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Human giant cell tumors of the bone (osteoclastomas) are estrogen target cells

(osteoclasts/bone resorption/lysosomal enzymes/steroid hormone/tartrate-resistant acid phosphatase)

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ABSTRACT **The decrease in estrogen levels that follows the onset of menopause results in rapid bone loss and osteoporosis. The major effect of estrogen deficiency on bone metabolism is an increase in the rate of bone resorption, but the precise mechanism by which this occurs remains unresolved. A recently developed technique for the isolation of avian osteoclasts has been modified to obtain highly purified multinucleated cells from human giant cell tumors. These osteoclast-like cells have been examined for evidence of estrogen receptors (ERs) and responses to 17β-estradiol (17β-E_2). Analysis of giant-cell RNA demonstrated expression of ER mRNA. Furthermore, immunoblot analysis revealed that the giant cells contained a 66-kDa protein that was recognized by a monoclonal antibody specific for the human ER. When isolated multinucleated cells were cultured on slices of bone, there was a dose-dependent decrease in resorption in response to treatment detectable at 10 pM 17β-E_2. Treatment with 10 nM 17α-estradiol or vehicle (control) did not inhibit resorption. Moreover, the multinucleated cells isolated from these tumors had decreased mRNA levels for cathepsin B, cathepsin D, and tartrate-resistant acid phosphatase (TRAP) as well as secreted cathepsin B and TRAP enzyme activity in response to treatment with 10 nM 17β-E_2. In contrast to these data, no change in gene expression was detected in mononuclear cells from these tumors in response to 17β-E_2 treatment. These data support the proposition that human osteoclasts are target cells for estrogen and that estrogen can inhibit bone resorption by human osteoclasts.**

Normal bone metabolism involves a balance between osteoclast-mediated resorption and osteoblast-mediated formation. This interaction, referred to as coupling, maintains overall bone mass throughout early adult life. For bone loss to occur in metabolic bone disease such as osteoporosis, alterations in this balance lead to a relative increase in resorption over formation. Bone resorption rates are determined by both the number and activity of osteoclasts. Many hormonal signals influence osteoclast differentiation, recruitment, and/or activity. In this context, osteoclast dysfunction appears to be a major factor in postmenopausal osteoporosis, and decreased estrogen levels, which are associated with menopause, have been implicated in the pathogenesis of this disease (1). Pilbeam *et al.* (2) have shown an estrogen-induced decrease in resorption in fetal mouse calvaria, and this laboratory has demonstrated that avian osteoclasts contain estrogen receptors (ERs) and respond to estrogen treatment with decreased resorption activity (3). Moreover, Pensler *et al.* (4) have presented evidence that human osteoclasts contain ERs, but the influence of estrogen on human osteoclast activity remained unresolved. To determine if there is an estrogen effect on osteoclast activity in humans, we have examined multinucleated cells from human osteoclast-like giant cell tumors of the bone for evidence of ERs and responses to 17β-estradiol (17β-E_2) treatment. These cells have been reported to exhibit all documented characteristics of authentic osteoclasts, including the ability to form pits when cultured on slices of cortical bone and are therefore appropriate osteoclast surrogates (5). An efficient technique for the isolation of highly purified avian osteoclasts was adapted to separate multinucleated cells from mononuclear cells in the human giant cell tumors (6). These multinucleated cells were examined for evidence of estrogen responsiveness.

