with FasL (Fig. 3) while addition of FasL did not have a significant effect on the proportion of apoptotic cells on day 7 of osteoclastogenic culture. These results showed similar pattern when using the NT method, although the increase in the proportion of apoptotic cells on day 3 was less prominent (Fig. 3A).

FIGURE 4. Effect of FasL on osteoblastic differentiation in vitro. Osteoblastogenic cultures were treated with 0.5 μg/ml rmFasL and 5 μg/ml anti-polyhistidine mAb (anti-6x-his) on days 5, 10, and 12. Cells treated only with 5 μg/ml anti-6x-his were used as a negative control. All experiments were repeated three times. A, FasL decreases formation of osteoblast colonies. Upper panels, Osteoblast colonies on day 14 of cell culture, stained for AP (red); lower panels, total colonies stained with methylene blue (blue). Histograms represent the number of osteoblast and total colonies per square centimeter plate surface (mean ± SD, n = 3; *, p < 0.05 vs control group, t test). B, Effect of rmFasL on osteoblastogenesis from wild-type mice (C57BL/6, B6) and Fas knockout mice (Fas<sup>−/−</sup>). Left panels, Osteoblast colonies on day 14 of cell culture, stained for AP (red); right panels, total colonies stained with methylene blue (blue). Histograms represent the number of osteoblast and total colonies per centimeter plate surface (mean ± SD, n = 3; *, p < 0.05 vs control group, t test). C, Caspase 8 inhibitor suppresses the effect of rmFasL on osteoblastogenesis in B6 mice. Thirty minutes before addition of FasL cells were treated with 20 μM caspase 8 inhibitor. Left panels, Osteoblast colonies on day 14 of cell culture, stained for AP (red); right panels, total colonies stained with methylene blue (blue). Histograms represent the number of osteoblast and total colonies per centimeter plate surface (mean ± SD, n = 3; *, p < 0.05 vs control group, t test). D, Expression of Runx2 in osteoblastogenic cultures after treatment with rmFasL. Values were calculated according to the standard curve of gene expression in the calibrator sample (cDNA from osteoblastogenic cultures) and normalized to the expression of the gene for β-actin (“endogenous” control). Results are arithmetic means ± SD of qPCR triplicates prepared from the same sample. Cell treatment was performed minimally in duplicate wells (*, p ≤ 0.05 vs control group, t test).
FIGURE 5. Deficiency of Fas or FasL in vivo enhances osteoblastogenesis. Osteoblastogenic cultures were prepared from bone marrow of wild-type mice (C57BL/6, B6), mice without functional FasL (gld) and Fas knockout mice (Fas−/−). All experiments were repeated three times. A, Osteoblast colonies on day 14 of cell culture, stained for AP (red). Histogram represents the number of osteoblast colonies per centimeter plate surface (mean ± SD, n = 3; ANOVA and the Student-Newman-Keuls post-hoc test; *, p < 0.05 vs B6 and Fas−/− mice; **, vs B6 and gld mice). B, Ratio of apoptotic cells in cultures from B6, gld, or Fas−/− mice normalized to apoptotic cells from B6 mice (mean ± SD of three repeated experiments). The proportion of apoptotic cells was determined by NT where apoptotic cells were labeled with avidin-FITC and Annexin V-FITC binding. For each group, cells were cultured in triplicates pooled for flow cytometry analysis. C, Gene expression pattern during in vitro osteoblastic differentiation. For each time point in each group, cells were cultured in triplicates pooled for RNA isolation. Day 0 represents gene expression in freshly isolated bone marrow cells. Values were calculated
FIGURE 6. Deficiency of Fas in vivo enhances osteoclastogenesis. Osteoclastogenic cultures were prepared from bone marrow of wild-type mice (C57BL/6, B6), mice without functional FasL (gld) and Fas knockout mice (Fas−/−). All experiments were repeated three times. A, Osteoclasts (mean ± SD, n = 6; t test, *p < 0.02 vs B6 mice) on day 6 of cell culture, stained red histochemically for the activity of TRAP. B, Ratio of apoptotic cells in cultures from B6, gld, or Fas−/− mice normalized to apoptotic cells from B6 mice (mean ± SD of three repeated experiments). The proportion of apoptotic cells was determined by NT where apoptotic cells were labeled with avidin-FITC and annexin V-FITC binding. Cells were cultured in quadruplicate for each group and pooled for flow cytometric analysis. C, Gene expression pattern in osteoclastogenic cultures from B6, gld, and Fas−/− mice. For each time point in each group, cells were cultured in quadruplicate and pooled for RNA isolation. Day 0 represents gene expression in freshly isolated bone marrow cells. Values were calculated according to the standard curve of gene expression in the calibrator sample (cDNA from osteoclastogenic cultures) and normalized to the expression of the gene for β-actin ("endogenous" control). Results are arithmetic means ± SD of qPCR triplicates prepared from the same sample. c-fms, M-CSF receptor; CTR, calcitonin receptor.

