RESEARCH COMMUNICATION

Retinoic acid directly stimulates osteoclastic bone resorption and gene expression of cathepsin K/OC-2

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Vitamin A metabolites such as all-*trans*-retinoic acid (all-*trans*-RA) affect several steps of metabolic processes in vertebrates. In the last few years, several studies have shown the effect of RA on bone formation and metabolism. However, mechanisms of its action still remain unclear, especially with respect to the regulation of bone cells. Therefore, this study was carried out to clarify how RA regulates the activity of osteoclasts. Using a pit assay involving unfractionated bone cells, including osteoclasts obtained from rabbits, we found that RA stimulated an increase in the bone-resorbing activity in a dose- and time-dependent manner. Furthermore, this effect occurred more rapidly than that

of treatments with 1 α ,25-dihydroxyvitamin D₃. However, this effect of RA may be partly related to cross-talk between osteoclasts and other types of cells. Therefore we studied the effect of RA on isolated osteoclasts. We found that all-*trans*-RA regulates the gene expression of cathepsin K/OC-2, a dominant cysteine proteinase, at the transcriptional level in mature osteoclasts isolated from rabbits. Moreover, retinoic acid-receptor α mRNA and retinoid X-receptor β mRNA were expressed in these mature osteoclasts. Our results indicate that osteoclasts are target cells for RA and that RA might regulate a part of bone formation and metabolism through osteoclasts.

INTRODUCTION

Vitamin A, including retinoic acid (RA), is a potent regulator of physiological actions such as vision, reproduction, growth and development in vertebrates [1]. It has been well demonstrated that RA enhances the proliferation and differentiation of many types of cells; e.g., it stimulates the proliferation of skin epidermal cells [2] and the differentiation of mouse embryonal carcinoma cells [3] and clonal human promyelocyte leukaemia cells (HL-60) [4]. Recently, the discovery of nuclear retinoid receptors consisting of two classes has revealed that RA actions are mediated through gene regulation [5], by the members of the steroid/thyroid hormone receptor superfamily, of a ligand-dependent transcription factor [6]. One class of retinoid receptors consists of three retinoic acid receptors (RAR α , β and γ), to which all-*trans*-RA and 9-*cis*-RA bind. The other class, comprising three retinoid X receptors (RXR α , β and γ), has 9-*cis*-RA as ligand. The RXRs form stable heterodimers with RARs, vitamin D receptor and thyroid hormone receptor [6]. Therefore, the action of vitamin A depends on tissue- and cell-specific expression of retinoid receptors [7].

Recently, an 'RAR knock out' mouse was established [8]. This study clearly showed that RARs play an important role in normal skeletal formation. Furthermore, it is well known that hyper- or hypo-vitaminosis A affects bone formation and metabolism [9–11]. Bone formation and metabolism are modulated by bone cells such as osteoblasts, osteocytes and osteoclasts [12].

The role of RA in osteoblasts has been well demonstrated by use of an osteoblastic cell line [13]. On the other hand, since there are no established cell lines for osteocytes and osteoclasts, the effect of RA on these cells remain unclear.

We reported earlier a method for the isolation of functional mature osteoclasts from long bones of rabbits, and using this method we found that cathepsin K/OC-2, which belongs to the cysteine proteinase gene family, was dominantly expressed in mature osteoclasts compared with its expression in other tissues such as kidney, liver, spleen and lung [14,15]. Cathepsin K/OC-2 might be considered to play a critical role in osteoclastic bone resorption by digesting bone matrix proteins.

Therefore, using a pit assay, we examined the effects of RA on osteoclastic bone resorption and the gene regulation of cathepsin K/OC-2 by RA as an indication of osteoclast activity, and also on the gene expression of RARs and RXRs in mature osteoclasts. In this study we demonstrated that RA directly regulates osteoclast activity.

