Induction of endothelin-1 synthesis by IL-2 and its modulation of rat intestinal epithelial cell growth

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Endothelin (ET) is a potent vasoconstrictor peptide expressed by various cell types, including vascular endothelial cells, cardiac muscle, and smooth muscle cells. ETs are synthesized and released by a variety of cell types in response to various stimuli, including inflammatory mediators and growth factors. The biological actions of ETs are mediated by specific receptors, which can be classified into ETA and ETB subtypes. ETA receptors are more potent vasoconstrictors than ETB receptors, and ET-1 is the strongest pressor of the ET family. ET-1 is a more potent agonist than ET-3 at the ETA receptor, while these two peptides have similar potencies at the ETB receptor. ETA receptor antagonists significantly enhanced cellular proliferation, suggesting involvement of the ETA receptor in modulation of IL-2-induced intestinal epithelial cell growth.

ETA receptor; mRNA expression

Endothelin (ET) is a potent vasoconstrictor peptide with 21 amino acid residues that was originally isolated from the supernatant of cultured porcine endothelial cells. Three ET isoforms (ET-1, ET-2, ET-3), identified in humans, pigs, and rats, have different pressor and vasoconstrictor activities. Among these, ET-1 is the strongest pressor and vasoconstrictor ever isolated. ETA receptor antagonists significantly enhanced cellular proliferation, suggesting involvement of the ETA receptor in modulation of IL-2-induced intestinal epithelial cell growth.
cells; ET-1, in turn, may be involved in regulating the growth of these cells. These findings suggest that ET can facilitate a coordinated response by epithelial cells and cellular constituents of the local immune system that is present in the intestinal mucosa.

**MATERIALS AND METHODS**

Cell cultures and IL-2 treatment. Cell lines IEC-6 and IEC-18, originating from rat intestinal epithelia (30), were purchased from American Type Culture Collection (Rockville, MD). The stock cultures were grown in an atmosphere of 5% CO₂ at 37°C in a culture medium composed of DMEM containing 5% FCS, 10 µg/ml insulin, 50 U/ml penicillin, 50 U/ml streptomycin, and 4 mM glutamine. IEC-18 from passages 8 or 9 were used. In experiments to assess the effects of IL-2 on cells, recombinant rat IL-2 (Sigma Chemical, St. Louis, MO) was added to subconfluent monolayers at a concentration of 10 U/ml, and the cells were cultured for 48 h.

Determination of ET release. Subconfluent monolayers were cultured in serum-free DMEM or in DMEM containing 5% FCS, and culture was maintained for 48 h. Culture media were cultured in serum-free DMEM or in DMEM containing 10% Block Ace, 0.4 M NaCl, and 2 mM EDTA.

RNA extraction and PCR amplification. Total RNA was isolated from intestinal epithelial cells using RNAzol (Biotect, Houston, TX). Cells were lysed with 1.0 ml RNAzol/dish. Isolation and extraction were performed according to the manufacturer’s suggested protocol. Briefly, RNA was extracted with chloroform and precipitated with isopropanol. The precipitated RNA was washed with 70% ethanol. The concentration of the extracted RNA was calculated by measuring the optical density at 260 nm. The ratio of the optical density at 260 nm to that at 280 nm was always > 1.9. The quality of RNA was assessed by the intactness of 28S and 18S bands and the lack of degradation on agarose-gel electrophoresis.

Aliquots of RNA (5 µg) were reverse-transcribed using an RT-PCR kit from Stratagene (La Jolla, CA). Briefly, 5 µg of RNA in 38 µl of diethyl pyrocarbonate-treated water was mixed with 0.3 µg of oligo(dT), heated at 65°C for 5 min, and then cooled slowly at room temperature. The following reagents were added to the tubes: 5 µl of 10X concentrated synthesis buffer (final concentration, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 1 µl of RNase block inhibitor (40 U/µl), 2 µl of 100 mM dNTPs, and 1 µl of Moloney murine leukemia virus-RT (50 U/µl). The reaction mixture was incubated for 1 h at 37°C before the reaction was terminated by incubating the tube at 90°C for 5 min and on ice for 10 min. The tube was stored at −80°C until PCR was performed using the Takara Taq kit (recombinant Taq DNA polymerase; Takara Biochemicals, Tokyo, Japan), with rat-specific primers prepared on a DNA synthesizer (Sawady Technology, Tokyo, Japan). The primers were designed according to cDNA sequences of rat preproET-1 (32) and preproET-3 (34) and are as follows:

**ET-1**

- Primer (antisense), bases 675–699:
  - 5'-AAGATCCCAGCCAGCATGGAGAGCG-3'
- Primer (sense), bases 157–181:
  - 5'-GTTGCTCTGTCCCTGATGG-3'