As osteoblasts and osteoclasts are adherent cells, their removal from the plastic substrate of the culture dish may damage the cell membrane and result in annexin V labeling of damaged nonapoptotic cells. To explore this possibility, we cultured both cell types on chamber slides, labeled them in situ with annexin V or NT, and examined their morphology (20). Using this approach, we found very few spontaneously apoptotic cells. The addition of FasL induced an weak and non significant increase in the number of apoptotic cells on day 14 of osteoclastogenic culture, and at both analyzed time points in osteoclastogenic cultures (Table I).

Treatment with FasL suppresses osteoblastic but not osteoclastic differentiation

In the previous set of experiments, we observed that the addition of FasL-induced apoptosis of only a small population of cells committed to the osteoblast lineage. To estimate whether FasL treatment would affect the final osteoblast number, we treated osteoclastogenic cultures with FasL during their differentiation in vitro, and analyzed the number of osteoblast colonies, visualized by AP staining, and total colonies, visualized by methylene blue staining. FasL treatment significantly reduced the number of osteoblast colonies but not total colonies (Fig. 4A). The reduction in the number of osteoblast colonies was most prominent when FasL was present continuously in the culture (Fig. 4A). Single treatment with FasL on day 10 also reduced the number of osteoblast colonies as well as on day 12, without influencing the number of total colonies (Fig. 4A). FasL treatment was ineffective in osteoclastogenic cultures of bone marrow from Fas gene knockout mice (Fig. 4B), confirming the specificity of the ligand-receptor interaction.

To establish whether the activation of the caspase pathway was involved in the inhibition of osteoblastic differentiation, we tested whether the activity of caspase 8, the first downstream enzyme activated upon ligand binding to Fas, is necessary for transduction of the apoptotic signal (21). FasL treatment was ineffective in decreasing the number of osteoblast colonies when the cells were preincubated with the inhibitor of caspase 8 Z-IETD-FMK before FasL treatment (Fig. 4C), confirming the involvement of the caspase 8 in the effect of FasL on osteoblastic differentiation. FasL treatment, with subsequent activation of caspases, had a direct inhibitory effect on osteoblastic differentiation because it suppressed the expression of Runx2 (Fig. 4D), a transcription factor required for commitment of bone marrow progenitors to the osteoblast lineage (22).

In contrast to its effect on osteoblastic differentiation, FasL had no effect on osteoclast number (377.5 ± 75.0 TRAP-positive osteoclasts in FasL-treated wells vs 403.3 ± 77.8 in control wells; p = 0.29, t test, n = 6 wells/group from a representative of three repeated experiments).