MATERIALS AND METHODS

Pit assay [16]

Unfractionated bone cells were isolated from tibiae, femora, humeri, ulnae and radii of 10-day-old rabbits. After the 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

Abbreviations used: RA, retinoic acid; all-*trans*-RA, all-*trans*-retinoic acid; 1,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; RAR, retinoic acid receptor; RXR, retinoid X receptor; ACT, β -actin; Act D, actinomycin D; CHX, cycloheximide; TRAP, tartrate-resistant acid phosphatase activity; α -MEM, α -minimum essential medium; FBS, fetal bovine serum.

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supernatant was used as unfractionated bone cells. In order to analyse the osteoclastic activity of these unfractionated bone cells, we performed a pit assay. For this assay, bone cells (1×10^6) were cultured on a dentine slice (6 mm in diameter) with α -MEM/5% charcoal-treated fetal bovine serum (FBS). After 2 h incubation at 37 °C, the medium was exchanged for fresh medium, and the desired factor {all-*trans*-RA or 1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]} dissolved in ethanol was added with a final concentration of 0.1% ethanol. Also, controls were prepared with ethanol alone added to a concentration of 0.1%. After being cultured for the indicated times, the cells were brushed off the dentine slices by using a 'handy labour policeman', and the slices were stained with acid haematoxylin (Sigma Chemical, St. Louis, MO, U.S.A.) for 3–4 min. The pit areas formed on the slices were determined under a microscope by counting the number of mesh squares covering the pit to evaluate possible osteoclastic bone resorption as osteoclast activity.

Isolation of mature osteoclasts [17,18]

Unfractionated bone cells obtained as described above were plated into 90 mm tissue culture dishes with α -MEM/5% FBS at 1×10^6 living cells/dish. After overnight culture, the cells were washed with PBS containing 0.001% Pronase E (Sigma Chemical) and 0.02% EDTA for 20 min at 37 °C to remove non-osteoclastic cells. After 24 h, the remaining multinucleate osteoclasts were used for further analysis as pure mature osteoclasts.

The multinucleate osteoclasts were positive for tartrate-resistant acid phosphatase activity (TRAP) and responsive to calcitonin, but were not responsive to parathyroid hormone and vitamin D, because non-osteoclastic cells were not present, as previously described [17–19].

Furthermore, the population of isolated osteoclasts was confirmed by TRAP staining using a leucocyte-acid-phosphatase kit (Sigma Chemical) in the presence of 50 mM L-tartrate by microscopy [18]. All animal experiments were conducted according to the guidelines of Meikai University, Saitana, Japan.

Northern blot analysis [7,14]

After 24 h culture in a 90 mm dish with 10 ml of α -MEM/5% FBS (dextran-coated-charcoal treated), the isolated mature osteoclasts was given 10 μ l of 10^{-7} M all-*trans*-RA (Sigma Chemical) dissolved in ethanol (controls were given 10 μ l of ethanol alone). Then after 6 h treatment with RA and control ethanol, the total RNA was extracted from the culture cells by ultracentrifugation. The RNA was blotted onto a nitrocellulose membrane after formaldehyde/agarose-gel electrophoresis, and Northern blotting was carried out as previously reported [7,14]. ³²P-Labelled radioactive probes were prepared by the random primer labelling procedure.

The hybridization probes were rabbit cathepsin K/OC-2 cDNA [14] and mouse α , β , γ RAR cDNAs and mouse α , β , γ RXR cDNAs, which were donated by Professor P. Chambon and Dr. P. Kastner (INSERM-U.184, CNRS-LGME, France). Human β -actin (ACT) cDNA probe was used as a reference.

