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Osteocytes Inhibit Osteoclastic Bone Resorption Through Transforming Growth Factor-β: Enhancement by Estrogen

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Abstract Osteocytes are the most abundant cells in bone and distributed throughout the bone matrix. They are connected to the each other and to the cells on the bone surface. Thus, they may also secrete some regulatory factors controlling bone remodeling. Using a newly established osteocyte-like cell line MLO-Y4, we have studied the interactions between osteocytes and osteoclasts. We collected the conditioned medium (CM) from MLO-Y4 cells, and added it into the rat osteoclast cultures. The conditioned medium had no effect on osteoclast number in 24-h cultures, but it dramatically inhibited resorption. With 5, 10, and 20% CM, there was 25, 39, and 42% inhibition of resorption, respectively. Interestingly, the inhibitory effect was even more pronounced, when MLO-Y4 cells were pretreated with 10^{-8} M 17-β-estradiol. With 5, 10, and 20% CM, there was 46, 51, and 58% of inhibition. When the conditioned medium was treated with neutralizing antibody against transforming growth factor-β (TGF-β), the inhibitory effect was abolished. This suggests that osteocytes secrete significant amounts of TGF-β, which inhibits bone resorption and is modulated by estrogen. RT–PCR and Western blot analysis show that in MLO-Y4 cells, the prevalent TGF-β isoform is TGF-β3. We conclude that osteocytes have an active, inhibitory role in the regulation of bone resorption. Our results further suggest a novel role for TGF-β in the regulation of communication between different bone cells and suggest that at least part of the antiresorptive effect of estrogen in bone could be mediated via osteocytes. J. Cell. Biochem. 85: 185–197, 2002. © 2002 Wiley-Liss, Inc.

Key words: osteocyte; osteoclast; estrogen; TGF-β; bone remodeling

Bone is a highly dynamic tissue, which undergoes continuous processes of modeling and remodeling. Bone remodeling is a complex process of tightly coordinated action of bone resorbing osteoclasts and bone forming osteoblasts. In the normal adult skeleton, bone formation takes place only at sites where bone has previously been resorbed, thereby preserving the integrity of the skeleton. Osteoblasts produce several factors by which they regulate the formation of osteoclasts and their resorption activity. In their final phase of differentiation, osteoblasts become embedded deep within the mineralized bone matrix during bone formation and become osteocytes [Palumbo et al., 1990]. Osteocytes are the most abundant cells in bone, and in normal human bone, there are approximately ten times as many osteocytes as osteoblasts [Parfitt, 1977]. During the maturation from osteoblast to osteocyte, the cellular volume and protein synthesis level is decreased [Nefussi et al., 1991], but they gain long cell processes with gap junctions in the tip [Doty, 1981].

Very little is known about the role of osteocytes in the control of bone remodeling. Osteocytes have a unique location in bone compared to osteoblasts and osteoclasts. They are regularly spaced throughout the matrix and still connected with each other via long processes. Osteocytes remain also in contact with bone surface and other cells [Kamioka et al., 2001]. This way they ensure the access of oxygen and nutrients and also the possibility of cell-to-cell signaling.

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The same origin of osteocytes and osteoblasts explains the many markers they share. Even though, there are some antibodies available for avian osteocytes [Nijweide and Mulder, 1986], the lack of a specific enzyme, cell- or matrix-protein marker for the osteocyte has made it even more difficult to isolate these cells. Due to the hard matrix around them, osteocytes have been extremely difficult to study. Kato et al. [1997] developed a unique osteocyte-like cell line, MLO-Y4 by targeting Simian Virus 40 (SV40) large T-antigen oncogene into osteocytic lineage using osteocalcin promoter. The cell line was isolated from the long bones of transgenic mice, and it appeared to have many properties of primary osteocytes. The cell line produced large amounts of osteocalcin but low amounts of alkaline phosphatase (ALP). The cells developed long dendritic cellular processes and were also positive for T-antigen, osteopontin, CD44, and connexin-43.

We have used the newly established cell line MLO-Y4 to identify potential signals between osteocytes and mature bone-resorbing osteoclasts. There are several signaling molecules, different cytokines, and chemokines produced by osteoblasts that regulate the formation and function of osteoclasts, but the production of only few of these has been described in osteocytes. Transforming growth factor-betas (TGF-βs) are multifunctional cytokines that regulate cell growth, differentiation, and adhesion [Massague et al., 2000]. The largest source of TGF-β in the body is bone and especially osteoblasts have been reported to produce large amounts of TGF-β [Robey et al., 1987, Sandberg et al., 1988, Bonewald et al., 1991]. Immunohistochemical studies have shown the presence of TGF-β also in osteocytes [Pelton et al., 1991, Thorp et al., 1992]. TGF-β in bone metabolism has been widely studied, but some controversies still exist. TGF-β stimulated the production of many bone matrix proteins including collagen and fibronectin [Massague, 1990] and it appeared to have a biphasic effect both on the differentiation of osteoclasts [Shinar and Rodan, 1990] and bone resorption [Dieudonne et al., 1991]. In this paper, we have identified TGF-β as an osteocyte-derived signaling molecule inhibiting bone resorption. Interestingly, the inhibitory effect on osteoclastic bone resorption by osteocytes was enhanced by 17-β-estradiol-treatment of MLO-Y4 cells. Thus, some functions and signaling activities of osteocytes could be mediated by estrogen. This suggests a new role for osteocytes in the control of bone remodeling cycle.

MATERIALS AND METHODS

Osteocyte and Osteoblast Cell Cultures

The immortalized mouse primary osteocyte-like cell line MLO-Y4 was kindly provided by Dr. Lynda Bonewald (University of Texas, Health Science Center at San Antonio, TX). Cell cultures were carried out in α-modified minimum essential medium (α-MEM, GIBCO BRL, Paisley, Scotland) supplemented with 5% fetal bovine serum (FBS, GIBCO BRL, Paisley, Scotland), 5% iron-supplemented calf serum (CS, HyClone Laboratories, Inc., Logan, UT), and antibiotics (penicillin/streptomycin; GIBCO BRL, Paisley, Scotland). All cultures were performed on type I collagen-coated plates (0.15 mg/ml, rat tail collagen type I; Becton Dickinson Labware, Bedford, MA). As a control, we used mouse calvaria-derived MC3T3-E1 cell that has the osteoblastic phenotype [Sudo et al., 1983]. They were cultured in α-MEM supplemented with 10% FBS and antibiotics.

