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Endothelin Modulates Osteopontin and Osteocalcin Messenger Ribonucleic Acid Expression in Rat Osteoblastic Osteosarcoma Cells

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Abstract Endothelins (ETs) are vasoconstrictive peptides produced mainly by endothelial cells. The ET receptors are expressed in many types of cells including osteoblast-like cells. The purpose of this study was to examine the effects of endothelin on the expression of osteoblastic phenotype-related genes. We found that endothelin-1 (ET-1) enhanced approximately two-fold the mRNA expression of both osteopontin and osteocalcin genes in rat osteoblastic osteosarcoma ROS17/2.8 cells. These effects were dose-dependent, peaking at 10^{-7} M. The ET-1 enhancement of the abundance of osteopontin and osteocalcin mRNAs was time-dependent, with a maximal effect at 24 h. ET-1 modulation of the expression of the two phenotype-related gene products of osteoblasts suggests that endothelin is one of the cytokines which modulate osteoblastic functions and that this molecule may play a role in the regulation of bone metabolism.

Key words: osteoblasts, endothelin, osteopontin, osteocalcin

Endothelin (ET) is a recently discovered vasoactive peptide, which is produced by endothelial cells and constricts capillary vessels [1]. Subsequent gene cloning studies revealed the presence of at least three related peptides, ET-1, ET-2, and ET-3 in human [2]. They all consist of 21 amino acids and have two intramolecular disulfide bonds. Propeptide form of ET consists of 38 amino acids and is called a big endothelin, which is then cleaved by an ET-converting enzyme to yield an active mature form [3,4].

ET binds to at least two types of receptors, ET-A and ET-B. Complementary DNAs for ET-A and ET-B were cloned and their sequences predict that the two receptors share about 65% amino acid sequence homology [5,6,7]. The affinity of ET-A for ET-1 is higher than that for ET-3. ET-A is expressed in vascular smooth muscle cells, and plays a key role in vascular constriction. On the other hand, the affinity of ET-B for ET-1 and ET-3 is similar. ET-B is expressed in vascular endothelial cells, which are thought to be important in the secretion of nitric-oxide (NO) and prostacyclines [8,9]. Furthermore, ET-B mRNA has been shown to be expressed in many tissues, including brain, lung, heart, adrenal gland, and kidney [5]. Rat osteoblast-like osteosarcoma ROS17/2 cells were reported to express abundant ET-B receptor message [5], and this observation suggested that ETs might play certain roles in bone metabolism.

The effects of ETs on osteoblasts or bone tissues have been reported by several groups. ET-1 at 10^{-7} M increases bone resorption and synthesis of collagenase-digestible and nondigestible proteins in organ cultures of bone [10]. ET-1 also increases inositol phosphate turnover at the range of 10^{-9} to 10^{-7} M, and stimulates DNA synthesis but decreases alkaline phosphatase activity in osteoblast-like MC3T3E1 cells and in osteoblast-enriched fetal calvaria cells [11–15]. In the experiments using isolated osteoclasts, ET-1 inhibits osteoclastic bone resorption and motility of these cells [16].

Noncollagenous bone proteins share about 10% of the organic component in bone, although the precise functions of most of them are still unclear. Osteocalcin (OC) is so far the only

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specific product of osteoblastic cells [17–19]. Osteopontin (OP) is also produced by bone cells and is one of the most abundant noncollagenous phosphoproteins with the Arg-Gly-Asp-Ser (RGDS) motif, which is involved in cell adhesion and has been shown to be the recognition site for the cell surface recertor, $\alpha_v\beta_3$ integrin [20,21].

In this report, we examined ET effects on the mRNA expression of these phenotype-related genes encoding OC and OP in osteoblast-like osteosarcoma ROS17/2.8 cells, which make bone when transplanted in animals and express many osteoblastic phenotypes including high alkaline phosphatase, parathyroid hormone receptor, type I collagen, OC, OP, and osteonectin [22,23]. We found that ET regulates the expression of OC and OP genes in these cells.

MATERIALS AND METHODS

Cell Culture

An osteoblast-like osteosarcoma cell line ROS17/2.8 was a kind gift from Dr. Gideon A. Rodan (Merck Research Laboratories). The cells were cultured in modified Ham's F12 medium, supplemented with 5% fetal bovine serum (Gibco Life Technologies, Inc., Grand Island, NY), at 37°C in a fully-humidified atmosphere of 95% air/5% CO_2.

Endothelins and Reagents

Human recombinant ET-1 and ET-3 were obtained from Peptide Institute (Osaka, Japan). ET was dissolved at 10^{-4} M in 0.1% acetic acid and stored in aliquots at −20°C as stock solutions. Dilutions of the stock solution were prepared immediately before use.