Absence of Fas or FasL stimulates osteoblastic differentiation

To confirm that Fas/FasL system is directly involved in the regulation of osteoblastic differentiation, we cultured bone marrow from mice with a gene knockout for Fas (Fas−/−) (6) or with spontaneous loss-of-function mutation in Fasl gene (gld mice) (4). Bone marrow from both Fas−/− and gld mice had greater osteoblastogenic potential than bone marrow from B6 control mice, with Fas−/− bone marrow forming significantly more osteoblast colonies than gld bone marrow (Fig. 5A). At the same time, there

according to the standard curve of gene expression in the calibrator sample (cDNA from osteoblastogenic culture) and normalized to the expression of the gene for β-actin ("endogenous" control). Results are arithmetic means ± SD of qPCR triplicates prepared from the same sample. Statistics: p < 0.001 (t test); *, vs B6 and gld mice; **, vs Fas−/− and gld mice; ***, vs B6 mice.
was no difference among B6, gld, or Fas\(^{-/-}\) mice in the proportion of apoptotic cells in osteoblastogenic cultures, detected by either the annexin V labeling or NT method (Fig. 5B). The expression of several gene markers for osteoblastic differentiation was significantly increased in Fas\(^{-/-}\) bone marrow cultures compared with control B6 cultures (Fig. 5C). The expression of Runx2, an early marker of osteoblast lineage commitment (23) was higher in osteoblastogenic cultures from Fas\(^{-/-}\) bone marrow than in osteoblastogenic cultures from gld or B6 bone marrow. The same was true for other osteoprogenitor markers, AP and BSP (24, 25). The expression of OC, marker of mature osteoblasts (26), was highest in Fas\(^{-/-}\) mature osteoblastogenic cultures. We also tested the expression of OPG, a soluble decoy receptor for RANKL produced by osteoblasts (27), and increased in bone tissue of gld mice as a part of their bone phenotype (8). OPG expression was higher and occurred earlier in osteoblastogenic cultures from Fas\(^{-/-}\) and gld mice than in B6 mice. The expression of RANKL (27) was slightly but not significantly lower in immature osteoblastogenic cultures from Fas\(^{-/-}\) or gld mice, compared with B6 mice, but unchanged in mature osteoblastogenic cultures.

**Absence of Fas or Fasl does not influence osteoclastic differentiation but increases osteoclast number**

Bone marrow cultures from Fas\(^{-/-}\) mice, but not gld mice, formed more TRAP-positive osteoclasts than those from control B6 mice, indicating an inhibitory effect of Fas on the proliferation of osteoclast precursors (Fig. 6A). There were no differences in the number of apoptotic cells on days 3 and 7 of osteoclastogenic cultures from gld or Fas\(^{-/-}\) mice, in comparison with wild-type mice. Only on day 7, annexin V labeling revealed a small nonsignificant reduction in the proportion of apoptotic cells in osteoclastogenic cultures from Fas\(^{-/-}\) mice (Fig. 6B). The expression of genes related to the osteoclastic differentiation was similar in all three groups of mice (Fig. 6C), indicating that the Fasl/FasL system does not have an effect on osteoclastic differentiation.

**Discussion**

The results of the present study demonstrated a novel nonapoptotic action of Fasl/Fasl system in bone. Activation of Fasl by its ligand inhibited commitment and differentiation of osteoblasts from bone marrow, without affecting the differentiation of osteoclasts from the same source of bone marrow. The inhibitory effect on osteoblastogenesis was demonstrated by the inhibition of differentiation after addition of Fasl to osteoblastogenic cultures in vitro and by increased osteoblastic differentiation in bone marrow from mice with a gene knockout for Fas or loss-of-function mutation of Fasl.

At the same time, apoptosis by Fasl/Fasl was not a dominant apoptotic mechanism for either osteoblasts or osteoclasts generated from normal bone marrow, as only approximately a third of cells from mature osteoblastogenic cultures and 10% of cells from mature osteoclastogenic cultures showed constitutive expression of Fasl and weakly expressed Fasl. Absence of this inhibitory effect on osteoblastic differentiation and, to a lesser extent, lack of apoptosis-inducing activity is thus at least partly responsible for the increased bone mass observed in the Fasl mutation phenotype of gld, mice (8), and Fas\(^{-/-}\) mice (our unpublished results).

