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Breast Cancer Cells Induce Osteoblast Apoptosis: A Possible Contributor to Bone Degradation

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Abstract Breast cancer cells exhibit a predilection for metastasis to bone. There, the metastases usually bring about bone loss with accompanying pain and loss of function. One way that breast cancer cells disrupt the normal pattern of bone remodeling is by activating osteoclasts, the bone degrading cells. Nevertheless, targeting the osteoclasts does not cure the disease or result in bone repair. These observations indicate that osteoblast function also may be compromised. The objective of this study was to investigate the interaction of metastatic breast cancer cells with osteoblasts. Human metastatic breast cancer cells, MDA-MB-435 or MDA-MB-231, or their conditioned media were co-cultured with a human osteoblast line hFOB1.19. The breast cancer cells caused an increase in the prevalence of apoptotic osteoblasts. Apoptotic osteoblasts detected by the TUNEL assay or by caspase activity increased approximately two to fivefold. This increase was not seen with non-metastatic MDA-MB-468 cells. In an investigation of the mechanism, it was determined that the hFOB1.19 cells expressed fas and that fas was functional. Likewise the hFOB1.19 cells were susceptible to TNF-α, but this cytokine was not detected in the conditioned medium of the breast cancer cells. This study indicates that osteoblasts are the target of breast cancer cell-induced apoptosis, but fas/fas-ligand and TNF-α, two common initiators of cell death, are probably not involved in this aspect of the metastases/bone cell axis. There are several mechanisms that remain to be explored in order to determine how breast cancer cells bring about osteoblast apoptosis. Even though the specific initiator of apoptosis remains to be identified, the results of this study suggest that the mechanism is likely to be novel. J. Cell. Biochem. 91: 265–276, 2004. © 2003 Wiley-Liss, Inc.

Key words: apoptosis; osteoblasts; breast cancer; metastasis; bone

Bone is the most common destination of breast cancer metastases. In nearly 50% of first metastasis and in greater than 70% of reoccurrences, breast cancer cells colonize the bone [Rubens and Mundy, 2000]. The result is extensive bone degradation with accompanying pain and loss of function. While the breast cancer cells themselves may degrade bone, especially late in metastasis [Sanchez-Sweatman et al., 1998] it is now widely accepted that the breast cancer cells also upset the normal bone remodeling process such that the osteoclasts become hyperactive. Guise and co-workers [Guise, 2000] present a model in which breast cancer cell-derived parathyroid hormone related protein (PTHrP) is indirectly responsible for the activation of osteoclasts. PHTrP activates osteoblasts to produce a receptor activator of nuclear factor κB ligand (RANKL) which, in turn, activates osteoclasts to degrade bone,

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thereby releasing TGF-β from the matrix. TFG-β in turn stimulates the cancer cells to produce more PTHrP setting up a "vicious cycle." More recently, other models have indicated that IL-8 [Bendre et al., 2002] and endothelin are critical molecules [Guise et al., 2003]. There are numerous other molecules in the metastatic environment that undoubtedly play a role [Mundy et al., 2002].

Because of their direct role in bone degradation, osteoclasts are the major target of pharmaceutical interventions. Several derivative drugs of the bisphosphonate family have succeeded in slowing lesion progression, but they do not bring about a cure. Moreover, the already existing lesions do not heal [Lipton et al., 2000]. Sasaki et al. [1995], using a rodent model and lytic MDA-MB-231 human breast cancer cells, carried out a histomophometric analysis following a bisphosphonate (residronate) treatment. Despite the fact that residronate reduced osteoclast numbers, slowed bone lysis, and reduced the tumor burden, there was no evidence for bone deposition and repair. This outcome suggests that normal osteoblast function also is impaired in osteolytic metastasis. In fact, very little is known about the osteoblast in osteolytic breast cancer metastasis. Furthermore, the clinical model is probably more complex than the mouse model. There are few studies of osteoblasts during bone metastasis. Stewart et al. [1982] carried out quantitative, histomorphometric analysis of bone biopsies of patients with hypercalcemia due to metastasis. Although there was the expected increase in osteoclast numbers overall, there was also a significant decrease in osteoblasts, osteoid surface, absolute osteoid volume, and an increase in empty osteocyte lacunae in bone adjacent to the tumor. Others report diminished or abnormal osteoblast activity near the site of the metastases [Taube et al., 1994]. These data taken together indicate a decrease in normal osteoblast function.

These reports led us to question the impact of breast cancer cells on osteoblasts. We developed a model co-culture system using an immortalized human fetal osteoblast cell line, hFOB1.19, and human metastatic breast cancer cells, MDA-MB-231 and MDA-MB-435 [Mercer et al., 2003]. Early in the course of these studies we observed an increase in osteoblast apoptosis in the presence of breast cancer cells. The results of the study to verify this observation are reported here.

MATERIALS AND METHODS

Cell Culture

The human fetal osteoblast line, hFOB1.19, immortalized with SV40 large T antigen [Harris et al., 1995], was a generous gift from Dr. Thomas Spelsberg. The cells were maintained in a 34°C, 5% CO_2, humidified chamber, with growth medium consisting of DMEM:Ham's F-12 (1:1), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin, hereafter referred to as "growth medium." To obtain differentiated osteoblasts, 85–90% confluent cultures were changed to differentiation medium and transferred to a 39.5°C, 5% CO_2, humidified chamber for 2 or 3 days before treatment as indicated in individual experiments. Differentiation medium consisted of DMEM:Ham's F-12 (1:1), 10% charcoal-stripped FBS, 50 μg/ml vitamin C (ascorbic acid), 10^{-8} M vitamin D_3, and 10^{-8} M vitamin K_3 (menadione).

Two human breast cancer metastatic cell lines, MDA-MB-231 and MDA-MB-435, and one non-metastatic breast cancer cell line, MDA-MB-468 [Price, 1996] were used. They were stably transfected with the plasmid pEGFP-N1 [Harms et al., 2002]. They could easily be distinguished from the hFOB cells in co-culture by fluorescent microscopy. MDA-MB-231 and MDA-MB-435 cells were maintained in DMEM supplemented with 5% FBS in a humidified 37°C chamber with 5% CO_2. Stock culture medium was supplemented with 500 μg/ml G418 to maintain pEGFP-N1. MDA-MB-468 cell cultures were maintained in DMEM:-Ham's F-12 supplemented with 10% FBS and 400 μg/ml hygromycin.