METHODS

Cell Isolation. Human giant cell tumors were obtained from the Department of Pathology after surgical procedures at the Mayo Clinic. The protocol has been approved by the Mayo Foundation Internal Review Board. Tumors were transported on ice in sterile buffer to the laboratory, and the cells were separated by modification of the technique of Collin-Osdoby *et al.* (6). Briefly, the tumors were minced in sterile Ca^{2+}/Mg^{2+}-free Tyrode's balanced salt solution and pelleted by centrifugation at 900 rpm (300 × *g*) for 10 min. The tumor pieces were suspended in dispase (Boehringer Mannheim) and digested at 37°C with occasional vigorous agitation for 20 min. Released cells were separated from undigested material by passage through a 350-μm nylon mesh. The cells were pelleted by centrifugation at 900 rpm for 10 min and an avian osteoclast-directed monoclonal antibody (121; a gift from P. Osdoby, Washington University, St. Louis) coupled to immunomagnetic beads (Dynal, Great Neck, NY) was used to obtain antigen 121-positive (121^+) cell populations that contain at least 90% pure multinucleated and 10% or less unidentified mononuclear cells. Avian osteoclasts purified by this method exhibit all of the phenotypic attributes of osteoclasts including multinucleation, ruffled border formation when cultured with bone particles, and the ability to form resorption pits when cultured on slices of cortical bone (6). The number of total cells, multinucleated cells (more than three nuclei), and 121^+ (bead-binding cells) were scored. The antigen 121-negative (121^-) cells were obtained from the nonmagnetically sorted cells.

Culture and Photography. Isolated 121^+ cells were cultured at 37°C for 24 hr in α minimal essential medium (MEM) without phenol red (GIBCO) supplemented with 10% (vol/vol) charcoal-stripped fetal calf serum under an atmosphere of 5% CO_2/95% air. Cultures were viewed on a Leitz Diaplan

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Abbreviations: 17β-E_2, 17β-estradiol; ER, estrogen receptor; TRAP, tartrate-resistant acid phosphatase; 121^+, antigen 121-positive.

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microscope by phase-contrast microscopy and photographed.

RNA Isolation and Reverse Transcription and Polymerase Chain Reaction (PCR) Analysis. Total RNA was purified from freshly isolated multinucleated cells, T-47D, and MCF7 human breast cancer cell line by the method of Chomczynski and Sacchi (7). Specific sequences in the reverse transcription product (1 μl) were analyzed essentially as described by Saiki et al. (8) with sequence-specific synthetic oligonucleotide primers (see Fig. 2). The product was electrophoresed on 1.5% agarose gels in 0.04 M Tris acetate/0.002 M EDTA buffer containing ethidium bromide at 1 μg/ml. PCR product bands were eluted, extracted with phenol/chloroform, and precipitated. The PCR product was sequenced by using a double-stranded DNA cycle sequencing system (GIBCO/BRL) and the second-round PCR upstream oligonucleotide primer.

Immunoblot (Western Blot) Analysis. Cell extracts from freshly isolated 121^+ cells were analyzed as described (3) for the presence of ER protein by using a purified monoclonal antibody (5 μg/ml) to the human ER (H222, a gift from G. Greene, University of Chicago) and an alkaline phosphatase-conjugated secondary antibody. Immunoreactive proteins were visualized by incubation in nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 20 min.

Quantitative Pit Resorption Assay. Separated 121^+ cell isolates were pelleted at 500 × g for 5 min, then resuspended in phenol red-free MEM containing 10% charcoal-stripped fetal calf serum (Irvine Scientific), and plated on 1-mm^2 slices of cortical bone prepared and analyzed as reported (3). The effect of treatment with each dose of steroid was compared with controls by the Student two-tailed paired t test.

Gene Response Studies. 121^+ cells were centrifuged at 500 × g for 5 min. Cell pellets were resuspended in phenol red-free MEM containing 10% charcoal-stripped fetal calf serum and were plated with 1 mg of unlabeled bone particles per 10^6 cells in the presence of vehicle (control) or 10 nM 17β-E_2 in a 5% CO_2/95% air chamber at 37°C for 24 hr. Cells were harvested by scraping the cells into the culture medium on ice and sedimenting them at 900 × g for 5 min. The cells were then used for RNA isolation as described below.