**ET-3**

- Primer (antisense), bases 479–499:
  - 5'-GCTGGTGACTTATCTGTCC-3'
- Primer (sense), bases 23–42:
  - 5'-TTCTCGGGCTCACAGTGACC-3'

The cDNA amplification products were predicted to be 543 bp in length for ET-1 and 477 bp for ET-3. To initiate the PCR, we added 2 µl of RT products to the PCR master mix, including 10X PCR reaction buffer diluted to final concentrations of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl₂, and 2.5 U of recombinant Taq DNA polymerase, 50 µM each of the primers, and 200 µM dNTPs. Tubes were placed in a Programmed Tempcontrol system (Applied Biosystems, Tokyo, Japan) that was programmed as follows: 1) incubation at 94°C for 3 min (initial denaturation); 2) 30 cycles of the following sequential steps: 94°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72°C for 3 min (extension); and 3) incubation at 72°C for 7 min (final extension). The PCR products were size-fractionated by agarose gel electrophoresis. After electrophoresis and ethidium bromide staining, DNA bands were visualized with an ultraviolet transilluminator. We confirmed the identity of the PCR products by direct DNA sequencing, using ABI PRISM linkage mapping sets (Perkin-Elmer Applied Biosystems) on an Applied Biosystems model 377 DNA sequencer.

In the case of ET receptors, PCR was performed with the following specific primers according to Cai et al. (3):

- ETₐ receptor primer (antisense), bases 384–403:
  - 5'-GGAGATCAATGACCAGCGTAG-3'
- ETₐ receptor primer (sense), bases 15–21:
  - 5'-AGAATCCAGCAGCATGGGAGC-3'
- ETₐ receptor primer (antisense), bases 479–499:
  - 5'-GCTGGTGAGACTTATCTGTCC-3'
- ETₐ receptor primer (sense), bases 23–42:
  - 5'-TTCTCGGGCTCACAGTGACC-3'

The cDNA amplification products were predicted to be 418 bp in length for the ETₐ receptor and 900 bp for the ETₐ receptor. The following conditions were used in the Programmed Tempcontrol system: ET receptors: 1) incubation at 94°C for 3 min (initial denaturation); 2) 30 cycles of the following sequential steps: 94°C for 1 min (denaturation), 55°C (ETₐ receptor) or 60°C (ETₐ receptor) for 1.5 min (annealing), and
72°C for 1.5 min (extension); and 3) incubation was done at 72°C for 7 min (final extension).

Expression of ET-1 in intestinal epithelial cells after IL-2 treatment was compared with that in nontreated cells by competitive PCR, which was performed using the PCR MIMIC Construction kit (Clontech Laboratories, Palo Alto, CA).

First, nonhomologous internal standard DNA fragments, called PCR MIMICs, were constructed for use in competitive PCR amplification to quantitate target mRNA levels. A PCR MIMIC consists of a heterologous DNA fragment with primer templates that are recognized by a pair of gene-specific primers. In the present study, we designed the PCR MIMIC for a PCR product 340 bp in size. Serial dilutions of PCR MIMICs were added to PCR amplification reactions containing the experimental cDNA samples.

Cell proliferation and DNA synthesis. Cells were cultured in 96-well multiwell plates in DMEM containing 10% FCS. After cells had attached to their substrates, the culture medium was changed to serum-free DMEM or DMEM containing 5% FCS for growth experiments at 24 h after plating. The number of cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 24 and 48 h after each treatment. On termination of incubation, reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salts) to formazan was assessed using a CellTiter 96 AQ nonradioactive cell proliferation assay kit (Promega, Madison, WI).

DNA synthesis was estimated with an immunocytochemical assay kit using monoclonal bromodeoxyuridine (BrdU) antibody to detect BrdU incorporation in cellular DNA (RPN 210; Amersham, Tokyo, Japan).

Agents studied. To assess the effect of IL-2 on cells, we added IL-2 (Sigma Chemical) at 10 U/ml to the culture media. The selective protein kinase C (PKC) inhibitor 1-(5-isouquinolinesulfonyl)-2-methylpiperazine (H-7) and the selective calmodulin inhibitor N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7) also were obtained from Sigma Chemical. These agents were added to culture media containing 5% FCS. Cell proliferation was determined at 24 h.

Statistical analysis. All results are expressed as means ± SE. Differences among groups were evaluated by one-way ANOVA and Fisher's post hoc test. Any difference for which \( P < 0.05 \) was considered statistically significant.