WR19L-A12, a mouse lymphoma cell line, stably transfected with human fas-ligand [Tanaka et al., 1998] was a generous gift from Dr. Laurie Owen-Schaub. WR19L-A12 cells were maintained in RPMI 1640 medium with 4 mM L-glutamine and 23.81 mM sodium bicarbonate, 10% FBS, 1 mM sodium pyruvate, 1% penicillin and streptomycin in a humidified 37°C chamber with 5% CO_2. Stock cultures were supplemented with 400 μg/ml G418 to select for fas-ligand expressing cells.

Breast cancer cell conditioned medium was prepared from breast cancer cells plated in T 75 cm^2 tissue culture flasks with 10 ml DMEM

supplemented with 5% FBS and incubated at 37°C. When the cells were nearly confluent, the medium was changed to serum-free DMEM:-Ham's F-12 (1:1). After 24 h the media were collected, aliquoted and frozen at −20°C. For most experiments they were diluted 1:1 with differentiation medium. In some experiments, as indicated, DMEM:Ham's F-12 was supplemented with 5% Serum Replacement 1 (Sigma, St. Louis, MO) and allowed to remain with the breast cancer cells for 3 days. Vehicle conditioned medium was prepared the same way as conditioned media except that it was not exposed to cells. Control conditioned medium was collected from NIH 3T3 fibroblasts cultured in the same way as for the breast cancer cells.

Apoptotic Analysis

Apoptosis was detected by one of two methods depending on the experiment. Caspase-3/7 activity was assayed with the APO-One™ Homogeneous Caspase-3/7 Assay (Promega, Madison, WI). Fragmented DNA was measured by a modified TUNEL assay [Jewell and Mastro, 2002]. Briefly, terminal deoxynucleotidyl transferase (TdT) from Promega was fluorescently linked to FluoroLink™ Cy5-dUTP (Amersham Pharmacia, Piscataway, NJ) for microscopy or to Biotin-21-dUTP (Clontech Laboratories, Inc., Palo Alto, CA) and R-phycoerythrin(RPE)-Cy5-conjugated streptavidin (DAKO, Copenhagen, Denmark) for flow cytometry. SYTOX® Orange Nucleic Acid Stain (Molecular Probes, Eugene, OR) was used to visualize the nuclei of all cells by fluorescence microscopy.

Caspase Activity

In order to assay caspase activity, hFOB1.19 cells were plated in T 75 cm^2 tissue culture flasks at 10^6 cells per flask and treated as described for individual experiments. After the final incubation, the cells were rinsed with PBS and harvested with 5 ml of Accutase™ (Innovative Cell Technologies, Inc., San Diego, CA). All media and washes were combined in order to collect all of the cells. Cells were centrifuged (4°C, 300*g*), resuspended in growth medium, and filtered through a 40 μm mesh nylon screen (Small Parts, Inc., Miami Lakes, FL) to ensure a single cell suspension. Cells were counted with a hemacytometer and 5×10^5 cells were placed in the wells of a 96-well plate in 100 μl of medium. Next, 100 μl of homogeneous caspase-3/7 reagent was added to each well. After shaking (30 s, 300 rpm), the plate was incubated (room temperature, dark) and readings (excitation/emission, 499/521 nm) were taken every hour for up to 6 h to obtain relative caspase 3/7 activity.

Flow Cytometric Analysis of TUNEL

Cells were plated and incubated as described for the caspase assay. After the final incubation in T-75 flasks, the cells were released with Accutase™, washed twice in PBS (4°C, 300*g*), fixed in 2% paraformaldehyde (20 min, 4°C), washed three times in PBS, and resuspended in 70% ethanol overnight at −20°C for permeabilization. The suspension was centrifuged to remove ethanol and the cells were washed three times with PBS before continuing with the TUNEL assay. At this point the cells were transferred to 12 by 75 mm plastic test tubes compatible with the CoulterXL flow cytometer in order to reduce cell loss. The protocol for DeadEnd™ Fluorometric TUNEL System (Promega) was followed for non-adherent cells with modifications for the fluorescent nucleotide mix. Biotin-21-dUTP (Clontech) was substituted for fluorescein dUTP in the nucleotide mix. A second incubation with streptavidin-RPE/Cy5 (488/670 nm) (DAKO) was carried out after the enzyme reaction was terminated and cells were washed in 0.1% Triton®X-100 in PBS containing 5 mg/ml bovine serum albumin. Propidium idiode staining was omitted. The cells were washed and resuspended in PBS for analysis.

Microscopic Analysis of TUNEL

In order to perform the TUNEL assay, hFOB1.19 cells were plated on gelatin, 0.5 mg/ml, coated glass coverslips (12 × 0.17 mm) in 24-well plates at 10^4 cells per well and treated as described for individual experiments. The cells were washed three times with PBS and fixed with 2% paraformaldehyde. Coverslips were processed in 24-well plates. Samples were rinsed three times with PBS before permeabilizing the cells with 2% Triton®X-100. The protocol for the DeadEnd™ Fluorometric TUNEL System (Promega) was followed for adherent cells except that the fluorescent nucleotide mix was replaced with the same mix containing Cy5-dUTP instead of fluorescein. After the samples were observed by microscopy and images captured for GFP (488/509 nm) and Cy-5 (649/670 nm), the slide was stained with SYTOX®

Orange in place of propidium iodide as described [Jewell and Mastro, 2002].

To calculate the percentage of apoptotic hFOB1.19 cells, digital images were captured on the Olympus BX-60 widefield digital microscope fitted with fluorescence optics. At least 700 fields per sample were captured for analysis of TUNEL positive cells. The number of breast cancer cells per sample was calculated by observing 10–50 images per slide and multiplying the average number of GFP positive cells per field by the total number of fields analyzed for apoptosis. The percentage of apoptotic cells was calculated from the total numbers of cells on the coverslip.