Detailed analysis of mediators of bone cell apoptosis is possible only in vitro because the apoptotic removal of osteoblasts and osteoclasts in vivo is a very rapid process and cannot be monitored well in intact bones (2). Weak expression of Fas on \(\sim 30\%\) of cells from freshly isolated bone marrow could be assigned to the cells of the erythroid, myelomonocytic or lymphoid lineages, which are known to express Fas (28, 29). In our culture conditions, according to the flow cytometry data, Fas was weakly expressed on 20–30% cells from the mature osteoblastogenic cultures, with little or no expression of Fasl during early stages of osteoblastic differentiation. Because Fas protein content was low in osteoblastogenic cultures and corresponded to the low expression pattern of Fas mRNA, a considerable percentage of cells that expressed Fas by flow cytometry may be explained by accumulation of Fas in a limited cell population. In addition, the intensity of fluorescence detected by flow cytometry within this population was low, indicating a weak expression of Fas per cell. Moderate membrane expression of Fas on osteoblasts has been reported in other human and mouse in vitro models (Refs. 11, 12, 30, and the Genome Anatomy Project for the Osteoprogenitor Lineage: http://skeletalbiology.uchc.edu/30_ResearchProgram/304_gap/index.htm).

Low very constitutive expression of the Fasl gene in osteoblastogenic cultures from normal mouse bone marrow in our study is supported by similar results from gene chip analysis of primary mouse osteoblasts (Genome Anatomy Project for the Osteoprogenitor Lineage: http://skeletalbiology.uchc.edu/ 30_ResearchProgram/304_gap/index.htm and Ref. 30). Despite very low mRNA expression compared with the ubiquitously expressed \(\beta\)-actin (Ct values between 18 and 20 cycles greater than for \(\beta\)-actin, depending on the timepoint), as well as low Fasl protein content, we found Fasl on the membrane of 10–30% cells from osteoblastogenic cultures. This finding may again be explained by accumulation of Fasl within a small cell population.

**Since the addition of Fasl induced apoptosis in a small proportion (<15%) of cells from mature osteoblastogenic cultures, apoptosis through the Fasl/Fasl system seems to be only partially responsible for the apoptotic removal of mature osteoblasts (2). Furthermore, our data showed that all cells in osteoblastogenic cultures were not equally sensitive to apoptosis. Even when they expressed Fasl (~30% cells on osteoblastogenic culture day 14), the addition of Fasl at the same time point was able to induce apoptosis in only 15% of cells or less. Although cells in osteoblastogenic cultures were relatively resistant to Fasl-induced apoptosis, cultures treated with Fasl had a significantly lower osteoblastic differentiation in vitro. This effect was not the result of decreased cell proliferation, as the number of total colonies in Fasl-treated cultures was similar to that in control cultures. Lower osteoblastogenesis in vitro may rather be a result of specific inhibition of differentiation, as shown by the decrease in the number of differentiated osteoblast colonies and down-regulation of Runx2, a transcription factor necessary for the commitment of mesenchymal stem cells to the osteoblast lineage (22). The inhibitory effect of Fasl on osteoblastogenesis was specific for the Fasl/Fasl interaction, because it was absent in osteoblastogenic cultures from mice without functional Fas. This finding is important because it confirmed that Fas is crucial for the Fasl regulation of osteoblastic differentiation.**

**Involvement of Fas in the cellular activation has been previously shown on human HEK293 cells (31) and those processes were initiated by caspases, which are primary effectors of apoptosis. Caspase 8 has been shown to be required for normal function of myeloid and B cell precursors in bone marrow and for maturation of macrophages in the presence of M-CSF (32). Our results indicate that activation of caspase 8 may also be involved in the transmission of the inhibitory Fasl/Fasl effect on osteoblastic differentiation, as the inhibition of caspase 8 activity abrogated the effect of Fasl on osteoblastogenesis.**

