Endothelins inhibit the mineralization of osteoblastic MC3T3-E1 cells through the A-type endothelin receptor

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Enthusiasts inhibit the mineralization of osteoblastic MC3T3-E1 cells through the A-type endothelin receptor. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1099–R1105, 1998.—We examined the effects of various endothelins on the mineralization of mouse clonal preosteoblastic MC3T3-E1 cells. MC3T3-E1 cells expressed mRNAs for endothelin (ET)-1 and the A-type receptor for ET (ET_

THREE ENDOTHELINS (ETs) have been identified, namely, ET-1, ET-2, and ET-3 (22, 30). Two types of receptor for ETs have been also cloned: the A-type (ET_

MATERIALS AND METHODS

Materials. Human ET-1, ET-2, and ET-3 were purchased from the Peptide Institute, Osaka, Japan. ETs were dissolved in 0.1% acetic acid. BQ-123 [cyclo-D-Trp-D-Asp-Pro-O-Val-Leu], an ET_

Cell cultures. MC3T3-E1 cells were a generous gift from Dr. M. Kumezawa (Melkai University, Sakado, Japan). Cells were maintained in 55-cm² dishes in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO² in air at 37°C. After reaching 70% confluence, cells were detached by treatment with 0.05% trypsin. The cells were replated in 12-well plates (3.8 cm²/well) or 6-well plates (9.4 cm²/well) at a density of 1 × 10⁴ cells/cm². During subculture, the medium, with or without ET, was replaced every 3 days.

RT-PCR. RNA was extracted from MC3T3-E1 cells by the acid guanidinium-phenol-chloroform method (5). Total RNA (1 µg) was reverse transcribed by Moloney murine leukemia virus reverse transcriptase. Superscript (200 units; Life Technologies), using oligo(dT) primers (5 nmol) in a 20-µl
reaction mixture. Amplification of the cDNA was performed with 35 cycles of PCR in 100 µl of Pfu DNA polymerase mixture (Toyobo, Tokyo, Japan) that contained 1 µM sense primer, 5'-TGTCTTGAGGAGCAACTCA-3', and antisense primer, 5'-GCTCGTGCTGTTGCATCTTGG-3', for mouse ET-B (537 bp) (20); 1 µM sense primer, 5'-ACAGACGTCTAGAAGACCTGAGG-3', and antisense primer, 5'-TTGTAAGTGAAAGCAGCTCAG-3', for mouse ET-2 (340 bp); 1 µM sense primer, 5'-TGAAGCTCGAGAGCATTAGG-3', and antisense primer, 5'-AGGACACAGCTGAGTCATC-3', for rat ET-3 (290 bp); 1 µM sense primer, 5'-GGCGCAATCTGCT-GACATGCTGAG-3', and antisense primer, 5'-CCACGTGATAAGGTCTCAGGG-3', for the rat ET-A receptor (343 bp) (25); or 1 µM sense primer, 5'-CGTCTGCGTCTAGGGC-3', and antisense primer, 5'-CGACCTCAAGAGGCAACAGCTCAGG-3', for the mouse ET-B receptor (293 bp) (25). The sequence of the gene for the mouse ET-B receptor is unknown. We referred to the sequence of the rat ET-A receptor in this experiment because Perkins et al (25) had previously detected the cDNA for the mouse ET-A receptor using fragments of the rat gene for the ET-A receptor. Each reaction cycle consisted of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. Products of PCR were subjected to electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide. DNA markers (molecular weight marker V; Boehringer Mannheim, Tokyo, Japan) were used as size markers.

Assay of binding of[^125^I]-ET-1. Cells, grown in 12-well plates (3.8 cm²/well), were washed with ice-cold PBS (pH 7.4; 20 mM sodium phosphate and 130 mM NaCl) and incubated in 0.5 ml of PBS that contained 0.2% (wt/vol) bovine serum albumin, sodium phosphate and 130 mM NaCl) and incubated in 0.5 ml (3.8 cm²/well), were washed with ice-cold PBS (pH 7.4; 20 mM phosphate, 50 µg/ml ascorbic acid, and ETs at various concentrations. We measured calcium, in hydroxyapatite, in the cell layer. The layers of cells in 12-well plates (3.8 cm²/well, polystyrene) were washed with PBS and incubated with 1 ml of 2 N HCl overnight with gentle shaking. The calcium ions in each sample were quantitated by the o-cresolphthalein complexone method with a Calcium C kit (Wako Pure Chemical Industries, Osaka, Japan) (10). This kit is specific for calcium, and the limit of sensitivity is 1 µg/ml. We used the standard solution of calcium (10 mg/dl) in the kit as the standard solution in our determinations.