Characterization of MLO-Y4 Cells

In order to confirm the phenotypic properties of MLO-Y4, we cultured the cells on collagen-coated 96-well plates with an initial cell density of 2,500 cells/well. After 72 h, the cells were fixed with 3% paraformaldehyde (PFA) for 10 min at room temperature. The immunohistochemical staining for osteocalcin (OCN) and connexin-43 (Cx43) was performed using goat antimouse OCN antibody (Paesel and Lorei, Hanau, Germany) and monoclonal mouse anti-Cx43 antibody (Zymed Laboratories, San Francisco, CA) as primary antibodies. The rest of the staining procedure was performed using a Histostain^{TM}-Plus kit (Zymed Laboratories) according to the manufacturer's instructions. Shortly, the samples were blocked for 10 min with blocking solution, the primary antibodies added as a 1:100 dilution in 1 × phosphate-buffered saline (PBS) and incubated for 1 h. Three 2-min washing steps were performed and a biotinylated secondary antibody was added. After a 10-min incubation, the plates were washed and enzyme conjugate added for 10 min. Finally, after washing, a chromogen/substrate solution (AEC) was added to the wells, incubated for 10 min, and rinsed with distilled

H_2O. All the steps were performed at room temperature (RT). Some wells were stained for alkaline phosphatase (ALP) using a histochemical kit (86-R, Sigma, St. Louis). The results were analyzed under the phase contrast microscope.

Serum-Free Conditioned Medium

MLO-Y4 and MC3T3-E1 cells were cultured near to confluence (48–72 h) and the media were changed into phenol red-free α-MEM without serum containing either 10^{-8} M 17-β-estradiol (E2, Sigma, St. Louis, MO) or carrier vehicle. The conditioned medium (CM) was collected after 24 h, centrifuged (5 min, 800 rpm), and kept at $+4^{\circ}$C. For Western blot analysis, 5 ml samples of CM were concentrated, using Ultrafree-15 concentrators (Millipore, Bedford, MA). The samples were loaded into the concentrators with Biomax membranes of nominal molecular weight limits of 10,000; 30,000; 50,000; and 100,000. Each concentrator was centrifuged for a certain period of time (from 15 up to 45 min; 1,800 rpm) and the concentrate was sterilized with 0.22 μm filters. The protein content was analyzed with the protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (BSA) as a standard.

Rat Osteoclast Culture

Boyde et al. [1984] and Chambers et al. [1984] originally introduced the method for osteoclast culture. The method was slightly modified and has previously been described in detail by Lakkakorpi et al. [1989]. Briefly, rat osteoclasts (ROC) were isolated from the long bones of 1–2-day-old pups and cultured on bovine bone slices. The culture medium was phenol red-free α-MEM with 10% FBS, antibiotics, and 20 mM HEPES (GIBCO BRL, Paisley, Scotland) with 0, 5, 10, or 20% MLO-Y4 CM. The same amount of serum-free α-MEM with antibiotics that had not been in contact with osteocytes was used as a control medium. Thus, the slight change in the percentage of serum caused by the addition of CM had no significance. ROC were cultured at $+37^{\circ}$C, 5% CO_2 for 24 h. In some experiments, 10 μg/ml of TGF-β neutralizing antibody (Pan-specific, R&D Systems, Abingdon, UK) was added to CM, before it was pipetted onto osteoclasts. After 24 h of culture, the bone slices were stained for tartrate-resistant acid phosphatase (TRAP) using a histochemical kit (386-A, Sigma Diagnostics, St. Louis, MO) and for nuclei with Hoechst 33258-stain (Sigma, St. Louis, MO), and the number of TRAP-positive multinucleated cells was counted. Then, the cells were removed by wiping the bone slices with a soft brush. After rinsing the slices in PBS, they were incubated with peroxidase-conjugated wheat germ agglutinin lectin (WGA lectin, Triticum vulgaris, Sigma, St. Louis, MO) diluted 1:50 in PBS for 20 min at RT. After rinsing with PBS for 5 min, the DAB solution (3,3′-DAB, 0.52 mg/ml in PBS containing 0.1% H_2O_2) was added and the bone slices were incubated for 20 min. The bone slices were rinsed in PBS and mounted on glass objective slides and the number of resorption pits was counted under the phase contrast microscope.

TGF-β1 Immunoassay

A solid phase enzyme-linked immunosorbent assay (ELISA) was used to detect TGF-β1 in MLO-Y4 CM. The kit (TGF-β1 ELISA, DRG Diagnostics, Marburg, Germany) uses both a polyclonal and a monoclonal antibody. For immunoassay, the cell culture samples were acidified with 1 M HCl and then neutralized with 1 M NaOH, thus activating the latent TGF-β1 in the sample. TGF-β1 determination was performed according to manufacturer's instructions.

Immunohistochemistry for TGF-β

The TGF-β isoform responsible for the inhibitory effect of osteocyte CM was determined by isoform-specific TGF-β1 and TGF-β3 antibodies (sc-146 and sc-82; Santa Cruz Biotechnology, CA). MLO-Y4 cells were cultured on type I collagen-coated coverslips for 5 days and then fixed with 3% PFA for 10 min at RT. The fixed cells were rinsed three times with PBS. The staining was performed using a Histostain™-Plus kit (Zymed Laboratories) as described above. The coverslips were mounted on objective slides and results analyzed under light microscope.