RNA Isolation and Northern (RNA) Blot Analysis. Total RNA was obtained for steady-state mRNA studies from the cell pellets obtained from the cell cultures (see above) by the method of Chomczynski and Sacchi (7). Each RNA (typically 1 μg) was separated on a 1% (wt/vol) agarose-glyoxal gel and transferred to a nylon membrane (Micron Separations). The RNA blots were hybridized for 18 hr at 43°C with cDNAs for tartrate-resistant acid phosphatase (TRAP) (a gift from Michael Roberts, University of Missouri, Columbia), cathepsin B (a gift from Donald F. Steiner, University of Chicago), or cathepsin D (a gift from Stuart Kornfeld, Washington University, St. Louis) labeled with ^{32}P by random primer extension using a Multiprime kit (Amersham). cDNA probes were stripped off, and the blots were reprobed with a cDNA for an 18S rRNA probe to determine if the sample loading was equivalent. The blot was washed twice with 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% SDS for 15 min at 43°C and was analyzed by autoradiography and subsequent densitometric analysis with a Shimadzu CS90004 dual-wavelength flying-spot scanning densitometer (Kyoto). Densitometric data are presented as means ± SEM of the ratio of treated to control samples after adjusting for lane loading, with 18S densitometry for all experiments performed.

Enzyme Activity Assays. Conditioned medium from 18-hr cultures of 121^+ cells treated with vehicle or 10 nM 17β-E_2 was harvested and microcentrifuged at 10,000 × g for 30 min. Cathepsin B activity was assayed by using N^{α}-carbobenzoxy-Arg-Arg 7-amido-4-methylcoumarin (Sigma) as the substrate as outlined by Barrett and Kirschke (9). TRAP activity was measured as the rate of hydrolysis of O-carboxyphenol by the method of Hofstee (10). Cell number was standardized between samples by DNA determination (11).

RESULTS

We have extended and adapted the procedure of Collin-Osdoby et al. (6) to purify human osteoclast-like cells from giant cell tumors of the bone. The cultured 121^+ cells isolated by this method were highly purified and estimated to contain at least 95% multinucleated cells (Fig. 1). The dark spherical particles visible on the cells are the 4.5-μm immunomagnetic beads. Table 1 details the age, sex, location, and characteristics of the tumors that were the source for the studies on bone resorption and gene expression reported here. Although there were a large number of multinucleated cells present in the one soft tissue-derived giant cell tumor that was included in these studies, no 121^+ multinucleated cells were obtained.

Nested PCR analysis revealed that human giant cell tumor cells contained ER mRNA (Fig. 2). DNA sequence analysis revealed that the PCR product was identical to that of the human ER (data not shown). Cell extracts from isolated 121^+ cells were probed by Western blotting for evidence of the ER protein by using a well-characterized monoclonal antibody (H222) that recognizes the 66-kDa human ER (12) (Fig. 3). There were two protein species recognized by H222: a 140-kDa protein and a 66-kDa protein. There were no bands

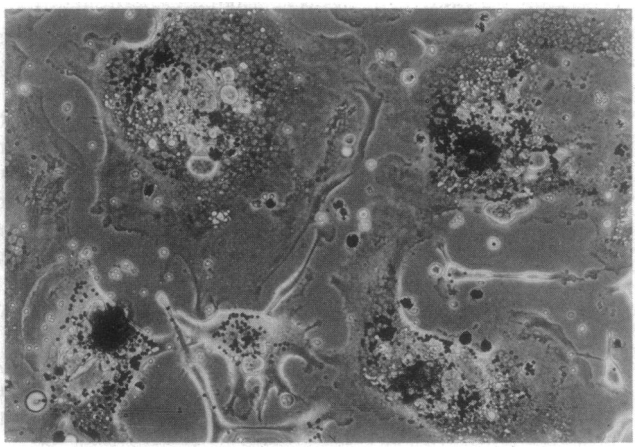

FIG. 1. Phase-contrast microscopy of 121^+ cells. The osteoclast-like cells were cultured for 24 hr. Dark spheres are the 4.5-μm-diameter immunomagnetic beads. (×220.)