Measurement of the production of inositol 1,4,5-trisphosphate. Cells that had been subcultured in 6-well plates (9.4 cm²/well) for 8 days were incubated with serum-free a-MEM for 3 h. This culture medium was supplemented with 10^-7 M ET-1 for various brief periods, as indicated, and then the medium was removed by aspiration and replaced with 0.5 ml of 4% perchloric acid. After incubation of the cells in perchloric acid on ice for 20 min, the supernatant was brought to pH 7.5 by titration with ice-cold 1.5 M KOH that contained 60 mM HEPES buffer. The inositol 1,4,5-trisphosphate (IP_3) generated was quantitated with a d-myo-[³H]IP_3 assay kit (Amersham Life Science).

Measurement of the accumulation of cAMP. Cells, grown in 12-well plates, were incubated with serum-free a-MEM that had been supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase, at 37°C for 15 min after washing with serum-free a-MEM. Osteoblastic cells were subsequently incubated at 37°C for 15 min with 10^-7 M ET. After incubation, the cells were lysed by addition of 200 µl of a 0.1 N solution of HCl that contained 5 mM EDTA. The amount of cAMP was measured with a radiomunnoassay kit from Yamasa (Chiba, Japan).

RESULTS

Presence of ET-1 and the ET_A receptor in osteoblastic cells. In a previous study that involved radiolabeled ligand-binding assays, we showed that osteoblast-like cells from calvariae of newborn rats (ROB cells) express the ET_A receptor (10). To identify ETs and the types of receptor present in MC3T3-E1 cells, we performed RT-PCR with primers for specific ET-1, ET-2, ET-3, and both the ETA and ET_B receptors. Messenger RNAs for ET-1 and the ETA receptor were detected in MC3T3-E1 cells, as shown in Fig. 1. We failed to detect the mRNAs for ET-2, ET-3, and the ET_B receptor in MC3T3-E1 cells. Binding assays on day 8 using radiolabeled ET-1 showed that ET-1, ET-2, and a specific antagonist of the ETA

Fig. 1. Detection of mRNAs for endothelin (ET)-1 and ET receptors by RT-PCR. Total RNA was isolated from MC3T3-E1 cells on days 8 and 10 and reverse transcribed. Products of amplification by PCR of cDNAs were subjected to electrophoresis on a 1.5% agarose gel and detected by staining with ethidium bromide. Arrows point to fragments of 537 bp, 343 bp, and 293 bp that correspond to cDNAs for ET-1, the ETA receptor, and the ET_B receptor, respectively. Results depicted are representative of results of 2 experiments.
receptor, BQ-123 (14), prevented the binding to receptors of radiolabeled ET-1 with similar potency (IC_{50} values: 5.4, 5.6, and 15.5 nM, respectively), whereas ET-3 was less potent (IC_{50}: 620 nM) (Fig. 2). ET_{A} receptors were detected in MC3T3-E1 cells during the entire culture period (data not shown).

Northern blotting analysis of the level of mRNA for osteocalcin. To examine whether ETs might be involved in the maturation of osteoblastic cells, we performed Northern blotting using, as probe, the cDNA for osteocalcin, which is a marker of osteoblastic maturation. MC3T3-E1 cells were treated continuously for 10 days with 10^{-7} M ET-1, ET-2, or ET-3. A final concentration of 0.0001% acetic acid was used as a control treatment. We found that ET-1 and ET-2 each markedly decreased the steady-state level of expression of the mRNA for osteocalcin in MC3T3-E1 cells (Fig. 3). ET-3 had a very weak inhibitory effect compared with that of ET-1 or ET-2.

Effects of ETs on mineralization by osteoblastic cells. We measured the deposition of calcium by MC3T3-E1 cells that had been treated with ETs at various concentrations. As shown in Fig. 4A, ET-1 and ET-2 inhibited the deposition of calcium by MC3T3-E1 cells in a dose-dependent manner. ET-1 and ET-2 had an inhibitory effect even at 10^{-11} M. ET-1 and ET-2 were much more effective than ET-3 (Fig. 4B). Furthermore, BQ-123, a specific antagonist of the ET_{A} receptor, attenuated the inhibitory effect of ET-1 (Fig. 5).

