Promotion of osteoclast apoptosis is one therapeutic approach to osteoporosis. Calmodulin, the major intracellular Ca\(^{2+}\) receptor, modulates both osteoclastogenesis and bone resorption. The calmodulin antagonist, trifluoperazine, rescues bone loss in ovariectomized mice (Zhang, L., Feng, X., and McDonald, J. M. (2003) Endocrinology 144, 4536–4543). We show here that a 3-h treatment of mouse osteoclasts with either of the calmodulin antagonists, tamoxifen or trifluoperazine, induces osteoclast apoptosis dose-dependently. Tamoxifen, 10 \(\mu\)M, and trifluoperazine, 10 \(\mu\)M, induce 7.3 ± 1.8-fold and 5.3 ± 0.9-fold increases in osteoclast apoptosis, respectively. In Jurkat cells, calmodulin binds to Fas, the death receptor, and this binding is regulated during Fas-mediated apoptosis (Ahn, E. Y., Lim, S. T., Cook, W. J., and McDonald, J. M. (2004) J. Biol. Chem. 279, 5661–5666). In osteoclasts, calmodulin also binds Fas. When osteoclasts are treated with 10 \(\mu\)M trifluoperazine, the binding between Fas and calmodulin is dramatically decreased at 15 min and gradually recovers by 60 min. A point mutation of the Fas death domain in the Lpr\(^{-}\) mouse renders Fas inactive. Using glutathione S-transferase fusion proteins, the human Fas cytoplasmic domain is shown to bind calmodulin, whereas a point mutation (V254N) comparable with the Lpr\(^{-}\) mutation in mice has markedly reduced calmodulin binding. Osteoclasts derived from Lpr\(^{-}\) mice have diminished calmodulin/Fas binding and are more sensitive to calmodulin antagonist-induced apoptosis than those from wild-type mice. Both tamoxifen- and trifluoperazine-induced apoptosis are increased 1.6 ± 0.2-fold in Lpr\(^{-}\)-derived osteoclasts compared with osteoclasts derived from wild-type mice. In summary, calmodulin antagonists induce apoptosis in osteoclasts by a mechanism involving interference with calmodulin binding to Fas. The effects of calmodulin/Fas binding on calmodulin antagonist-induced apoptosis may open a new avenue for therapy for osteoporosis.

Healthy adult bone requires a balance between bone formation by osteoblasts and bone resorption by osteoclasts. An alteration in osteoclast apoptosis has been shown to contribute to the pathogenesis of postmenopausal osteoporosis (3, 4). Fas, a death receptor of the tumor necrosis factor family, has a cysteine-rich domain and a highly conserved death domain 1 (DD1), critical for death signaling. Mice with defective Fas signaling have mutations leading to either decreased Fas expression (lymphoproliferative (Lpr)) or Fas inactivation (Lpr complementing Gld (Lpr\(^{cg}\))) (5, 6). We have shown that mice with defective Fas signaling develop a low bone mass phenotype (7), indicating that the Fas/Fas Ligand (FasL) system is involved in the mechanism of osteoclast apoptosis in the immune cell-rich bone marrow microenvironment. However, the levels of Fas expression and Fas-mediated apoptosis in osteoclasts are reduced in the presence of high levels of receptor activator of nuclear factor-κB ligand (RANKL) (8), which has been reported to be increased in many osteolytic situations, such as estrogen depletion and rheumatoid arthritis. The inability of Fas to induce apoptosis in osteoclasts, under osteolytic conditions, led us to search for a novel mechanism by which osteoclast apoptosis is mediated.

Fas interacts with various proteins, such as Fas-associated phosphatase-1, Fas-associated death domain protein (FADD), and Fas/FADD-interacting serine/threonine kinase (9–11). These Fas-binding proteins primarily participate in the regulation of apoptosis. Cross-talk between the Fas and the calmodulin (CaM) apoptotic signaling pathways has been reported recently by Ahn et al. (2, 12) who demonstrated a direct interaction between Fas and CaM in Jurkat cells (2). Using glutathione S-transferase (GST) fusion proteins containing the intracellular domain of human Fas and Fas with mutations and deletions at the putative CaM-binding site, they showed that CaM binds to the DD of the cytoplasmic region of Fas.

CaM is a 148-amino acid protein composed of 4 helix-loop-helix protein folding motifs, called EF hands. As the main intracellular Ca\(^{2+}\) concentration sensor, CaM is responsible for mediating numerous Ca\(^{2+}\)-triggered processes (13), including gene expression, protein synthesis, secretion, cell motility, and chemotaxis (14, 15). CaM antagonists bind to CaM, blocking the binding of substrates and thus preventing activation of CaM (16) and of its downstream signals, many of which are cell type-specific. In osteoclasts, the inhibition of CaM or its downstream pathways has impact on both differentiation and function. One of the main downstream target molecules for CaM is calcineurin. Inhibition of the calcineurin/nuclear factor of activated T cells (NFAT) signaling pathway blocks RANKL-induced osteoclast differentiation (17). Zhang et al. (1) showed that the CaM antagonist, trifluoperazine (TFP), inhibits osteoclastogenesis when added on day 3 of a 6-day differentiation.

