Interleukin 6 Stimulates the Production of Immunoreactive Endothelin 1 in Human Breast Cancer Cells


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Abstract

To investigate the potential regulation of endothelin 1 production in human breast cancer cells, we measured the release of immunoreactive endothelin 1 (ir-ET-1) from the MCF-7 and ZR-75-1 breast cancer cell lines in response to various agents including estrogen and tamoxifen as well as several cytokines. ir-ET-1 was detected in conditioned medium of MCF-7 cells and ZR-75-1 cells by specific radioimmunoassay. Among the agents tested, estrogen, tamoxifen, tumor necrosis factor, γ-interferon, interleukin (IL) 1, and transforming growth factor β had no effect on ir-ET-1 secretion by these breast cancer cells. However, IL-6 (20 ng/ml) treatment of MCF-7 cells and ZR-75-1 cells caused maximal increases in the amount of ir-ET-1 secreted into the culture medium to 206 and 314% of basal values after 6 h, respectively. This effect of IL-6 on ir-ET-1 secretion was inhibited by actinomycin D and cycloheximide, indicating that IL-6 stimulates de novo synthesis of ir-ET-1 at a transcriptional level.

Reverse-phase high performance liquid chromatography coupled with radioimmunoassay in the conditioned medium from IL-6-treated cells revealed one major ir-ET-1 component corresponding to human standard ET-1. The present study demonstrates the potential for IL-6 to stimulate ir-ET-1 production in human breast cancer cells, which may participate in the process of acute phase reactant-like expression of this peptide and/or in the process of IL-6 enhanced breast cancer cell motility, the latter being recently clarified.

Introduction

ET-1 is a novel vasoconstrictive peptide originally isolated from the spent medium of cultured endothelial cells of porcine vessels (1). Several investigators have reported that ET-1 is produced by a wide variety of nonendothelial cultured cell types, i.e., vascular smooth muscle cells (2), renal epithelial cells (3), endometrial glandular epithelial cells (4), and normal human breast epithelial cells (5), as well as several human breast cancer cell lines including breast cancer cell lines (6, 7).

Recently, we have demonstrated that a large amount of ET-1 is present in tissue extracts from human breast cancer (8). Several investigators including us have demonstrated that ET-1 produced by tumor cells may play a role stimulating growth of tumor cells in an autocrine or paracrine fashion (7, 9–11). However, no significant association was found between ir-ET-1 concentration in tissue extracts from human breast cancer and clinicopathological factors such as tumor size, lymph node involvement, histological type, and hormone receptor status (8). These results suggested that ET-1 expressed in human breast cancer cells has biological significance other than growth stimulation. In the present study, we explored whether estrogen, tamoxifen, and various cytokines influence ET-1 production in two human breast cancer cell lines, MCF-7 and ZR-75-1, to investigate the mechanisms mediating ir-ET-1 production by human breast cancer cells. We report here that ir-ET-1 production was stimulated by IL-6 among the agents tested. A possible significance of ET-1 expression in human breast cancer cells is discussed.

Materials and Methods

Chemicals. 17-β-Estradiol, actinomycin D, and cycloheximide were purchased from Sigma Chemical Co., St Louis, MO. Tamoxifen (1-β-dimethoxyethoxyphenyl-trans-1,2-diphenylbut-1-ene) was a gift from ICI Pharmaceutical Co., London, United Kingdom. Recombinant TNF and IFN-γ were kindly supplied by Dainippon Pharmaceutical Co., Osaka, Japan, and Shionogi Co., Osaka, Japan, respectively. Recombinant human IL-1α, IL-6, and TGF-β were purchased from Genzyme Co., Cambridge, MA.

Cell Culture. Human breast cancer cell line MCF-7 was kindly provided by Dr. Yasuo Nomura (National Kyushu Cancer Center Hospital, Fukuoka, Japan), and ZR-75-1 (passage 82) was obtained from the American Type Culture Collection (Rockville, MD). All of these cell lines were maintained in 25-cm² plastic tissue culture flasks by using RPMI 1640 (GIBCO Laboratories, Detroit, MI) at 37°C in a 95% room air-5% CO₂ humidified incubator. After planting at 1 × 10⁴ cells/flask, the culture medium for all these cell lines was supplemented with 10% fetal bovine serum. When the cells had grown to 90% confluence, the culture medium of each flask was replaced with a serum-free RPMI medium by washing with the same medium. Each culture flask was subjected to further incubation for 3 days at 37°C in the serum-free medium prior to the experiment.

