

Detection of estrogen receptor α , carbonic anhydrase II and tartrate-resistant acid phosphatase mRNAs in putative mononuclear osteoclast precursor cells of neonatal rats by fluorescence *in situ* hybridization

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ABSTRACT

Increasing evidence suggests that estrogen deficiency in women promotes the expansion of populations of bone marrow cells that differentiate into osteoclasts under the influence of osteotropic hormones and local factors. A progressive cytoplasmic accumulation of osteoclastic bone resorbing enzymes, such as tartrate-resistant acid phosphatase (TRACP) and carbonic anhydrase II (CA II), characterizes osteoclast differentiation. To evaluate the possibility that estrogen may have a direct effect on osteoclast precursor cells, we investigated the mRNA levels of estrogen receptor α (ER α), TRACP and CA II genes in neonatal rat bone imprints by fluorescence *in situ* hybridization and confocal microscopy. Morphological assessment of bone imprints has shown that the putative mononuclear osteoclast precursor cells (MOPC) display strongly basophilic cytoplasm and a low nuclear/cytoplasmic ratio, while some of these cells possess pale-staining ruffled border regions similar to those observed in

osteoclasts. Both CA II and TRACP mRNAs were detected in putative MOPC as well as multinuclear osteoclasts. The gene transcripts were mainly located in the cytoplasm of these cells. To determine whether these putative MOPC possess ER mRNA, a 637 base pair antisense ER riboprobe was used. The results indicated that MOPC which show TRACP reactivity express high levels of ER gene transcripts in their cytoplasm. In contrast, only a few multinuclear osteoclasts in the bone imprints possessed ER gene transcripts. Interestingly, the levels of ER mRNA in these multinuclear osteoclasts were very low compared with those in the putative MOPC. Treatment with RNase prior to hybridization resulted in a significant loss of signal in these cells. The results of these studies suggest that estrogen may have a direct role in modulating the recruitment of osteoclast precursor cells during osteoclastogenesis.

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INTRODUCTION

Postmenopausal osteoporosis is characterized by a reduction in bone mass and increased susceptibility to fractures (Riggs *et al.* 1969, Raisz 1988) and is a major cause of morbidity and mortality in elderly women. The bone loss appears likely to be due to estrogen deficiency, and can be prevented by administration of estrogen (Ettinger *et al.* 1985, Stevenson *et al.* 1989). The major mechanism by which estrogen protects against bone loss is through suppression of bone resorption by either reduction of osteoclastogenesis or decrease of osteoclast activity (Horowitz 1993).

Tartrate-resistant acid phosphatase (TRACP) and carbonic anhydrase II (CA II) are believed to be key enzymes for osteoclastic bone resorption (Vaes 1988, Baron 1989, Zaidi *et al.* 1989, Roth *et al.* 1992). These enzymes have been used as specific markers for osteoclasts in bone and their progressive accumulation in mononuclear osteoclast precursor cells (MOPC) signals osteoclast differentiation (Baron *et al.* 1986, Kurihara *et al.* 1990). Our previous studies showed that estrogen deficiency after ovariectomy in rats enhances the levels of TRACP and CA II mRNA in bone, whereas administration of 17 β -estradiol to ovariectomized

rats reduces gene expression of these enzymes (Zheng *et al.* 1995b). However, it is unclear whether the variation of TRACP and CA II mRNA in these investigations was the result of changes in osteoclastogenesis or due to a direct effect on TRACP and CA II gene expression. In this study, we postulated that estrogen has a direct action on osteoclastogenesis by suppressing the generation of MOPC. We have therefore used fluorescence *in situ* hybridization (FISH) with confocal microscopic assessment to determine the presence of estrogen receptor α (ER α) mRNA in putative MOPC in rat bone imprints. Thus FISH for TRACP and CA II gene, TRACP cytochemistry and light microscopic appearances were correlated and used in the characterization of putative MOPC.

MATERIALS AND METHODS

Materials

The mouse CA II cDNA was kindly provided by Dr P J Venta (Department of Small Animal Clinical Sciences, Michigan State University, MI, USA). A rat TRACP cDNA was obtained from Dr G Andersson (Karolinska Institute, Huddinge University Hospital, Huddinge, Sweden). Animals were obtained from the Animal Resource Center of Western Australia, Perth, Australia. The procedure for animal experimentation has been approved by the Animal Ethics Committee of the University of Western Australia.