Materials

Sources of materials not already indicated are provided here. Biotin conjugated mouse anti-human monoclonal antibody CD95 (clone DX2), biotin conjugated mouse anti-human fas-ligand monoclonal antibody (clone NOK-1), biotin-conjugated mouse IgG1,κ immunoglobulin isotype control (clone MOPC-21), and purified mouse anti-human TNF-α monoclonal antibody (no azide, low endotoxin, MABTNF-A5), were purchased from BD Biosciences (San Diego, CA). Vitamin D_3 was purchased from BIOMOL Research Laboratories, Inc., (Plymouth Meeting, PA). Caspase-3 Inhibitor (Ac-DEVD-CHO) and GM-6001, a metalloproteinase inhibitor, were obtained from Chemicon International (Temecula, CA); and human CD95 biotin conjugate (Clone B-G27) and mouse isotype control for flow cytometry (mouse IgGγ2a biotin) were from Cymbus Biotechnology Ltd., (Chandlers Ford, Hants, UK). Hygromycin B and G418 were from Calbiochem (EMD Biosciences, Inc., La Jolla, CA). From Immunotech (Beckman Coulter, Brea, CA), we purchased mAb CD95 fas (clone CH-11). Cell Titer 96® Aqueous Assay was purchased from Promega. Heat inactivated FBS, MTT, menadione (vitamin K_3), and human recombinant tumor necrosis factor-alpha (TNF-α) were purchased from Sigma. Vectashield Mounting Medium was a product of Vector Laboratories (Burlingame, CA).

RESULTS

Detection of Apoptotic Osteoblasts

In order to determine if metastatic breast cancer cells were able to attach and grow on monolayers of osteoblasts, GFP expressing MDA-435 or MDA-231 cells were added to confluent monolayers of hFOB1.19 cells. Before viewing with a fluorescence microscope, the cultures were stained with propidium iodide as a means of identifying dead cells. The green fluorescent breast cancer cells could clearly be seen growing over the hFOB cells (Fig. 1A) which were evident when a phase contrast objective was used. The MDA-435 cells were elongated and followed the underlying osteoblasts; the MDA-231 cells remained more compact (Fig. 1B). Although most osteoblasts had a typical morphology, the propidium iodide stain revealed the presence of apoptotic nuclei in some osteoblasts (Fig. 1 arrows). Apoptotic nuclei also were apparent when the cultures were stained with DAPI or Hoechst stain (micrographs not shown).

Quantification of Apoptotic Osteoblasts

In order to quantify the percentage of apoptotic nuclei and to ascertain that they belonged to hFOB cells and not breast cancer cells, we carried out TUNEL assays of the culture using Cy5-dUTP to distinguish the fluorescence of the apoptotic nuclei from that of the GFP. In addition Sytox® Orange was used to stain all the cells in the culture in order to calculate the percentages of apoptotic osteoblasts and breast cancer cells. The monolayers of hFOBs cultured alone contained about 2% apoptotic cells. Breast cancer cell cultures were similar, between 1 and 2%. In co-culture the percentage of apoptotic hFOB cells increased several fold (8–12%) while the percentage of apoptotic breast cancer cells did not change (Fig. 2). Similar results were obtained with MDA-MB-231 cells. However, co-culture with non-metastatic MD-MBA-468 cells did not bring about an increase in apoptotic hFOB cells.

Quantitation of TUNEL positive hFOB1.19 cells after incubation with conditioned medium was also carried out by flow cytometry (Table I). Compared to vehicle conditioned medium, i.e., medium prepared like cell conditioned medium, but without cells, apoptosis increased about sixfold.

Caspase activity also increased when the osteoblasts were co-cultured with the metastatic breast cancer cells (data not shown).

Apoptosis is Induced by Breast Cancer Cell Conditioned Medium

In order to determine if the apoptosis-inducing activity of the breast cancer cells was