The serum-free medium was supplemented with test agents that included estrogen (10⁻⁸ M), tamoxifen (10⁻⁶ M), TNF (10 ng/ml), IFN-γ (10 ng/ml), IL-1β (20 ng/ml), IL-6 (20 ng/ml), and TGF-β (2 ng/ml). Finally, the cells were sustained for various times as indicated in “Results” to obtain the conditioned medium used for the ensuing analyses. The cells were counted manually, and nonviable cells were estimated by trypan blue staining.

Extraction Method. The conditioned medium (2 ml) acidified with trifluoroacetic acid was applied to Oxytrol cartridge. The materials adsorbed to the cartridge were eluted with 60% acetonitrile/0.09% trifluoroacetic acid and evaporated by a centrifugal concentrator. The dried residues were reconstituted in the assay buffer and subjected to RIA. The assay buffer for RIA was 0.01 M phosphate buffer, pH 7.4, containing 0.01 M EDTA, 0.02 M glycine, 0.01 M ε-aminocaproic acid, 0.001 M sodium azide, 0.1% heat-inactivated human albumin, and 0.1% Triton X-100.

Human ET-1 RIA. Immunoreactive ET-1 was determined by specific RIA for ET-1 as described by Ando et al. (12). Briefly, the incubation mixture consisted of 0.1 ml sample or standard, 0.1 ml assay buffer, and 0.1 ml anti-human ET-1 serum. Incubation was carried out at 4°C overnight, followed by the late addition of 0.05 ml 125I-human ET-1 and further incubation at 4°C for 2 days. The bound from free ligands were separated by adding 0.5 ml assay buffer containing 10 µl goat anti-rabbit γ-globulin, 1 µl normal rabbit serum, and 5% polyethylene glycol and incubated at 4°C for 15 min. The radioactivity in the precipitate was counted in a gamma spectrometer.

Reverse-Phase HPLC. Reverse-phase HPLC was performed in conditioned medium from IL-6-treated MCF-7 cells and ZR-75-1 cells using a column (0.46 × 25 cm; Capcell Pak C18SG 120) eluted with a linear gradient of acetonitrile from 10 to 60% in 0.09% TFA for 60 min at a flow rate of 1.0 ml/min. After evaporation, each elute was subjected to ET-1 RIA.

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1To whom requests for reprints should be addressed.

2The abbreviations used are: ET-1, endothelin 1; ir-ET-1, immunoreactive endothelin 1; RIA, radioimmunoassay; HPLC, high performance liquid chromatography; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor.
Results

Production of ir-ET-1 in Human Breast Cancer Cells. As shown in Fig. 1, ir-ET-1 was secreted into the culture medium of two breast cancer cell lines, MCF-7 and ZR-75-1. In these cell lines, ir-ET-1 was detected as early as 6 h after the replacement of the culture medium, followed by almost a plateau up to 24 h. The amount of ir-ET-1 released into the culture medium of MCF-7 cells was about twice as much as that of ZR-75-1.

A reverse-phase HPLC profile of ET-1 in the conditioned medium from IL-6-treated MCF-7 cells and ZR-75-1 cells is depicted in Fig. 2 and shows a major peak in the position of human standard ET-1. A serial dilution curve of extracted conditioned medium obtained from MCF-7 and ZR-75-1 exhibited parallelism with that of standard ET-1 in the RIA (data not shown), indicating that these cells release ir-ET-1.