RESULTS

All-*trans*-RA induces a increase in bone resorption by mature osteoclasts

To clarify whether RA would stimulate bone resorption by mature osteoclasts, we examined the effects of RA on bone resorption by pre-existing mature osteoclasts contained in a population of unfractionated bone cells cultured on dentine

Figure 1 All-*trans*-RA induces osteoclastic bone-resorbing activity

(a) Dose-dependent effect of all-*trans*-RA on osteoclastic bone-resorbing activity. Unfractionated bone cells (10^6 cells) were cultured for 18 h on dentine slices. The number of meshes covering the pit area was counted, values are means $\pm$ S.D. of at least six samples. (b) Time-course study of osteoclastic bone-resorbing activity on treatment with all-*trans*-RA (10^{-10} M) or 1,25(OH)₂D₃ (10^{-8} M), controls had ethanol alone added to 0.1%. * $P < 0.05$ compared with the corresponding group of controls.

Figure 2 Isolated osteoclasts on tissue-culture dish

Light micrographs of isolated osteoclasts from rabbit long bone stained for TRAP.

slices. Figure 1(a) shows that all-*trans*-RA stimulated the pit formation in a dose-dependent manner, the pit area being maximal at 10^{-10} M, representing about a 3-fold increase over that of the control at 18 h. RA might be toxic for osteoclasts at a high concentration (10^{-8} M). Next we examined the time-dependency of this effect of RA on the pit formation. At 6 h, the pit area was 2.5-fold increased over that of the control. On the other hand, 1,25(OH)₂D₃ at 10^{-8} M did not affect pit formation activity by 18 h but did increase it about 2.5 times over the control by 24 h (Figure 1b). These results indicate that physiological concentrations of all-*trans*-RA up-regulate bone resorption by mature osteoclasts more rapidly than vitamin D₃ does, suggesting the direct effect of RA on osteoclasts.

Gene expression of cathepsin K/OC-2 in osteoclasts is regulated at the transcriptional level by all-*trans* RA

In order to verify the direct effect of RA on osteoclasts, we examined whether RA would affect the gene expression of

Figure 3 Positive regulation of mRNA levels for cathepsin K/OC-2 by all-trans-RA

(a) Northern blot analysis. Total RNA (5 μ g) from isolated mature osteoclasts treated or not with all-trans-RA (10^{-10} M) for 6 h was used for samples. (b) Relative abundance of cathepsin K/OC-2 mRNA. The relative abundance of cathepsin K/OC-2 mRNA on Northern blotting was evaluated from the values of densitometric scanning. The values shown were normalized with respect to the abundance of ACT transcript and expressed as means $\pm$ S.D. of samples from four different experiments.

cathepsin K/OC-2 in isolated mature osteoclasts. The purity of isolated osteoclasts was checked by microscopy; mature osteoclasts as TRAP-positive multinucleated cells were $> 95\%$ of the population (Figure 2). As shown in Figure 3, RA (10^{-10} M) stimulated an increase in the cathepsin K/OC-2 mRNA level in these cells within 6 h. In fact, this induction could first be detected as early as 3 h after addition of RA (results not shown). Next we sought to elucidate at which step RA regulates the gene expression of cathepsin K/OC-2 in osteoclasts by using inhibitor drugs. An RNA synthesis inhibitor, actinomycin D (Act D,

Figure 4 All-trans-RA regulates the gene expression of cathepsin K/OC-2 at the transcriptional level

Northern blot analysis. Act D (5 μ g/ml), CHX (10 μ g/ml) or control saline was added to isolated mature osteoclasts, and after 1 h all-trans-RA (10^{-10} M) was added to them and incubation was continued for 6 h.

Figure 5 RAR α and RXR β genes were expressed in isolated mature osteoclasts

Total RNA (100 μ g) from mature osteoclasts and total RNA (30 μ g) from lung tissue of normal rabbit were used for Northern blotting.

5 μ g/ml), a protein synthesis inhibitor, cycloheximide (CHX, 10 μ g/ml), or control PBS was added to isolated mature osteoclasts, and all-trans-RA (10^{-10} M) was added 1 h later. After 6 h of culture, cathepsin K/OC-2 expression was examined by Northern blot analysis. Act D, but not CHX, blocked the induction of cathepsin K/OC-2 gene expression by RA (Figure 4). Also, the addition of inhibitors only did not affect the RNA level of cathepsin K/OC-2 in the osteoclasts (results not shown). These results clearly show that RA regulates cathepsin K/OC-2 gene expression at the transcriptional level without *de novo* protein synthesis in osteoclasts.