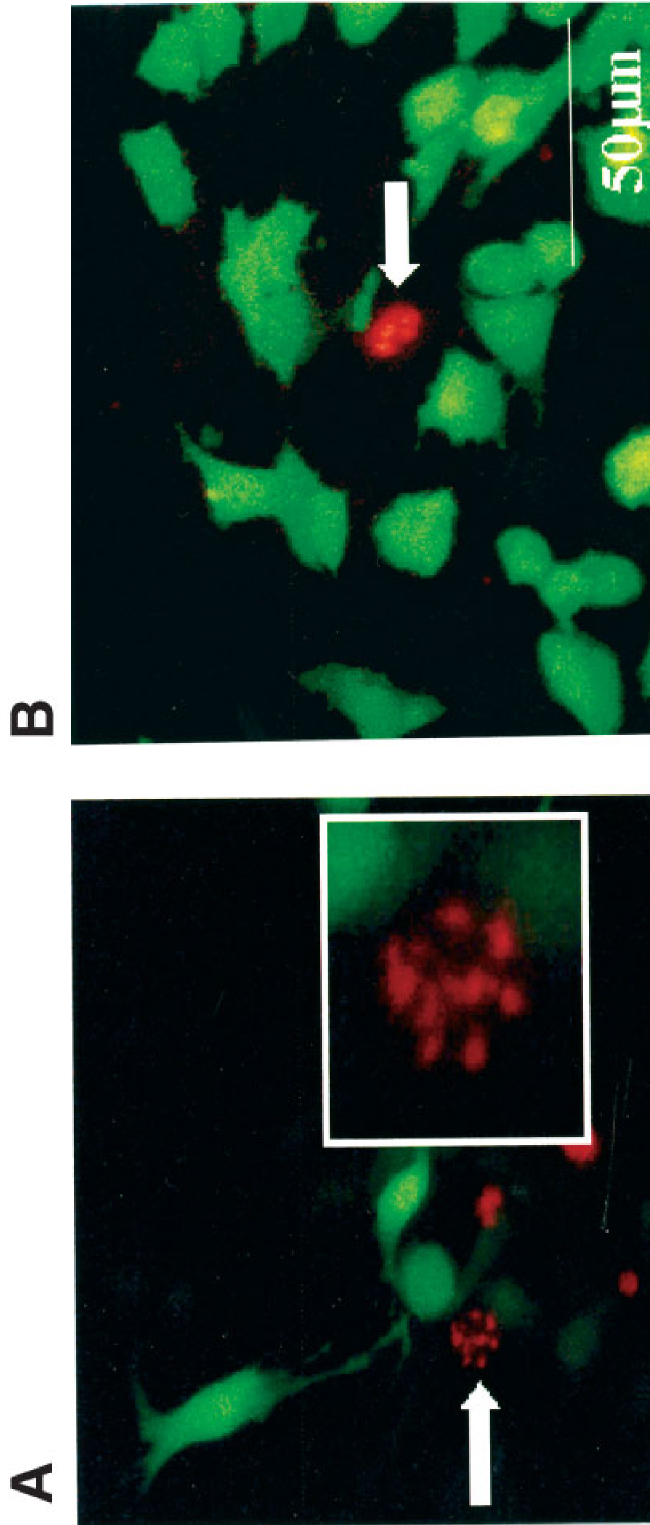

Fig. 1. Apoptotic nuclei in hFOB1.19 cells cultured with MDA-MB-435 or MDA-MB-231 cells. MDA-MB-435 cells (**A**) or MDA-MB-231 cells (**B**) expressing GFP were added to a confluent monolayer of hFOB1.19 cells (39°C) at a ratio of 1:100. After 48 h of co-culture the cells were stained with propidium iodide to detect dead cells, and immediately viewed with a fluorescence microscope. The arrows point to a fragmented nucleus (A) or condensed nucleus (B) indicative of apoptotic cells.

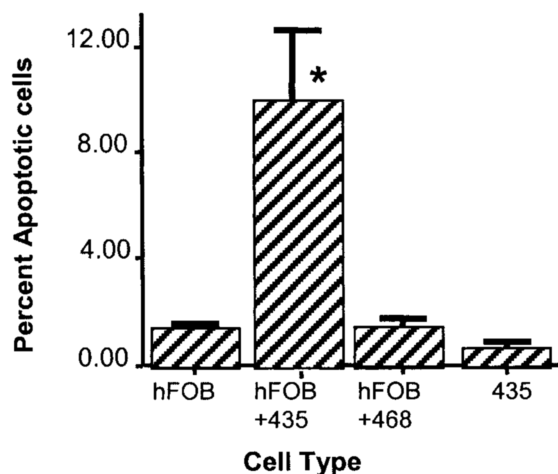

Fig. 2. Percentage of apoptotic hFOB1.19 cells in co-culture with breast cancer cells. Co-cultures of MDA-MB-435 or MDA-MB-468 cells expressing GPF were carried out as described in the legend to Figure 1. The cultures were fixed with a 2% paraformaldehyde and prepared for TUNEL assay of apoptotic nuclei using Cy5-dUTP [Jewell and Mastro, 2002]. Sytox® Orange was used as a counterstain. The cells were viewed with an Olympus BX-60 widefield digital microscope. A total of 700 fields were photographed for later analysis of TUNEL positive, GFP negative (hFOB1.19) and TUNEL positive, GFP positive (cancer cells). The percentages of apoptotic cells were calculated from the total number of cells on the coverslip. The experiment was done in triplicate and repeated. Shown are the mean ± SD. $*P < 0.01$ comparing hFOB cells plus MDA-MB-435 cells with hFOB cells alone.

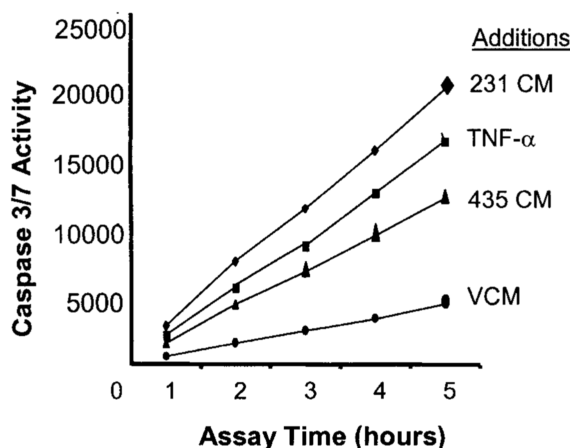

Fig. 3. Caspase activity in hFOB1.19 cells treated with breast cancer cell conditioned medium. The hFOB1.19 cells were plated at 1×10^6 per T 75 cm^2 tissue culture flask. Conditioned medium from the breast cancer cells was mixed 1:1 with normal differentiation culture medium and added to confluent, differentiated cultures of hFOBs and allowed to remain for 2 days at 39°C. Vehicle conditioned medium (VCM) was used for comparison. One set of samples was incubated with normal growth medium plus 0.3 ng/ml of TNF-α. After 48 h of incubation the cells were harvested and assayed for caspase 3/7 as described in the "Materials and Methods" section. The samples were assayed in duplicate; shown are the average values. The conditioned medium from MDA-MB-231 cells was tested seven times; from MDA-MB-435 cells three times, and TNF-α, six times with similar results.

relayed through a soluble factor, conditioned medium from the breast cancer cells lines was tested. Conditioned medium was diluted 1:1 with normal differentiation culture medium and added to confluent monolayers of hFOB1.19 cells. A vehicle control medium was mixed with culture medium as a control. After 48 h, caspase activity was assayed (Fig. 3). In this assay, conditioned medium from both MDA-MB-231 and MDA-MB-435 cells increased caspase activity two to fourfold over vehicle control medium (Fig. 3). On average there was about a twofold increase in caspase activity in the presence of conditioned medium from breast cancer cells (Table II) equivalent to treatment with 0.3 ng/ml TNF-α used as a positive control. In other

TABLE I. Quantitation of Apoptotic Osteoblasts by TUNEL Assay and Flow Cytometry

Treatment with media	Percent TUNEL positive cells	No. of expts.
[a]Breast cancer cell	4.5 ± 1.73	(6)
[b]Vehicle control	0.6 ± 0.3	(7)

The hFOB1.19 cells were treated with conditioned medium for 48 h as described in the legend to Figures 1 and 2 and the "Materials and Methods" section. The TUNEL protocol for flow cytometry (Promega) was used. Shown are the mean ± standard deviations. The number of experiments are given in parentheses. For comparison, treatment with TNF-α, 0.3 ng/ml, resulted in 5.6% TUNEL positive cells.
[a]Conditioned medium was 3 day MDA-MB-231 conditioned medium.
[b]Vehicle conditioned medium was the same as cell conditioned medium except it was not exposed to cells.