1. The abbreviations used are: DD, death domain; Lpr, lymphoproliferation; Lpr\(^{cg}\), Lpr complementing Gld; RANKL, receptor activator of nuclear factor-κB ligand; FADD, Fas-associated-death domain protein; CaM, calmodulin; GST, glutathione S-transferase; NFAT, nuclear factor of activated T cells; TFP, trifluoperazine; TXM, tamoxifen; HIV, human immunodeficiency virus; M-CSF, macrophage-colony-stimulating factor; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
period and that it rescues ovariectomy-induced bone loss in mice (1). Another CaM antagonist, tamoxifen (TMX), is also an estrogen antagonist (18). Paradoxically, TMX has been reported to preserve bone mass, as does estrogen. TMX decreases osteoclastic bone resorption most likely via its CaM-antagonistic properties (19). One of the diverse functions of CaM is mediating cell apoptosis. Whether CaM antagonists can induce osteoclast apoptosis in addition to their known inhibition of osteoclastogenesis and osteoclast activity is unknown.

The importance of CaM in apoptosis has been suggested before (20). In different cell lines, CaM exerts either an anti-apoptotic or a pro-apoptotic influence by regulating various downstream targets (21). For example, in HIV-infected T cells CaM binds to glycoprotein 160, the envelope protein of HIV, enhancing Fas-mediated apoptosis; thus CaM antagonists inhibit Fas-mediated apoptosis in these cells (22). In contrast, in the human cholangiocarcinoma cell line, SK-ChA-1, where the Fas protein is heterogeneously expressed at the cell surface (23), Pan et al. (23) demonstrated that only Fas-high expressing cells undergo apoptosis in response to the CaM antagonists, TFP and TMX; Fas-low expressing cells are unaffected.

In this study, we focus on the interaction between Fas and CaM, the involvement of CaM in osteoclast apoptosis, and more importantly, how the interaction between CaM and Fas affects CaM antagonist-induced apoptosis. Our findings suggest a novel therapeutic approach to shorten osteoclast life span under osteolytic conditions in which the levels of Fas are likely to be decreased.

EXPERIMENTAL PROCEDURES

Animals—Five 8-week-old, male, B6.mrl-tnftrsf6/lpr/J, MRL.CBAJms- Tnfrsf6lpr-cg/tg, and control mice for each strain were purchased from Jackson Laboratories (Bar Harbor, ME). All procedures involving animal care and use were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Cells, Antibodies, and Reagents—Primary mouse osteoclast precursors were isolated from bone marrow and differentiated into mature osteoclasts in vitro in the presence of RANKL and M-CSF, as described previously (7). Osteoclasts were also derived, as described previously, from RAW264.7 cells, a macrophage lineage, purchased from the ATCC (Manassas, VA) (7). CaM-Sepharose 4B and GST antibody were purchased from Amersham Biosciences, and CaM-Sepharose 4B was from Sigma. Fas (polyclonal, M-20) and caspase-3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CaM monoclonal antibody to CaM was developed as described previously (24).

Generation of Fas Fragments by the Polymerase Chain Reaction—The cytoplasmic domain (191–335) of human Fas was generated by PCR with the following forward (F) and reverse (R) primers containing EcoRI (underlined) and XhoI (bold) sites: F1, 5'-ccatcaatttggtgaagagaaag-3'; R1, 5'-catactataaqctgaatggcttcg-3'. The Fas point mutation, V254N, was generated using the QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers for the mutagenesis were purchased from Invitrogen. Wild-type or mutated Fas cytoplasmic regions were inserted into the pGEX5–1 vector using the EcoRI and XhoI sites. Expression and purification of the GST fusion proteins were performed according to the manufacturer's directions (Amersham Biosciences).

Apoptosis Assay—Apoptosis was measured by Hoechst 33258 staining of condensed chromatin (25). Cells, differentiated on coverslips, were treated with various concentrations of TFP and TMX, as indicated, and stained with Hoechst 33258 fluorescent dye (25 μg/ml) (Sigma) for 2 min at room temperature. The percentage of apoptotic cells was determined by fluorescence microscopy and calculated as apoptotic multinucleated cells/total number of multinucleated cells × 100%. Only cells containing more than three nuclei were included in the count.

Fluorescent Caspase-3 Activity Assay—Caspase-3 activity was measured using the ApoAlert caspase fluorescent assay kit (Clontech Laboratories, Inc., Palo Alto, CA). Briefly, cells were lysed in 50 μl of buffer and incubated on ice for 10 min. Cell lysates were centrifuged and supernatants collected. Reaction buffer/dithiothreitol mix (50 μl) and 5 μl of 1 mM caspase-3 substrate were added to each reaction and incubated for 1 h at 37 °C. A Fusion™ universal microplate analyzer (PerkinElmer Life Sciences, Inc.) was used to measure fluorescence (excitation at 400 nm and emission at 505 nm).

CaM-Sepharose Pull-down Assay—Cell lysates were incubated with equal amounts of either control Sepharose CL-4B or CaM-Sepharose 4B at 4 °C for 1–3 h. After incubation, beads were washed six times with 1% Triton X-100 in PBS to remove nonspecific binding and heated in SDS sample buffer at 95 °C for 5 min, and proteins were separated by SDS-PAGE, followed by Western blotting.

