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Detection and Characterization of Endothelin in Transformed Human Osteoblast Cell Culture Medium

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Endothelin-1 (ET-1), a 21 amino acid peptide originally purified from conditioned medium of cultures of porcine aortic endothelial cells, is recognized also as a product of many other cells such as epithelial cells, glial cells, and neurons. It is now recognized that at least ET-1 plays an important role in bone metabolism. It has been shown that ET-1 inhibits osteoclast bone resorption by a direct effect on cell motility and it can also activate phospholipase C in the osteoblast. Furthermore, several studies have shown that ET-1 stimulates the formation of inositol phosphates, the synthesis of DNA, the mobilization of calcium from extra- and intracellular pools, the activation of phospholipase D, and the stimulation of tyrosine phosphorylation in osteoblast-like (MC3T3-E1 and UMR-106) cells. The aim of the present study was to detect and characterize the presence of endothelin in transformed human osteoblast cell culture medium (HTb96) by radioimmunoassay and chromatography methods. Immunoreactive endothelin (IR-ET) was undetectable in the medium incubated at 0.5 and 1 h and was 3.2 ± 0.2 fmol/10^5 cells (mean ± SEM, n = 6) at 2 h, 9.5 ± 0.5 fmol/10^5 cells at 6 h, 19.8 ± 2.1 fmol/10^5 cells at 24 h, and 23.7 ± 2.0 fmol/10^5 cells at 48 h, respectively. Sephadex G-25 superfine chromatography and fast protein liquid chromatography studies showed that >90% of IR-ET in the culture medium coeluted with synthetic ET-1. These results show that ET-1 could be formed by transformed human osteoblasts. Further studies should be conducted to elucidate the physiological role of endothelins as possible autocrine, paracrine, or endocrine factors in calcium and bone metabolism.

Key Words: Chromatography; endothelin; osteoblast; radioimmunoassay.

Introduction

Although it is well known that some kinds of interaction between osteoblasts and osteoclasts exist in the regulation of bone resorption, the exact mechanism underlying these interactions is not clearly understood. It is assumed that locally produced paracrine factors are of central importance in this control system in bone. It has been shown that osteoblasts respond to mechanical forces *(1)* as well as systemic factors such as parathyroid hormone (PTH) *(2)* by producing local factors that influence osteoclast function and hence the rate of bone resorption.

Endothelin-1 (ET-1), a 21 amino acid peptide originally purified from conditioned medium of cultures of porcine aortic endothelial cells *(3)*, is recognized as a product of many other cells as well *(4)*. Recently, substantial evidence has shown that endothelins may have a role in bone metabolism *(5)*. Immunostaining for ET-1 has been observed over osteoclasts, osteoblasts, and vascular endothelial cells *(6)*. Both endothelin receptors type A and B (ET_A and ET_B, respectively) have been demonstrated in osteoblastic cells by ligand binding and these receptors are functional *(7)* and could be downregulated by 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$) *(8)*. It has been shown that ET-1 elicited a dose-dependent stimulatory effect on interleukin-6 expression in primary human osteoblastic cells; thus, it may participate in the regulation of bone resorption *(9)*. Hiruma et al. *(10)* have demonstrated that mouse clonal preosteoblastic MC3T3-E1 cells expressed mRNAs for ET and the A-type receptor for endothelin. They also demonstrated that ET might inhibit the mineralization of osteoblastic MC3T3-E1 cells *(10)* as well as rat calvarial osteoblast-like cells *(11)* via an interaction with the ET_A receptor, with generation of inositol triphosphate as the intracellular signal.

The aim of the present study was to detect the presence of immunoreactive endothelin (IR-ET) by radioimmunoassay (RIA) and to characterize IR-ET by chromatography in a transformed human osteoblast-like cell preparation (HTb 96).