TABLE II. Caspase Activity of Osteoblasts in the Presence of Breast Cancer Conditioned Medium or TNF-α

Additions to hFOB1.19	Fold increase in caspase activity	No. of expts.
TNF-α	2.1 ± 0.4	(6)
Conditioned medium (231)	2.2 ± 0.50	(7)
Conditioned medium (435)	2.5 ± 0.2	(3)

Cells, hFOB1.19, were allowed to differentiate before they were treated with conditioned medium from MDA-MB-231 or MDA-MB-435 cells or with TNF-α (0.3 ng/ml) for 72 h. Caspase activity was assayed as described in the "Materials and Methods." Activity was compared to the values of cells incubated with vehicle conditioned medium. Shown are the average ± SD of activities. The number of experiments is given in parentheses.

experiments we determined that conditioned medium from 3T3 fibroblasts did not cause apoptosis (data not shown).

Fas is Expressed by hFOB1.19 Cells

It is reported that fas is expressed by primary cultures of osteoblasts [Kawakami et al., 1997] and that fas-ligand is expressed by breast cancer cells [Mullauer et al., 2000] and MDA-231 cells in particular [Keane et al., 1996]. Therefore, the interaction of fas with fas-ligand would seem a likely mechanism for breast cancer cell mediated apoptosis of hFOB cells. We also determined that the MDA-231 cells expressed fas-ligand mRNA. In order to ascertain that hFOB1.19 cells expressed fas, a non-cross linking biotin conjugated antibody to CD95 (anti-fas) and streptavidin RPE-Cy5 were used for flow cytometric analysis.

We found that fas was expressed both by proliferating and differentiated hFOB cells (Fig. 4). Unstained cells, cells incubated with an isotype control or cells incubated with streptavidin, but without biotinylated CD95 all showed the same low amount of background staining (Fig. 4). The breast cancer cells treated under the same conditions were negative for fas expression (data not shown). The differentiated cells always expressed greater levels of fas than the proliferating cells. The increase in fas expression in the differentiated cells versus the growing ones ranged from 2.3 to 44-fold (average of 16 within 5 experiments) depending on the length of time in differentiation medium.

Although the hFOB1.19 cells expressed fas, we considered that fas may be unable to transmit the death signal. To test this possibility, hFOB cells were incubated with cross-linking IgM CD95 alone or together with cycloheximide, an inhibitor of protein translation which sensitizes cells to fas-induced apoptosis [Mullauer et al., 2000]. CD95 alone caused about 17% apoptosis; while CD95 plus cycloheximide increased this value to about 25% when compared to cells treated with cycloheximide alone (Fig. 5). Cycloheximide consistently slightly decreased apoptosis (>100% viability) compared to cells with no treatment.

A fas-ligand over-expressing cell line, WR19L-A12 [Tanaka et al., 1998] was used to determine the sensitivity of fas expressing hFOB1.19 cells to fas-ligand induced cell death. Co-culture of hFOB1.19 cells with WR19L-A12

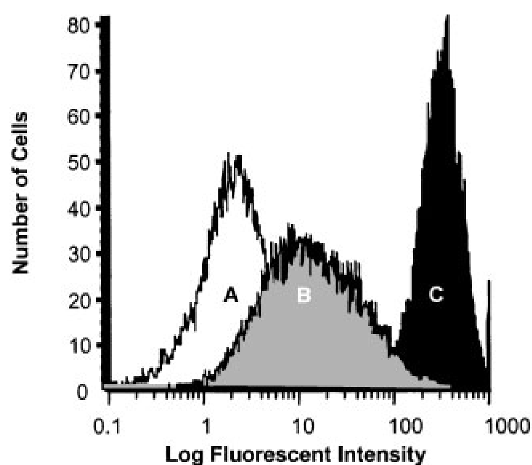

Fig. 4. Expression of fas by hFOB1.19 cells. The hFOB1.19 cells were grown at 34°C until confluent. Proliferating cells were kept at 34°C in growth medium. The other cells were switched to differentiation medium and cultured for 2 more days at 34°C. The cells were removed from the plate with Accutase^{TM} (Phoenix Flow Systems, San Diego, CA), resuspended and washed in staining solution (5% calf serum, 2.5% goat serum, 0.1% NaN_3 in complete PBS). The cells were incubated for 30 min at 4°C in the dark with biotin conjugated anti-fas (CD95, Pharmingen, Bedford, MA) 10 μl/10^6 cells; washed and resuspended with steptavidin RPE-CY5 (DAKO) 1/10 dilution (10 μl) for 20 min, 4°C, in the dark; washed and either analyzed immediately with the flow cytometer (Coulter XL-MCL) or fixed (0.2% paraformaldehyde in PBS) and analyzed the next day. An isotype matched antibody was used as a control. **A**: Cells with isotype control antibody; **B**: Proliferating cells; **C**: Differentiating cells.

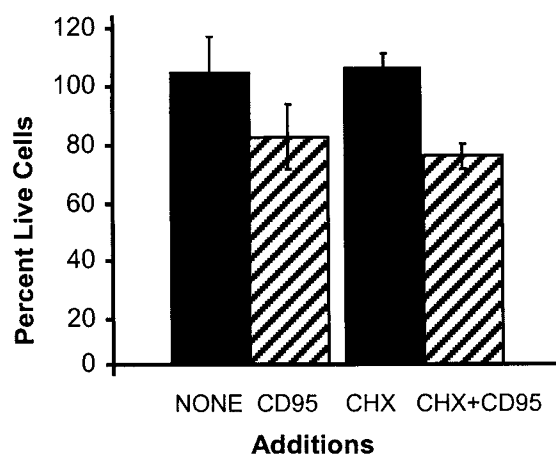

Fig. 5. Induction of hFOB1.19 cell death by anti-fas (CD95) antibody. The hFOB1.19 cells (5.4×10^4 cells) were grown in 24-well plates at 34°C until 80% confluent (about 3 days), changed to differentiation medium, and incubated at 39°C for 2 more days. Cycloheximide (10 ng/ml) and/or CD95 (anti-CD95 IgM, CH-11 Immunotech Marseille, Cedex, France) (100 ng/ml) were added and the cells incubated for an additional 48 h. Viable cells were assayed by the MTT assay following the protocol provided by Sigma. Data are expressed as percentage live cells compared to cultures with no treatment. The assay was done in duplicate and variance is indicated by the bars.

