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Fas/Fas ligand system inhibits differentiation of murine osteoblasts but has a limited role in osteoblast and osteoclast apoptosis[1]

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Abstract

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: a) the expression of Fas/FasL during osteoblastogenesis and osteoclastogenesis *in vitro*, b) the effect of FasL on apoptosis and osteoblastic/osteoclastic differentiation, and c) 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 osteoblastogenic cultures. In a colony forming unit assay, FasL decreased the proportion of osteoblast 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, 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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Keywords

Apoptosis; Cell Differentiation; Monocytes/Macrophages; Stromal Cells; Transgenic/Knockout Mice

Introduction

The immune system and bone are anatomically and functionally closely related, sharing common progenitor cells and various cytokine networks (1). Furthermore, development and function of both systems is regulated by apoptosis. Apoptosis is an important regulator of the immune reaction and development of hematopoietic cells, and also determines the lifespan and number of bone cells. All osteoclasts and 60–80% of osteoblasts eventually die by apoptosis (2). One of major apoptotic mediators in the immune system is the cell surface receptor Fas (CD95). Binding of Fas ligand (FasL[3], CD95L, CD178) to Fas leads to the recruitment and activation of initiator caspases, such as caspase 8, which start a series of intracellular events culminating in cell death (3). The importance of the Fas/FasL system is reflected in mice lacking functional Fas or FasL who develop a systemic autoimmune disease marked by lymphadenopathy and splenomegaly, production of auto antibodies and formation of immunocomplexes, lymphoproliferation, and a shortened life span (4-7). We have previously shown that a constitutive component of the phenotype of *gld* mice, which lack functional FasL, is increased bone mass *in vivo* and increased number of osteoblasts *in vitro* (8). *Gld* mice also form more bone and cartilage during endochondral bone formation *in vivo* (9, 10). Although these studies point to the importance of the Fas/FasL system in the

[3]Abbreviations used in this text:

AP	alkaline phosphatase
BSP	bone sialoprotein
Ct	cycle threshold
DAPI	4′,6-diamidine-2′-phenylindol dihydrochloride
FasL	Fas ligand
NT	nick-translation
qPCR	quantitative PCR
OC	osteocalcin
OPG	osteoprotegerin
OPN	osteopontin
RANK	receptor activator of NF-κB
RANKL	RANK ligand
rm	recombinant mouse
Runx2	runt-related transcription factor 2
TRAP	tartrate-resistant acid phosphatase

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control of bone homeostasis, cellular and molecular mechanisms underlying the bone phenotype in *gld* have not been clarified. As apoptosis of bone cells is a rapid process and cannot be assessed *in vivo* during bone turnover in a normal adult organism (2), expression and function of the Fas/FasL system on bone cells has been investigated *in vitro*, with controversial results from different experimental models and methods. Several studies reported constitutive or inducible expression of Fas and FasL on human and mouse osteoblasts (11-13). The studies on the expression of Fas and FasL ligand on osteoclasts reported both positive (14, 15) and negative (16) findings. To elucidate cellular and molecular mechanisms underlying increased bone formation *in vivo* in the absence of active Fas, we systematically investigated the expression and activity of Fas and FasL during *in vitro* osteoblastogenesis and osteoclastogenesis from wild-type murine bone marrow cells, as well as bone marrow cells from mice with a gene knockout for Fas (6) or loss-of-function FasL mutation in *gld* mice (4). We report here that, although Fas is expressed on cells from mature osteoblastogenic and osteoclastogenic cultures, it plays a limited role in the regulation of their apoptosis, but has a novel inhibitory effect on osteoblastic differentiation.

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 kind gift from Prof. Dr. Markus Simon (Max Planck Institute for Immunobiology, Freiburg, Germany). Mice homozygous for the *gld* mutation on a C57BL/6 background were originally obtained from Prof. Dr. Eckhard R. Podack (Miami University, Miami, FL). All animal protocols were approved by the Ethics Committee of the Zagreb University School of Medicine, Croatia.