Materials and Methods

Cell Culture Medium

Conditioned media of transformed human osteoblast-like cells (HTb-96) *(12)* collected at defined culture times

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were obtained from Prof. S. R. Bloom of Hammersmith Hospital of London. These cells are derived from a moderately differentiated sarcoma and have been shown to have osteoblast-like properties *(13)*, including the production of alkaline phosphatase [AP] *(12)*. The cells were grown as monolayers in Dulbecco's modified Eagle's medium containing fetal calf serum (10% [w/v]; BRL, Paisley, Renfrewshire, Scotland) and gentamycin (100 μg/mL); (Flow Laboratories, Rickmansworth, Herts,UK). The cells were grown in 75- or 125-cm^2 Falcon flasks (Becton Dickinson, Oxford, UK) and were maintained in a humidified atmosphere of 7.5% CO_2 at 37°C. They were subcultured at confluence by washing in 0.5 mmo/L of EDTA (disodium salt) followed by treatment with trypsin-EDTA (0.025% [w/v]; Flow Laboratories) for 2 min at 37°C, addition of complete medium before centrifugation (150*g*) and resuspension in complete medium. The medium was sampled before and after 0.5, 1, 2, 6, 24, and 48 h of incubation with cells. Monolayers were washed three times in phosphate-buffered saline-A, and the media were frozen in aliquots at –70°C until used for RIA of ET-1 and chromatography studies.

Radioimmunoassay

IR-ET in conditioned medium was extracted by Sep-Pak C_{18} cartridges (Waters, Milford, MA) and then assayed by RIA as described previously *(14)*. The assay could detect changes of 0.05 fmol/assay tube at 95% confidence with duplicate tubes. Crossreaction with big ET-1 (human 1-38; Nova Biochem, Nottingham, UK) and ET-2 and ET-3 (Peptide Institute, Minoh-Shi, Japan) was 0.1, 60, and 70%, respectively. The intra- and interassay coefficients of variation were 12% ($n = 9$) and 19% ($n = 7$), respectively.

Gel Permeation Chromatography

Gel permeation chromatographic characterization of the IR-ET in culture medium was carried out using Sephadex G-25 superfine column chromatography (0.9 × 60 cm), (Pharmacia, Uppsala, Sweden) as described prevoisly *(15)*. Briefly, reconstituted Sep-Paked culture medium extracts were centrifuged to remove particulate materials and the supernatants were loaded onto the column. The column was eluted at a flow rate of 3.5 mL/h at 4°C in 60 m*M* phosphate buffer (pH 7.4) containing 10 m*M* EDTA, 7 m*M* sodium azide, 0.2 *M* NaCl and 0.3% (w/v) bovine serum albumin. The positions of void volume (V_0) and total volume (V_T) were determined by dextran blue ($Mr\ 2 \times 10^6$) and $Na^{125}I$, respectively. Cytochrome-*c* (*Mr* 12,384) was included as an internal marker. Fractions of 0.7 mL each were collected for RIA of ET-1. The recovery of IR-ET in culture medium extracted from the column was >80%.

Fast Protein Liquid Chromatography (FPLC)

Fractionation of the IR-ET from the culture medium was carried out by FPLC using a high-resolution reverse-phase 5/5 (Pep Rpc HR 5/5) C_{18} column (Pharmacia) as described previously *(15)*. The pooled culture media were extracted by Sep-Pak C_{18} cartridges, reconstituted in water containing 0.1% (v/v) trifluoroacetic acid (TFA), and centrifuged for 15 min in a microfuge. The supernatants were then loaded onto the column, which was first equilibrated with 15% (v/v) acetonitrile in water (each with 0.1% [v/v] TFA). After injection of the samples, the column was eluted with a gradient of acetonitrile from 15 to 35% (v/v) in water with 0.1% TFA over 1 h at a flow rate of 1 mL/min per fraction. Samples of each fraction were dried in a Savant vacuum centrifuge, reconstituted in assay buffer, and assayed. The recovery of IR-ET in culture medium extracted from the column was >95%.

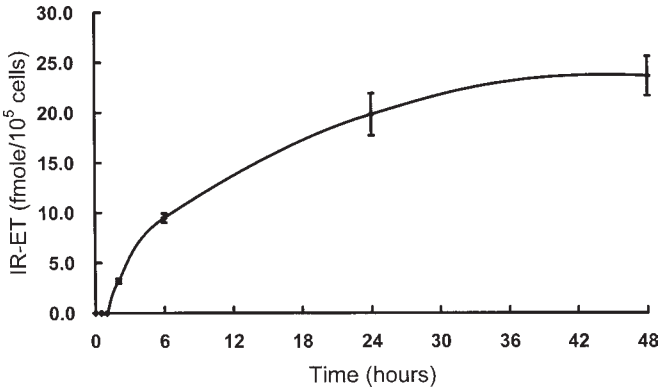

Fig. 1. Time course showing the progressive production of IR-ET in conditioned media obtained from transformed human osteoblast cell culture. Vertical bars are the mean ± SEM ($n = 6$).