Preparation of tissue imprints

To obtain rat osteoclasts and putative MOPC, long bones from one-day-old male Wistar rats were dissected free of soft tissue as previously described (Zheng *et al.* 1994), then sectioned longitudinally and placed in phosphate-buffered saline (PBS) in pyrocarbonate (DEPC)-treated water. Adherent bone marrow cells were cleaned off with a fine paint brush. The bones were then imprinted with minimal pressure onto RNase-free salinated glass slides. The slides were air-dried at room temperature for 15 min. For the morphological assessment of imprinted cells, slides were fixed with 10% formalin and stained with Giemsa. They were viewed with a Zeiss light microscope using a 60 \times objective. For FISH, slides were air-dried and fixed in 4% paraformaldehyde in DEPC-treated PBS for 30 min.

Rat ER α cDNA cloning

For rat ER α cloning, total RNA from rat uterus was extracted with RNazolB (Biotecx, Houston, TX,

USA) using the manufacturer's modification of the acid guanidinium thiocyanate-phenol-chloroform procedure (Chomczynski & Sacchi 1987). Single-stranded DNA was generated by incubating total RNA with avian myeloblastosis virus reverse transcriptase (AMV-RT) in the presence of oligo dT (10 ng/ μ l), mM dNTPs, 8 mM MgCl₂ and ribonuclease inhibitor (Promega, Sydney, Australia). A 637 base pair fragment of the ER was synthesized using the published rat sequence. The primer sequences for rat ER were 5'-GAC CAG ATG GTC AGT GCC TT-3' and 5'-AGA TGC TCC ATG CCT TTG TT-3'. The PCR mixture contained single-stranded DNA, 10 pmol of each primer and 0.2 mM of each dNTP in 50 mM KCl, 10 mM Tris HCl (pH 9.0), 2.0 mM MgCl₂, 0.1% Triton X-100 and 1–5 units of thermus aquaticus (Taq) DNA polymerase. Twenty-five cycles of 94 °C (1 min) and 72 °C (1 min) were performed. The ER cDNA fragment was then subcloned into the plasmid vector pGEM1 (Promega). The nucleotide sequence of the cDNA insert was determined by the Sanger method of dideoxy-mediated chain termination using Sequencer (United States Biochemical, Cleveland, OH, USA).

Synthesis of riboprobes

Probes were labeled with digoxigenin using a DIG RNA labeling kit (Boehringer Mannheim, Sydney, Australia) according to the manufacturer's instructions. A 0.451 kb EcoRI fragment of mouse CA II cDNA in pBluescribe(+/-) plasmid was linearized with SmaI and transcribed into antisense riboprobe with T7 RNA polymerase. Similarly, a 0.832 kb EcoRI/PstI insert of rat bone TRACP in pT7T3 18/19 plasmid and 0.637 kb SmaI insert of rat ER in pGEM1 plasmid were linearized with EcoRI and BamHI respectively. Both of them were transcribed with T7 polymerase to generate the antisense strand.

Fluorescence *in situ* hybridization

In situ hybridization was performed as previously described by Zheng *et al.* (1993) with minor modifications. Slides were washed in 0.2% Triton X-100 in DEPC-treated PBS for 5 min after fixation. The cells on the slides were digested with proteinase K at a concentration of 2 μ g/ml in 0.1 M Tris buffer (pH 8.0) and 50 mM EDTA for 20 min at 37 °C in a humidified chamber, followed by 0.1% glycine in PBS for 2 min. The cells were post-fixed in 4% paraformaldehyde in PBS for 15 min, and then pretreated with RNase-free DNase (1 U/ml) for 30 min at 37 °C before hybridization to ensure

specificity of mRNA hybridization. Slides were rinsed in PBS between each pretreatment, and all procedures were carried out at room temperature unless otherwise indicated.

The hybridization solution consisted of 45% deionised formamide, 10% dextran sulfate, 5-fold SSC, and 1 mg/ml denatured and sonicated salmon sperm DNA. For negative controls, slides were incubated with 100 μ g/ml RNase at 37 °C for 60 min before prehybridization. Prehybridization was performed at 37 °C for 1 h in hybridization solution. The digoxigenin-labeled probes of TRACP, CA II and ER were diluted with hybridization solution to a final concentration of 0.35 ng/ μ l, 0.4 ng/ μ l and 0.2 ng/ μ l respectively. The mixtures were heated at 65 °C for 10 min and then 30 μ l placed on each slide. The slides were covered with coverslips and incubated at 37 °C for 16 h in a humidified chamber. Coverslips were removed, and the slides were washed twice in 2-fold SSC at 37 °C, then twice in 1-fold SSC at room temperature, and finally twice in 0.1-fold SSC at 37 °C for 15 min for each wash. The slides were then incubated with 1% blocking solution for 30 min at room temperature. Detection of hybridization probes was performed with a fluorescent antibody enhancer set according to the manufacturer's instructions (Boehringer Mannheim). Briefly the slides were incubated in the first antibody solution (anti-DIG) for 60 min at 37 °C in a humidified chamber, then the second antibody (anti-mouse-Ig-DIG) added under the same conditions. Finally the third antibody (anti-DIG-fluorescein) was added again at 37 °C for 60 min in a humidified chamber and in the dark. Slides were washed briefly three times in washing buffer (at 37 °C) after each incubation. Some of slides were counterstained with 3 μ g/ml propidium iodide for 30 min at room temperature to visualize the nuclei of cells. After air-drying in the dark the slides were covered with anti-fade solution and a coverslip. Signal was then detected by confocal microscopy.

