Estrogen directly down-regulates the bone-resorbing activity of mature osteoclasts through nuclear estrogen receptor α

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Abstract

The decrease in estrogen level that follows the onset of menopause causes rapid bone loss, resulting in osteoporosis. However, the mechanism remains unclear, especially concerning the regulation of bone-resorbing osteoclasts. Here we analyzed the function of estrogen and its receptor in mature osteoclasts. We found that estrogen directly inhibited bone-resorption by purified rabbit mature-osteoclasts. Moreover, using a RT-PCR technique, we report that nuclear estrogen receptor (ER) α but not ER β is expressed in mature osteoclasts. The antisense oligodeoxynucleotide for ER α inhibited the reduction in osteoclastic bone-resorbing activity caused by estrogen. We conclude that in part estrogen directly inhibits the bone-resorbing activity of mature osteoclasts through the ER α .

Introduction

Osteoclasts are hemopoietic in origin. These cells are characterized by a unique combination of properties; i.e., they are multinucleate giant cells that possess tartrate-resistant acid phosphatase (TRAP) activity, an abundance of calcitonin receptors, and highly developed ion transport systems (Hakeda and Kumegawa, 1996). Osteoclasts are involved in bone metabolism, such as bone-modeling and bone-remodeling. Especially osteoclasts resorb mineralized bone surfaces under both physiological and pathological conditions. Post menopausal osteoporosis (bone loss) is a major health problem in woman. Estrogen deficiency has long been recognized as a cause of this osteoporosis, and estrogen replacement therapy is an effective treatment for the prevention of bone loss (Rifkin, 1993).

Estrogen modulates estrogen-targeted genes through its nuclear receptors. Now, two types of estrogen receptor (ER) are known, ER α and ER β . The tissue- and cell-specific expression of these ERs has been reported (Kuiper et al., 1997), and the data sug-

gest different roles for these receptors in each tissue and cell. Also, mRNAs of both ER α and ER β are expressed in osteoblasts, the bone-forming cells (Arts et al., 1997). However, whether ER α and ER β are expressed in mature osteoclasts remains unclear.

Evidences obtained from *in vitro* studies demonstrate that the differentiation of osteoclasts is indirectly controlled by various factors, such as vitamin D, macrophage colony-stimulating factor and interleukin 6 (IL-6), and that non-osteoclastic stromal cells are involved in the formation of osteoclasts (Ishizuka et al., 1988; Kodama et al., 1991). For instance, many studies have shown that estrogen loss promotes IL-6 secretion in non-osteoclastic stromal cells and that this IL-6 up-regulates osteoclast formation (Rifas et al., 1995; Stein and Yang, 1995; Kassem et al., 1996). However, the direct effect of estrogen on mature osteoclasts remains unclear, because of the difficulty in preparing purified mature osteoclasts for cell culture on mineralized materials.

Therefore, we sought to examine the direct effect of estrogen and its analogs on mature osteoclasts purified from rabbit long bones by use of collagen gel,

Figure 1. Effect of calcitonin, vitamin D, and estrogen on unfractionated bone cells and purified osteoclasts. Pit assay of calcitonin (10^{-6} M), $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-8} M) and 17β -estradiol (10^{-10} M) for their effect on unfractionated bone cells (upper panel) and purified osteoclasts (lower panel). Values are means $\pm$ SD of three cultures.

as well as the expression and function of ERs in these mature osteoclasts. Using purified mature osteoclasts, we clarified that estrogen directly regulates osteoclast bone-resorbing activity. Moreover, we showed that nuclear ER α is expressed in mature osteoclasts and is involved in the regulation of osteoclast bone-resorbing activity by estrogen.

Materials and methods

Isolation of osteoclasts for pit assay

Unfractionated bone cells were isolated from tibiae, femora, humeri, ulnae, and radii of ten-day-old rabbits. After removal of soft tissues, these bones were minced in α -minimum essential medium (α -MEM). Cells were dissociated from the bone fragments by sedimentation under normal gravity for 1 min, and the supernatant was used as unfractionated bone cells (crude osteoclasts). These cells were plated in 10-cm tissue-culture dishes coated with 0.24% collagen gel (Nitta Gelatin, Tokyo, Japan) in α -MEM supplemented with 5% fetal bovine serum (FBS). After 3 h incubation, cells other than osteoclasts were removed from the gel by sequential treatment with 0.001%

pronase E (Sigma Chemical, St. Louis, MO, USA) and 0.01% collagenase. The remaining osteoclasts were collected by 0.1% collagenase treatment, and washed with Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline (PBS) three times. The isolated osteoclasts were replated onto dentine slices in phenol red-free α -MEM supplemented with 0.1% bovine serum albumin (BSA) for use in the pit assay.