Cell culture

Bone marrow was flushed out of the medullar cavity of long bones from at least 6 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 per well in 3 mL of α-MEM supplemented with 10% FCS (HyClone, Logan, UT) (17). Osteoblastic differentiation was induced by the addition of 50 μg/mL ascorbic acid, 10^{-8} 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 Corp., Milwaukee, MI). For the induction of apoptosis, cells were cultured in 24-well culture plates (Corning-Costar Corp., Acton, MA), at a density of 1×10^6 cells per well in 0.7 mL α-MEM with 10% FCS per well. For fluorescence microscopy, cells were plated in 4-well chamber slides at a density of 1×10^6 cells in 0.7 mL culture medium per well. Osteoblast characteristics and purity of cultures were checked on day 14 of osteoblastogenic culture, when AP-positive colonies comprised minimally 75% of total colonies, compared with less then 20% of AP-positive colonies in cultures without addition of ascorbic acid, dexamethasone and β-glycerophosphate. The total number of colonies was determined after destaining 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) receptor activator of NF-κB ligand (RANKL) (gift from Amgen, Thousand Oaks, CA) and rmM-CSF (R&D systems, Minneapolis, MN) to the 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 calcitonin receptor gene expression as a marker of mature osteoclasts (18). Labeling with mAb to receptor activator of NF-κB (RANK) was used to confirm the presence of osteoclast progenitors on day 2 of osteoclastogenic culture. At this time point, there were minimally 25% RANK-positive cells, indicating at least 25% osteoclast progenitors. Osteoclasts did not differentiate in cultures without the addition of RANKL and M-CSF, and there were less than 5% of RANK-positive cells on culture day 2, as well as no calcitonin receptor mRNA. For TRAP staining, cells were cultured in 48-well culture plates (1×10^6 cells in 1 mL α-MEM with 10% FCS per well). For RNA isolation, flow cytometry for Fas and FasL, and induction of apoptosis, cells were cultured in 24-well culture plates (2×10^6 cells in 1 mL MEM with 10% FCS per well). For the morphologic estimation of apoptotic cells and immunofluorescence staining of Fas and FasL, cells were cultured in 4-well chamber slides, at a density of 2×10^6 cells in 1 mL culture medium per well.

Induction of apoptosis

For the induction of apoptosis, 0.5 μg/mL of 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 on days 6 and 13, and days 2 and 6, respectively, and incubated for 16 h at 37°C. Cells treated with 5 μg/mL mAb to polyhistidine tag or 0.5 μg/mL BSA were used as negative controls. For a positive control, lymph node lymphocytes were stimulated with 2 μg/mL mitogen Con A (Sigma) for 48 h in RPMI/10% FCS at 37°C with 5% CO_2. After that lymphocytes were treated with 0.5 μg/mL of 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 less the 15% of apoptotic cells in cultures treated only with anti-6x-histidine mAb. Furthermore 1.0 μg/mL of rmFasL induced apoptosis of minimally 65% of Jurkat cells. There were less than 5% of apoptotic Jurkat cells after treatment with anti-6x-histidine mAb only.

For inhibition of caspase 8 activity, cells were preincubated with 20 μM of the caspase 8 inhibitor Z-IETD-FMK (BD Pharmingen, San Diego, USA) for 30 min at 37°C, and then treated with FasL.

Flow cytometry

Cells (1×10^6) were suspended in 100 μL PBS with 0.1% NaN_3, 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 (Syrian hamster IgG 2λ, conjugated with FITC or PE, BD Pharmingen) for 30 minutes on ice in the dark. To obtain a positive control for anti-Fas and anti-FasL mAb labeling, lymph node lymphocytes were stimulated with 2 μg/mL mitogen Con A (Sigma) for 48 h in RPMI/10% FCS at 37°C with 5% CO_2 and analyzed by flow cytometry. Expression of RANK in osteoclast cultures was analyzed by staining with a goat-anti-RANK Ab (R&D Systems) and detecting with a PE-anti-goat Ab. Data for the expression of cell markers were collected on a FACSCalibur flow cytometer (Beckton Dickinson, San Jose, CA) and analyzed using the CellQuest software (Beckton Dickinson), 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 (Beckton Dickinson).