Effects of Various Agents on ir-ET-1 Production in Human Breast Cancer Cells. Table 1 showed the effects of various agents including estradiol, tamoxifen, TNF, IFN-γ, IL-1β, TGF-β, and IL-6 on ir-ET-1 secretion into the conditioned medium of MCF-7 cells and ZR-75-1 cells. In both cell lines, estradiol, tamoxifen, TNF, IFN-γ, IL-1β, and TGF-β had no effects on ir-ET-1 concentration of the culture medium at any times tested. However, IL-6 (20 ng/ml) treatment of MCF-7 cells and ZR-75-1 cells stimulated ir-ET-1 release after 6 h to 206% (P < 0.005) and 314% (P < 0.001) of control values, respectively. Similarly, IL-6-stimulated release of ir-ET-1 was observed both after 12 and 24 h in these cell lines.

To test further whether the stimulated production of ir-ET-1 in MCF-7 cells and ZR-75-1 cells was dependent upon IL-6 concentration, ir-ET-1 concentration 6 h after IL-6 treatment was compared among the media treated with various concentrations of IL-6. As shown in Table 2, an increase in ir-ET-1 secretion was not seen with 2 ng/ml of IL-6. However, an increase in dose (200 ng/ml) resulted in a further production of ir-ET-1 in MCF-7 cells and ZR-75-1 cells, indicating that IL-6-stimulated production of ir-ET-1 in these cells was dose dependent.

Effects of Actinomycin D and Cycloheximide on ir-ET-1 Secretion Stimulated by IL-6. To determine whether IL-6-stimulated ir-ET-1 secretion required de novo protein synthesis, either actinomycin D (2.5 μg/ml) or cycloheximide (10 μg/ml) was simultaneously added to the culture medium with IL-6. As compared with ir-ET-1 concentration 6 h after the medium replacement, cycloheximide inhibited both the basal ir-ET-1 secretion and IL-6-stimulated ir-ET-1 secretion in MCF-7 cells and ZR-75-1 cells (Table 2). Similarly, the presence of actinomycin D effectively prevented both the basal ir-ET-1 secretion in ZR-75-1 cells and IL-6-stimulated ir-ET-1 secretion in two breast cancer cell lines (Table 2). However, the inhibitory effect of actinomycin D on the basal ir-ET-1 secretion in MCF-7 cells does not reach statistical significance (Table 2).

Discussion

The mechanisms mediating ET-1 production are poorly understood. TGF-β, which has been shown previously to cause ET-1 release and ET-1-mRNA induction in several cells, e.g., endothelial cells (13),
IL-6 Stimulation of Endothelin 1 Production

Table 1: Effects of various agents on ir-ET-1 production in human breast cancer cells

<table>
<thead>
<tr>
<th>Agents</th>
<th>MCF-7</th>
<th>ZR-75-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>46.1 ± 13.8</td>
<td>18.3 ± 5.4</td>
</tr>
<tr>
<td>Estradiol (10^-8M)</td>
<td>45.3 ± 5.3</td>
<td>14.3 ± 5.4</td>
</tr>
<tr>
<td>Tamoxifen (10^-8M)</td>
<td>52.6 ± 6.3</td>
<td>17.4 ± 4.9</td>
</tr>
<tr>
<td>TNFα (10 ng/ml)</td>
<td>40.6 ± 15.0</td>
<td>15.1 ± 7.4</td>
</tr>
<tr>
<td>IFN-γ (10 ng/ml)</td>
<td>49.7 ± 10.7</td>
<td>24.9 ± 5.5</td>
</tr>
<tr>
<td>IL-1β (20 ng/ml)</td>
<td>42.5 ± 7.4</td>
<td>22.4 ± 6.2</td>
</tr>
<tr>
<td>TGF-β (2 ng/ml)</td>
<td>57.3 ± 6.1</td>
<td>19.9 ± 3.3</td>
</tr>
<tr>
<td>IL-6 (20 ng/ml)</td>
<td>95.0 ± 17.5b</td>
<td>102.4 ± 8.8c</td>
</tr>
</tbody>
</table>

* P < 0.05 compared with control.
+ P < 0.005 compared with control.
- P < 0.001 compared with control.