Co-immunoprecipitation—Anti-CaM monoclonal antibody and non-immune mouse IgGx were coupled to AminoLink Plus coupling gel ( Pierce), as described in the manufacturer's protocol. Cells were lysed in M-PER reagent ( Pierce) and cell debris removed by centrifugation at 16,000 × g for 10 min. Equal amounts of protein (100–200 μg) were incubated with anti-CaM antibody or mouse IgGx antibody (15 μg)–coupled gel in a Handee spin cup column ( Pierce) at 4 °C overnight. Samples were washed three times with immunoprecipitation buffer ( Pierce). Co-immunoprecipitated proteins bound to the antibody-coupled gel were eluted with 50 μl of ImmunoPure elution buffer ( Pierce) at 12,000 × g for 1 min.

125I-CaM Preparation—IODO-BEADS (Pierce) were washed in 0.5 mM CaCl2, 100 mM Tris-HCl, pH 7.5, dried, and added to a solution containing 0.5 mM CaCl2, 100 mM Tris-HCl pH 7.5, and 1 μCi of 125I, for 5 min at room temperature. The IODO-BEAD-containing solution was incubated with 1 μg/ml of CaM (16,000 μl) for 2 min at room temperature. The reaction was stopped by applying the solution to a Bio-Rad 10DG column (Bio-Rad), which had been pre-equilibrated with PBS, pH 7.5. Seven 1-ml fractions were collected. Total 125I cpm in 5 μl were counted in a γ-counter. Trichloroacetic acid-precipitated 125I cpm were measured by adding 900 μl of 1% BSA/PBS and 100 μl of 100% trichloroacetic acid to 5-μl aliquots, incubating on ice for 15 min, washing with 16,000 × g for 15 min, and counting the pellets in the γ-counter. Total 125I and trichloroacetic acid-precipitated 125I versus fraction number was plotted. The percentage of trichloroacetic acid-precipitated 125I/total 125I was greater than 90%.

125I-CaM Overlay—125I-CaM overlay was performed as described (26). Briefly, following 7.5% SDS-PAGE, protein bands were fixed in 40% ethanol and 7% acetic acid (Hunhung, MA). After being washed in 10% ethanol overnight with shaking, and then rinsed in water. After incubation with buffer A (100 mM imidazole, pH 7.0) for 30 min, and then with buffer B (20 mM imidazole, 0.1% BSA, 0.2 M KCl, pH 7.0 plus 1 mM CaCl2 or 1 mM EGTA) for 10 min, the gels were incubated with buffer B containing 1 × 106 cpm/ml of 125I-CaM for 12–16 h at 4 °C. The nonspecifically bound 125I-CaM was removed by several washes with buffer B at 4 °C. The gels were rinsed with water five times for 5 min each, washed in 40% methanol, 7% acetic acid for 15 h to remove BSA, stained for 2 h in Commassie Brilliant Blue G-250, destained, dried, and subjected to autoradiography at −70 °C.

Western Blotting—Western blotting was performed as described previously (7). Briefly, equal amounts of protein were subjected to 10% SDS-PAGE and subsequently electrotransferred to a polyvinylidene difluoride membrane (Millipore Co., Bedford, MA). The membranes were blocked with 5% nonfat milk, the membrane was incubated with purified anti-Fas (4 μg/ml), followed by peroxidase-conjugated anti-rabbit IgG (1:2000 dilution). Immunoreactive proteins were visualized with ECL Western blotting detection reagents (Amersham Biosciences), following the manufacturer's instructions. As an additional control to ensure equal protein loading of gels, after transfer, gels were stained with Coomassie Blue R-250, destained, and examined for any abnormal protein band densities.

RESULTS

CaM Antagonists Induce Osteoclast Apoptosis—The CaM antagonists, TFP and TMX, induce apoptosis in osteoclasts, concentration-dependently. TFP inhibits osteoclastogenesis when added on day 3 of a full 6-day differentiation period and does not induce apoptosis in osteoclast progenitors (1). TMX, an anti-estrogen and also a potent CaM antagonist, inhibits bone resorption in avian osteoclasts (19). Whether CaM antagonists decrease osteoclastogenesis and bone resorption by inducing apoptosis in osteoclasts, especially at the later stages of osteoclastogenesis, is unknown. Here, using Hoechst 33258 staining of fragmented nuclei, we showed that in vitro differentiated mouse osteoclasts undergo apoptosis after a 3-h treatment with the CaM antagonists, TFP and TMX, in concentration-dependant manners (Fig. 1). TFP, 10 μM, induces a 5.3 ± 0.9-fold
Fas/CaM in Osteoclasts

Fig. 1. The CaM antagonists, TMX and TFP, induce apoptosis in osteoclasts. Mouse osteoclasts were differentiated in vitro on chamber slides in the presence of 1.1 nmol/liter RANKL and 10 ng/ml M-CSF for 5–7 days, then treated with 0–10 μM TMX (A) and TFP (B), as indicated, for 3 h. Cells were fixed in 3% paraformaldehyde and stained with Hoechst 33258, 25 μg/ml. Apoptotic cells were counted as visualized by fluorescence microscopy and reported as apoptotic multinucleated cells/total multinucleated cells × 100. C, a representative fluorescent image of normal (arrow) and apoptotic (double arrow) osteoclast nuclei is shown. Results from A and B are presented as mean ± S.E., n = 4; *, p < 0.05; ***, p < 0.01 versus vehicle (Me2SO).