Western Blot Analysis

Western blots with TGF-β isoform-specific antibodies (Santa Cruz Biotechnology) were performed to study the expression of different TGF-β isoforms in MLO-Y4 cells. Cells were cultured on collagen-coated plates as described above, and after 72 h in culture, they were lysed using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet® P40, 0.5% sodium deoxycholat, 0.1% SDS and protease inhibitors,

(Boehringer Mannheim, Mannheim, Germany)). The sample preparation procedure for Western blotting is described in detail in Santa Cruz Biotechnology research applications instructions that were provided together with the antibodies. The protein concentration in the samples was analyzed as above. SDS–PAGE gel with 12% lower gel and 5% upper gel was prepared according to standard protocols (Bio-Rad Laboratories). The samples (cell lysates, 20 μg of protein per lane, and CM concentrates, 6.5 μg of protein per lane), controls (rhTGF-β1 or rhTGF-β3, 20 ng of protein per lane, R&D Systems), and standards (Biotinylated Protein Marker and Prestained Broad Range Protein Marker, New England Biolabs, Beverly, MA) were boiled in non-reducing sample buffer for 4 min at + 95°C and loaded into the gel. Proteins were blotted onto a nitrocellulose membrane (Protran®, Schleicher & Schuell, Dassel, Germany) using a semidry electroblotter (Bio-Rad Laboratories). The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (20 mM Tris base, 137 mM NaCl, pH 7.6) overnight at + 4°C. Subsequently, the membranes were incubated at RT for 1 h in 1:200 dilution of primary antibody (rabbit polyclonal anti-TGF-β1 or anti-TGF-β3; Santa Cruz Biotechnology), and bound antibodies were detected with horse radish peroxidase (HRP)-conjugated anti-rabbit (1:2,000) antibody and standards with anti-biotin (1:1,000) antibody (New England Biolabs) with a further incubation of 45 min at RT. The membranes were exposed to X-ray film (Kodak, Rochester, NY) using enhanced chemiluminescense detection system (LumiGLO reagent, New England Biolabs).

RNA Isolation and RT–PCR

To further evaluate the expression levels of TGF-β isoforms in MLO-Y4 and MC3T3-E1 cells, RT-PCR was performed. Cells were plated at 2×10^4 cells/cm^2 in 100-mm-diameter tissue culture plates and cultured to confluence (48–72 h). In some experiments, MLO-Y4 cells were treated with 10^{-10}, 10^{-9}, or 10^{-8} M estrogen for 24 h prior to RNA isolation. Total RNA was isolated by a single-step method using RNA STAT-60™ reagent (Tel-Test, Inc., Friendswood, Texas) according to manufacturer's instructions. Briefly, 5 μg of total RNA was subjected to cDNA synthesis in a 20 μl reaction mixture containing 0.5 μg of oligo d(T) primer, 1 × PCR-buffer, 2.5 mM $MgCl_2$, 1 mM dNTP mixture, 10 mM DTT, 1 U RNAse H, and 2 U of Superscript II reverse transcriptase (all reagents from Gibco, Superscript II kit). cDNA was amplified using PCR in a 50-μl-reaction mixture containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 1 mM dNTP mixture, 3 ng of primers, and 1 U of Tag DNA polymerase. Amplifications were performed in a Mastercycler® (Eppendorf, Hamburg, Germany) for 30 cycles following the reaction profile: 95°C for 1 min, 55°C for 50 min, and 72°C for 1 min. The following primers were used: TGF-β1: 5′-TGA GTG GCT GTC TTT TGA CG, 3′-AC GAA GTC GAG GTG TCT CTC; TGF-β3: 5′-CTC TCT GTC CAC TTG CAC CA, 3′-CA GTG TGG AAA GTC GGG TTA. The final products were analyzed on 1.5% agarose gel.

RESULTS

Characterization of MLO-Y4 Cells

Kato et al. [1997] reported in their original publication that MLO-Y4 cells produced large amount of osteocalcin (OCN) but low amounts of alkaline phosphatase (ALP), had stellate morphology, and gap junctions between the cells. The cell line also expressed large amount of connexin-43 (Cx43), a gap junction protein. In our hands, the cell line displays the similar phenotypic characteristics. As shown in Figure 1a, the cells have long dendritic processes and cell-to-cell contacts. No positive staining for ALP is seen (Fig. 1b). The positive staining for OCN and Cx43 was detectable in nearly all cells (Fig. 1c,d).

Inhibition of Osteoclastic Bone Resorption by Osteocyte Conditioned Medium (CM)

The osteocyte CM had no effect on ROC number in 24-h cultures (Fig. 2A), but dramatically inhibited pit formation in a dose dependent manner. With 5, 10, and 20% CM, there was 25, 39, and 42% inhibition of resorption, respectively (Fig. 2B). Conditioned medium from control cells (MC3T3-E1) had no effect on resorption (Fig. 3).

Inhibition of Resorption Enhanced by Estrogen

When MLO-Y4 cells were treated with 10^{-8} M 17-β-estradiol (E2) for 24 h and then this E2-treated CM was added in osteoclast culture, there was no effect on ROC number (data not shown), but the inhibition of resorption was even more pronounced. With 5, 10, and 20% CM,