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 PBS, fixed in 4% formaldehyde in PBS for 15 minutes, washed again with PBS, and then non specific binding was blocked by incubation for 15 minutes 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 minutes at room temperature (RT). After three washes with PBS, cell nuclei were stained with 2 μg/mL of 4′,6-diamidine-2′-phenylindol dihydrochloride (DAPI, Sigma) for 15 minutes. Apoptosis was detected by examining the morphology of cell nuclei stained with DAPI, membrane binding of annexin V, and staining of nuclei by the nick translation (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 hour on ice in buffer (300 mM NaCl, 50 mM Tris-Cl, 0.5% Triton-X100, pH 7.6,) containing protease inhibitors (Protease Inhibitor Cocktail Tablets, Roche Diagnostic Corp., Manheim, Germany). After centrifugation at 1500 g for 15 minutes at 4°C, the supernatant was collected and centrifuged again for 15 minutes at 14000 g at 4°C. Protein concentration in the samples was determined using a commercial kit (BCA protein assay, Pierce Biotechnology, Rockford, IL). The concentration of Fas and FasL proteins in cell lysates 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 hours at RT on a horizontal orbital microplate shaker, washed five times and incubated for the next 2 hours with horseradish peroxidase conjugated Fas or FasL specific Ab. After further washing the reaction was visualized with tetramethylbenzidine, and arrested with hydrochloric acid. Optical density was determined within 15 minutes, on a microplate reader (BioRad, Hercules, CA) set to 450 nm excitation wavelength.

Gene expression analysis

Total RNA was extracted from cultured cells using a commercial kit (TriPure; Roche, Basel Switzerland). For PCR amplification, 2 μg of total RNA was converted to cDNA by reverse transcriptase (Applied Biosystems, Foster City, CA). 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′CATTGCTGACAGGATGCAGAA3′, antisense 5′GCTGATCCACATCTGCTGGA3′), RANK (sense 5′GACACTGAGGAGACCACCCAA3′, antisense 5′ACAACGGTCCCCTGAGGACT3′), RANKL (sense 5′TGCAGCATCGCTCTGTTCC3′, antisense 5′CCCACAATGTGTTGCAGTTCC3′), c-fms (sense 5′AGTCCACGGCTCATGCTGAT3′, antisense 5′TAGCTGGAGTCTCCCTCGGA3′), and osteocalcin (OC) (sense 5′CAAGCAGGAGGGCAATAAGGT3′, antisense 5′AGGCGGTCTTCAAGCCATACT3′), with SYBR Green chemistry (SYBR Green Master Mix, Applied Biosystems). Expression of Fas, FasL, runt-related transcription factor 2 (Runx2), AP, osteopontin (OPN), 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 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 fluorescence signal ΔRn vs. the 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 osteoblastogenic or 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 3 wells of a 6-well plate. Differences in the number of osteoclasts or osteoblast colonies between B6, Fas –/– 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 (Figures 1A and B). Anti-Fas mAb labeled approximately 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 approximately 30% of freshly isolated bone marrow cells and after that less than 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 less than 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 (Figure 1A, two left panels). Weak signal was apparent in comparison with the clear signal obtained on activated T lymphocytes used as a positive control (Figure 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, Figure 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 (Figure 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 (Figure 1D). The difference in the Ct between Fas/FasL and β-actin was about 15 cycles for Fas and 20 cycles for FasL, indicating a 2^{15}- and 20^{20}-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 (Figure 1D).

Weak expression of Fas and FasL during osteoclastic differentiation

During osteoclastic differentiation, membrane expression of Fas was continuously weak (Figure 1A, two middle panels), compared with the signal of corresponding isotype controls and activated T lymphocytes (Figure 1A, two right panels). Anti-Fas mAb stained 30% of freshly isolated bone marrow cells (day 0) and about 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 (Figure 1E). ELISA revealed varying levels of Fas and low levels of FasL during the culture (Figure 1F). Fas and FasL mRNA expression was also low, about 2^{20}- and 2^{19}-fold lower than that of β-actin and corresponded to the expression levels observed by ELISA (Figure 1G). Immunofluorescence staining of osteoclastogenic cultures grown in chamber slides revealed no expression of Fas and FasL on mature osteoclasts but rather on surrounding non-osteoclastic cells (Figure 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 receptor to mediate apoptosis. Spontaneous apoptosis occurred during osteoblastic differentiation *in vitro*, with approximately 8% apoptotic cells on day 7 and no increase after the addition of FasL (Figures 2A and B). 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 (Figure 2B).

During osteoclastic differentiation *in vitro*, 5-15% of control cells were labeled with annexin V on flow cytometry (Figures 3A and B). The proportion of annexin V labeled cells increased 2- to 3-fold on day 3 of osteoclastogenic culture after treatment with FasL (Figures 3A and B) 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 (Figure 3A).

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 non-apoptotic 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 osteoblastogenic culture, and at both analyzed time points in osteoclastogenic cultures (Table 1).