TRACP enzyme cytochemistry

In order to characterize further the cells with signal for ER mRNA, a relatively weak staining of TRACP by enzyme histochemistry was employed (Zheng *et al.* 1994). In brief, after the FISH for ER, mRNA slides were incubated with acetate buffer (pH 5.0) containing Naphthol AS-BI phosphate (0.2 mg/ml; Sigma, St Louis, MO, USA), 100 mM sodium tartrate and Fast Garnet GBC (0.5 mg/ml; Sigma) for 10 min at 37 °C. Thus signals for both ER mRNA and a very weak staining of TRACP were assessed in the same cell using confocal

microscopy. It is noteworthy that a strong TRACP staining could obscure the fluorescence signal.

Confocal microscopic assessment

The detection of fluorochromes for mRNA signals was carried out by a confocal laser scanning microscope (MRC-1000, Biorad), equipped with a krypton argon laser coupled to an epifluorescence Nikon Diaphoto 300 inverted microscope. Samples were viewed with a 40 \times Plan objective lens (NIKON, NA=0.7), and images were recorded in a 512 \times 512 pixel format. Optical sections were collected along the z axis with 1 to 2 μ m z-step. The 488 nm line of the Kr/Ar laser for excitation and 522 nm emission filter were employed for detection of FITC. For the assessment of TRACP activity in ER mRNA-positive MOPC, transmitted light was used. For the measurement of ER mRNA levels in MOPC, the intensity of signal in each cell was evaluated by particle analysis and expressed as intensity per mean area of cell.

RESULTS

Morphological assessment of bone tissue imprints

Giemsa staining of bone imprints showed that several cell types (including multinuclear osteoclasts and mononuclear cells) varying in shape, intensity of cytoplasmic staining and nuclear/cytoplasmic ratio were present. Among the mononuclear population, oval-shaped cells with strongly basophilic cytoplasm and low nuclear/cytoplasmic ratio were found. The nuclei of these cells often possessed one to two nucleoli. Several of these cells displayed pale staining ruffled border regions similar to those observed in osteoclasts. Moreover, they were often situated in close proximity to multinuclear osteoclasts (Fig. 1A). These oval-shaped cells were considered to be putative MOPC. Spindle-shaped cells with a pale staining juxtannuclear cytoplasmic region (Golgi-rich zone) were identified as stromal cells or preosteoblasts (Fig. 1B). Osteoblasts were often seen as round or triangular shaped with a pale staining juxtannuclear cytoplasmic region (Golgi-rich zone) and round nuclei (Fig. 1C). Chondroblasts were round to oval in shape with strong and compact cytoplasmic staining (Fig. 1D). Other cells such as granulocytes, monocytes and lymphocytes were also observed in the imprints. The numbers of these various cells in the bone imprints were counted under a 40 \times objective and the results are shown in Table 1.

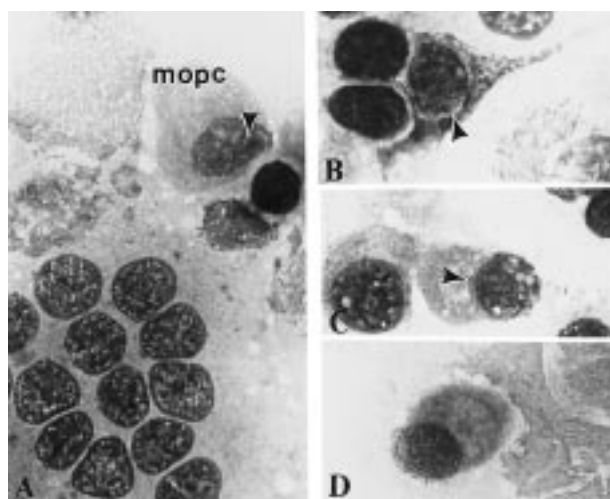


FIGURE 1. Wright-Giemsa-stained bone imprints from one-day-old rats. (A) Putative MOPC (mopc) are oval-shaped and exhibit strongly basophilic cytoplasm and low nuclear/cytoplasmic ratio. Distinct nucleoli are seen in the nuclei (arrow). A multinuclear osteoclast is seen next to the MOPC. (B) Stromal cells are spindle-shaped with strongly staining cytoplasm. (C) Osteoblasts are triangular with a pale staining perinuclear cytoplasmic region (Golgi-rich zone). (D) Chondroblasts are oval to round-shaped with a prominent cytoplasmic staining. Magnification $\times 600$.