**Alteration in the differentiation of cells of the osteoblast lineage was less prominent in mice deficient in Fasl than in those with Fas deficiency. Fasl-deficient mice used in our experiments were produced by a gene knockout for the Fas gene and had full penetration**
of the phenotype (6), whereas FasL-deficient mice are spontaneous gld mutants (4). Incomplete penetration of a spontaneous mutation has been described for the lpr mouse strain which carries a spontaneous mutation for Fas (33). There is no specific study confirming the completeness of penetration of the gld mutation, but more severe autoimmune syndrome in Fasl knockout mice than in gld mice (7) favors incomplete penetration of the spontaneous mutation. A recent systematic study on the interactions between TNF and TNFR superfamily members could not identify ligands for Fas other than FasL (34). However, Balkow et al. (35) observed the presence of apoptotic cells in the liver of lymphocytic choriomeningitis virus-infected gld mice, and suggested the possibility of an undiscovered ligand that may trigger Fas-mediated apoptosis. This may be an alternative explanation for the smaller effect on osteoblastogenesis and unaltered osteoclast numbers observed in gld mice in our study.

Constitutive expression and activity of the Fas/FasL system on cells in osteoclastogenic cultures was limited in our experimental model, contrasting the reports from some research groups, such as Wu et al. (14), who described a strong expression of Fas on mouse osteoclasts. However, their culture conditions included 5-fold higher doses of RANKL for the stimulation of osteoclastogenesis than in our culture system. RANKL is necessary for osteoclastic differentiation in culture, but higher doses may increase Fas expression on osteoclast progenitors (36). Our findings are similar to that of Park et al. (15), who described weak expression of both Fas and FasL on osteoclasts, and of Ogawa et al. (16), who could not detect Fas on mature mouse osteoclasts. Similarly to Ogawa, who detected Fas only on the surrounding lymphocytes in culture, anti-Fas mAb-labeled cells in osteoclastogenic cultures in our study were small supportive stromal-like cells, and not osteoclasts. However, addition of FasL to cell cultures in our study did not affect the number of osteoclasts, which contrasts with the finding of enhanced osteoclastogenesis after Fasl treatment reported by Park et al. (15) and ascribed to the stimulation of IL-β and TNF-α production (15). Such differences may be explained by the higher amount of RANKL, which, in turn may increase production of IL-1 (37). We believe that our in vitro cultures are more likely to resemble physiological in vivo conditions, in which cytokines are effective in picomolar concentrations. Despite the fact that FasL treatment did not affect osteoclastogenesis in vitro, TRAP-positive osteoclastic cell number was increased in Fas-deficient mice, and the proportion of apoptotic cells in osteoclastogenic cultures from Fas- or FasL-deficient mice was only weakly decreased. Because osteoclast progenitors comprise a minor proportion of hematopoietic bone marrow cells, the effect of single dose of FasL in vitro may not be noticeable. Another explanation may lie in the fact that osteoclast progenitors are susceptible to FasL-induced apoptosis only at a certain stage of development (possibly at the stage of GM-CFU before exposure to RANKL) and those cells could not be investigated in our culture conditions. This is supported by the finding that the number of apoptotic cells increased after the addition of FasL early to the osteoclastogenic culture. However, prolonged deficiency of FasL in vivo may cause accumulation of osteoclast progenitors in bone marrow and subsequent increase in osteoclastogenesis ex vivo. Taken together, our results favor the explanation that Fas/FasL has a limited role in the apoptosis of osteoclast progenitors, while having no direct effect on osteoclastic differentiation.

In conclusion, the Fas/FasL system, although an important regulator of the immune system, is only partially involved in the apoptotic death of bone cells or their progenitors. Rather, it has a direct and specific regulatory effect on osteoblastic differentiation. This finding provides an important contribution to the understand-
to identify subpopulations of cells at different stages of the osteoblast lineage. J. Bone Miner. Res. 17: 15–25.