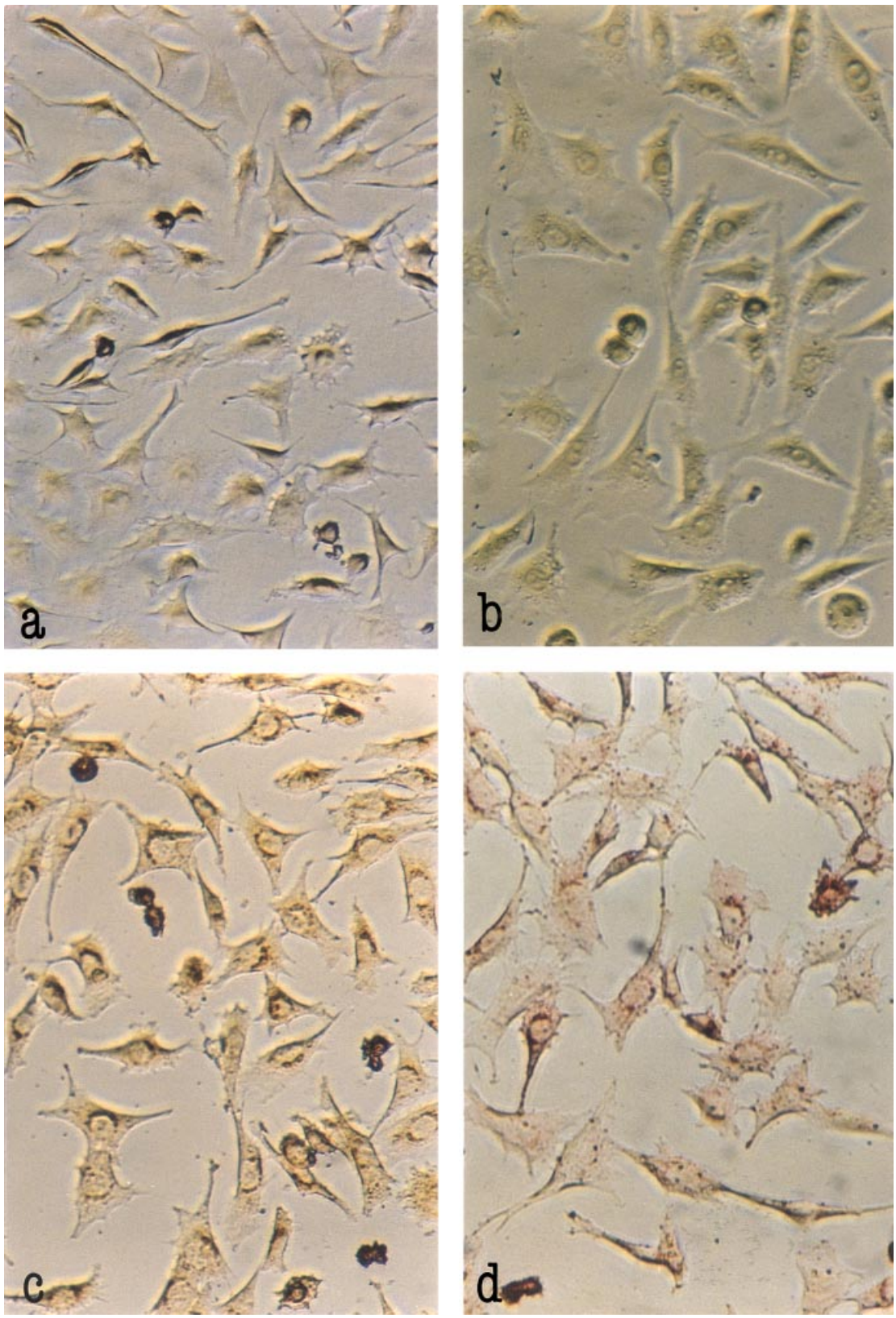

Fig. 1. Phase contrast photomicrographs of osteocytic MLO-Y4 cells. Note the stellate morphology and extensive dendritic processes (**a**). The cells were grown on collagen-coated plates for 72 h, fixed and stained for ALP (**b**), OCN (**c**), or Cx43 (**d**) as described in the text. Original magnification is 20 ×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

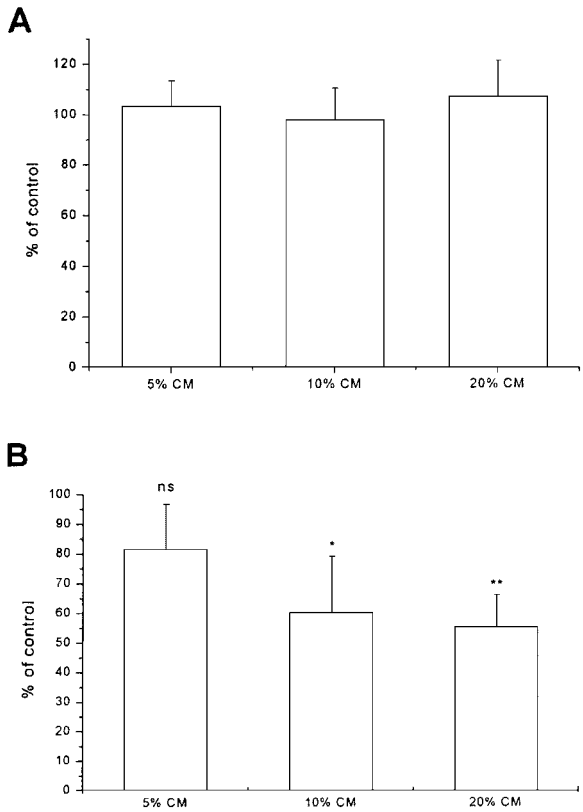

Fig. 2. The effects of MLO-Y4 CM on rat osteoclast number (**A**) and resorption activity as pits per osteoclast (**B**) in 24-h culture. The means + SD values are represented as percentages of control. The control level of resorption is 91 pits/bone slice for (B). The results are obtained from eight separate experiments with four replicate bone slices each. Serum-free α-MEM with antibiotics that had not been in contact with osteocytes was used as a control medium. In (B), the statistical differences were determined using One-Way ANOVA with the significance level of 0.05; ns = not significant; $*P < 0.05$; $**P < 0.01$.

there was 46, 51, and 58% of inhibition (Fig. 3). With the E2-treated CM from MC3T3-E1 cells, there was no inhibition of resorption. Treatment of MLO-Y4 cells with 10^{-8} M parathyroid hormone (PTH) did not have any effect on the capacity of CM to inhibit osteoclastic bone resorption (data not shown).