Results

Immunoreactive endothelin was undetectable in the culture medium incubated at 0.5 and 1 h, but the concentration of IR-ET increased to 3.2 ± 0.2 fmol/10^5 cells (mean ± SEM, $n = 6$) at 2 h, 9.5 ± 0.5 fmol/10^5 cells at 6 h, 19.8 ± 2.1 fmol/10^5 cells at 24 h, and 23.7 ± 2.0 fmol/10^5 cells at 48 h. (Fig. 1). Both Sephadex G-25 column chromatography (Fig. 2) and fast protein liquid chromatography (FPLC) (Fig. 3) showed that >90% of IR-ET in the cultured medium eluted in an identical position to synthetic ET-1.

Discussion

The regulation of bone metabolism depends on paracrine interactions among bone cells (osteoblasts, osteoclasts, lining cells) and between bone cells and other mesenchymal cell types such as endothelial cells, in the bone microenvironment. Originally identified as a product of endothelial cells *(3)*, endothelin is now known to be a product of many other cells including epithelial cells *(4)*. Although the physiological functions of ET-1 in bone remain to be elucidated, the growing literature documents the presence and effects of endothelins in bone. By using the immunostaining method, Sasaki and Hong *(6)* demon-

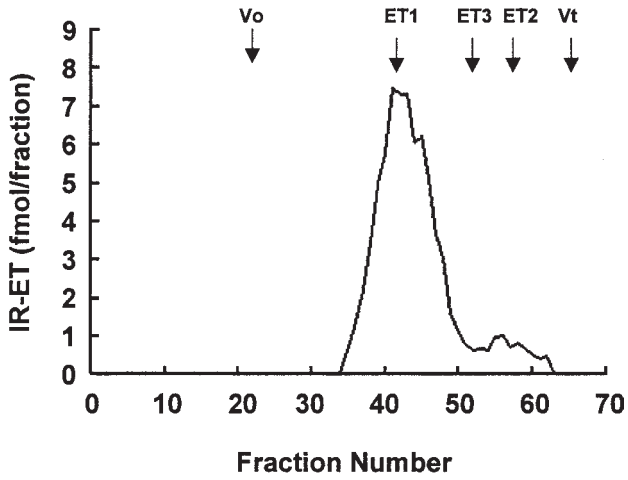

Fig. 2. Sephadex G-25 superfine column chromatographic profile of transformed human osteoblast cell–conditioned media. ET1, ET2, and ET3 indicate the elution positions of ET-1, ET-2, and ET-3, respectively. Molecular size markers are as follows : V_0, dextran blue; V_t, $Na^{125}I$.

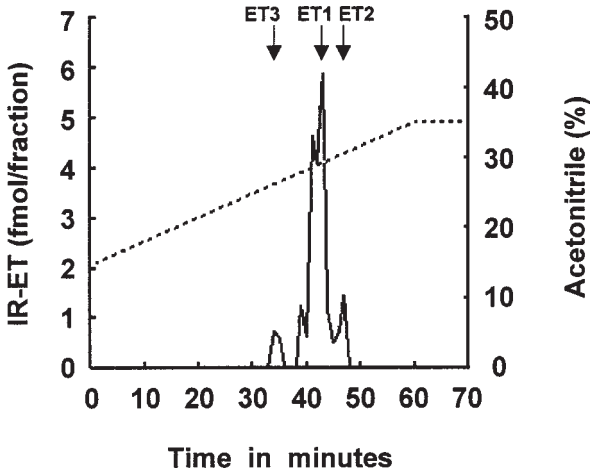

Fig. 3. FPLC profile of transformed human osteoblast cell–conditioned media. The dotted line shows a linear gradient of acetonitrile from 15 to 35%. ET1, ET2, and ET3 indicate the elution positions of ET-1, ET-2, and ET-3, respectively.