TABLE 1. Differential counts of various cell types in bone imprints. Number is expressed as the mean \pm s.d. per $40\times$ objective field. More than five fields per slide were counted and the results of four experiments were assessed

Cell type	Number
Osteoclasts	2.4 ± 1.5
Osteoblasts	6.2 ± 5.5
Putative MOPC	3.4 ± 2.3
Stromal cells/preosteoblasts	9.4 ± 3.7
Chondroblasts	1.4 ± 1.5
Granulocytes	19.4 ± 14
Monocytes/lymphocytes	24.8 ± 3.6

Localization of CA II, TRACP and ER mRNA in putative MOPC

To determine if the oval-shaped mononuclear cells with strong basophilic staining in their cytoplasm were putative MOPC, FISH was used to assess the presence of TRACP and CA II mRNA in these cells. Both CA II and TRACP mRNAs were detected in these oval-shaped mononuclear cells as well as in multinuclear osteoclasts (Figs 2 and 3). Gene transcripts for both CA II and TRACP were

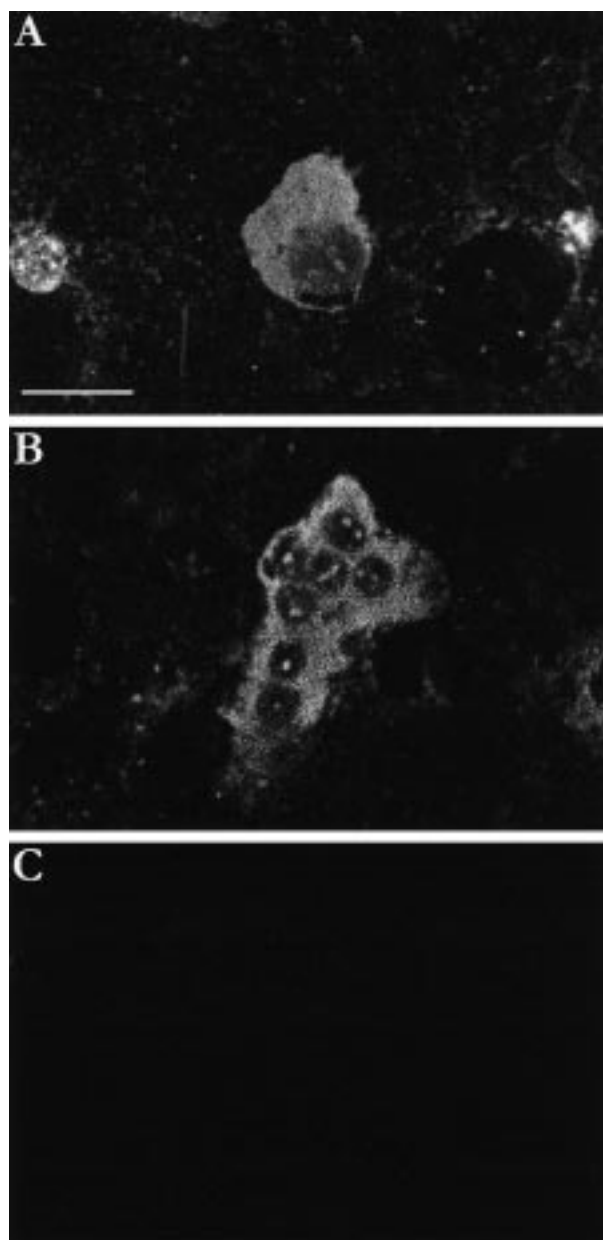


FIGURE 2. Localization of CA II mRNA in oval-shaped mononuclear cells (putative MOPC) and osteoclasts by FISH and confocal microscopic assessment. Oval-shaped mononuclear cells (A) and multinuclear osteoclasts (B) showed cytoplasmic distribution of CA II mRNA. A small amount of CA II mRNA was also detected in their nuclei (arrow). The CA II mRNA signal disappeared when the cells were incubated with 1 U/ml RNase at 37°C for 60 min prior to hybridization. Scale bar represents $20\ \mu\text{m}$.

mainly located in the cytoplasm of these cells. However, CA II mRNA was also detected in the nuclei of these cells but at a lower level than in the