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 osteoblastogenic 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 (Figure 4A). The reduction in the number of osteoblast colonies was most prominent when FasL was present continuously in the culture (Figure 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 (Figure 4A). FasL treatment was ineffective in osteoblastogenic cultures of bone marrow from Fas gene knockout mice (Figure 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 (Figure 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 (Figure 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 per group from a representative of 3 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 (Figure 5A). At the same time, there 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 (Figure 5B). The expression of several gene markers for osteoblastic differentiation was significantly increased in Fas –/– bone marrow cultures compared with control B6 cultures (Figure 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 (Figure 6A). There were no differences in the number of apoptotic cells on day 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 non-significant reduction in the proportion of apoptotic cells in osteoclastogenic cultures from Fas –/– mice (Figure 6B). The expression of genes related to the osteoclastic differentiation was similar in all three groups of mice (Figure 6C), indicating that the Fas/FasL system does not have an effect on osteoclastic differentiation.

Discussion

The results of the present study demonstrated a novel non-apoptotic action of Fas/FasL system in bone. Activation of Fas 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 Fas/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 Fas 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 approximately 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 Fas during early stages of osteoblastic differentiation. Since Fas protein content was low in osteoblastogenic cultures, and corresponded to the low expression pattern of Fas mRNA, 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 vitr*o models (11, 12, 30, 31).

Very low 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 (30, 31). Despite very low mRNA expression compared with the ubiquitously expressed β-actin (Ct values between 18 and 20 cycles smaller than for β actin, depending on the time point), 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 Fas/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 Fas (about 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 Fas-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 Fas/FasL interaction, because it was absent in osteoblastogenic cultures from mice without functional Fas. This finding is important because it confirmed that Fas receptor is crucial for the FasL regulation of osteoblastic differentiation.

Involvement of Fas in the cellular activation has been previously shown on human HEK293 cells (32) 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 (33). Our results indicate that activation of caspase 8 may also be involved in the

transmission of the inhibitory Fas/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. Fas 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 (34). 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 TNF receptor superfamily members could not identify ligands for Fas other than FasL (35). However, Balkow and co-workers observed the presence of apoptotic cells in the liver of LCMV-infected *gld* mice, and suggested the possibility of an undiscovered ligand that may trigger Fas mediated apoptosis (36). 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 five 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 (37). 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 and ascribed to the stimulation of IL-1β 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 (38). 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. Since 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 prior to 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 understanding of the functional interactions

between anatomically adjacent hematopoietic and bone/stromal compartments in the bone marrow. FasL expressed on differentiating hematopoietic cells may directly regulate osteoblastic differentiation, which in turn constitutes an important part of the hematopoietic stem cell niche (39). During inflammatory conditions, a decrease in osteoblastic differentiation and subsequent bone loss may be caused by increased Fas/FasL activity in the immune system, where the immune reaction is restricted by Fas/FasL mediated apoptosis primarily in cytotoxic T lymphocytes. Direct regulatory effect on osteoblastic differentiation may also be a potential target for therapeutic modulation of the Fas/FasL system. In the approaches aimed at stimulation of Fas/FasL induced apoptosis, such as in anti-tumor therapy (40), the inhibitory effect of this system on osteoblastic differentiation must be addressed. In contrast, therapeutic strategies to reduce Fas/FasL induced apoptosis, such as in autoimmunity (41), have an important protective effect on bone.

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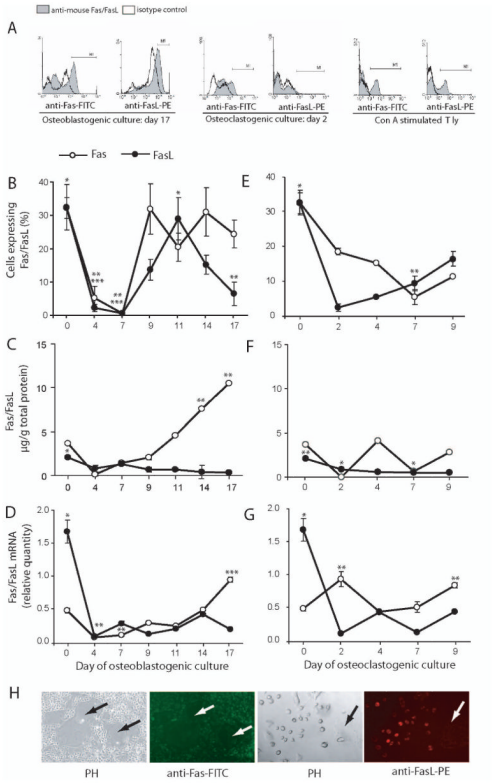