Inhibitory Factor Is TGF-β

Resorption was brought back to the control level when CM was incubated with pan-specific TGF-β neutralizing antibody (Fig. 4). This antibody recognizes all the main isoforms of TGF-β, i.e., TGF-β1, pTGF-β1.2, pTGF-β2, TGF-β3, and raTGF-β5. Hence, on the basis of this result it was not possible to conclude which isoform was responsible for this effect. We used both immunohistochemical staining and RT–PCR to study in more detail the expression of

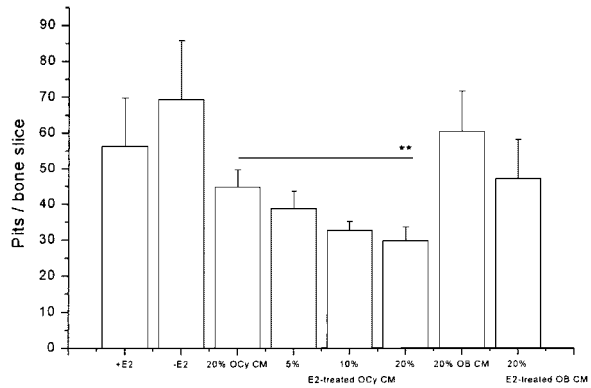

Fig. 3. The effect of estrogen (E2) treatment of conditioned medium (CM) on rat osteoclast resorption. The first two bars represent the controls with (+ E2) or without (− E2) 10^{-8} M estrogen. The 20% osteocyte (OCy) CM control and E2-treated OCy CM (5, 10, or 20%) are followed by 20% MC3T3-E1 osteoblast (OB) CM control, and the final column represents the 20% of E2-treated OB CM. The means ± SD values are from two separate experiments with four replicate bone slices each. The statistical difference between 20% normal and 20% E2-treated OCy CM was determined using One-Way ANOVA with the significance level of 0.05; $**P > 0.01$.

TGF-β1 and TGF-β3 isoforms in osteocytes. The immunohistochemical staining of MLO-Y4 cells with isoform-specific antibodies showed background level of staining for TGF-β1 (Fig. 5a,b), but the staining with TGF-β3 antibody was very intense (Fig. 5c). The osteoblastic MC3T3-E1 cells used as a control showed the most intense staining with TGF-β1 antibody (data not shown). By RT–PCR, the expression of TGF-β1 and TGF-β3 was detected in both cell lines, MLO-Y4, and MC3T3-E1 (Fig. 6A). When MLO-Y4 cells were treated with 10^{-8}, 10^{-9}, or 10^{-10} M estrogen, the level of TGF-β3 expression seemed to be higher than the level of TGF-β1 (Fig. 6B). Even though the RT–PCR is not a quantitative method, this result suggests that 17-β-estradiol actually has an isoform-specific effect in MLO-Y4 cells. However, it is important to remember that the cells produce TGF-β in a latent form. Thus, using only mRNA levels for quantitation may not be relevant.

TGF-β1 Immunoassay

In order to quantitate the amount of TGF-β1 isoform produced by MLO-Y4 cells, TGF-β1 immunoassay was performed. In the amounts of TGF-β1, there were no statistically significant differences between vehicle- and estrogen-treated CM. In vehicle-treated CM, the levels between four replicates were ranging from 199–266 pg/ml (mean 233 pg/ml, SD 29.3) and in E2-

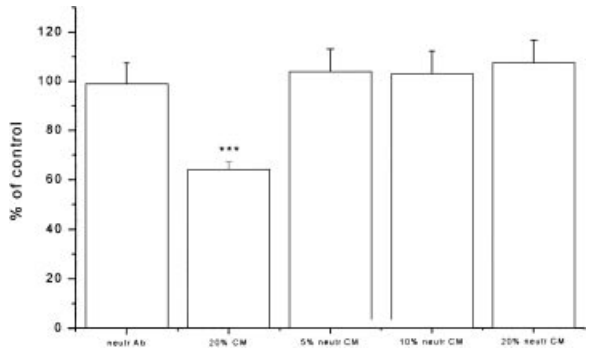

Fig. 4. The effect of TGFβ-neutralization of MLO-Y4 CM on rat osteoclast resorption activity as pits per osteoclast. The means + SD values are represented as percentages of control. The results are obtained from four separate experiments with four replicate bone slices each. The control level of resorption is 93 pits/bone slice. The first bar (neutr Ab) represents the neutralizing antibody alone and the following bar (20% CM), the non-neutralized CM control. The statistical difference between neutralizing Ab control and 20% CM was determined using One-Way ANOVA with the significance level of 0.05; $^{***}P < 0.001$. The last three bars represent the increasing (5, 10, and 20%) concentrations of TGFβ-neutralized CM that brings the resorption back to the control level.

treated CM 235–277 pg/ml (mean 259 pg/ml, SD 19.8), respectively. This further supports the RT–PCR result that in osteocytes TGF-β1 is not the isoform that is being upregulated with estrogen.

Western Blot for TGF-β

Because there is no functional immunoassay available for TGF-β3, we used Western blot to further compare the protein levels of TGF-β1 and TGF-β3 in osteocyte cell lysates and CM concentrates (Fig. 7). Western blot with TGF-β1-specific antibody showed very weak or no bands in all samples (Fig. 7A, lanes 1–4 and 5–7). The rhTGF-β1 used as a control in turn showed one prominent band, approximately 25-kDa in size representing the mature, active form of TGF-β1 (Fig. 7A, lane 8). Western blot with TGF-β3-specific antibody showed a prominent 25-kDa band of rhTGF-β3 (Fig. 7B, lane 8). Surprisingly, in all MLO-Y4 cell lysates (Fig. 7B, lanes 5–7), a band of approximately 75-kDa in size was seen. The concentrates of CM