Table 2: Effects of actinomycin D and cycloheximide on IL-6-stimulated production of ir-ET-1 in MCF-7 cells and ZR-75-1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCF-7</th>
<th>ZR-75-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>46.1 ± 13.8</td>
<td>18.3 ± 5.4</td>
</tr>
<tr>
<td>IL-6</td>
<td>51.8 ± 16.1b</td>
<td>29.6 ± 10.1b</td>
</tr>
<tr>
<td>20 ng/ml</td>
<td>95.0 ± 17.5b</td>
<td>57.5 ± 5.0b</td>
</tr>
<tr>
<td>200 ng/ml</td>
<td>126.5 ± 7.3c</td>
<td>80.4 ± 15.5c</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>26.2 ± 9.3b</td>
<td>10.0 ± 2.2b</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>7.3 ± 3.1b</td>
<td>2.5 ± 1.5b</td>
</tr>
<tr>
<td>IL-6 (20 ng/ml) + actinomycin D</td>
<td>20.8 ± 8.6c</td>
<td>20.7 ± 6.8c</td>
</tr>
<tr>
<td>IL-6 (20 ng/ml) + cycloheximide</td>
<td>9.1 ± 3.0c</td>
<td>8.4 ± 3.9c</td>
</tr>
</tbody>
</table>

normal breast epithelial cells (5), HepG-2 cells (6), and HEC-1A cells (4) had no effect on ir-ET-1 release from the MCF-7 and ZR-75-1 breast cancer cell lines. Furthermore, IL-1, which has been reported to increase the production of ET-1 by cultured endothelial cells (14), also had no effect on ir-ET-1 secretion from two breast cancer cell lines tested in the present study. Very recently, Schrey et al. (10) reported that bombesin and glucocorticoid stimulate T47D human breast cancer cell line to produce ir-ET-1, suggesting a possible autocrine/paracrine role for ET-1 in breast cancer. However, nothing is known about the effect of IL-6 on ET-1 production.

The present study clearly demonstrated that ir-ET-1 was produced by two breast cancer cell lines, MCF-7 and ZR-75-1, and that this ir-ET-1 production was stimulated by IL-6 in these cell lines which have been reported to possess the IL-6 binding high affinity receptors (15). IL-6 is the major systemic mediator of the early host response (the acute phase response) to infection and injury (16–18). Furthermore, IL-6 is often detected in the systemic circulation and in the local neoplastic tissue in cancer patients (19–21). Recently, Inoue et al. (22) have cloned the whole length of the human preproendothelin 1 gene and the corresponding complementary DNA and determined the complete nucleotide sequences. The 5’-flanking region of the gene contains hexanucleotide sequences for the acute phase reactant-regulatory elements, CTGGGA, which is thought to mediate the induction of gene expression under acute physical stress in vivo (23, 24). Furthermore, previous observations have indeed indicated that plasma levels of ET-1 in humans are transiently elevated after a major physical stress such as acute myocardial infarction (25). Although most acute phase reactants are produced by hepatocytes, it is tempting to hypothesize that breast cancer cells mimic the acute phase reactant-like expression of ET-1 in response to the progression of breast cancer itself and IL-6 is involved in this process.

Another possible explanation is that ET-1 may be a participant in the process of IL-6-enhanced cell motility. In human breast cancer cells, IL-6 has been shown to have a unique effect on cellular morphology. Recently, Tamm et al. (26, 27) reported that IL-6-treated cells convert from an epithelial shape to a fibroblastoid shape of ZR-75-1 breast cancer cells, which is accompanied by a decrease in cell-cell association and an increase in cell motility. Furthermore, among many cytokines, IL-6 has been confirmed to be the only one that causes a striking conversion of ductal breast carcinoma cells from an epithelial to a fibroblastoid phenotype, which is attended by the acquisition of the ability to migrate over considerable distances (28). However, it is at yet unknown what the IL-6-mediated alterations are in cellsurface or extracellular matrix proteins that underlie the increase in motility. At present, there are no data showing the direct linkage of ET-1 to an increase in cancer cell motility. However, it seems conceivable to expect that ET-1 produced by breast cancer cells may participate in the process of IL-6-enhanced cancer cell motility. Furthermore, Tamm et al. (28) reported that ET-1 acts as an increase in intracellular Ca2+ levels, which may promote cell replication and motility (4).

References

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