RNA Extraction and Northern Blot Analysis

Subconfluent cells were treated with ET or vehicle in fresh media. Total RNA was extracted by acid-guanidium thiocyanate phenol chloroform extraction method (AGPC method), as described by Chomczynski et al. [24]. Total RNA was separated by electrophoresis in 1% agarose/0.22 M formaldehyde gel and electroblotted to Hybond N filters (Amersham Corp., Arlington Heights, IL) and the filters were exposed to UV light to cross-link RNA.

The filters were prehybridized in a buffer containing 50% formamide, 5 × S.S.C. (1 × S.S.C. = 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt's solution, 50 ng/ml sheared and denatured herring sperm DNA, and then hybridized overnight at 42°C in a fresh buffer containing all the ingredients in the prehybridization buffer in addition to 1×10^6 cpm/ml of radiolabeled cDNA probes for rat OP [25], rat OC, or human glycer-aldehyde-3-phosphate dehydrogenase (GAPDH). The rat OC cDNA (pOC918) used in this experiment was cloned from ROS17/2.8 library by M. Noda using the oligonucleotide sequence published previously [26]. At the end of the hybridization period, filters were washed in 2 × S.C.C. with 0.1% sodium dodecyl sulfate at 56°C for 20 min and exposed to X-ray films at −80°C. Autoradiograms were quantitated using an Ultrascan XL laser densitometor (Pharmacia, LKB, Uppsala, Sweden).

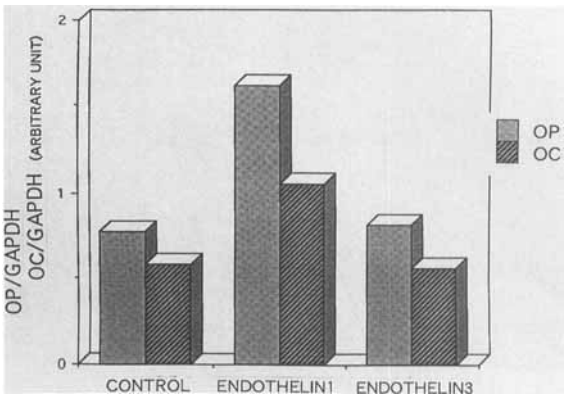

Fig. 1. The ET-1 and ET-3 effects on the expression of OP and OC mRNAs in ROS17/2.8 cells. The cells were plated at 2×10^4 cells/cm^2 and were grown for 5 to 7 days before the initiation of the treatment. Subconfluent cells were treated with 10^{-7} M ET-1, ET-3, or vehicle (control). Twenty-four hours later, total RNA was extracted by AGPC methods and Northern blot analysis was conducted as described in Materials and Methods. The bands on the autoradiograms were quantitated by using laser densitometer. The values for OP (plain shaded bar) and OC (diagonal bar) were normalized against those for glycer-aldehyde-3-phosphate dehydrogenase (GAPDH). The figure represents one of two experiments with similar results.

RESULTS

Treatment with 10^{-7} M ET-1 for 24 h enhanced the mRNA expression of OP gene by at least two-fold (Fig. 1). A similar enhancement was also observed for OC mRNA expression in these cells (Fig. 1). In contrast, ET-3 had little effect compared to ET-1 after either 24 h (Fig. 1) or 72 h (data not shown). Therefore, in the following experiments, we examined the effects of only ET-1 on these cells.

The time course of the ET^{-1} effect is shown in Figure 2. The basal levels of OC and OP mRNAs were enhanced by the medium change by itself.

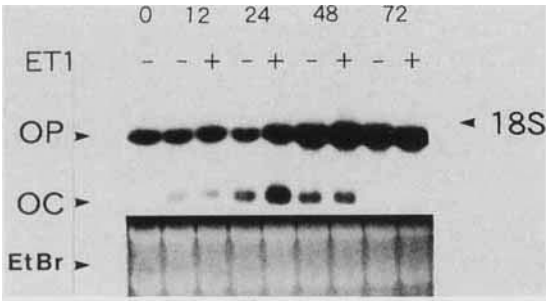

Fig. 2. Time course of the endothelin-1 effects on OP and OC mRNA levels. Subconfluent ROS17/2.8 cells were treated with vehicle (−) or 10^{-7} M endothelin-1 (+) for the indicated number of hours. Northern blot analysis was conducted as described in Materials and Methods. The bottom panel indicates the ethidium bromide stained gel shown as control. The figure represents one of two experiments with similar results.