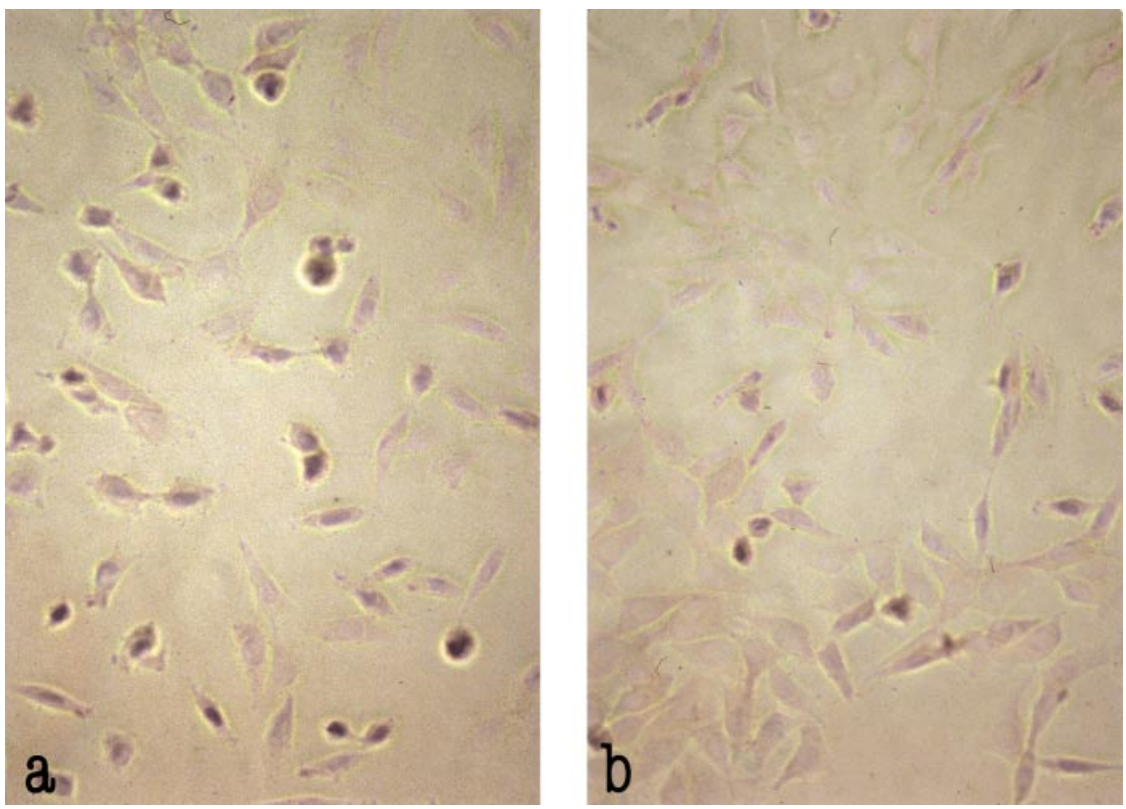

Fig. 5. Photomicrographs of TGF-β isoforms in MLO-Y4 cells. The staining was performed with isoform-specific TGF-β antibodies as described in the text. **a**: No primary antibody (negative control), (**b**) staining with TGF-β1 antibody, and (**c**) staining with TGF-β3 antibody. Original magnification is 10 ×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

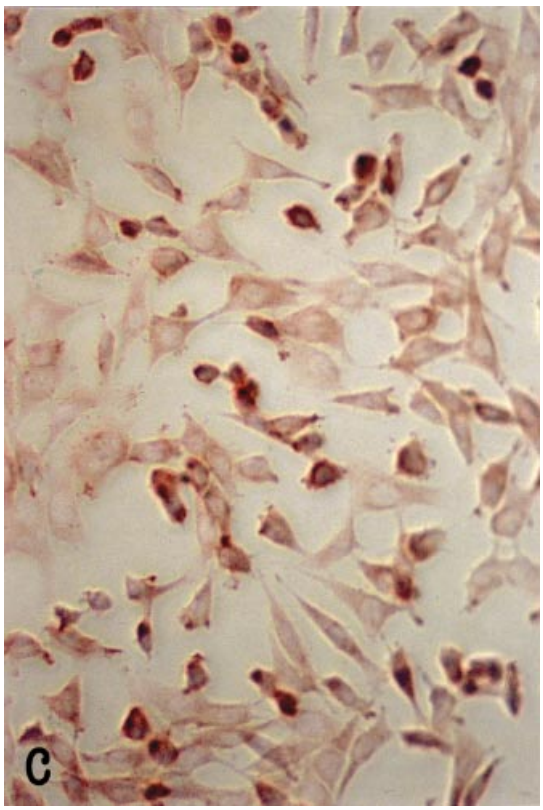

Fig. 5. (*Continued*)

(Fig. 7A,B, lanes 1–4) did not show very clear bands with either antibody, suggesting that there is not enough specific protein to be detected. The 75-kDa fragment in cell lysates probably represents the precursor homodimer from which the mature form has been cleaved.

DISCUSSION

We describe here that MLO-Y4 cells can be used as a model in studying the biochemical interactions between osteocytes and osteoclasts. MLO-Y4 cells produce TGF-β that is responsible for inhibiting osteoclastic bone resorption without affecting the number of mature osteoclasts. Interestingly, TGF-β production seems to be upregulated by 17-β-estradiol. We have shown previously that MLO-Y4 cells express estrogen receptors [Matikainen and Väänänen, 1999], and our new data suggests that in osteocytes these receptors are functional and respond to E2 by producing active TGF-β.

Bone is a highly dynamic tissue, which undergoes continual processes of modeling and remodeling [Parfitt, 1994], and these actions must be tightly controlled. It has been suggested that osteocytes have a central role in controlling bone turnover as an "operations center" (Marotti, 2000), also via biochemical means. Transforming growth factor-betas (TGF-βs) are multifunctional cytokines with a wide distribution in the body. The largest

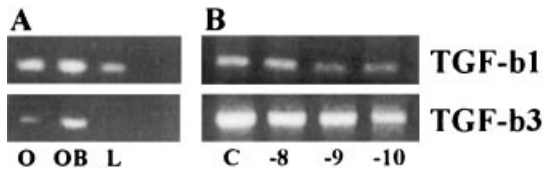

Fig. 6. RT-PCR for analysis of TGF-β1 and TGF-β3. Five micrograms of total RNA was subjected to RT–PCR using specific primers for TGF-β1 and TGF-β3. **A**: Both TGF-β1 and TGF-β3 mRNA can be detected in MLO-Y4 (O) and MC3T3-E1 (OB) cells. In liver (L) only TGF-β1 mRNA is present. **B**: Subconfluent MLO-Y4 cells were treated with vehicle (c) or 10^{-8}, 10^{-9}, or 10^{-10} M estrogen for 24 h prior to RNA isolation. Both TGF-β1 and TGF-β3 mRNA can be detected in all MLO-Y4 samples, but TGF-β3 shows more intense bands than TGF-β1.