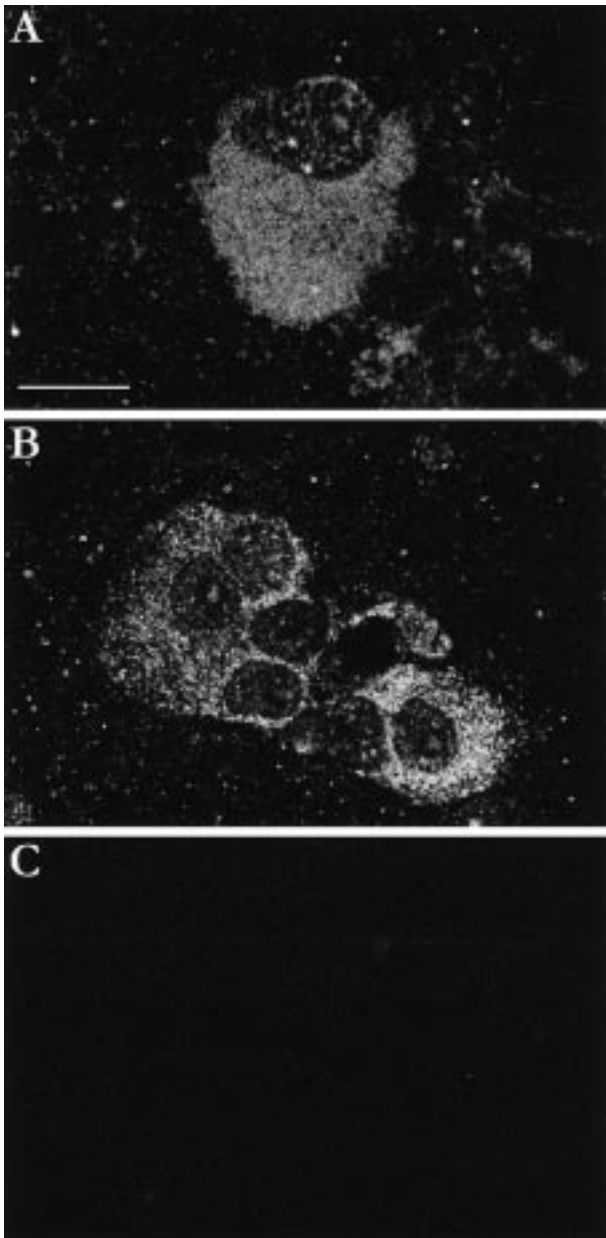


FIGURE 3. Localization of TRACP mRNA in oval-shaped mononuclear cells (putative MOPC) and osteoclasts by FISH and confocal microscopic assessment. Oval-shaped mononuclear cells (A) and multinuclear osteoclasts (B) showed cytoplasmic distribution of TRACP mRNA. The TRACP mRNA signal disappeared when the cells were incubated with 1 U/ml RNase at 37 °C for 60 min prior to hybridization (C). The scale bar represents 20 μ m.

cytoplasm. No signal was seen in the ruffled border regions of these cells. Treatment with RNase prior to hybridization resulted in a significant loss of

signal (Figs 2 and 3). In addition, samples treated with the anti-DIG-fluorescein detection system in which the cells had not been incubated with DIG-labeled riboprobes also showed no signal (not illustrated).

To determine whether these oval-shaped mononuclear cells also possessed ER mRNA, a 637 base pair antisense ER riboprobe was used. The results indicated that these oval-shaped mononuclear cells expressed a high level of ER gene transcript in their cytoplasm (Fig. 4). In contrast, only a few multinuclear osteoclasts in the bone imprints possessed a weak signal of ER gene transcripts (Fig. 4). From a total of 200 osteoclasts examined in the bone imprints only 19 possessed a weak ER mRNA signal. To confirm that ER mRNA-positive oval-shaped mononuclear cells were putative MOPC, a double staining technique for ER mRNA and TRACP enzyme activity was used. By using confocal microscopy these ER mRNA-positive oval-shaped mononuclear cells were also found to express TRACP activity in their cytoplasm (Fig. 5). It is noteworthy that the enzyme reaction for TRACP was shortened to 10 min to avoid the obscurity of fluorescence signal. Further to determine if these MOPC expressed ER mRNA at the same level, the intensity of signal in each TRACP-positive MOPC was evaluated by particle analysis and expressed as intensity per mean area of cell. Figure 6 shows the distribution of ER mRNA levels in TRACP-positive MOPC. As expected, the results indicated that ER mRNA levels in MOPC were present in a close to normal distribution. In addition, based on the morphological assessment, stromal cells and osteoblasts also express ER mRNA detected by FISH (not illustrated).