We next examined the effects of the pulsed administration of ET-1 on the deposition of calcium by MC3T3-E1 cells. The presence of 10^{-7} M ET-1 on days 6–8 exclusively suppressed mineralization by MC3T3-E1 cells to the same extent as the continuous presence of 10^{-7} M ET-1 from day 0 to day 16 (Fig. 6).

Transduction of the ET-1 signal in MC3T3-E1 cells. To elucidate the signaling pathway triggered by ET-1 in MC3T3-E1 cells, we measured the rates of production of both IP_{3} on day 8 and cAMP on day 9 when ET-1 most effectively influenced the mineralization by MC3T3-E1 cells, as shown in Fig. 6. The production of IP_{3} in MC3T3-E1 cells was stimulated by ET-1, with a peak between 15 and 30 s after exposure to ET-1 (Fig. 7A). By contrast, ET-1 and ET-3 had no significant effect on the level of cAMP compared with the control (Fig. 7B). Moreover, ET-1 had no effect on the production of cAMP during the entire culture period (data not shown).

DISCUSSION
In this study, we demonstrated that ET-1 might affect osteoblastic metabolism via the ET_{A} receptor, acting as an autocrine/paracrine regulator. We next demon-
strated that ETs decreased the level of mRNA for osteocalcin, which is known as a marker of the maturation of osteoblastic cells. ETs also acted, via the ETA receptor, to inhibit mineralization by MC3T3-E1 cells in a dose-dependent manner. It appeared that phospha-
tidylinositol turnover might be involved in the action of ET-1 in MC3T3-E1 cells, and all our results together suggest that ETs might be local modulators of osteoblastic function in bone.

There are some reports of the regulation by ETs of the metabolism of cartilage and bone. ET-1 and the ETA receptor have been shown to be involved in the formation of bone and cartilage that is derived from the branchial arch by use of mice deficient in ET-1 (20) and the ETA receptor. Such mice have poorly developed mandibular and thyroid cartilage and mandibular and temporal bone. We also reported the presence of ETA receptors on the perichondrium in rat tracheal and xiphoid cartilage and in the fetal rat epiphysis and showed that ET-1 increased the rate of incorporation of thymidine into cartilage tissue (21). ET-1 also regulates osteoclastic bone resorption (1, 34, 37). Localization of ET-1 in the osteoclasts indicates that osteoclasts are target cells for ET-1 (28). Perkins et al. (25) showed that ET-1 stimulates osteoblastic production of interleukin-6 (IL-6), an active mediator of osteoclasts. Their result indicates that ET-1 might regulate the genera-

**Fig. 4. Effects of ETs on mineralization by MC3T3-E1 cells.** Cells in 12-well plates were cultured with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 µg/ml L-ascorbic acid, and 10^{-7} M ET or 0.0001% acetic acid (control). Quantitative analysis of calcium ions derived from hydroxyapatite was performed as described in MATERIALS AND METHODS. A: dose-dependent decreases in the deposition of calcium in MC3T3-E1 cells. Cells were treated with ET-1 and ET-2 at various concentrations for 14 days. B: effects of ETs on calcium deposition in MC3T3-E1 cells. Cells were treated with 10^{-7} M ET for 12 days. Data are means ± SD of results from 3 or 4 wells and are typical of results of 3 separate experiments. *P < 0.01 vs. control.

**Fig. 5. Attenuation by the ETA receptor-specific antagonist BQ-123 of the ET-1-induced inhibition of mineralization by MC3T3-E1 cells.** Cells in 12-well plates were cultured for 15 days with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 µg/ml L-ascorbic acid, and 10^{-9} M ET-1 and/or 10^{-6} M BQ-123, as indicated. Quantitative analysis of calcium ions derived from hydroxyapatite was performed as described in MATERIALS AND METHODS. Data are means ± SD of results from 3 wells. *P < 0.01 vs. control; **P < 0.01 vs. results obtained with ET-1.

**Fig. 6. Effects of pulsed treatment with ET-1 on mineralization by MC3T3-E1 cells.** Cells in 12-well plates were cultured with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 µg/ml L-ascorbic acid, and they were treated with 10^{-7} M ET-1 continuously or for the indicated periods. On day 16, calcium ions derived from hydroxyapatite were quantitated as described in MATERIALS AND METHODS. Data represent means ± SD of results from 3 wells and are typical of results of 3 separate experiments. *P < 0.01 vs. control.
The maturation stage. These results strongly suggest that ET-1 inhibits the differentiation of osteoblastic cells. Recently, ET was reported to increase the level of mRNA for osteocalcin in rat osteosarcoma cells (29). Differences in the effects of ET-1 on the expression of osteocalcin might reflect differences in cell culture conditions (methods of administration of ET-1), differences between species (rat versus mouse), and the type of osteoblast model used (osteosarcoma cells versus normal osteoblastic cells).