The CaM antagonists, TMX and TFP, induce apoptosis in osteoclasts. Presented as mean ± S.E., we investigated the Ca2+ with 0–10 μM M-CSF for 5–7 days, then treated in vitro with 0–10 μM TMX (A) and TFP (B), as indicated, for 3 h. Cells were fixed in 3% paraformaldehyde and stained with Hoechst 33258, 25 μg/ml. Apoptotic cells were counted as visualized by fluorescence microscopy and reported as apoptotic multinucleated cells/total multinucleated cells × 100. C, a representative fluorescent image of normal (arrow) and apoptotic (double arrow) osteoclast nuclei is shown. Results from A and B are presented as mean ± S.E., n = 4; *, p < 0.05; ***, p < 0.01 versus vehicle (Me2SO).

Caspase-3 Is Activated by CaM Antagonists in Osteoclasts—Caspase-3, the major executioner in the apoptosis pathway, is activated by CaM antagonists. Caspase-3 is produced as a catalytically inactivezymogen being activated by proteolytic cleavage during apoptosis (27). To demonstrate that caspase-3 is involved in osteoclast apoptosis induced by CaM antagonists, we treated differentiated osteoclasts with various concentrations of TFP and TMX, demonstrating activation of caspase-3 by the disappearance of the noncleaved form, detected by Western blotting. Treatment with increasing concentrations of drug decreases the amount of procaspase-3 in a concentration-dependent manner (Fig. 2, A and B). These results were confirmed by measuring caspase-3 activity, a surrogate marker for apoptosis, by a fluorescent caspase-3 activity assay (Fig. 2C).

CaM Interacts with Fas in Osteoclasts—CaM interacts with Fas in osteoclasts, as detected by CaM-Sepharose 4B pull down, co-immunoprecipitation, and 125I-CaM overlay. CaM has recently been reported to bind to the death receptor, Fas, in Jurkat cells by Ahn et al. (2) and the human cholangiocarcinoma cell line SK-ChA-1. In osteoclasts, CaM-Sepharose 4B was able to pull down Fas from lysates of differentiated mouse cells. Sepharose CL-4B was used as a negative control. Fas was detected only in the CaM-Sepharose-bound proteins (Fig. 3A). As many CaM-target protein interactions are Ca2+-dependent (28), we investigated the Ca2+-dependence of the CaM/Fas binding in osteoclasts by adding Ca2+ (1 mM) or EGTA (1 mM) to the binding buffer and showed a partial calcium dependence (Fig. 3A). To verify whether CaM and Fas interact in vivo we incubated CaM antibody, conjugated to AminoLink Plus gel, with lysates from differentiated osteoclasts. Mouse IgG1κ was used as a nonimmune control. As shown in Fig. 3B, Fas was co-immunoprecipitated with CaM, confirming the presence of the complex in cells. The binding of CaM to Fas was confirmed by 125I-labeled CaM overlay of renatured SDS-PAGE-separated cell lysates from differentiated osteoclasts. After washing to remove nonspecifically bound 125I-CaM, gels were dried and subjected to autoradiography at −70 °C. A band was detected at a molecular mass of 45–48 kDa, the size of Fas. Addition of EGTA during the incubation results in decreased intensity of this band (Fig. 3C).

Treatment with CaM Antagonists Decreases the Binding between CaM and Fas—CaM/Fas binding is decreased by treatment with the CaM antagonist, TFP. The addition of a Fas-neutralizing antibody cannot block CaM antagonist-induced apoptosis in SK-ChA-1 cells (2). This observation suggests that CaM antagonist-induced apoptosis does not require a direct interaction between the CaM antagonist and the trimerized Fas antigen. To further explore the mechanisms by which CaM antagonists induce apoptosis in osteoclasts, we treated osteoclasts derived from RAW264.7 cells with 10 μM TFP for various times. By co-immunoprecipitating Fas with gel-conjugated CaM antibody, we detected a marked decrease in CaM/Fas binding at 15 min in osteoclast lysates (Fig. 4A), which recovered, in part, by 60 min (Fig. 4A). Treatment of RAW264.7-derived osteoclasts with various concentrations of TFP for 15 min resulted in decreased CaM/Fas binding at 5 and 10 μM but not at 2.5 μM (Fig. 4B). These observations are consistent with CaM antagonists inducing apoptosis in osteoclasts by inhibiting CaM/Fas binding. TFP, 10 μM, treatment for 15 min yields a 66.1 ± 9% inhibition of CaM binding to Fas, obtained by calculating the ratio of the band intensities in each experiment (n = 5).

