Avian osteoclasts as estrogen target cells

(steroid hormone/steroid receptor/bone resorption/protooncogene expression)

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ABSTRACT Although in vivo effects of 17β-estradiol (estrogen) on bone turnover have been shown to occur mainly through influences on osteoclast-mediated bone resorption, the mechanism by which estrogen reduces bone resorption is unclear. To approach this question, we have examined authentic osteoclasts for evidence of a direct osteoclast response to estrogen in vitro. Highly purified (>90%) viable avian osteoclasts from birds maintained on a low calcium diet were obtained using an osteoclast-specific monoclonal antibody coupled to magnetic beads. Isolated cells were either analyzed directly for estrogen receptor (ER) levels or cultured to assess the biological effects of estrogen. Northern blot analysis revealed a 5.2-kilobase mRNA that hybridized with a cDNA to human ER mRNA in the osteoclasts. An anti-human ER antibody recognized proteins of 66 kDa and 140 kDa in osteoclast extracts by Western blot analysis. The 66-kDa size is in close agreement with the reported size of the human ER. Nuclear binding of estrogen to intact viable osteoclasts was steroid-specific and saturable, with 5662 ± 1420 molecules bound per nucleus (mean ± SEM). In vitro estrogen responses in osteoclasts included a dose-dependent decrease in resorption as well as an increase in nuclear protooncogene mRNA levels. These observations indicate that osteoclasts are capable of directly responding to estrogen in vivo.

Postmenopausal osteoporosis is a major health problem in the United States resulting in 1.5 million fractures and costing $7 to $10 million each year (1). 17β-Estradiol (estrogen) deficiency has long been recognized as a cause of postmenopausal osteoporosis and estrogen replacement therapy is an effective treatment for the prevention of bone loss (2–6). Formerly, it was believed that the effects of estrogen on bone were indirect since early studies were unable to identify estrogen receptors (ERs) in bone tissue. Recent reports, however, have demonstrated that cells of the osteoblast lineage contain ERs and respond physiologically to estrogen (7, 8). These findings were paradoxical because the primary biological effect of estrogen on bone in vivo is to decrease bone resorption (2–6) and resulted in the hypothesis that estrogen effects on osteoblast activity may be regulated through the osteoblast.

On the basis of immunohistochemical localization and radioimmunoassay studies of bone samples from four children, Pensler et al. (9) have suggested that human osteoclasts may contain ERs. However, these studies were not conclusive and, to date, the direct effects of estrogen on osteoclasts have yet to be demonstrated. This report describes the use of an immunomagnetic separation method (10) for the isolation of highly purified viable avian osteoclasts to assess the potential for a direct estrogen effect on osteoclasts in vitro. The results presented show unequivocally that not only are functional ERs and their mRNAs present in these osteoclasts, but also estrogen rapidly modulates steady-state mRNA levels of osteoclast nuclear protooncogenes c-fos and c-jun. We also demonstrate that estrogen directly inhibits osteoclast resorption activity in vitro.

METHODS

Cell Isolation. Osteoclasts were isolated from birds maintained on a low calcium diet for a period of 4 weeks (11). An osteoclast-directed monoclonal antibody (12) coupled to immunomagnetic separation beads was used to purify osteoclasts from cultures that contain at least 90% osteoclasts and 10% or less unidentifiable mononuclear cells. These cells have all 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 (10). Purified cells were either analyzed directly for ER mRNA or cultured for estrogen-response studies (see below). Marrow-derived multinucleated giant cells were obtained by culturing isolated marrow mononuclear cells for 12 days as described (13).

Resorption Assay. Osteoclast cell pellets were resuspended in phenol red-free α minimal essential medium (α-MEM, GIBCO) containing 10% (vol/vol) charcoal-stripped fetal calf serum (Irvine Scientific). Cells (20,000 osteoclasts per sample) were cultured with 0.1 ml (100 μg) of [3H]proline-labeled bone particles (14) per sample in a 5% CO2/95% air chamber at 37°C for the required period of time in the presence of vehicle (control) or the indicated concentration of estrogen. The released [3H]proline was quantitated as directed in Blair et al. (14). The specific activity of the bone particles was determined by acid hydrolysis and counting replicates of 100 μg of bone particles.