Table 1. Characteristics of tumors used in gene response studies

Age, yr	Sex	Tumor site	Giant cells %*	Giant cells No. × 10^{-6}†	Giant cells % 121^+‡
39	F	Left proximal fibula	2	1.2	97
18	M	Left tibia	10	4.6	95
29	F	Left proximal femur	5	1.8	96
22	M	Soft tissue left leg	21	9.7	0
37	M	Right ischium	1	1.5	94
37	F	Sacrum	7	2.3	98

Characteristics of giant cell tumors that were examined for the effects of 17β-E_2 on lysosomal enzyme steady-state mRNA levels.
*The percent giant cells were determined from cell counts of the original cell suspension. Cells with three or more nuclei were scored as giant cells.
†The number of giant cells were determined from cell counts of the original cell suspensions.
‡The percentage of giant cells that were 121^+ was determined from cell counts of the final 121^+ isolates and comparison with the number of giant cells (second column from right).

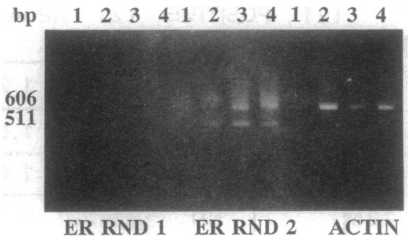

FIG. 2. PCR analysis for ER mRNA. RNA (5 μg) was analyzed by PCR for the presence of ER or actin mRNA. PCR products were analyzed as described in text. Locations of primers in the nucleotide sequence were as follows: first-round ER, GenBank locus *HUMERMCF* positions 1550–1565 (5′-GGCATGGTGGAGATCT-3′) and 2091–2077 (5′-CCAGGGAGCTCTCAG-3′); second round ER, GenBank locus *HUMERMCF* positions 155–1565 and 2076–2061 (5′-ACTGTGGGAGGGAAA-3′); and actin, GenBank locus *HUMACCYBA* positions 2096–2114 (5′-TCATGTTTGAGACCTTCAA-3′) and 2072–2684 (5′-GTCTTTGCGGATGTCCACG-3′). Lanes: 1, no RNA control; 2, 121$^+$ giant cell tumor cells; 3, MCF7 cells; 4, T-47-D cells; ER RND1, PCR analysis for ER round 1 using first-round primers; ER RND2, PCR analysis of a 1:100 dilution of ER RND1 product using second-round primers; Actin, PCR analysis for using actin primers. The expected size of the actin PCR product is 606 bp, and the expected size of the ER RND2 PCR product is 511 bp.

observed in the identical companion blot, which received no H222 antibody.

Isolated 121$^+$ cells were cultured for 18 hr on slices of cortical bovine bone in the presence of 17β-E_2 in a range of concentrations or vehicle (control). There was a dose-dependent decrease in the number of pits per cell detectable at 10 pM 17β-E_2 (Fig. 4 *Upper*). Quantitative examination of the areas of excavation formed by these cells revealed that 10 μM 17β-E_2 treatment resulted in a significant decrease in the mean surface area of the pits when compared with vehicle control samples (Fig. 4 *Lower*). In contrast with these data, 10 nM 17α-estradiol-treated 121$^+$ cells formed resorption pits that were not significantly different in surface area when compared with vehicle control.

Since 17β-E_2 treatment of the 121$^+$ cells resulted in a decrease in bone resorption, we examined 10 nM 17β-E_2 regulation of 121$^+$ cell lysosomal enzymes after 18 hr of estradiol treatment. To determine the hormone responsiveness of the mononuclear 121$^-$ cells present in the tumor, separate cultures of these cells were examined. Cathepsin B mRNA was decreased in the 121$^+$ cells in response to 17β-E_2 (Fig. 5). In contrast, there was no apparent response in the 121$^-$ cells from the bone-derived cell isolates or in the 121$^-$ mixed mononuclear and multinucleated cells obtained from the soft tissue-derived giant cell tumor (Fig. 5). The blots

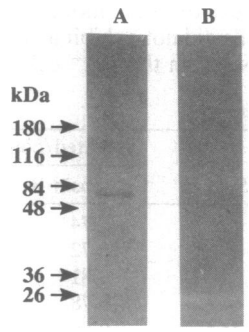

FIG. 3. Western blots for the ER. Isolated 121$^+$ multinucleated cells were analyzed by SDS/PAGE and subsequent protein blotting. Lanes: A, sample incubated with a monoclonal antibody to human ER; B, sample incubated with normal rat serum. Both lanes A and B were then incubated with the subsequent reaction components. Sizes are indicated in kDa.