Figure 1. Expression of Fas and FasL during osteoblastic and osteoclastic differentiation *in vitro*

(A) Representative histograms of cells from osteoblastogenic and osteoclastogenic cultures, and Con A stimulated lymph node lymphocytes, 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 β-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. days 0, 4, and 9; ** FasL expression vs. all other time points. **(G)** Fas/FasL mRNA expression in osteoclastogenic cultures. Expression was calculated according to the standard curve for Fas/FasL expression in the calibrator sample

(cDNA from osteoclastogenic culture) and normalized to the mRNA quantity of β-actin ("endogenous" control). Statistics: $p<0.001$ (t-test); * FasL expression vs. all other time points; ** Fas expression vs. days 0, 4, and 7. **(H)** Representative micrographs of mature osteoclasts on day 6 of culture (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, E); arithmetic means (±SD) of duplicate samples analyzed by ELISA (C, F); or arithmetic means (±SD) of qPCR triplicates prepared from the same sample (D, G).

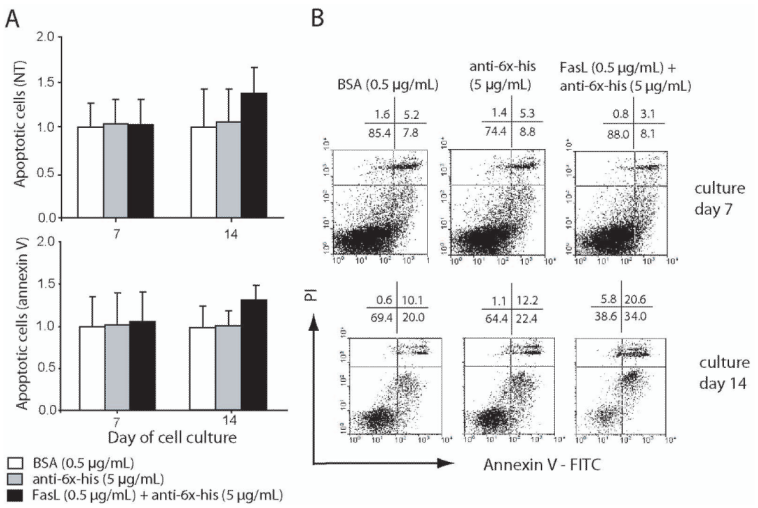

Figure 2. Proportion of apoptotic cells after addition of rmFasL during osteoblastic differentiation *in vitro*

Osteoblastogenic cultures were treated with 0.5 μg/mL of rmFasL and 5 μg/mL anti-polyhistidine mAb (anti-6x-his) on day 6 or day 13 after seeding, for 16 hours. Control cells were treated with 5 μg/mL anti-6x-his or 0.5 μg/mL BSA. The proportion of apoptotic cells was determined by annexin V-FITC combined with propidium iodide (PI) labeling, or the nick translation (NT) method where apoptotic cells were labeled with avidin-FITC. Cell treatment was performed in triplicate and pooled for flow cytometric analysis. **(A)** The ratio of apoptotic cells in cultures treated with BSA, anti-6x-his, or FasL, normalized to cultures treated with BSA (mean±SD of three repeated experiments). **(B)** Representative data of the annexin V-FITC labeling of apoptotic cells. Numbers represent percentages of cells in each quadrant, and apoptotic cells are in the lower right quadrant.

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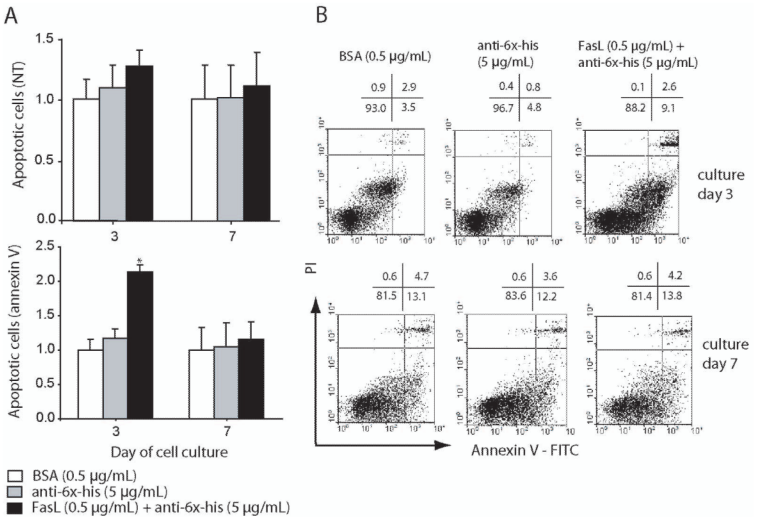