The gene expression of nuclear retinoid receptors in osteoclasts

Since RA regulates osteoclastic bone resorption and gene expression of cathepsin K/OC-2 at the level of transcription, we speculated that some types of nuclear retinoid receptors would exist in osteoclasts. The gene expression of α , β , γ RARs and α , β , γ RXRs in isolated rabbit osteoclasts was examined by Northern blotting. Figure 5 shows that RAR α mRNA and RXR β mRNA were expressed, but RAR β , γ mRNA and RXR α , γ mRNA were not, in rabbit mature osteoclasts. Lung was used as a positive control [7]. These results indicate that at least a part of vitamin A signalling in osteoclasts is modulated via nuclear retinoid receptors.

DISCUSSION

Osteoclasts function as bone- and cartilage-resorbing cells to modulate bone formation and metabolism. It is generally believed that osteoclasts differentiate from haematopoietic cells and that osteoclast precursors fuse with each other to form multinucleate mature osteoclasts. Therefore, the regulation of

formation and activation of osteoclasts is very important for vertebrate development and growth, because skeletal development is essential for normal growth. Although RA is involved in the modulation of osteoclast formation and activation, several lines of evidence suggests that the effect of RA on osteoclasts is very complicated: an excess amount of RA (10^{-8} M) inhibits bone resorption by chick osteoclasts [20]. On the other hand, RA stimulates the formation of osteoclasts from its precursor [21,22]. In the present study, we found that unfractionated bone cells from rabbit bones responded to RA, resulting in an increase in pit formation on dentine slices. These discrepancies might be partly due to the difference in species or culture conditions.

Our present data, that RA stimulated an increase in pit formation by unfractionated bone cells, suggest that RA directly affected the activation of mature osteoclasts, because RA already acted on the cells by 6 h, whereas $1,25(\text{OH})_2\text{D}_3$ required 24 h to show its effect. Mature osteoclasts have no nuclear receptors for $1,25(\text{OH})_2\text{D}_3$ [23], and the effect of $1,25(\text{OH})_2\text{D}_3$ is considered to occur indirectly via stromal cells, including osteoblasts [24,25]. However, there is a possibility that RA may also affect osteoclasts indirectly via non-osteoclastic cells, because there are types of cells other than osteoclasts in an unfractionated bone cell population and osteoblasts might have the nuclear receptors for all-*trans*- and/or 9-*cis*-RA [26]. But an indirect effect of RA on mature osteoclasts is completely ruled out, however, because of up-regulation of cathepsin K/OC-2 in rabbit-isolated mature osteoclasts by RA at the transcriptional level. This result greatly supports the idea that RA might have a direct effect on mature osteoclasts. Polyclonal antibody to cathepsin K/OC-2 blocked osteoclastic bone resorption in the pit assay (O. Ishibashi and M. Kumegawa, unpublished work). Moreover in this study we also demonstrated the existence of nuclear retinoid receptors ($\text{RAR}\alpha$ and $\text{RXR}\beta$) in the osteoclasts, as the mRNA levels of retinoid receptors probably correspond to their protein levels [27,28]. These results also indicate the possibility of the direct effect of RA on mature osteoclasts. Thus, we conclude from these results that RA might be intimately involved in bone formation and metabolism via the direct up-regulation of osteoclast activity.

In conclusion, using a pit assay we demonstrated that RA up-regulates osteoclast activity. We also demonstrated the gene expression of cathepsin K/OC-2 in response to RA and the existence of $\text{RAR}\alpha$ and $\text{RXR}\beta$ in mature osteoclasts. Thus, RA has a direct effect on osteoclastic activity, whereby it partly regulates bone formation and metabolism.

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