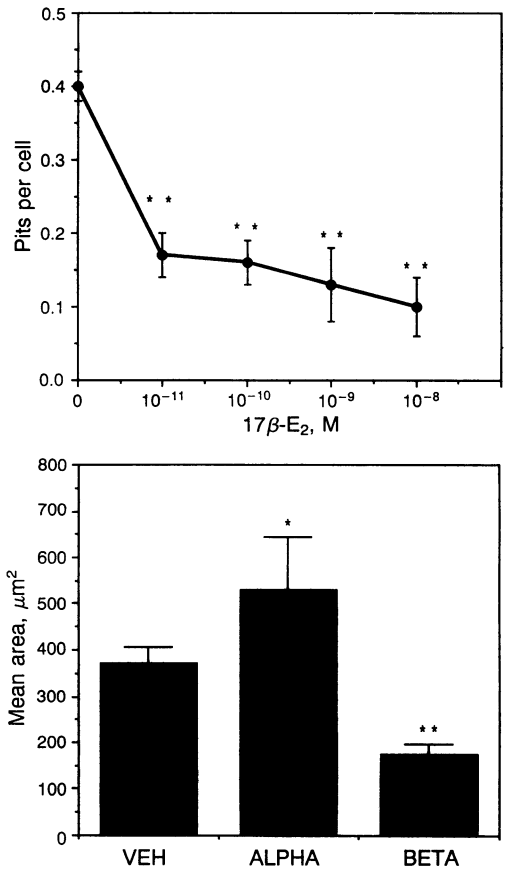

FIG. 4. Quantitative pit formation assay for osteoclastic bone resorption activity. (*Upper*) Isolated 121$^+$ cells from the 37-year-old male (see Table 1) were cultured with ethanol (0) or the indicated molar concentration of 17β-E_2. (*Lower*) Isolated 121$^+$ cells from the 37-year-old female (see Table 1) were cultured on bone slices with ethanol [vehicle (VEH)], 10 nM 17α-E_2 (ALPHA), or 10 nM 17β-E_2 (BETA). After 18 hr, bone slices were harvested and processed as described in text. Data are presented as the mean (n = four slices) ± SEM and are representative of three separate experiments from three separate patients. *, $P < 0.1$; **, $P < 0.01$—both compared with vehicle control.

were probed with an 18S ribosomal RNA probe to assure equal relative lane loading. Cathepsin D mRNA levels in all 121$^+$ cultures examined were similarly decreased in response to 17β-E_2 (Fig. 6). Regulation of cathepsin D mRNA was not observed in the 121$^-$ cells (Fig. 6). TRAP steady-state mRNA levels were examined in the cell populations after 17β-E_2 treatment. After 18 hr of culture with 10 nM 17β-E_2, the 121$^+$ cell steady-state mRNA levels for TRAP were decreased, whereas the 121$^-$ cells showed no apparent modulation of mRNA levels for TRAP (Fig. 7). Densitometric analyses confirmed these observations (Figs. 5–7).

Cathepsin B and TRAP activity levels were measured in the conditioned medium from 121$^+$ cells from five separate tumors (Table 2). Secreted cathepsin B and TRAP levels were uniformly decreased in 17β-E_2-treated cultures when compared with vehicle (control)-treated cultures. There appeared to be no influence of patient sex or age on the responsiveness of the cell cultures at either the steady-state mRNA or secreted protein levels.