Gene Response Studies. Osteoclast cell pellets were resuspended in α-MEM supplemented with 10% fetal calf serum (as above) and plated with unlabeled bone particles (1 mg per 10⁶ cells) in the presence of vehicle (control) or the indicated concentration of estrogen for 30 min in a 5% CO2/95% air chamber at 37°C. Cells were harvested by scraping cells in culture medium on ice and centrifuging at 900 × g for 5 min.

RNA Isolation and Northern Blot Analysis. Total RNA was obtained for ER mRNA studies from freshly isolated osteoclasts, separated mononuclear contaminating cells, avian liver tissue, and oviduct by the method of Chomczynski and Sacchi (15). Studies of osteoclast gene responses utilized osteoclast pellets from cultures (see above) for a source of total RNA. Each RNA (10 μg) was separated on a 1% agarose/glyoxal gel and transferred to a nylon membrane (MSI, Westboro, MA). The RNA blots were hybridized for 18 hr at 43°C with a cDNA to the human ER (for ER message

Abbreviation: ER, estrogen receptor.
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Nuclear Binding Assay. Steroid receptor nuclear binding assay was performed as described (16) on freshly isolated osteoclasts. This assay measures the relative amounts of functional ER [i.e., the level that can be activated and bound to the nuclear acceptor sites in isolated cells (16)]. Briefly, freshly isolated cells were suspended in basal medium Eagle (BME) (Irvine Scientific). Replicate samples of the cell suspension (20,000 cells per replicate) were incubated with [3H]estradiol (New England Nuclear) and a 10-fold excess of unlabeled progesterin (a synthetic progesterone used to block nonspecific steroid binding) or with [3H]estradiol, a 10-fold excess of unlabeled progesterin, and a 100-fold excess of unlabeled estradiol for estimation of total and nonspecific binding, respectively. Incubations were performed for 45 min at room temperature. After the incubation, the cells were pelleted by centrifugation, washed in BME, and suspended in homogenization buffer [50 mM Tris-HCl, pH 7.5/10% (vol/vol) glycerol/10 mM KCl/0.1% Triton X-100/0.1% bovine serum albumin]. The cells were homogenized in a Teflon pestle/glass homogenizer (Thomas type A) and the homogenate was layered over a cushion of 1.4 M sucrose in homogenization buffer and centrifuged for 20 min at 7000 × g.

Each nuclear pellet was extracted with ethanol and the radioactivity was determined by liquid scintillation spectrometry in an LS-5801 liquid scintillation counter (Beckman). The DNA in each replicate was quantitated by a microversion (17) of the diphenylamine assay of Burton (18) with calf thymus DNA (Calbiochem) treated similarly as a standard. Control experiments indicated that a minimum of 1 μg of DNA per replicate was required for a reliable diphenylamine assay. The specific nuclear steroid binding was determined by subtracting the average of the replicate assays for the total binding of [3H]estrogen (expressed as dpm/μg of DNA) minus the corresponding average of the replicate assays for the nonspecific binding. For calculation of molecules per cell nucleus, a value of 6 μg of DNA per 106 nuclei was used.