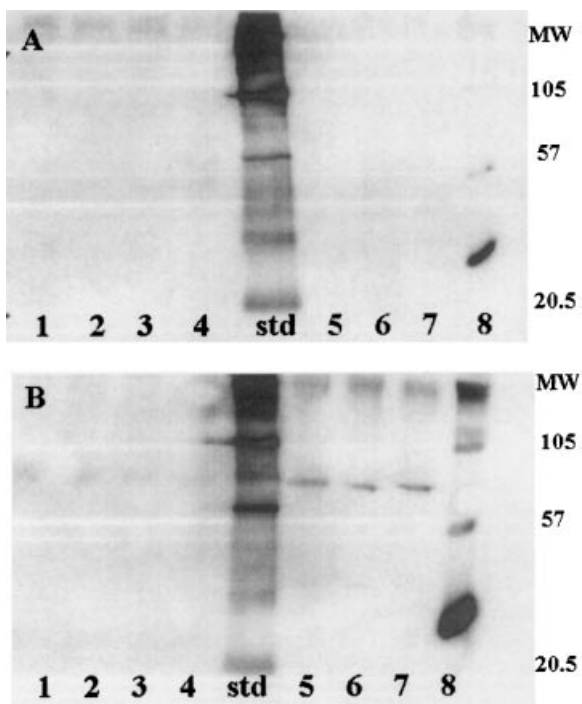

Fig. 7. Western blot analyses of TGF-β1 (**A**) and TGF-β3 (**B**). CM concentrates (6.5 μg total protein) were loaded into **lanes 1–4**, the biotinylated protein marker was loaded into the middle and MLO-Y4 cell lysates (20 μg total protein) into **lanes 5–7**. As a control, rhTGF-β1 or rhTGF-β3 (20 ng) was loaded into the **lane 8**. Isoform-specific rabbit polyclonal anti-TGF-β1 or anti-TGF-β3 was used as a primary antibody. The most prominent molecular weight (MW) markers are indicated. A: Only the mature 25-kDa rhTGF-β1 in the control can be visualized. B: In all cell lysate samples, the 75-kDa precursor form of TGF-β3 can be visualized.

source, however, is bone, and the concentrations of TGF-βs correlate positively with the levels of bone remodeling [Pfeilschifter et al., 1998, Bismar et al., 1999]. The expression of different TGF-β isoforms and responses to exogenous TGF-βs vary in different cell types and stages of development. In developing mouse embryo, the isoform TGF-β1 has been localized in the periosteum of bones and the maturing chondrocytes, while TGF-β2 and TGF-β3 isoforms were found in osteocytes [Pelton et al., 1991]. Thorp et al. [1992] described that most of the cells on the bone surface showed staining for all three TGF-β isoforms, but the staining for TGF-β3 was generally more intense than that for TGF-β1 and -β2. Osteocytes located near the osteoid surface were positive for TGF-β, but osteocytes located deep in the bone matrix were frequently negative. Our finding that TGF-β3 is the major isoform in MLO-Y4 osteocytes, is particularly interesting, as TGF-β1 is the major isoform produced by osteoblasts. The change from the isoform TGF-β1 to TGF-β3 may represent a switch from osteoblastic to osteocytic phenotype. The osteoblastic MC3T3-E1 control cell line expresses also TGF-β3 as determined by RT–PCR, immunohistochemistry and Western blot, but to a much lesser extent than the osteocytic MLO-Y4 cell line (data not shown). Unfortunately, there is no functional immunoassay available to measure the TGF-β3 isoform in these cell lines.

The role of TGF-β in bone resorption is still controversial and most likely dependent on the relative prevalence of precursor and mature osteoclasts in the culture system used. The effect of TGF-β on the differentiation of osteoclasts has been described to be biphasic [Shinar and Rodan, 1990], while other investigators have reported that TGF-β had only an inhibitory effect on osteoclast-like cell formation in human and mouse bone marrow cultures [Chenu et al., 1988; Hattersley and Chambers, 1991]. However, at the same time, TGF-β simultaneously stimulated the resorption activity. Dieudonne et al. [1991] demonstrated that low concentrations were inhibitory and high concentrations stimulatory on bone resorption. In our in vitro assay, the MLO-Y4 CM did not have any effect on the number of mature osteoclasts during the 24-h culture period, but the inhibition of resorption was already significant (42% inhibition of resorption with 20% CM). This report is the first one to analyze the TGF-β in osteocytic cell line and involves a totally new experimental set-up combining MLO-Y4 cells and ROC resorption cultures. Thus, it is difficult to have a straight comparison with other studies. Maejima-Ikeda et al. [1997] have described an 18.5-kDa chick osteocyte-derived protein that inhibited osteoclastic bone resorption. They used mouse, rabbit, or human giant tumor cell osteoclasts for bone resorption assay but did not see any effect with primary chick osteocyte CM. Lately, a totally new mechanism involving RANKL and OPG has been proposed for the regulation of osteoclast differentiation and function [Takahashi et al., 1999]. Both OPG and RANKL can themselves also be regulated by TGF-β [Murakami et al., 1998; Takai et al., 1998]. Also in these studies, the effect of TGF-β1 on osteoclast formation seems to be dependent on the culture system used [Sells Galvin et al., 1999].Very recently, Fuller et al. [2000] suggested a role for TGF-β1 acting together with RANKL in enhancing the osteoclastogenesis from bone marrow precursors and monocytes. Interestingly, it has been reported that MLO-Y4 cells express both RANKL and OPG [Zhao et al., 1999]. In this study, the osteoclast activity was assayed in a mixed cell population and it is possible that other cell types and molecules may partially mediate the inhibitory action of TGF-β. Especially OPG, being a soluble factor, may in turn contribute to the inhibition of resorption seen by MLO-Y4 CM, either directly or via TGF-β.