DISCUSSION

To better understand steroid hormone influences on human osteoclast activity, we have initiated studies using human giant cell tumors of the bone as surrogate human osteoclasts. More detailed discussions of the characteristics of these

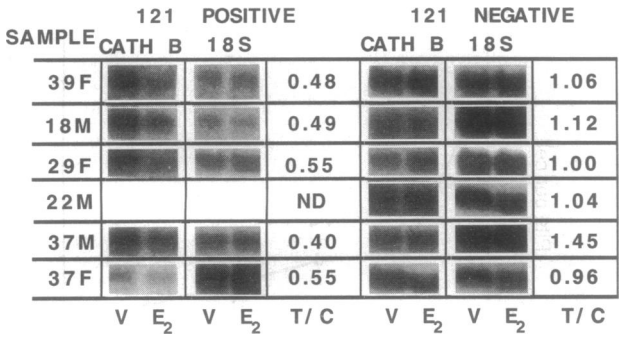

FIG. 5. Northern blot analysis of cathepsin B steady-state mRNA levels. Isolated 121^+ cells and 121^- cells from a series of patient tumors were cultured for 24 hr, and total RNA was isolated and blotted as described in text. Blots were probed to determine the steady-state mRNA level of cathepsin (CATHB) in either ethanol [vehicle (V)]- or 10 nM 17β-E_2 (E_2)-treated cultures followed by reprobing to determine the amount of 18S ribosomal RNA in each lane. Densitometric analysis (T/C) is calculated as described in *Methods*.

tumors have been presented by Hanaoka *et al.* (13), Steiner *et al.* (14), Kasahara *et al.* (15), Goldring *et al.* (16), Joyner *et al.* (17), Wood *et al.* (18, 19), and Burmester *et al.* (20). In this report we have extended these studies to demonstrate that monoclonal antibody directed against avian osteoclasts also recognizes multinucleated cells from human giant cell tumors of the bone. PCR analysis demonstrated that the 121^+ multinucleated cells expressed mRNA for the ER. Furthermore, Western blot analysis using a monoclonal antibody directed against the human ER demonstrated that the human 121^+ cell extracts detected a 66-kDa protein, as reported previously for avian osteoclasts (3), which is the appropriate size for the ER.

Osteoclasts resorb bone by forming an external lysosome-like compartment juxtaposed to the bone surface (21, 22). To explore the mechanism of estrogen action on resorption activity, we investigated 17β-E_2 modulation of three osteoclastoma lysosomal proteins—i.e., TRAP and two cathepsins, B and D. TRAP is a lysosomal-associated enzyme that is present in large amounts in both osteoclasts and osteoclastomas (19). Although the precise role of TRAP in osteoclast activity is poorly understood, antibodies directed against TRAP inhibit bone resorption (23), and elevated TRAP levels have been associated with increased resorption activity (24). Several lines of evidence have suggested that cathepsins are likely to be involved in the resorption process. Studies using protease inhibitors support the possibility that cysteine-rich proteases are implicated in the bone resorption

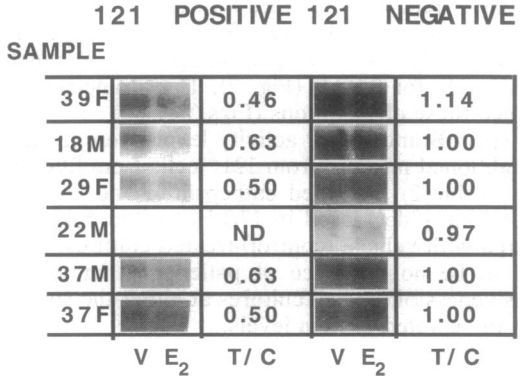

FIG. 6. Northern blot analysis of cathepsin D steady-state mRNA levels. The same blots examined in Fig. 5 were stripped and reprobed to determine the steady-state mRNA level of cathepsin D in either ethanol [vehicle (V)]- or 10 nM 17β-E_2 (E_2)-treated cultures. Densitometric analysis (T/C) is calculated as described in *Methods*.