strated the localization of ET-1 in osteoclasts, osteoblasts, and vascular endothelial cells. In osteoblasts, they noted that ET-1 immunoreactivity was found mainly along the plasma membranes, cisterns of rough-surfaced endoplasmic reticulum, mitochondria, and cytoplasmic matrices. The present study demonstrates the presence of IR-ET-1 in conditioned culture medium from transformed human osteoblast-like cells (HTb 96) by means of RIA and chromatography. Actually, ET-1 mRNA in the culture medium had been measured by means of Northern blot hybridization as described previously *(16)*. However, no hybridization could be shown in the culture medium (S. R. Bloom, personal communication). This may simply reflect the insensitivity of the Northern blot analysis. The production of immunoreactive endothelin was progressively increased in culture in a time-dependent manner, and Sephadex G-25 chromatography and FPLC both demonstrated that the major IR-ET in the conditioned media was eluted in the position of standard ET-1, which suggests that cultured human osteoblast-like cells are also capable of producing ET-1 as do the endothelial cells *(14)*.

Both ET_A and ET_B have been demonstrated in osteoblastic cells by ligand binding, and these receptors are functional *(7)* and could be downregulated in response to $125(OH)_2D_3$ *(8)*. Kasperk et al. *(17)* demonstrated that ET-1 exerts its potent stimulatory effects on human osteoblastic cell proliferation and differentiated functions (including alkaline phosphatase [AP] and type I collagen production) mainly mediated by the ET_A receptor. Schvartz et al. *(18)* showed that ET-1, acting via its rapid stimulation on the tyrosine phosphorylation, has potent mitogenic activity in osteoblast-like cells. In addition, ET-1 has been shown to regulate osteopontin and osteocalcin mRNA expression in rat osteoblastic osteosarcoma cells *(19)*, and there are reports demonstrating the regulatory effects of ET-1 on the metabolism of osteoblastic cells. In osteoblast-like MC3T3-E1 cells, ET-1 has been shown to induce stimulation of phospholipase C–mediated phosphoinositide hydrolysis, mobilization of calcium from extra- and intracellular pools and stimulation of DNA synthesis *(20)*, reduction of cellular AP activity *(21)*, stimulation of tyrosine phosphorylation *(18)*, and activation of phospholipase D *(22)*. In osteoblast-like UMR-106 rat osteosarcoma cells, it has been shown that ET-1 modulates calcium signaling by epidermal growth factor, thrombin, prostaglandin E_1 (PGE_1)*(23)*, and PTH *(24)* and activates calcium and inositol phosphate second-messenger systems *(25)*. Hence, it is possible that ET-1 may modulate growth and functions of osteoblasts in an autocrine manner. Furthermore, it also provides a basis for a regulatory role of ET-1 in bone formation because the number of differentiated osteoblasts has been suggested by Gruber et al. *(26)* to be the most important determinant for the bone-forming capacity of a bone compartment.

Besides having osteoanabolic actions on the osteoblasts, ET-1 has also been shown to have antiresorption effects on osteoclasts. Alam et al. *(27)* showed that ET-1 exerts its inhibitory effect on osteoclastic bone resorption by a direct effect on osteoclast cell motility and osteoclast margin ruffling (quiescence or Q effect) at concentrations similar to those effective for their primary vasoconstrictive action. On the other hand, ET-1 has also been shown to be able to induce bone resorption in cultured neonatal mouse calvaria, and this resorption is prone to inhibition by indomethacin, thus suggesting a role for prostaglandins in the actions of ET-1 on bone *(28)*. Recently, Leis et al. *(29)* demonstrated that in mouse osteoblast-like MC3T3-E1 cells, ET-1-induced PGE_2 synthesis is attributable to ET_A receptor

occupancy and is mediated through a protein tyrosine kinase–dependent and protein kinase C–dependent pathway. Furthermore, Eguchi et al. *(30)* showed that endothelin inhibits the secretion of PTH from human adenoma cells and in an in vivo parathyroid gland perfusion model *(31)*, and Lee and Stern *(24)* showed that ET-1 enhances calcium signals elicited by PTH in UMR-106 cells via endothelin-B receptor activation. These findings suggest that endothelin may also have a regulatory role in PTH secretion and action.

In conclusion, the present study demonstrated that the mature ET-1 peptide is produced by transformed human osteoblast cell lines (HTb 96). Because Hiruma et al. *(10)* recently also clearly demonstrated the presence of ET-1 mRNA in mouse clonal preosteoblastic MC3T3-E1 cells, further studies should be performed to elucidate the physiological role of endothelins as possible autocrine, paracrine, or endocrine factors in calcium and bone metabolism.

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