The population of isolated osteoclasts was confirmed by microscopic observation of TRAP staining in the presence of 50 mM L-tartrate with a leukocyte-acid-phosphatase kit (Sigma Chemical, St. Louis, MO, USA) (Kakudo et al., 1996).

When TRAP-positive multinucleate cells isolated from collagen gels were placed on dentine slices, they showed compact and round shape, and the purity of TRAP-positive multinucleate cells was almost >99% (data not shown).

These TRAP-positive multinucleate cells (100 cells) were cultured in dishes for one week to assess the formation of colonies of non-osteoclastic stromal cells. Only 0.4 ± 0.49 ($n = 5$) colonies were formed after one-week culture. Thus we concluded that the purity of the TRAP-positive multinucleate cells is more than 99%. When these cells were cultured on dentine slices, re-

Figure 2. Effects of estrogen on isolated osteoclasts. Pit assay of estrogen analogs for their effect on purified osteoclasts. 10^{-9} M TAM or 10^{-9} M ICI was added to osteoclast cultures in the presence or absence of 10^{-10} M 17β -estrogen. Values are means $\pm$ SD of three cultures.

sorption pits were formed around these cells as observed under a scanning-electron-microscope. Moreover, transmission-electron-microscopic observation revealed a ruffled border-like membrane and clear zone on the side of these cells attached to the surface of the dentine (Kakudo et al., 1996). Thus, these cells isolated from collagen gels exhibited the typical morphology of authentic osteoclasts, and the multinucleate cells had the ability to excavate the dentine even when stromal cells were absent from the culture. We concluded that these TRAP-positive multinucleate cells were indeed mature osteoclasts.

Isolation of osteoclasts for RNA isolation

Unfractionated bone cells were plated onto 90-mm tissue-culture dishes with α -MEM/5% FBS at 1×10^8 living cells/dish. After overnight culture, the cells were washed with PBS containing 0.001% pronase E and 0.02% EDTA for 20 min at 37 °C to remove non-osteoclastic cells. These detached non-osteoclastic cells were used as stromal cells. After further incubation for 16 hours, the remaining multinucleate osteoclasts were used as pure mature osteoclasts for RNA isolation. The purity of the osteoclasts in the dishes was more than 99%.

Pit assay for assessment of bone-resorbing activity

To determine the bone-resorbing activity of osteoclasts, we measured the area and number of resorption pits formed on dentine slices by isolated mature os-

teoclasts, or unfractionated bone cells (Takada et al., 1992). Briefly, isolated osteoclasts or unfractionated bone cells were replated on dentine slices (circular, 6 mm in diameter) in 96-well plates (Beckton Dickinson) at 150 isolated osteoclasts/well or 1×10^4 unfractionated bone cells/well, respectively, and cultured for one hour in phenol red-free α -MEM supplemented with 0.1% BSA. The medium was then replaced with fresh medium containing various concentrations of each factor. After 20 h, the dentine slices were brushed with a rubber policeman to remove the cells, and were stained with acid hematoxylin for 5 min. The total number of pits on a dentine slice was counted under a light microscope, and the total area of pits was quantified by densitometric analysis of dentine slice images using a videomicroscope and NIH image software (Kameda et al., 1997).