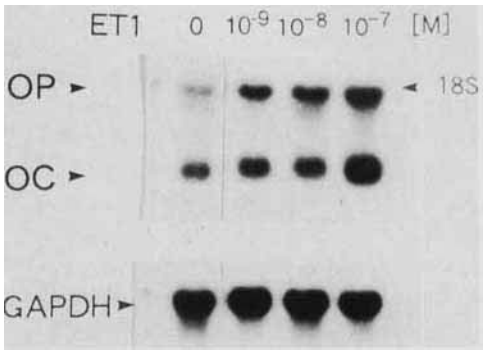

Fig. 3. Dose dependence of the ET-1 effects on the abundance of OP and OC mRNAs. Subconfluent ROS17/2.8 cells were treated with vehicle (0) or the indicated doses of ET-1 for 24 h. Northern blot analysis was carried out as described in Materials and Methods. The positions of OP, OC, glycer-aldehyde-3-phosphate dehydrogenase (GAPDH), and 18S ribosomal RNA are indicated. The figure represents one of two experiments with similar results.

The abundance of both OP and OC mRNA was further enhanced by the treatment with 10^{-7} M ET-1. This effect peaked at 24 h after the initiation of the treatment for both OP and OC mRNAs. The ET effect was less at a later time point (72 h) when the basal message levels were higher (OP) or lower (OC) than those at the earlier time point (24 h) (Fig. 2). The 28S and 18S ribosomal RNA bands in ethidium bromide stained gel are shown to visualize the equality of the loading (Fig. 2).

The dose dependence of the ET effect is shown in Figure 3. ET-1 upregulated OP and OC mRNA expression in a dose dependent manner starting at 10^{-9} M. The maximal effects on mRNA expression of both OP and OC genes were observed at 10^{-7} M. GAPDH mRNA levels on the same filter served as a control.

DISCUSSION

In this report, we have demonstrated that ET-1 modulates mRNA expression of osteoblastic phenotype-related genes, encoding OP and OC. The products of these two genes are the major constituents of noncollagenous proteins in bone matrix, and the expression of these gene products has been shown to be under the control of calcitropic hormones and cytokines. The fact that ET is among those effectors which enhance mRNA expression of OP and OC genes, supports the idea that ET is at least one of the regulators of osteoblastic functions in the local environment in bone. The direction of the modulation of OP expression is dependent on the regulatory molecules, although many of them which enhance bone resorption also have been shown to enhance OP production. ET has been observed to suppress osteoclastic bone resorption [16], and this may suggest that ET may be acting in a similar way as those which enhance OP expression, but suppress osteoclastic resorption. TGF-β is one of those molecules which fall into this category. TGF-β is also produced not only in bone cells but also in endothelial cells, where it might interact with ET with regard to their regulation of osteoblastic functions by the endothelial cells [27].

It is still to be determined which type of cells produce ET in the local milieu where osteoblast-osteoclast interaction is taking place. Vascularization is necessary for the normal bone development both on the formation and resorption sides. Bone formation, but not cartilage formation, requires vascularization, and the precursor cells for osteoclasts are thought to be recruited through vascularization. These observations suggest that certain communication may exist between the endothelial cells in the vasculatures and bone cells in the local environment. This interaction may be either direct or indirect via vascularization. Further experiments are necessary to examine whether ET may be playing a role in this interaction.

OC is one of the specific products of osteoblasts, and the enhancement of OC gene expression by ET is supporting the idea that ET could be one of the mediators of the coupling phenomenon between bone formation and bone resorption. Upon certain stimuli, ETs could be produced by endothelial cells which, in turn, inhibit bone resorption and stimulate bone formation.

Osteoblast-like osteosarcoma cells ROS17/2, have been shown to express ET-B receptor mRNA [5,28]. Takuwa et al. reported that MC3T3E1 cells are sensitive to ET-1 more than ET-3 [12]. ET-1 has higher affinity for ET-A than ET-B receptor [12] and these observations suggest the possible variation in the profile of the receptor composition which could depend on the stage of differentiation, or the type of osteoblast-like cells. Our data indicate that in ROS17/2.8 cells, ET-1 enhanced OP and OC mRNA expression while ET-3 did not modulate the expression of these two genes. This result may imply that ROS17/2.8 cells might express ET-A receptor in addition to ET-B receptor. Alternatively, ROS17/2.8 cells may express only ET-A receptor while ROS17/2 could express ET-B receptor. This point has to be clarified by examining the profile of receptors for ET in these osteoblast-like cells.

Relationship between vascularization and bone formation has been well described morphologically in many cases, including the embryonic development, fracture healing, and normal bone remodeling in adults. However, the molecular and cellular mechanisms in the interaction of these two tissues have not yet been well understood. Further investigation should elucidate the signaling molecules acting between the two tissues, which make the coordination of these heterogeneous cell societies, to maintain homeostasis of bone tissues in the normal environment as well as in pathological situations.

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