The Lpr<sup>−/−</sup> Mutation Abrogates the Binding between CaM and Fas—A point mutation (I225N) in the DD of Fas decreases CaM/Fas binding. To further explore the mechanisms by which CaM antagonists induce apoptosis, we treated osteoclasts derived from RAW264.7 cells with 10 μM TFP for various times. By co-immunoprecipitating Fas with gel-conjugated CaM antibody, we detected a marked decrease in CaM/Fas binding at 15 min in osteoclast lysates (Fig. 4A), which recovered, in part, by 60 min (Fig. 4A). Treatment of RAW264.7-derived osteoclasts with various concentrations of TFP for 15 min resulted in decreased CaM/Fas binding at 5 and 10 μM but not at 2.5 μM (Fig. 4B). These observations are consistent with CaM antagonists inducing apoptosis in osteoclasts by inhibiting CaM/Fas binding. TFP, 10 μM, treatment for 15 min yields a 66.1 ± 9% inhibition of CaM binding to Fas, obtained by calculating the ratio of the band intensities in each experiment (n = 5).

The Lpr<sup>−/−</sup> Mutation Abrogates the Binding between CaM and Fas—A point mutation (I225N) in the DD of Fas decreases binding to CaM in osteoclasts derived from Lpr<sup>−/−</sup> mice. This mutation blocks the Fas-FADD interaction causing a defect in Fas signaling that leads to a lymphoproliferation phenotype in Lpr<sup>−/−</sup> mice (5). Based on sequence alignment analysis of mouse and human Fas, isoleucine 225 of mouse Fas, which is mutated to asparagine in the Lpr<sup>−/−</sup> mouse (29), is equivalent in position to valine 254 of human Fas. A GST fusion protein of the Fas intracellular domain, containing a similar point mutation in the DD of human Fas (V254N), was generated and had reduced binding to CaM in vitro compared with the nonmutated sequence (Fig. 5A). The potential significance of CaM binding to

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2 Y. Chen, P. Pawar and J. M. McDonald, unpublished observations.
**FIG. 2.** CaM antagonists activate caspase-3. Osteoclasts were differentiated in vitro in the presence of 1.1 nmol/liter RANKL and 10 ng/ml M-CSF for 5–7 days. Cells were treated with various concentrations of TMX (A) and TFP (B), as indicated, for 4 h. Equal amounts of cell lysates were separated by SDS-PAGE, electrotransferred, and immunoblotted for procaspase-3. A representative of three experiments is shown here. C, caspase-3, activated by TMX, 10 μM, and TFP, 10 μM, was measured by a fluorescent activity assay. Results are presented as mean ± S.E., n = 6 (TMX) and n = 5 (TFP); *, p < 0.01 versus vehicle.

**FIG. 3.** CaM interacts with Fas in osteoclasts. A, CaM-Sepharose 4B pulls down Fas protein in osteoclasts, in a partially calcium-dependent manner. Osteoclasts were lysed in 1% Triton X-100. Lysates were incubated with CaM-Sepharose beads at 4 °C for 1 h, in the presence of either 1 mM Ca²⁺ or 1 mM EGTA. Fas protein that was pulled down was detected by Western blotting. An equal amount of lysate was incubated with Sepharose CL-4B as a negative control. A representative of three experiments is shown. B, co-immunoprecipitation of CaM and Fas from osteoclasts. Osteoclasts were lysed in a mammalian protein extraction buffer. Lysates were incubated with anti-CaM antibody coupled to AminoLink Plus coupling gel overnight at 4 °C. Fas protein, co-immunoprecipitated with CaM, was detected by Western blotting. Incubation of the same amount of lysate with mouse IgG1 was used as a negative control. A representative of at least three experiments is shown. C, the interaction between Fas and CaM detected by ¹²⁵I-labeled CaM overlay. Osteoclasts were lysed and proteins separated by 7.5% SDS-PAGE. Gels were fixed, proteins were re-natured, and gels were blocked with 0.1% BSA and then incubated with ¹²⁵I-CaM at 4 °C for 12–16 h. After nonspecifically bound ¹²⁵I-CaM was washed away, gels were dried and subjected to autoradiography at −70 °C. EGTA, 1 mM, was added to the blocker and buffer containing ¹²⁵I-CaM, during incubation with one gel (right lane). CaCl₂, 1 mM, was added to the other gel (left lane). IP, immunoprecipitation; IB, immunoblot; Con, control.

**FIG. 4.** TFP treatment interferes with Fas/CaM binding. Osteoclasts were derived from RAW264.7 cells in the presence of RANKL, 2.22 nmol/liter, for 5–7 days. A, Osteoclasts were treated with 10 μM TFP for various times, as indicated. Co-immunoprecipitation of Fas with CaM by gel-conjugated anti-CaM antibody was performed and analyzed by Western blotting. B, treatment of osteoclasts with various concentrations of TFP, as indicated, for 15 min. Co-immunoprecipitation of Fas with CaM by gel-conjugated anti-CaM antibody was performed and analyzed by Western blotting. A and B, one-tenth the amount of protein used for the co-immunoprecipitation was electrophoresed and Western blotted for Fas as a control for each condition. Shown are representative results from two independent experiments for both A and B. IB, immunoblot.