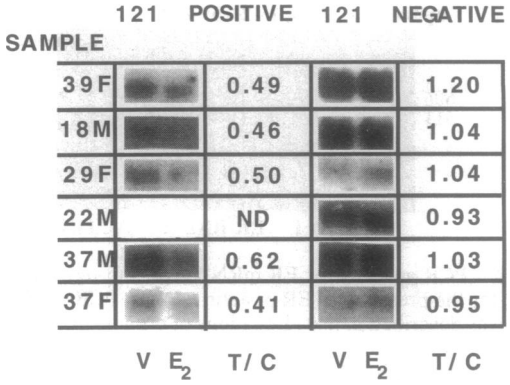

FIG. 7. Northern blot analysis of TRAP mRNA levels. The same blots examined in Fig. 5 were stripped and reprobed to determine the steady-state mRNA level of TRAP in either ethanol [vehicle (V)]- or 10 nM 17β-E_2 (E_2)-treated cultures. Densitometric analysis (T/C) is calculated as described in *Methods*.

process (25). Moreover, an acidic collagenase related to cathepsin B has been isolated from avian osteoclasts (26). Further, Rifkin *et al.* (27) have demonstrated high levels of cathepsin B and L in isolated avian osteoclasts, thus strengthening the potential for lysosomal enzyme involvement in bone resorption.

The above considerations resulted in our investigation of TRAP, cathepsin B, and cathepsin D mRNA levels after 17β-E_2 treatment of two separate cell types from human giant cell tumors—i.e., the 121^+ and the 121^- cells. We have demonstrated steroid dose-dependent decreases in the steady-state mRNA levels of two lysosomal-associated proteins, lysozyme and a lysosomal membrane protein (LEP-100), after 18 hr of treatment in avian osteoclasts (3, 28). The native hormone, 17β-E_2, causes a comparable decrease in lysosomal protein mRNAs in human osteoclast-like cells. Since the inactive stereoisomer 17α-estradiol did not cause a similar gene response in the 121^+ cells, these effects appear to be specific to the active isomer, 17β-E_2. Based on our more extensive data relative to avian osteoclast responses to 17β-E_2, we selected 18 hr of treatment with either vehicle or 10 nM 17β-E_2. Since the affinity of 17β-E_2 for its receptor is in the nanomolar range, treatment with 10 nM 17β-E_2 results in receptor saturation and, thus, a rapid and maximal response. Furthermore, 17β-E_2 has a very low affinity for other steroid hormone receptors; thus, it is unlikely that we are measuring a response mediated by these other receptors.

The 121^+ cells from giant cell tumors obtained from both males and females uniformly demonstrated a 17β-E_2-induced decrease in lysosomal steady-state mRNA and protein secretion levels, whereas the 121^- mononuclear and the mixed soft tissue tumor cells did not exhibit a comparable response. The lack of response from the 121^- cells suggested that the

Table 2. Enzyme activity

Age, yr	Sex	Treated/control ratio	
		Cathepsin B	TRAP
26	F	0.74	0.72
26	F	0.72	0.46
40	M	0.81	0.75
30	M	0.78	0.60
28	M	0.80	0.87

Analysis of Cathepsin B and TRAP enzyme activity levels in conditioned media from 121^+ cultures obtained from five patients. The age and sex of the patients are indicated. Measured enzyme activity was standardized to the amount of DNA recovered from each culture to correct for plating efficiency. Data are presented as the ratio of 10 nM 17β-E_2-treated samples to control (vehicle) values.

responding cells in the 121^+ cell isolates were the 121^+ cells and not the 5% contaminating 121^- cells. These data were strengthened by the absence of detectable ER protein in the mononuclear cells. We conclude from these data that human surrogate osteoclasts are estrogen target cells and respond to estrogen treatment *in vitro* with decreased resorption activity. These responses may involve, at least in part, a modulation of lysosomal enzyme mRNAs. These data are consistent with the current postulate that physiological estrogen levels in premenopausal women suppress osteoclast-mediated resorption activity and that the decreased estrogen levels in postmenopausal women aggravate the negative bone balance often seen in these patients. Furthermore, these data suggest that one effect of estrogen on bone metabolism is to directly regulate bone resorption activity.

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