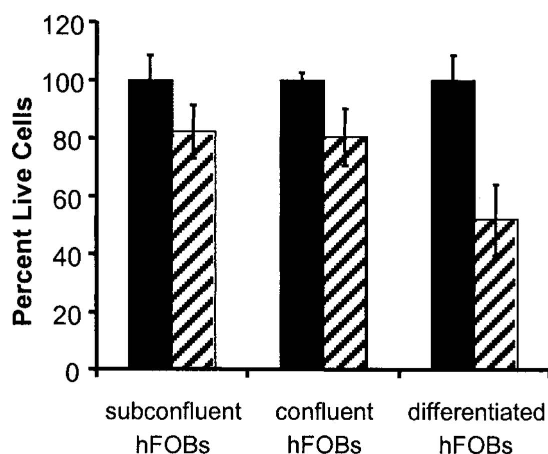

Fig. 6. Death of hFOB1.19 cells induced by fas-ligand over-expressing WR19L-A12 cells. The h-FOB1.19 cells were plated in growth medium at 5×10^3/well or 1×10^4/well in 24-well plates. After the cells plated at 1×10^4 became confluent, one half of the wells were incubated for 2 more days in differentiation medium at 39°C (differentiating) or at 34°C in regular growth medium (confluent). The cells plated at 5×10^3/well were treated when subconfluent, with either cycloheximide alone (10 ng/ml) (solid bars) or cycloheximide plus 5×10^4 WR19L-A12 cells (striped bars). After 48 h the media were removed and all the plates washed with PBS to remove non-adherent cells. The viable, attached cells, were assayed according to the MTT protocol. The experiment was carried out with triplicate samples. Shown are the average values compared to cultures without treatment.

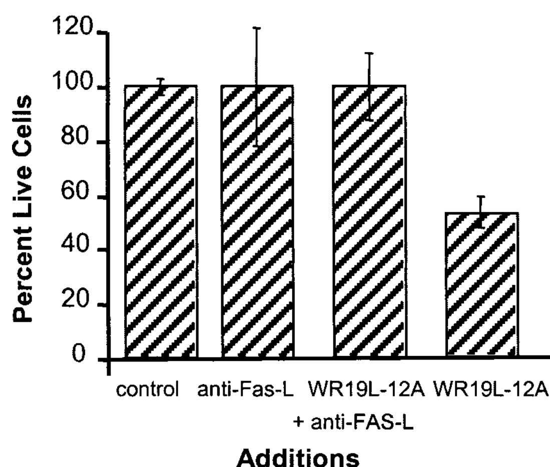

Fig. 7. Fas-ligand induced apoptosis in hFOB1.19 cells. The hFOB1.19 cells were plated at 5×10^4/well in 24-well plates in growth medium and incubated at 34°C for 3 days. The medium was changed to differentiation medium and to 39°C at 3 days when the cultures were nearly confluent. After 2 days, WR19L-A12 cells were added at 5×10^4/well to some wells. In one case the WR19L-A12 cells were incubated with anti-fas-ligand antibody (NOK-1, BD Pharmingen, 1 μg/ml) for 15 min before addition to the hFOBs. The co-cultures were incubated at 39°C for 48 h; the medium removed; and all plates washed with PBS. Remaining viable cells were assayed by the MTT assay. Shown are the average of duplicate samples with the variance.

cells for 2 days resulted in up to about 50% cell death (Fig. 6). As predicted from the data shown in Figure 4, the differentiated hFOB were more sensitive than growing or even confluent but non-differentiated cells. Cycloheximide alone did not cause apoptosis and did not enhance fas-ligand induced apoptosis by these cells.

To ascertain that the apoptosis induced by the WR19L-A12 cells was due to fas-ligand, a blocking antibody to fas-ligand was added. It prevented the WR19L-A12 cell-induced apoptosis (Fig. 7).

In order to compare the levels of fas-ligand expressed by the MDA-MB-231 and MDA-MB-435 cells with the WR19L-A12 cells, we carried out flow cytometry using a biotin conjugated, anti-fas-ligand monoclonal antibody and RPE-Streptavidin. The WR19L-A12 cells were clearly positive compared to isotype controls, but the breast cancer cells were not (data not shown). Because cancer cells often express metalloproteinase activity which may cleave fas-ligand [Kayagaki et al., 1995], they were incubated for 48 h with a metalloproteinase inhibitor, GM-6001. Nonetheless, expression of cell surface fas-ligand was not detected.

RNA was collected from the breast cancer cell lines and RTPCR was carried out to determine if fas-ligand mRNA were expressed. We detected fas-ligand mRNA in the MD-MBA-231 cells, but we were unable to detect it in the MD-MBA-435 cells (data not shown).

TNF-α Causes Apoptosis of hFOB1.19 Cells

It has been reported that human osteoblasts were sensitive to fas-induced death in the presence of TNF-α, apparently because TNF-α up-regulates fas [Tsuboi et al., 1999]. The same was true for hFOB1.19 cells. When these cells were incubated with 0.3 ng/ml TNF-α for 48 h they showed a dramatic increase in fas expression (Fig. 8). In addition it was seen that TNF-α alone induced cell death in hFOB1.19 cells (Fig. 9). Death was mediated by caspases (Fig. 10) and thus likely to occur by apoptosis. This TNF-α induced cell death was reversed by a blocking antibody to TNF-α (Fig. 11).

Conditioned medium from both MDA-MBA-231 cells and MDA-MBA-435 cells were tested by ELISA for the presence of TNF-α. None was detected at the level of 10 pgm/ml.

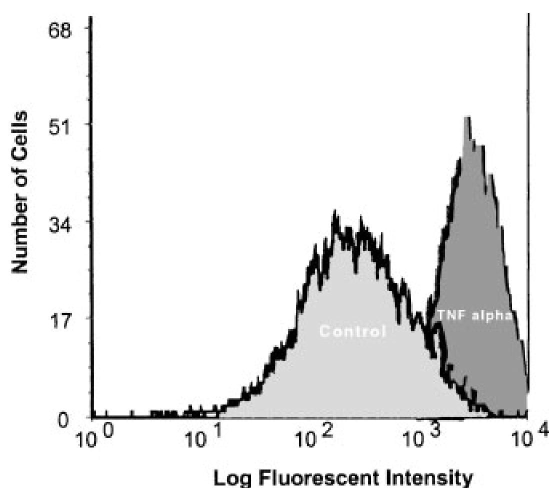

Fig. 8. Up-regulation of fas expression in hFOB1.19 cells by TNF-α. hFOB1.19 cells were plated at 5×10^5 in T 25 cm^2 flasks and incubated at 34°C overnight. The next morning the medium was changed to differentiation medium and cultures incubated at 39°C. At this time TNF-α (0.3 ng/ml) was added and 48 h later the cells were harvested and analyzed for fas expression by flow cytometry and biotin conjugated anti-fas as described in the "Materials and Methods" section and in the legend to Figure 4.