Figure 3. Proportion of apoptotic cells induced by rmFasL during osteoclastic differentiation *in vitro*

Osteoclastogenic cultures were treated with 0.5 μg/mL of rmFasL and 5 μg/mL anti-polyhistidine mAb (anti-6-x-his) on days 2 or 6 after seeding, for 16 hours. Control cells were treated with 5 μg/mL anti-6x-his or 0.5 μg/mL BSA. The proportion of apoptotic cells was determined by annexin V-FITC binding combined with propidium iodide (PI) staining, or the nick translation (NT) method where apoptotic cells were labeled with avidin-FITC. Cell treatment was performed in four wells and pooled for flow cytometric analysis. **(A)** The ratio of apoptotic cells in cultures treated with BSA, anti-6x-his, or FasL normalized to cultures treated with BSA (mean±SD of three repeated experiments; * $p<0.05$ vs. control groups, ANOVA and Student-Newman-Keuls post hoc test) **(B)** Representative data of the annexin V labeling of apoptotic cells. Numbers represent percentages of cells in each quadrant, and apoptotic cells are in the lower right quadrant.

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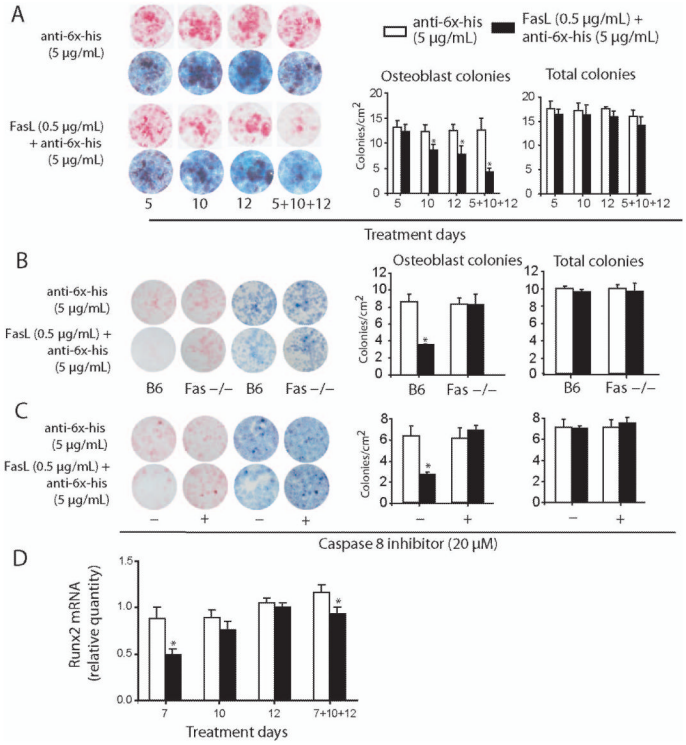

Figure 4. Effect of FasL on osteoblastic differentiation *in vitro*

Osteoblastogenic cultures were treated with 0.5 μg/mL of rmFasL and 5 μg/mL anti-polyhistidine mAb (anti-6-x-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 –/–). 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 of 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 reaction triplicates prepared from the same sample. Cell treatment was performed minimally in duplicate wells. (* $p\leq 0.05$ vs. control group, t-test)