The DD of Fas is illustrated in vivo by comparing the CaM/Fas binding in osteoclasts derived from wild type with those from Lpr⁻/⁻ mouse bone marrow. Using both co-immunoprecipitation of Fas by gel-conjugated CaM antibody and CaM-Sepharose 4B pull down, we detected less CaM/Fas binding in lysates from Lpr⁻/⁻-derived osteoclasts (Fig. 5, B and C) compared with those from wild-type mice. Therefore, the DD of Fas is critical for CaM/Fas binding both in vitro and in vivo.

Enhanced Apoptosis by CaM Antagonists in Fas-mutated Cells—CaM antagonists induce more apoptosis in Lpr⁻/⁻-derived osteoclasts than in osteoclasts from wild-type mice. To investigate the impact of CaM/Fas binding on apoptosis induced by CaM antagonists, we treated osteoclasts derived from both wild-type and Lpr⁻/⁻ mice with various concentrations of TFP and TMX. The CaM antagonists induced apoptosis concentration-dependently in both wild-type and Lpr⁻/⁻-derived osteoclasts. However, the Lpr⁻/⁻-derived osteoclasts were more sensitive to CaM-antagonist-induced apoptosis than wild type. Treatment with TMX, 10 μM, caused 27 ± 2.2% of the wild-type
osteoocytes to undergo apoptosis; in comparison, 42 ± 6% of the Lpr−/−-derived osteoclasts became apoptotic after TMX treatment. This represents a 1.6 ± 0.2-fold increase in apoptosis (apoptosis rate in Lpr−/−/apoptosis rate in wt). Similarly, TFP (10 μM)-induced apoptosis is increased 1.6 ± 0.2-fold in Lpr−/−-derived osteoclasts (37 ± 0.8% apoptotic cells) compared with wild type (24 ± 2.8% apoptotic cells) (Fig. 6). The basal apoptosis levels in Lpr−/− and wild-type-derived osteoclasts are not statistically different. Results were confirmed by measuring the activation of caspase-3 (data not shown).

In contrast to the Lpr−/− mouse, which expresses a mutated inactive Fas molecule, the Lpr−/− mouse has diminished Fas expression due to the insertion of an early transposable element in the Fas gene (6). When osteoclasts derived from Lpr mice were treated with the CaM antagonists, TMX, 10 μM, or TFP, 10 μM, caspase-3 activation was increased 2.1 ± 0.6-fold and 1.3 ± 0.1-fold, respectively, compared with wild type (data not shown). This result suggests that disruption of CaM/Fas binding sensitizes osteoclasts to CaM antagonist-induced apoptosis.

**DISCUSSION**

Promoting apoptosis in osteoclasts is potentially an important therapeutic strategy for treating osteolysis. We reported previously that osteoclast apoptosis is regulated by the Fas system. However, RANKL decreases Fas expression and Fas-mediated apoptosis, and in most osteolytic situations RANKL is up-regulated (7, 8). Therefore, it is important to identify other mechanisms by which osteoclast apoptosis is regulated, especially under osteolytic conditions.

CaM, a small calcium-binding protein, recognizes different target proteins, including enzymes, receptors, G-proteins, and transcription factors (30–32), thus regulating signaling and function in a variety of cells, including osteoclasts. In osteoclasts, CaM participates in the regulation of both formation and function (1, 19). However, the role of CaM in regulating osteoclast apoptosis is unknown. In this study, we demonstrate that there is a dose-dependent induction of apoptosis by CaM antagonists and a dynamic interaction between CaM and Fas in mature osteoclasts. A point mutation in the DD of the Fas molecule that abolishes Fas-mediated apoptosis also abolishes the binding between Fas and CaM. The disruption of CaM/Fas binding sensitizes osteoclasts to CaM antagonist-induced apoptosis.

CaM participates in the regulation of osteoclastogenesis and of osteoclastic bone resorption. A downstream target of CaM, the calcineurin/NFAT signaling pathway is essential for osteoclastogenesis (17, 33). Inhibition of CaM by the specific antagonist, TFP, inhibits osteoclastogenesis at an early stage of differentiation (1). CaM is enriched at the acid-secreting ruffled membrane, where the H+/ATPase, an essential apparatus for osteoclastic bone resorption, is located. In avian osteoclasts, TMX inhibits osteoclastic bone resorption functioning as a CaM antagonist (19), possibly by inhibiting the H+/ATPase (34).

CaM is also involved in the regulation of apoptosis in many cell types (35). An in vivo study showed that treatment with TFP rescues the bone loss in ovariectomized mice (1). In addition to the known inhibitory effects on osteoclast formation and bone resorption, the antiresorptive effect of CaM antagonists could result from the induction of osteoclast apoptosis. CaM antagonists mediate apoptosis in a cell-specific manner. For example, TMX and TFP exert an anti-apoptotic effect in HIV-infected CD4(+)/T cells (36) and pro-apoptotic effects in breast cancer cells (37). The maturation stage of the cells has an impact on the apoptotic response to CaM antagonists. In osteoclast precursors, TFP does not induce apoptosis (1)3; however, in differentiated osteoclasts, CaM antagonists induce apoptosis and activate caspase-3 in concentration-dependent manners (Figs. 1 and 2). The demonstration of TMX- and TFP-induced apoptosis in osteoclasts suggests that CaM is protective, revealing another possible mechanism by which CaM antagonists inhibit bone resorption, by inducing osteoclast apoptosis.