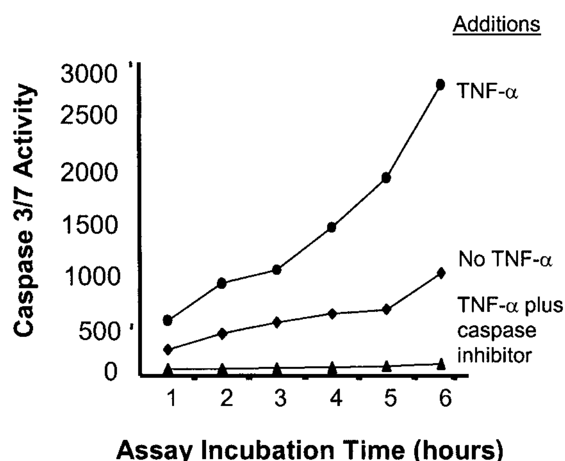

Fig. 10. Caspase activity following TNF-α treatment of hFOB1.19 cells for 48 h. hFOB1.19 cells were plated at 10^6 cells per T 75 cm^2 tissue culture flasks. The cells were allowed to differentiate at 39°C; TNF-α (3 ng/ml) was added and the flasks were incubated for 48 h more. To one flask 0.025 nM caspase inhibitor (Ac-DEVD-CHO) was added. Shown is one experiment out of five.

DISCUSSION

Osteolytic bone diseases including metastatic breast cancer disrupt the normal skeletal equilibrium so that bone formation and bone resorption are no longer coupled. As metastatic breast cancer progresses, there is severe loss of bone. Understanding exactly how that balance is upset is critical for devising methods for restoring it. It is clear that osteoclast activity increases probably due to increased osteoclastogenesis, increased osteoclast activity, and

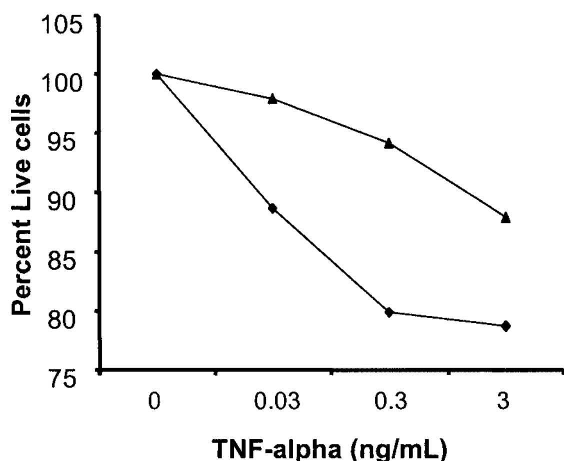

Fig. 9. Induction of apoptosis by TNF-α of hFOB1.19 cells. hFOB1.19 cells were plated at 5×10^3 well in 96-well plates and incubated at 34°C overnight. The medium in one group was then changed to differentiation medium (triangles) and cultures incubated at 39°C. The medium was replaced with growth medium (squares) in the second group and cultures were incubated at 34°C. TNF-α was added over a range of concentrations as indicated. After 48 h the cells were assayed for viability using Cell Titer 96® Aqueous Assay (Promega). Shown are the averages of triplicate samples. The standard deviations were all <0.06%.

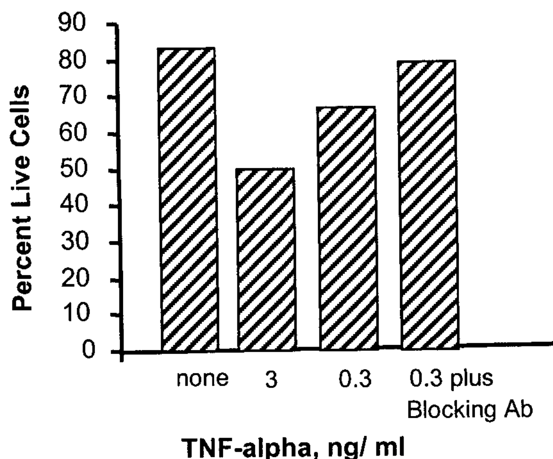

Fig. 11. Reversal of TNF-α mediated cell death with a blocking antibody to TNF-α. The hFOB1.19 cells were plated at 5×10^5 in T 25 cm^2 flasks and incubated at 34°C overnight. The next day TNF-α, pre-incubated for 10 min with neutralizing antibody to TNF-α (15 μg/ml) was added to one flask. The remaining cultures received TNF-α at two concentrations as indicated. Cells were incubated for further 48 h. Viable cells were assayed by trypan blue exclusion.

increased life span [Thomas et al., 1999]. However, we believe that the osteoblast also contributes to the imbalance.

The data presented in this study indicate that osteolytic, metastatic breast cancer cells increased the prevalence of apoptosis in a human osteoblast cell line, hFOB1.19. Apoptosis was detected by several mechanisms including TUNEL and caspase activity. Quantitation indicated that the percentage of apoptotic osteoblasts increased several fold, from about 2% to an average of 10%, in the presence of metastatic breast cancer cells or their medium. This finding is consistent with other studies of diseases or conditions that lead to bone loss such as osteoporosis [Clohisy and Ramnaraine, 1998; Clohisy, 1999], rheumatoid arthritis [Tsuboi et al., 1999; Manolagas, 2000], glucocorticoid-induced bone loss [Weinstein et al., 1998], and *Staphylococcus aureus* infection [Alexander et al., 2001]. To consider one example, glucocorticoid-induced bone loss is associated with a change in osteoblast apoptosis in vivo from about 0.7 to about 2% [Weinstein et al., 1998]. This change combined with decrease osteoblastogenesis and increased osteoclast activity is sufficient to account for the observed overall bone loss.