Continuous culture in the presence of ET-1 or ET-2 resulted in inhibition of the deposition of calcium by MC3T3-E1 cells. Moreover, ET-1 inhibited the mineralization of MC3T3-E1 cells when present only from day 6 to day 8, as shown in Fig. 5. We also showed that the level of the ETA receptor did not change in MC3T3-E1 cells during the culture period. These results suggest that ET-1 might regulate the expression of mineralization-related genes from day 6 to day 8. We are now using the technique of differential-display PCR to identify the genes whose expression is regulated by endothelins. Transforming growth factor (TGF)-α has also been reported to inhibit the formation of nodules after a short pulse in the initial culture (12). It will be of interest to pursue the relationship, in terms of signaling, between ET-1 and TGF-α in osteoblastic cells.

In a previous study (16), we showed that 8-bromo-cAMP inhibits both the synthesis of alkaline phosphatase (ALPase) and the formation of mineralized nodules, in a model of bone formation in vitro. Parathyroid hormone (PTH), which activates adenylate cyclase in osteoblastic cells, has been reported to inhibit the activity of ALPase and the mineralization of osteoblastic cells via production of cAMP, depending on the exposure time in vitro (17). Therefore, we postulated that cAMP might be a candidate for a second messenger of ET-1 in MC3T3-E1 cells. However, ET-1 did not affect the production of intracellular cAMP in MC3T3-E1 cells during culture from day 3 to day 14. Takuwa et al. (32) reported similar results with confluent MC3T3-E1 cells. These results indicate that ET-1 does not stimulate adenylate cyclase in MC3T3-E1 cells, unlike PTH and prostaglandin (PG) E2 (6). However, ET-1 can modulate calcium signaling via phospholipase C in various types of osteoblastic cell (31, 32). In our cell culture system, ET-1 stimulated the production of IP3 (Fig. 7A). PGF2α has been shown to decrease the activity of ALPase, via the accumulation of inositol phosphate, in MC3T3-E1 cells (11). Signaling by inositol phosphate in response to ET-1 might be involved in the inhibition of differentiation of osteoblastic cells and of mineralization by osteoblastic cells. Recently, the presence of phospholipase D was demonstrated in MC3T3-E1 cells, and the enzyme was shown to hydrolyze phosphatidylcholine (31).

In the present study, we found that ETs retarded the maturation and mineralization of osteoblastic cells. ET-1 from vascular endothelial cells might act on osteoblasts since bone is rich in blood vessels. However, we showed that osteoblastic cells expressed mRNAs for ET-1 and the ETA receptor. These results suggest that ET-1 derived from osteoblasts might regulate some...
aspect of osteoblastic differentiation. Our present findings suggest that a relationship might exist between bone cells and ETS in the local environment.

Perspectives

The results obtained in this study suggest that ET-1 might inhibit the differentiation and mineralization of preosteoblastic MC3T3-E1 cells via the ET\textsubscript{A} receptor, with generation of IP\textsubscript{3} as the intracellular signal. Upregulation of the level of intracellular calcium ions at a certain time might regulate the differentiation and mineralization of MC3T3-E1 cells. To clarify whether increases in levels of cytosolic calcium ions reflect a common mechanism for inhibition of mineralization, we will attempt to examine the role of calcium-sensing receptors of osteoblastic cells and the effects of calcium channel blockers and calcium ionophores on mineralization by osteoblastic cells.

We have proposed that vasoconstrictors, such as angiotensin II (9) and ET-1, might retard the differentiation and formation of bone by osteoblastic cells. Spontaneously hypertensive rats are known as models of osteoporosis (3, 35). The vasocontractile effects of ET-1 are greater in spontaneously hypertensive rats than in normotensive Wistar-Kyoto rats (23). These observations suggest that certain relationships are likely to exist between bone cells and vasoactive peptides in the local environment. It might even be useful to examine whether antagonists of the ET\textsubscript{A} receptor, such as BQ-123, could be effective in the treatment of osteoporosis.

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