Reverse transcription-polymerase chain reaction (RT-PCR) and DNA sequencing

Total RNA was extracted from purified osteoclasts and stromal cells cultured on tissue culture dishes by the acid-guanidinium-phenol-chloroform method (Saneshige et al., 1995), and cDNA was synthesized with moloney murine leukemia virus reverse transcriptase. In order to detect ER α transcripts, we synthesized a set of primers (5' CACCAGATCCAAGG-GAA 3', and 5' GCTGTTGAGCTGGGGGAA 3') based on the reported rabbit ER α cDNA sequence (Kaluz et al., 1997). For detection of ER β transcripts, a set of primers (5' GAATGGTCAAGTGTGGAT 3',

Figure 3. Expression of ER α mRNA in mature osteoclasts. Results of RT-PCR for detection of nuclear ER α and ER β mRNAs in stromal cells and mature osteoclasts are shown.

and 5' GGCCTTCACACAGAGATA 3') was synthesized based on the reported rat ER β cDNA sequence (Kuiper et al., 1996). Amplification was carried out for 38 cycles, each of 94 °C, 1 min; 56 °C, 1 min; 72 °C, 2 min in a 20 μ l reaction mixture containing 2 μ l of each cDNA, 20 pmoles of each primer, 0.2 mM dNTP, 1 U of Taq DNA polymerase, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, and 10 mM Tris-HCl; pH 8.3. PCR products were analyzed on 1% agarose gel, and purified and subcloned into pCR 3.1 (Invitrogen, USA) for sequencing.

S-Oligodeoxynucleotide uptake by mature osteoclasts

Rabbit ER α antisense (5' GTGGAGCAGGGCCATGCCGGA 3') and sense (5' TCCGGCATGGCCCT-

GCTCCAC 3') S-Oligodeoxynucleotides (S-ODNs) were synthesized on an automated nucleotide synthesizer. The sense S-ODN for ER α was used as a negative control. Isolated osteoclasts were cultured on dentine slices in the presence or absence of β -estradiol, and antisense or sense S-ODNs in α -MEM containing 0.1% BSA and 100 nM Tfx-50 (Promega, WI, USA) as a carrier (Inui et al., 1997). After 20 h of culture, the pit assay was carried out.

Results

Effect of estrogen on purified osteoclasts

The unfractionated bone cells containing osteoclasts and non-osteoclastic stromal cells were cultured on dentine slices with or without calcitonin, 1 α , 25-dihydroxyvitamin D₃, or 17 β -estradiol. Calcitonin and estrogen down-regulated osteoclastic bone resorption; and vitamin D₃ up-regulated the resorption (Figure 1, upper panel). However, whereas calcitonin and estrogen inhibited bone resorption by purified osteoclasts, vitamin D₃ did not affect it (Figure 1, lower panel). These data suggest that both calcitonin receptor and ER may be expressed in mature osteoclasts but that the vitamin D receptor (VDR) is not.

Next we examined the effect of estrogen analogs on purified osteoclasts to confirm the existence of ERs in mature osteoclasts. Figure 2 shows that tamoxifen (TAM) partially prevented 17 β -estradiol-dependent inhibition of osteoclastic bone resorption, and inhibited osteoclastic bone-resorbing activity when used alone. On the other hand, the pure estrogen antagonist ICI164,384 (ICI) almost completely prevented the inhibition of osteoclastic bone resorption by 17 β -estradiol.

Expression of ER α in mature osteoclasts

Since we observed the direct effect of estrogen on bone-resorbing activity of mature osteoclasts, we tried to examine the existence of estrogen receptors in osteoclasts. Using a RT-PCR technique, we found that ER α mRNA was expressed in both osteoclasts and stromal cells obtained from rabbits (Figure 3). We confirmed this fragment to be rabbit ER α cDNA by sequencing (data not shown). The mRNA of ER β was detected in rabbit stromal cells, but not in osteoclasts, by RT-PCR (Figure 3). Also, ER β mRNA was not detectable in mature osteoclasts by use of other sets of ER β primer (5' TGTAGCCAGTCCATCCTA3', 5'

TAGAACTTCTCTGCCTCC3'; 5' GCGTAGAAGG-GATTCTGG 3', 5'TCTCTGTAGTCTGTCCGC 3').

ER α participates in the regulation of osteoclastic bone-resorbing activity by estrogen

As our data suggested that 17 β -estradiol acts on osteoclasts through ER α but not ER β , we performed a pit assay using antisense S-ODN for ER α and purified osteoclasts to confirm the above data. Previously we demonstrated that the production of cathepsin K protein and bone-resorbing activity in osteoclasts treated with antisense S-ODN for cathepsin K was inhibited (Kuiper et al., 1996).