DISCUSSION

Osteoclasts are derived from bone marrow hematopoietic stem cells. A body of evidence suggests that the myeloid progenitor cells, probably granulocyte-macrophage colony-forming units (CFU-GM), generate immature osteoclast precursor cells which migrate to the bone surface where they become mature osteoclast precursor cells. The latter then differentiate and fuse into osteoclasts (see review by Zheng *et al.* 1991, Athanasou 1996). A progressive accumulation of osteoclastic bone-resorbing enzymes, such as TRACP and CA II, characterizes osteoclast differentiation (Baron *et al.* 1986, Kurijara *et al.* 1990).

Although the identification and purification of osteoclast precursors has always been difficult, several investigators have shown that putative

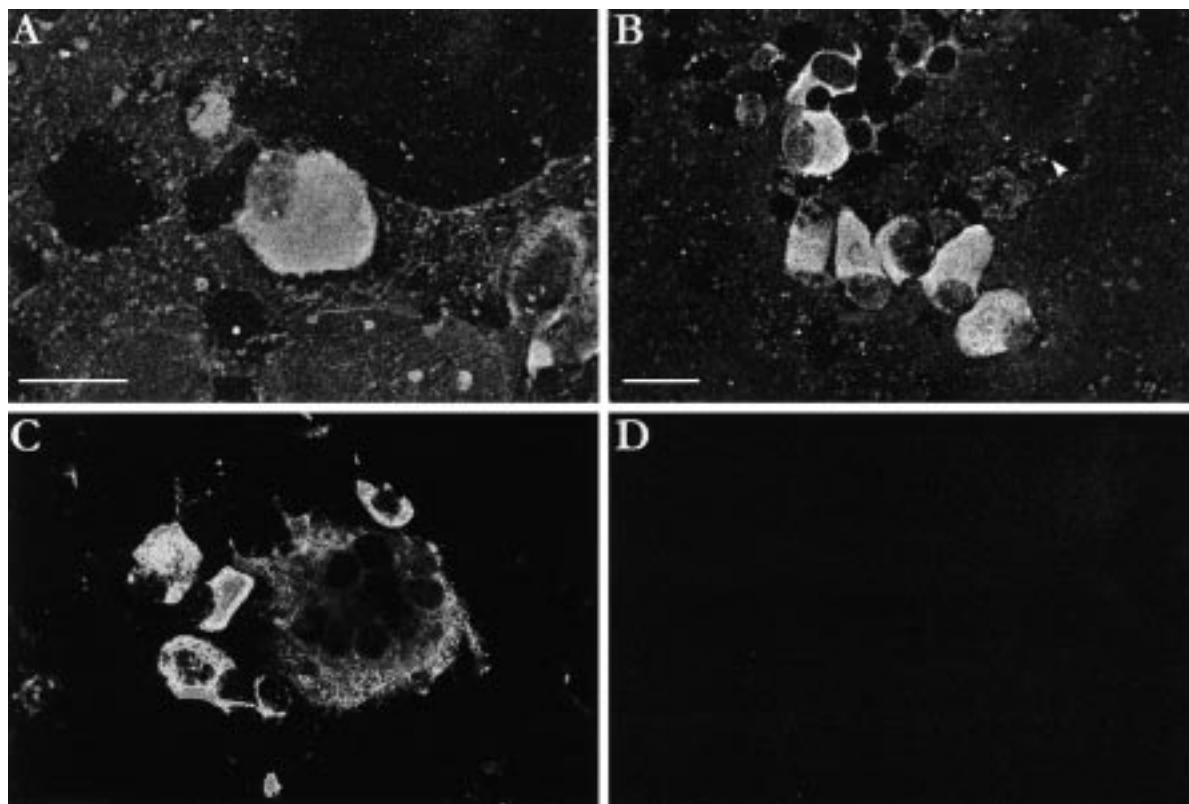


FIGURE 4. Localization of ER α mRNA in oval-shaped mononuclear cells (putative MOPC) and osteoclasts by FISH and confocal microscopic assessment. (A) Oval-shaped mononuclear cells showed cytoplasmic distribution of ER α mRNA. (B) In the same bone imprint the majority of osteoclasts (arrow) did not possess ER α gene transcripts in their cytoplasm, in contrast to the oval-shaped mononuclear cells (putative MOPC). (C) A few osteoclasts (less than 10% of total) displayed very low levels of ER α mRNA in their cytoplasm compared with the oval-shaped mononuclear cells that stained strongly and frequently. (D) The ER α mRNA signal disappeared when the cells were incubated with 1 U/ml RNase at 37 °C for 60 min prior to hybridization. A: scale bar represents 10 μ m; B, C and D: scale bar represents 20 μ m.