When studying the biological effects of TGF-β, one must consider the mechanisms by which it transmits the signals from cell to cell. TGF-β is usually secreted and stored by osteoblasts mainly in its latent form [Bonewald and Dallas, 1994; Dallas et al., 1994]. It has been described that greater homology exists between the mature regions as compared to the precursor regions, suggesting that the precursor regions may provide the distinct functions of different TGF-β isoforms. On the basis of our Western blot analysis, it seems that MLO-Y4 cells contain the precursor form of TGF-β3 because only the 75-kDa band in the cell lysate samples was detected. In an effort to analyze TGF-βs in concentrated CM, no signal was detected. The reason may be that the amount of TGF-β in CM is so small that the detection limit of Western blot is not high enough. We also do not know in which part of the TGF-β molecule is the epitope where isoform-specific antibodies bind. Latent

TGF-β may be produced and secreted by one cell type and activated in conjunction with another adjacent cell type in vivo, representing a paracrine mode of action. Thus, the latent TGF-β produced by osteocytes may be further activated in rat osteoclast cultures by osteoclastic enzymes such as matrix metalloproteinase-9 (MMP-9) or TRAP. It has been reported that TGF-β is released locally in the bone microenvironment when osteoclasts degrade bone matrix [Pfeilschifter and Mundy, 1987], and both the acidic environment and osteoclastic enzymes can activate TGF-β [Oursler, 1994]. MMP-9 is a crucial enzyme in the bone resorption process [Kusano et al., 1998], and recently it has been reported that MMP-9 proteolytically activated TGF-β in tumor-associated tissue remodeling [Yu and Stamenkovic, 2000].

E2-treatment of MLO-Y4 cells enhanced the inhibitory effect on osteoclastic bone resorption by almost 20%, which suggests that E2 upregulates TGF-β production by MLO-Y4 osteocytes. Estrogen has been reported to negatively regulate osteoclast formation and function but how this takes place, is far from clear. Because TGF-β also decreases osteoclast-mediated bone resorption, these effects may be linked. The presence of estrogen receptors (ER) has been demonstrated in osteocytes [Braidman et al., 1995; Hoyland et al., 1997; Vidal et al., 1999]. The deficiency of ovarian steroids and idiopathic osteoporosis has been found to be associated with the ERα expression in osteocytes [Hoyland et al., 1999; Braidman et al., 2000]. The interactions of estrogen and TGF-β seem to be relevant also in vivo as it has been reported that ovariectomy lowers both the TGF-β mRNA and protein concentration in rat bone, but the treatment with E2 eliminates the TGF-β deficit [Finkelman et al., 1992; Ikeda et al., 1993]. In vitro data suggests that this results from the increased osteoblast activity [Oursler et al., 1991; Hughes et al., 1996]. Our results suggest that in osteoblasts 10^{-8} M E2 does not regulate the production of TGF-β3. However, when using only one dose of E2, it is not possible to conclude that E2 would be totally ineffective in MC3T3-E1 cells. Komm et al. [1988] reported that E2 enhanced TGF-β mRNA levels in cultured human osteoblast-like cells. It is possible that the same kind of mechanism is functional also in osteocytes, especially when the cells are modulating the effects of mechanical loading and hormonal status. This is further supported by the results of Bodine et al. [1998], who reported that E2 upregulates TGF-β mRNA in rat calvarial osteoblasts in late mineralization stage. Also parathyroid hormone (PTH) has been reported to behave similarly stimulating TGF-β protein and mRNA levels [Oursler et al., 1991]. In our experiments, PTH treatment of MLO-Y4 did not enhance the inhibition of resorption, suggesting that the mechanisms of TGF-β upregulation may differ between different cell types and TGF-β isoforms. Interestingly, 17-β-estradiol, as well as tamoxifen and TGF-β1, have been reported to promote apoptosis of murine osteoclasts and these effects were abolished by anti-TGF-β antibody, indicating that TGF-β1 may mediate this effect [Hughes et al., 1996]. The response of different TGF-β isoforms to different hormones varies. Robinson et al. [1996] reported that estradiol was found to stimulate TGF-β production by osteoclasts, and interestingly caused a shift from TGF-β2 to TGF-β3. Besides, estradiol and raloxifene stimulated TGF-β3 expression in bone and TGF-β3 inhibited osteoclasts [Yang et al., 1996), which is in agreement with our results, because we found that TGF-β3 seems to be the major isoform responsible for the inhibitory effect. So far it has been unclear why in cultured cells only certain SERMs and E2 metabolites, and not E2, have a potent effect on the TGF-β3 promoter. The new data by Lu and Giguere [2001] shows that E2 acts as a potent inducer of the TGF-β3 promoter via a novel ER-α-mediated mechanism. This action requires the dual activation of Ras-dependent pathways by one or more growth factors produced locally in bone. All this suggests a central role for estrogen, estrogen receptors, and osteocytes in the control of bone turnover.

Bone is a specialized tissue, which undergoes continuous remodeling in terms of osteoclastic bone resorption and osteoblastic bone formation. These events occur in temporally and spatially coordinated ways, and thus they must be tightly regulated. Osteocytes are the only type of bone cell that is enclosed within the bone matrix. They contact each other and cells on the bone surface with long cytoplasmic processes, and therefore, it is possible that they transmit signals both to osteoblasts and osteoclasts. This mechanism may have significance in vivo e.g., during aging and after osteocyte apoptosis. When osteocytes grow old or die, the inhibitory effect of osteocytes is removed, and osteoclastic

bone resorption may proceed in an accelerated manner. There are reports about glucocorticoid-induced osteocyte apoptosis [Weinstein et al., 1998] and that bisphosphonates and calcitonin can prevent these events [Plotkin et al., 1999]. The death of osteocytes has also been associated with estrogen withdrawal [Tomkinson et al., 1997, 1998] and bone with high remodeling activity [Noble et al., 1997].

In this article, we take advantage of a newly developed osteocyte-like cell line, MLO-Y4, and describe the use of in vitro assay, which may be relevant for studying both soluble and physical signaling between osteocytes and osteoclasts. On the basis of literature and our present data, we conclude that osteocytes have an active, inhibitory role in the regulation of bone resorption. This would suggest a link between osteocyte apoptosis, estrogen levels, and increased bone turnover. Our results further suggest a novel role for TGF-β in the regulation of communication between bone cells.

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