The skeleton is a very dynamic organ. During development, the skeleton is modeled, i.e., bone is deposited and removed in order for the skeleton to achieve its mature structure. However, the mature skeleton continues to be replaced [Manolagas, 2000]. This remodeling process that takes place in normal bone as well as damaged bone. It is estimated that the skeleton regenerates itself every 10 years [Parfitt, 1994]. Because remodeling involves a complicated interplay between osteoblasts and osteoclasts, lack of coordination results in too much bone deposition (osteopetrosis) or too much bone loss (e.g., osteopetrosis, osteoporosis). After the relatively short-lived osteoclasts degrade bone, the osteoblasts are recruited to repair it. Osteoprogenitor cells proliferate and differentiate into mature osteoblasts which produce the matrix for mineralization. At the end of this process an estimated 30–50% of the osteoblasts remain as bone lining cells or become trapped in the matrix as osteocytes. The remaining 50–70% undergo apoptosis [Jilka et al., 1998]. Apoptosis is a rapid process and there is evidence that in vivo the apoptotic osteoblasts are quickly disposed of [Cerri et al., 2003]. Nevertheless apoptotic osteoblasts can be detected under conditions of excessive loss [Jilka et al., 1998; Weinstein et al., 1998] or rapid turnover [Cerri et al., 2003]. In addition, in vitro analysis of osteoblast proliferation supports the idea that apoptosis occurs as part of the normal life cycle.

In osteolytic breast cancer metastasis the role of apoptosis of osteoblasts has not been previously investigated [Mastro et al., 2003]. Activated osteoblasts are important intermediaries in the model in which PTHrP produced by tumor cells leads to osteoclast activation. The PTHrP causes increased RANKL expression, but down-regulates OPG produced by osteoblasts. The osteoblasts in turn cause osteoclast activation. While this activation may occur in humans as part of the metastatic process, there is also clinical evidence of osteoblast loss at sites near the tumors [Stewart et al., 1982]. We suggest that osteoblasts undergo apoptosis in the presence of breast cancer cells, and loss by apoptosis must be considered in the overall equation.

What is the mechanism by which osteoblast apoptosis is induced? The specific initiators of osteoblast apoptosis under various physiological conditions are not well known. The literature suggests several initiators of osteoblast apoptosis under inflammatory conditions or during normal bone remodeling. In vitro studies indicate the necessity for fas–fas ligand interactions [Kawakami et al., 1997] in human osteoblasts apoptosis. Primary osteoblasts normally express fas. It is not clear what cells display fas-ligand. Under conditions of inflammation, activated T-cells could play this role and participate in inflammatory bone loss. Because many cancer cells including MDA-MB-231 cells have been reported to express fas-L [Keane et al., 1996; Mullauer et al., 2000] it seemed likely that the fas–fas ligand pathway is involved in breast cancer mediated osteoblast apoptosis. In order to test this, we determined that hFOB1.19 cells expressed fas; expression was greater in the more differentiated compared with less differentiated cells; and apoptosis was induced by fas-ligand over-expressing cells or through cross linking of fas by anti-fas antibody. Nevertheless we were unable to demonstrate that the cancer cells expressed fas-ligand by flow cytometry. It is known that fas-ligand can be rapidly cleaved from the surface with metalloproteinases. This is consis-

tent with our inability to detect fas-ligand with flow cytometry. Further examination by RT-PCR for the mRNA for fas-L was negative in the MDA-MB-435 cells although it was expressed in MDA-MB-231 cells. Taken together the data suggests that in this model fas-ligand is not the initiator of apoptosis.

TNF-α also has been linked to osteoblast apoptosis in vivo [Kimble et al., 1997] and in vitro [Tsuboi et al., 1999]. In this present study, we saw that the hFOB cells were susceptible to TNF-α induced apoptosis (Fig. 10). TNF-α also up-regulated fas expression in hFOB cells as previously reported for primary osteoblasts and other osteoblast lines [Tsuboi et al., 1999]. Pederson et al. [1999] report that MDA-MB-231 cells secrete TNF-α, but we were unable to detect it in the conditioned medium of either MDA-MB-231 or of MDA-MB-435 cells by ELISA. The difference may be that Pederson et al. [1999] collected three-day conditioned medium and fractionated it prior to testing, whereas we used 24 h unfractionated conditioned medium. It also is likely that in vivo, under conditions of metastatic growth where there is an inflammatory response, cytokines such as TNF-α or IL-6 would be produced by immune cells at the site.

There are other postulated inducers of osteoblast apoptosis. Apoptosis is part of the normal bone remodeling process so matrix degradation products may play a role. Inorganic phosphate [Adams et al., 2001] released from bone and RGD sequences from fibronectin and osteopontin are potent apoptogens in vitro [Perlot et al., 2002]. OPN is produced by the cancer cell lines, but without a method to detect or block such proposed apoptogens, the hypothesis remains untested.

Alexander et al. [2001] reported the up-regulation of TRAIL in bone in the presence of *Staphylococcus aureus*. We also found TRAIL expression in hFOB cells using RTPCR (data not shown). However, an inhibitor of TRAIL, DR4, did not prevent metastatic cell induced apoptosis of the hFOB cells. Further, breast cancer cell conditioned media did not up-regulate TRAIL expression by the osteoblasts. Therefore, TRAIL does not seem to be important in this case.

Other reported possibilities include bone morphogenetic protein-2 (BMP-2) [Hay et al., 2001], oxygen radicals under inflammatory conditions [Kelpke et al., 2001], or fibroblast growth factor FGF 2 [Mansukhani et al., 2000].

Fromigue et al. [2001] using a stromal cell line showed that MCF-7 breast cancer cells secrete factors that brought about osteoblast apoptosis and necrosis. However, MCF-7 cells are weakly invasive and do not normally metastasize to bone [Thomas et al., 1999]. Saunders et al. [2001] also reported that metastatic MDA-MB-435 cells form heterotypic gap junctional communication with hFOBs. Whether gap junction disruption is associated with the apoptotic process is not clear. There are many possible mechanisms for initiation of osteoblast apoptosis both in the normal remodeling process and in disease states. At this time we do not know which contribute to bone loss by osteolytic breast cancer metastases, but it is clear that breast cancer cells cause abnormally high rates of apoptosis in osteoblasts.

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