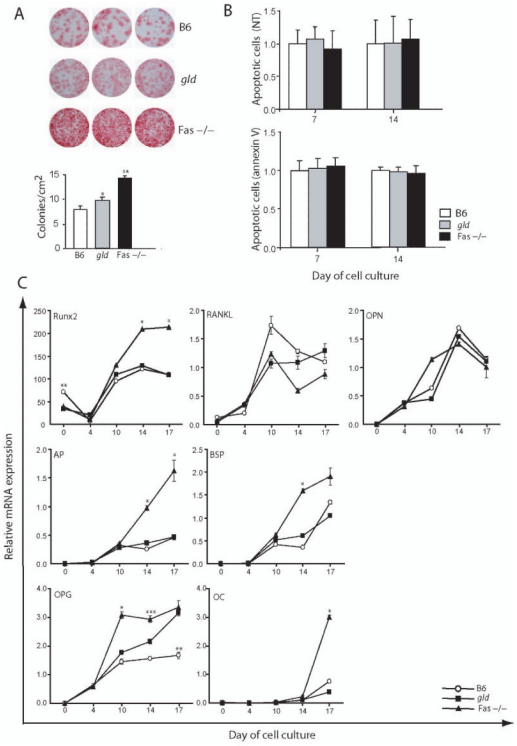

Figure 5. Deficiency of Fas or FasL *in vivo* enhances osteoblastogenesis

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 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 nick translation (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 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 reaction 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.

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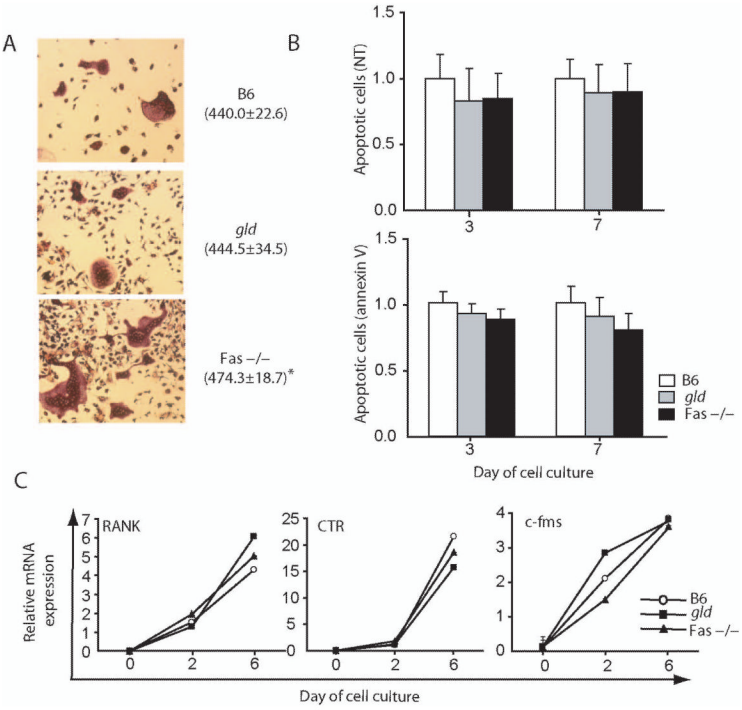

Figure 6. Deficiency of Fas *in vivo* enhances osteoclastogenesis

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 nick translation (NT) where apoptotic cells were labeled with avidin-FITC, and annexin V-FITC binding. Cells were cultured in quadriplicates 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 quadriplicates 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 reaction triplicates prepared from the same sample. c-fms, M-CSF receptor; CTR, calcitonin receptor.

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Table 1

Induction of apoptosis by rmFasL during osteoblastic and osteoclastic differentiation *in vitro*[*]

Day of cell culture	Treatment	Apoptotic cells (%, DAPI)	Apoptotic cells /20 fields (annexin V)	Apoptotic cells /20 fields (NT)
OSTEOBLASTOGENIC CULTURES				
7	BSA	3.4±0.5	25±3	9±3
	anti-6x-his	3.0±0.8	28±3	10±2
	FasL	3.8±1.5	27±4	10±3
14	BSA	1.4±0.5	13±3	6±2
	anti-6x-his	1.2±0.8	11±3	8±3
	FasL	2.8±0.8	18±2	11±5
OSTEOCLASTOGENIC CULTURES				
3	BSA	4.4±1.8	20±6	16±5
	anti-6x-his	3.4±2.8	21±6	15±6
	FasL	8.8±1.6	25±5	20±3
7	BSA	5.6±2.5	9±2	13±2
	anti-6x-his	6.1±2.5	10±3	10±4
	FasL	6.2±1.3	13±3	17±4

[*]Bone marrow cells were cultured in chamber slides and treated overnight with 0.5 μg/mL of 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 nick translation (NT) method. In osteoclastogenic cultures, only cells with three or more nuclei were analyzed.

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