CaM antagonist-induced apoptosis activates caspase-3 in osteoclasts. Caspase-3 is a member of a family of cysteine proteases that cleave substrates after an Asp residue. In the breast cancer cell lines, MDA-MB-231 and BT-20, 5 μM TMX activates caspase-3 in a time-dependent manner, preceding the appearance of apoptotic morphology (38). Caspase-3 has been reported to be activated during Fas-mediated apoptosis in osteoclasts (7). Here, by both Western blot analysis and a fluorescent caspase-3 assay, we demonstrate that CaM antagonists activate caspase-3 in osteoclasts.

Cross-talk between the Fas and CaM pathways is of interest

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3 X. Wu and J. M. McDonald, unpublished observations.
in many systems and is the focus of our ongoing investigations in many cells. The direct interaction between Fas and CaM regulates both the Fas and the CaM pathways. CaM interacts with a variety of proteins and serves diverse functions. Fas also interacts with various proteins, including the Fas-associated phosphatase-1 and FADD, both of which participate in the regulation of apoptosis (9, 10). In Jurkat cells, the binding of CaM to the Fas DD is altered during Fas-mediated apoptosis. Fas activating antibody treatment first up-regulates (at 30 min) and then down-regulates (at 60 min) the binding between Fas and CaM (2), suggesting that this interaction regulates Fas signaling, possibly by binding to cFLIP, the inhibitory molecule in the Fas-mediated apoptotic pathway (2).

In osteoclasts, CaM binds to the Fas DD, and this binding has an impact on CaM antagonist-induced apoptosis. Osteoclasts derived from Lpr<sup>cg</sup> mice with a point mutation, 1225N, in the mouse Fas DD (5, 29) have less CaM/Fas binding than those generated from wild-type mice. Furthermore, CaM antagonists induce apoptosis probably in part by interfering with CaM/Fas binding. Treatment of osteoclasts derived from RAW264.7 cells with 10 µmol/liter TFP caused a decrease in CaM/Fas binding at 15 min, which gradually recovered over 60 min. Treatment with various concentrations of TMX for 15 min in RAW264.7-derived osteoclasts decreased CaM/Fas binding dose-dependently. This suggests that in osteoclasts from control mice, Fas decreases CaM antagonist binding to CaM by competitively binding to CaM; however, in osteoclasts from Lpr or Lpr<sup>cg</sup> mice, the deficiency in CaM-Fas binding allows more binding of CaM to CaM antagonists, thus sensitizing osteoclasts from these mice to CaM antagonist-induced apoptosis.

To support this hypothesis, we generated osteoclasts from both strains of mutant mice, Lpr and Lpr<sup>cg</sup>, which are deficient in Fas signaling due to lack of Fas expression and expression of nonfunctional Fas, respectively (5, 6, 39), and showed greater induction of apoptosis and increased caspase-3 activation in response to the CaM antagonists, TMX and TFP, compared with wild-type osteoclasts (Fig. 6). Therefore, the decrease in Fas/CaM binding enhances CaM antagonist-induced osteoclast apoptosis. The exact mechanism by which Fas affects CaM antagonist-induced apoptosis requires further study.

CaM antagonists are potentially an effective treatment for osteoporosis. Both post-menopausal osteoporosis and rheumatoid arthritis are associated with increased levels of RANKL, which down-regulates Fas expression and Fas-mediated apoptosis in osteoclasts (8). The decreased Fas-mediated apoptosis in osteoclasts contributes to exacerbated bone loss. This is consistent with the report that defective Fas-mediated apoptosis of osteoclasts in Lpr mice leads to an osteopenic phenotype (7). The reduced levels of Fas lead to decreased CaM/Fas binding resulting in an increased response to CaM antagonists.

This observation suggests that the level of Fas expression and the degree of binding to CaM regulate the sensitivity of the response of osteoclasts to CaM antagonists. Therefore, we speculate that CaM antagonists may be therapeutic agents for osteoporotic bone without affecting normal bone. Further study comparing the levels of CaM antagonist-induced apoptosis in osteoclasts differentiated in vitro, in the presence of different concentrations of RANKL, is important, and animal models such as the ovarietomized Lpr or Lpr<sup>cg</sup> mice will be invaluable for further investigation of the significance of CaM/Fas binding in osteoclasts.