MOPC have abundant free ribosomes, mitochondria and extensive Golgi apparatus associated with dense specific granules (Scott 1967, Baron *et al.* 1986, Nicholson *et al.* 1986, Akisaka *et al.* 1989, Simpson & Horton 1989, James *et al.* 1996). Under the light microscope these cells have been identified as oval-shaped mononuclear cells with strong basophilic cytoplasm (Ries 1984). They are often situated next to multinuclear osteoclasts and express calcitonin and vitronectin receptors (Scott 1967, Baron *et al.* 1986, Zheng *et al.* 1994, James *et al.* 1996).

In this study, we have used FISH with confocal microscopic assessment to determine the gene expression of CA II and TRACP (both of which have been established as specific markers for osteoclasts) in these oval-shaped mononuclear cells. We have demonstrated that both oval-shaped mononuclear cells and multinuclear osteoclasts

express abundant CA II and TRACP mRNA in their cytoplasm. *In situ* hybridization for ER revealed that these oval-shaped mononuclear cells also express high levels of ER mRNA in their cytoplasm. Double staining for ER mRNA by FISH and TRACP activity by enzyme cytochemistry showed that these cells are positive for both ER mRNA and TRACP activity. Thus, our results have indicated that the ER-positive oval-shaped mononuclear cells may be putative MOPC. However, the precise stage along the osteoclast differentiation pathway occupied by these oval-shaped mononuclear cells is unclear. It is possible that the oval-shaped mononuclear cells may be mature osteoclast precursor cells (or the so called mononuclear osteoclasts) that fuse and form multinuclear osteoclasts. To determine convincingly the exact stage of differentiation of these oval-shaped mononuclear precursor cells, double or triple FISH

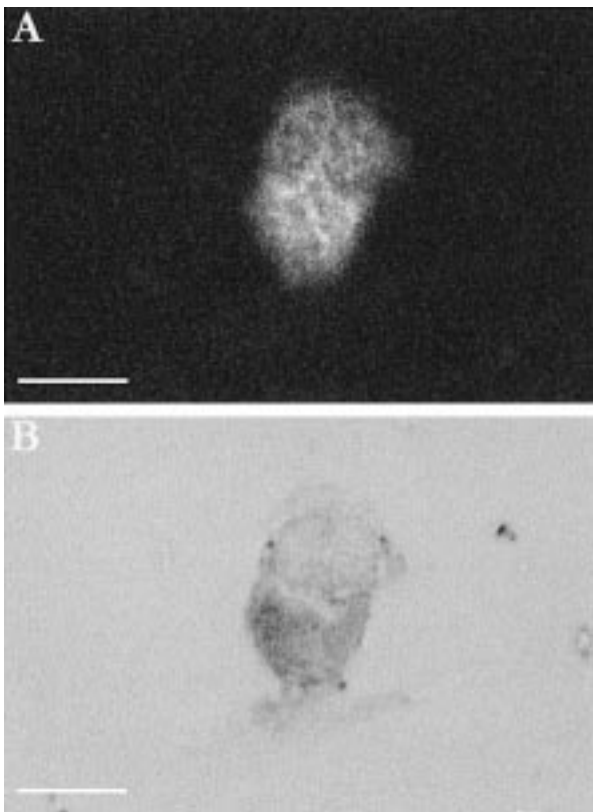


FIGURE 5. Localization of ER α mRNA in TRACP-positive mononuclear osteoclast precursor cells (MOPC). (A) Putative MOPC showed cytoplasmic distribution of ER α mRNA. (B) TRACP staining was observed in MOPC. Scale bars represent 25 μ m.

for ER, CA II and TRACP would need to be performed on individual cells.

Because target cell sensitivity to steroid action is generally dictated by the specific steroid receptors that are trans-regulators of the hormone-responsive genes in cells, the detection of ER α mRNA enables the identification of cells directly targeted by estrogen. Our previous studies showed that estrogen receptor mRNA detected by RT-PCR was expressed in long bones obtained from sexually immature rats (Zheng *et al.* 1995c). In male rats, ER mRNA was seen at day 1 and the level increased in the following 4 weeks and then decreased as they matured. Using immunohistochemistry, ER α protein was not found in bones from either sexually immature or mature rats (Zheng *et al.* 1995c). Although the mechanism in which sexually immature male rats express ER mRNA is not clear, the findings have provided a model system for examination of ER mRNA in bone by using sexually immature rats. In the present study, by