Protein Blot Analysis. Cell extracts from freshly isolated osteoclasts and marrow-derived multinucleated giant cells were concentrated by 40% ammonium sulfate precipitation and SDS/PAGE was carried out by the method of Laemmli (19) using a 10% polyacrylamide resolving gel with a 5% stacking gel region. Proteins were transferred to nitrocellulose by the method of Towbin et al. (20) using a Genie electrotransfer unit (Idea Scientific, Corvallis, OR). The blots were blocked for 16 hr at 4°C with 5% (wt/vol) nonfat dry milk in phosphate-buffered saline containing 0.05% Tween 80 (PBST). All subsequent steps were at 22°C. The blots were incubated for 2 hr with purified monoclonal antibody (5 μg/ml) to the human ER (H222) (21) in blocking solution, washed with PBST three times over 1 hr, and further incubated for 2 hr with PBST containing biotin-conjugated goat anti-rat IgG antibody (Sigma) at 1 μg/ml. The blots were washed with PBST three times over 1 hr and incubated for 2 hr with PBST containing rabbit anti-biotin conjugated with alkaline phosphatase at 1 μg/ml. After a 60-min wash with three changes of 0.1 M Tris-buffered saline (pH 7.9), immunoreactive proteins were visualized by incubation in nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 20 min.

RESULTS

Initially, we examined osteoclast RNA for expression of ER mRNA. Total RNA (10 μg) from avian osteoclasts, marrow mononuclear cells, liver, and oviduct was analyzed on a Northern blot. The osteoclast preparations were 90–95% pure osteoclasts with 5–10% contaminating marrow mononuclear cells. We, therefore, also examined marrow mononuclear cell RNA for ER mRNA. The blots were incubated with probes generated from a full-length cDNA to the mRNA for human ER. Specific hybridization to a band migrating at 5.2 kilobases (kb) was present in avian osteoclasts, liver, and oviduct (Fig. 1). The contaminating mononuclear cells present in the osteoclast preparation displayed faint hybridization or no band depending on the individual blot. This 5.2-kb mRNA is smaller than that of the human ER mRNA that was reported (22) to be 6.4 kb and may simply reflect an animal species difference.

To assess whether functional ER protein was present in osteoclasts, specific nuclear binding for [3H]estradiol was determined. Specific saturable nuclear binding of [3H]estradiol in avian osteoclasts was measured at 5662 ± 1420 binding sites per nucleus (Fig. 2A). The nuclear binding of [3H]estradiol was also shown to be steroid specific (Fig. 2B). In these studies, the osteoclasts were incubated with 10 nM [3H]estradiol alone or [3H]estradiol plus a 100-fold excess of nonradiolabeled estrogen, dexamethasone, promegestone (a synthetic progestin), testosterone, or tamoxifen. Only nonradiolabeled estrogen competed to an appreciable level with [3H]estradiol for the nuclear binding sites.

To determine the presence of ER protein in the avian osteoclasts, Western blot analysis was performed. Both osteoclasts and the closely related marrow-derived giant cells contained a large molecular mass protein that was recognized by the anti-ER antibody (Fig. 3). However, only the osteoclasts contained a 66-kDa protein that was recognized by the anti-ER antibody. The 66-kDa protein, but not the large

![Fig. 1. Expression of ER mRNA. Total RNA (10 μg) from avian osteoclasts (lane 5), contaminating mononuclear cells (lane 2), liver (lane 3), and oviduct (lane 4) were analyzed on a Northern blot with a cDNA for the entire coding region of the ER. The blots were washed twice at 43°C in standard saline citrate/0.1% SDS and then analyzed by autoradiography after a 48-hr exposure. Lane 1 contains λ phage HindIII DNA fragments as size markers.](image-url)
cells received no estrogen with longed recover of activity. A with Isolated assay. examined using was
to block nonspecific binding alone or also in the presence of a 100-fold excess of unlabeled estradiol for total or nonspecific binding, respectively. The data points represent means of nuclear [3H]estradiol binding from five experiments; bars indicate SEM. (B) Isolated osteoclasts were incubated with 10 nM [3H]estradiol alone or with a 100-fold excess of unlabeled estrogen, progestin (Progest), testosterone (Testos), tamoxifen (Tamox), or dexamethasone (Dex). The results are presented as percent of inhibition of binding (of control assays) without competitive steroids.

molecular mass protein, was observed in similarly processed liver or oviduct samples (data not shown).