We made two S-ODNs, one sense and one antisense for the rabbit ER α , and used them in the pit assay. Figure 4 shows that 17 β -estradiol did not inhibit bone-resorbing activity in the osteoclasts that had been treated with antisense S-ODN for ER α . On the other hand, ER α sense S-ODN did not affect 17 β -estradiol reducing osteoclastic bone-resorption activity. Also, ER α sense S-ODN did not affect pit formation activity of mature osteoclasts compares with non-treated control one.

Discussion

We showed that calcitonin directly and vitamin D indirectly modulated osteoclastic bone-resorbing activity. Previous reports showed that the calcitonin receptor, but not the vitamin D receptor (VDR), is expressed in mature osteoclasts (Hakeda and Kumegawa, 1996; Ishizuka et al., 1988). The bones of the VDR-null mouse are abnormal, as the function of bone cells is impaired. However, the function of osteoclasts from this mouse was normal, when they were cultured with stromal cells from normal mice (Yoshizawa et al., 1997). These results indicate that function of osteoclasts was directly and/or indirectly controlled by each factor.

Several reports have indicated that estrogen modulates osteoclasts indirectly through factors such as IL-6 and transforming growth factor- β (TGF- β) mediated by stromal cells (Rifas et al., 1995; Yang et al., 1996). However, our data suggest that estrogen can also regulate osteoclast bone-resorbing activity regardless the presence of stromal cells. This discrepancy might be due to the difference of species and differentiation stage of osteoclasts. We used rabbit osteoclasts, while others used mouse or human osteoclasts. Moreover, we used mature osteoclast, whereas

others used precursor or immature osteoclasts.

We demonstrated the expression of ER α , but not that of ER β , in rabbit mature osteoclasts by RT-PCR. Previously we showed the expression of ER α but not ER β in mature osteoclasts by Northern blot analysis (Kameda et al., 1997). In this study, ER α -antisense S-ODN inhibited estrogen-induced down-regulation of osteoclastic bone resorption.

Using Northern blot analysis we checked the level of ER α mRNA in mature osteoclasts treated with the ER α antisense S-ODN. The mRNA levels of ER α in osteoclasts were partially reduced by ER α antisense S-ODN treatment in osteoclasts (data not shown). However, it is well known that S-ODN mostly affects post-transcriptional process. We speculated that the levels of ER α protein decreased in ER α antisense S-ODN treated osteoclasts. These suggest that ER α plays a critical role in estrogen signalling in mature osteoclasts.

However, it has been reported that both α and β ER mRNAs are expressed in osteoblasts, which are bone-forming cells in bone tissue (Arts et al., 1997; Onoe et al., 1997; Hoshino et al., 1995). Both ERs may play important roles in bone tissue. Recently an ER α null mouse was established (Das et al., 1997). The bones of such mice were slightly abnormal. Therefore, the function of osteoclasts is modulated by estrogen both directly and indirectly through ER α and ER β *in vivo*.

Many estrogen analogs have been developed as anti-cancer or anti-osteoporosis reagents. These estrogen analogs modulate estrogen-dependent gene expression through ERs and its co-factors. We showed that TAM is a partial estrogen agonist activity for mature osteoclasts and that ICI is a complete one for them. It is reported that TAM is the partial estrogen antagonist activity in some tissues (Yang et al., 1996). Recently many orphan nuclear receptors were discovered (Blumberg and Evans, 1998). Thus we could not exclude the possibility of orphan nuclear receptors in mature osteoclasts. It is important to develop cell-specific estrogen analogs for prevention of side effects. The mechanisms by which estrogen acts in each cell type should be clarified.

We conclude that estrogen directly down-regulates the bone-resorbing activity of mature osteoclasts through their ER α . These findings may shed new light on our understanding of the cellular mechanism by which estrogen provides protective effects on the skeleton. They also support the validity of using estrogen replacement therapy for treating postmenopausal osteoporosis.

Figure 4. ER α is involved in the regulation of osteoclastic bone resorption by estrogen. The results of pit assay utilizing antisense S-ODN for ER α and purified mature osteoclasts are given. Each S-ODN (5 μ M) was added to osteoclast cultures in the presence or absence of 10^{-10} M 17 β -estrogen. Values are means $\pm$ SD of three cultures.

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