REFERENCES

1. Zhang, L., Feng, X., and McDonald, J. M. (2003) Endocrinology 144, 4536–4543
2. Ahn, E. Y., Lim, S. T., Cook, W. J., and McDonald, J. M. (2004) J. Biol. Chem. 279, 5661–5666
3. Manolagas, S. C. (2000) Endocr. Rev. 21, 115–137
4. Weinistein, R. S., Chen, J. R., Powers, C. C., Stewart, S. A., Landes, R. D., Bellido, T., Jilka, R. L., Parfitt, A. M., and Manolagas, S. C. (2002) J. Clin. Invest. 109, 1041–1048
5. Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992) Nature 356, 314–317
6. Adachi, M., Watanabe-Fukunaga, R., and Nagata, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1756–1760
7. Wu, X., McKenna, M. A., Fung, X., Nagy, T. R., and McDonald, J. M. (2003) Endocrinology 144, 5545–5555
8. Wu, X., Pan, G., McKenna, M. A., Zayzafon, M., Xiong, W.-C., and McDonald, J. M. (2005) J. Bone Miner. Res. 20, 107–116
9. Chinnaiyan, A. M., O’Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
10. Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995) Science 268, 411–415
11. Rochat-Steiner, V., Becker, K., Micheau, O., Schneider, P., Burns, K., and Tschopp, J. (2000) J. Exp. Med. 192, 1165–1174
12. Ahn, E. Y., Pan, G., Oh, J. H., Tyler, E. M., and McDonald, J. M. (2003) Am. J. Pathol. 163, 2053–2063
13. Mamar-Bachi, A., and Cox, J. A. (1987) Cell Calcium 8, 473–482
14. Cohen, P., and Klee, C. B. (1988) Calmodulin (Cohen, P., and Klee, C. B., eds) Elsevier Science Publishers B.V., Amsterdam, the Netherlands
15. Van Eldik, L. J., and Wattersen, D. M. (1998) Calmodulin and Signal Transduction (van Eldik, L. J., and Wattersen, D. M., eds) Academic Press, San Diego, CA
16. Evans, J. S., Levine, B. A., Williams, R. J. P., and Wormald, M. R. (1988) in Calmodulin (Cohen, P., and Klee, C. B., eds) pp. 57–82, Elsevier Science Publishers, New York
17. Takayama, H., Kim, S., Koga, T., Sushina, H., Ishiki, M., Yashida, H., Saita, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E. F., Mak, T. W., Kodama, T., and Taniuchi, I. (2002) Dev. Cell 3, 889–901
18. Love, R. R., Mazess, R. B., Barden, H. S., Epstein, S., Newcomb, P. A., Jordan, V. C., Carbone, P. P., and DeMets, D. L. (1992) N. Engl. J. Med. 326, 852–856
19. Williams, J. P., Biir, H. C., McKenna, M. A., Jordan, S. E., and McDonald, J. M. (1996) J. Biol. Chem. 271, 12488–12495
20. Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) Nature 396, 584–587
21. Franklin, R. A., and McCulrey, J. A. (2000) Leukemia (Basingstoke) 14, 2019–2024
22. Micoli, K. J., Pan, G., Wu, Y., Williams, J. P., Cook, W. J., and McDonald, J. M. (2000) J. Biol. Chem. 275, 1233–1240
23. Pan, G., Vickers, S. M., Pickens, A., Phillips, J. O., Ying, W., Thompson, J. A., Siegel, G. P., and McDonald, J. M. (1999) Am. J. Pathol. 155, 193–203
24. Sacks, D. B., Porter, S. E., Ladenson, J. H., and McDonald, J. M. (1991) Anal. Biochem. 194, 369–377
25. Kameda, T., Ishikawa, H., and Tsutsui, T. (1995) Biochem. Biophys. Res. Comm. 219, 481–486
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26. Radding, W., Williams, J. P., McKenna, M. A., Tummala, R., Hunter, E., Tytler, E. M., and McDonald, J. M. (2000) AIDS Res. Hum. Retroviruses 16, 1519–1525
27. Shi, Y. (2002) Mol. Cell 9, 459–470
28. Rhoads, A. R., and Friedberg, F. (1997) FASEB J. 11, 331–340
29. Ish, N., and Nagata, S. (1993) J. Biol. Chem. 268, 10982–10987
30. Lee, A., Wong, S. T., Gallagher, D., Li, B., Storm, D. R., Scheuer, T., and Catterall, W. A. (1999) Nature 399, 155–159
31. Liao, B., Paschal, B. M., and Luby-Phelps, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6217–6222
32. Zuhlke, R. D., Pitt, G. S., Deisseroth, K., Tsien, R. W., and Reuter, H. (1999) Nature 399, 159–162
33. Hiratani, H., Tuohy, N. A., Woo, J. T., Stern, P. H., and Clipstone, N. A. (2004) J. Biol. Chem. 279, 13984–13992
34. Williams, J. P., McKenna, M. A., Thames, A. M., 3rd, and McDonald, J. M. (2000) Biochem. Cell Biol. 78, 715–723
35. Nicotera, P., and Orrenius, S. (1998) Cell Calcium 23, 173–180
36. Pan, G., Zhou, T., Radding, W., Saag, M. S., Mountz, J. D., and McDonald, J. M. (1998) Immunopharmacology 40, 91–103
37. Frankfurt, O. S., Sugarbaker, E. V., Robb, J. A., and Villa, L. (1995) Cancer Lett. 97, 149–154
38. Mandlekar, S., and Kong, A. N. (2001) Apoptosis 6, 469–477
39. Kimura, M., and Matsuzawa, A. (1994) Int. Rev. Immunol. 11, 193–210