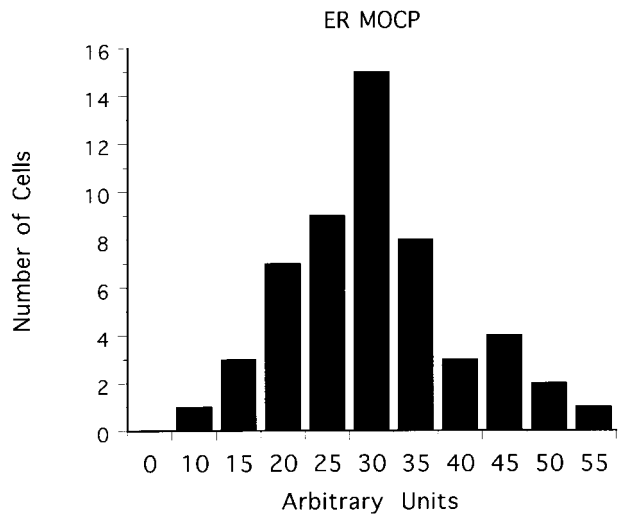


FIGURE 6. Histogram of ER α mRNA levels in MOPC. The intensity of FISH signals in MOPC was measured by particle analysis under the confocal microscope. The results are expressed as mean intensity per area of cell. The x axis represents the number of MOPC examined. The y axis represents the mean intensity per area of cell. $n=48$.

using FISH we observed that putative MOPC obtained from one-day-old rats expressed abundant ER mRNA. These results suggest that estrogen may play a more complex role in osteoclastogenesis than previously recognized. At least two pathways are involved in the mechanism of estrogen action on osteoclastogenesis. First, estrogen may have an indirect effect on osteoclasts by controlling the production of various cytokines, including interleukin-1, interleukin-6, tumour necrosis factor, transforming growth factor β and insulin-like growth factor in bone marrow stromal cells and osteoblasts (Jilka *et al.* 1992, Horowitz 1993, Manolagas *et al.* 1993, Hughes *et al.* 1996). Secondly, estrogen may directly modulate the recruitment of osteoclast precursor cells.

The possibility of direct estrogen action on osteoclastogenesis is supported by recent findings that estrogen is capable of modulating the number of early osteoclast progenitor cells (CFU-GM) in rats, through a stromal cell-independent mechanism that involves apoptosis (Shevde & Pike 1996). A significant increase in CFU-GM number was observed as early as seven days following ovariectomy, and correlated directly with an increase in the number of osteoclast-like cells generated in marrow cultures. It appears then that estrogen has a direct effect on the differentiation of osteoclast precursor cells.

Although the majority of osteoclasts do not express ER mRNA in their cytoplasm, as

determined by FISH, the existence of low levels of ER gene transcripts in some multinuclear osteoclasts is in contrast to our previous finding that human osteoclast-like giant cells in giant cell tumors of bone did not express ER α when tested by DIG-alkaline phosphatase *in situ* hybridization (Zheng *et al.* 1995a). Possibly those weak ER α mRNA-positive osteoclasts are newly formed syncytia which, with time, dramatically reduce the level of ER α gene expression. Alternatively, it is possible that there are two populations of osteoclasts existing in bone. This is supported by the finding of two types of osteoclast in *c-src*-deficient mice—a vast majority with absent ruffled border activity, and a small number that are functionally active (G Mundy, personal communication).

Even though a small proportion of osteoclasts possess a low level of ER α mRNA, the direct estrogen action on these osteoclasts is still unclear. Although studies by Oursler *et al.* (1991, 1993, 1994) indicated that avian and human osteoclasts contain ER α mRNA and respond to estrogen treatment *in vitro* with decreased osteoclastic bone resorbing activity, many investigators, ourselves included, have failed to demonstrate a direct estrogen action on mammalian osteoclasts. It seems reasonable to accept the explanation that osteoclasts may express ER α depending on the species of origin. However, further studies need to be conducted to determine whether the small proportion of osteoclasts with low levels of ER α mRNA expression are indeed responsive to estrogen action.

In short, the results of these studies have shown that oval-shaped mononuclear cells possess both morphological and biochemical characteristics of osteoclast precursor cells, including the expression of CA II and TRACP gene transcripts. Thus, we considered these cells as putative MOPC and further examined the expression of ER α gene transcripts in these cells. We showed the expression of ER α mRNA in putative MOPC and suggested that estrogen may have a direct role in modulating the recruitment of osteoclast precursor cells during osteoclastogenesis. Hence, our previous observations that ovariectomy enhanced and estrogen suppressed TRACP and CA II mRNA in rats may, at least in part, be due to the direct suppression by estrogen of the osteoclast precursor cells.

ACKNOWLEDGEMENTS

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