The influence of estrogen on osteoclast resorption activity was examined using a [3H]proline-labeled rat bone particle assay. Isolated osteoclasts were found to respond to estrogen treatment with a dose-dependent decrease in resorption activity. A significant inhibition of resorption was observed even at concentrations as low as 10 pM estrogen after 72 hr of exposure (Fig. 4A). To examine the ability of osteoclasts to recover from estrogen treatment, osteoclasts were challenged with 1 nM estrogen for 24 hr and then washed free of estrogen for the subsequent 48 hr (Fig. 4B). Control cells that received no estrogen resorbed high levels of bone whereas cells that were cultured for 24–72 hr with 1 nM estrogen resorbed significantly less bone. The osteoclasts that were exposed to estrogen for 24 hr resorbed a significant amount of bone after the removal of the estrogen. These data demonstrate that the inhibitory action of estrogen on osteoclast resorption activity was reversible, at least in these time periods and at this concentration.

Modulation of osteoclast nuclear protooncogene expression by estrogen was assessed. Treatment of isolated osteoclasts with estrogen for 30 min followed by mRNA blot analysis of the total RNA from these cells revealed a dose-dependent increase in the steady-state levels of c-fos and c-jun mRNA. Fig. 5 presents a densitometric analysis of RNA from osteoclasts cultured for 30 min with vehicle or various concentrations of estrogen that has been probed for steady-state levels of c-fos, c-jun, and 18S rRNA levels. The 18S rRNA level was determined to normalize for lane-loading variability. In the absence of estrogen treatment, there was significantly more c-fos than c-jun mRNA. There appeared to be no detectable modulation of these messages at 1 nM estrogen whereas a 2-fold change in the message was seen at 0.1 nM estrogen. At 10 nM estrogen, there was an ≈4-fold change in the steady-state mRNA levels of these two nuclear protooncogenes.

**DISCUSSION**

This report has provided clear evidence for the presence of functional ERs in avian osteoclasts. We have utilized a method for osteoclast isolation that reproducibly yields highly purified cell populations (12). To further ensure that the receptor mRNA we were studying was from authentic osteoclasts, we examined the contaminating mononuclear cell RNA for evidence of ER mRNA. Northern blot analysis supports that the 5–10% mononuclear contaminating cells could not be responsible for the ER mRNA observed in the
Osteoclast RNA. On the basis of this observation, we are confident that the estrogen responses detailed here are not the result of contaminating mononuclear cell estrogen responses but, instead, are osteoclast estrogen responses. In addition to demonstrating ER mRNA, these data also document that osteoclasts contain a protein that has the ability to localize $[^{3}H]$estradiol in the nuclei in a steroid-specific manner. The nuclear binding assay employed for these studies provides added information when compared to the less sensitive charcoal-binding assay since the former measures functional steroid receptors, defined here as those that bind steroid, become activated, and bind to the nuclear acceptor site (16). Moreover, the nuclear binding assay has been shown to mimic the patterns of nuclear binding of $[^{3}H]$steroid in vivo (16). The inability of tamoxifen (in birds, a complete anti-estrogen) to compete with $[^{3}H]$estradiol for nuclear binding may be attributable to the fact that tamoxifen has a relatively low affinity for ER when compared with either 17$\beta$-estradiol or hydroxytamoxifen. The protein blot data revealed that the osteoclast extracts contained a 66-kDa protein that was recognized by a well-characterized monoclonal antibody to the human ER (21). This size was in close agreement with the size of the human ER (22). The nature of the 140-kDa protein species was unclear, but similarly probed avian liver and oviduct extracts did not appear to contain this protein (data not shown). When closely related marrow-derived giant cells were examined for ER protein, a faint band at 140 kDa was the only immunoreactive species observed. Since these cells did not appear to contain ER mRNA (data not shown), the relationship between this 140-kDa band and the 66-kDa ER remains unknown.

Although ERs and gene responses to estrogen have been reported in osteoblasts in vitro (7, 8), the observed in vivo effects of estrogen treatment appear to involve osteoclast recruitment and activity (2–6). This paradox could be resolved if estrogen influences on osteoclast recruitment and activity were mediated by osteoblasts. In a similar manner, other modulators of osteoclast recruitment and activity, such as parathyroid hormone, are thought to influence osteoclasts through osteoblasts (23). However, the data presented here demonstrating that the level of osteoclast nuclear estrogen binding sites was 3-fold higher than was observed in human osteoblast-like cells using the same methods (7), suggest that estrogen may directly influence osteoclast activity in vivo. Furthermore, these studies demonstrate the direct inhibition by estrogen of osteoclast resorption. We have demonstrated that isolated viable osteoclasts bind to bone particles and form an extensive ruffled membrane juxtaposed to the bone surface (11). This and other properties of these cells including the ability of the isolated cells to form pits on slices of cortical bone in vitro support the view that these cells are indeed authentic osteoclasts (11). It is, therefore, reasonable to conclude that authentic osteoclasts resorb bone particles in a manner indistinguishable from in vivo bone resorption. Estrogen modulation of bone resorption is measurable between 1 nM and 10 pM estrogen, concentrations within physiological range. We further demonstrated that treatment of osteo-

**Fig. 4.** Osteoclast resorption activity. Isolated osteoclasts (25,000 cells per sample) were incubated with 100 $\mu$g of $[^{3}H]$proline-labeled rat bone particles. Quadruplicate samples were analyzed. (A) Osteoclasts and bone particles were incubated with the indicated concentration of estrogen for 72 hr, the supernatant was aspirated, and radioactivity was measured. (B) Osteoclasts and bone particles were incubated with vehicle (●) or 1 nM estradiol (○ and △) for the indicated time period. After 24 hr of culture, estrogen-containing medium was replaced with fresh estrogen-free medium in one set of samples for the subsequent 48 hr (○). Control samples had medium replaced with fresh estrogen-containing (△) or estrogen-free media (●) for the subsequent 48 hr. Samples were analyzed at 24 hr and after the subsequent 48 hr.

**Fig. 5.** Northern blot analysis for estrogen responses. Isolated osteoclasts were cultured for 30 min in the presence of rat bone particles with the indicated dose of estrogen. Total RNA was isolated, blotted, and probed to determine RNA levels for c-fos, c-jun, and 18S rRNA.

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clasts with estrogen at 1 nM for 24 hr results in a reversible inhibition of bone resorption, suggesting that the estrogen doses employed within this time period were not detrimental to cell viability.

Estrogen rapidly modulated the steady-state mRNA levels of c-fos and c-jun nuclear protooncogenes. The mechanism by which estrogen influences bone cell activity are unknown; however, the rapidity of the response of these nuclear protooncogene mRNAs to estrogen treatment suggests that this is likely to be a primary response of osteoclasts to estrogen treatment. The protein products of c-fos and c-jun form a complex in the nucleus that binds the AP-1 upstream regulatory regions as a transcription factor for the regulation of gene transcription (for review, see ref. 24). Therefore, estrogen appears to rapidly regulate the expression of genes whose protein products in turn regulate the expression of other genes (25, 26). Rapid alterations in nuclear protooncogene expression levels after steroid treatment have been reported in other steroid target tissues and thus appear to be a widespread estrogen response mechanism (27). These data are important since, in addition to gaining insight on the mechanism of estrogen action on osteoclasts, modulation of osteoclast genes in response to estrogen further supports the concept that osteoclasts are estrogen target tissues. We conclude from the data presented here that osteoclasts contain significant numbers of functional ERs, likely to be an estrogen target tissue, and may respond directly to estrogen in vivo. These data explain previous observations that bone loss as the result of postmenopausal osteoporosis may be, at least in part, due to estrogen effects directly on osteoclast activity.

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