RESULTS

Figure 1 depicts changes over time in ET-1 concentration in the culture media of IEC-6 and IEC-18 cells. Both cell lines were shown to release a small amount of ET-1 (\( < 10 \) pg/ml, \( 4 \times 10^{-9} \) M at 48 h) under unstimulated conditions. IL-2 significantly enhanced release of ET-1 into culture media in a time-dependent manner for both IEC-6 and IEC-18 cells. In serum-free medium, IL-2 administration increased the release of ET-1 to \( 50 \) pg/ml (\( 2 \times 10^{-8} \) M) at 48 h. In DMEM containing 5% FCS, ET-1 release was significantly greater, reaching 400 pg/ml (\( 1.6 \times 10^{-7} \) M) and 300 pg/ml (\( 1.2 \times 10^{-7} \) M) under IL-2 stimulation for IEC-6 and IEC-18 cells, respectively. ET-3 was not detectable in the culture media of IEC-6 or IEC-18 cells either before or after IL-2 treatment.

Fig. 1. Time-course changes in endothelin-1 (ET-1) concentrations in culture media of IEC-6 (A) and IEC-18 cells (B). Subconfluent monolayers were cultured in serum-free DMEM or in DMEM containing 5% FCS, and concentrations of ET-1 in culture media were determined by enzyme immunoassay. Recombinant rat interleukin-2 (IL-2) was added to the subconfluent monolayers at a concentration of 10 U/ml, and the incubation was continued for 48 h. Values are expressed as means ± SE of 6 experiments. * \( P < 0.05 \) vs. IL-2 (−) FCS (−). # \( P < 0.05 \) vs. IL-2 (+) FCS (−).

Fig. 2. Expression of ET-1 and ET-3 mRNA by IEC-6 and IEC-18 cells as determined by RT-PCR. Both cell lines express specific ET-1 mRNA at 543 bp. In contrast, there is no detectable ET-3 mRNA in these cell lines.
As determined by RT-PCR, both IEC-6 and IEC-18 cell lines specifically expressed ET-1 mRNA at 543 bp (Fig. 2). In contrast, no mRNA for ET-3 was detectable in these cell lines. Expression of ET-1 by IEC-6 and IEC-18 cells 2 h after IL-2 treatment was compared with that in nontreated cells using competitive PCR (Fig. 3). In both cell lines, a significant increase in ET-1 mRNA expression was observed after addition of IL-2.

ET receptor mRNA expression by IEC-6 and IEC-18 cells was determined by RT-PCR (Fig. 4). Both cell lines were shown to express ET\textsubscript{A} receptor mRNA at 418 bp and ET\textsubscript{B} receptor mRNA at 900 bp. Figure 5 shows the effect of the PKC inhibitor H-7 and the calmodulin inhibitor W-7 on the release of ET-1 into culture media. Cells were grown in media containing 5% FCS. H-7 treatment significantly attenuated ET-1 release from both IEC-6 and IEC-18 cell cultures. W-7 similarly inhibited ET-1 release in both cell lines.

We used the MTT assay to determine the effect of ET-1 administration on proliferation of IEC-6 and IEC-18 cells in serum-free media (Fig. 6). Addition of ET-1 induced a slight but consistent proliferative response that was significant at 24 h in both cell types at low concentrations ($10^{-11}$–$10^{-9}$ M, 0.025–2.5 pg/ml); ET-1 inhibited cell growth at a higher concentration ($10^{-7}$ M, 250 pg/ml).

We also used the MTT assay to determine the effect of IL-2 on proliferation of IEC-6 and IEC-18 cells in serum-free media. In Fig. 7, the rate of proliferation is
expressed as the percent increase from control values without IL-2. IL-2 time dependently increased cell proliferation up to 48 h in both cell lines. Addition of ET-1 at a concentration of $10^{-7}$ M significantly attenuated IL-2-induced cell proliferation at 48 h, while ET-1 at a lower concentration ($10^{-9}$ M) did not affect IL-2-induced proliferation. IL-2 did not produce any significant increase in cell proliferation when either cell type was cultured in medium containing 5% FCS (data not shown). The effect of IL-2 on DNA synthesis by IEC-6 and IEC-18 cells was assessed by BrdU uptake (Fig. 8). In serum-free media, IL-2 significantly promoted DNA synthesis in both cell lines at 24 h. Again, addition of ET-1 at a concentration of $10^{-7}$ M, but not at $10^{-9}$ M, significantly suppressed the IL-2-induced increase in DNA synthesis at 24 h. Moreover, promotion of DNA synthesis at 24 h also was attenuated when either cell line was cultured in the medium containing 5% FCS. Cells were examined by light microscopy using the trypan blue exclusion test at the end of the experiments and were found to be morphologically intact. With or without IL-2 treatment, the number of detached cells was negligible after 48 h.

Figure 9 displays the effect of ET-receptor antagonists on proliferation of IEC-6 and IEC-18 cells. Treatment with BQ-123, a selective ETA-receptor antagonist, significantly induced proliferation of these cells. Bosentan, an ET_A- and ET_B-receptor antagonist, similarly enhanced cell proliferation.

**DISCUSSION**

Our present results revealed that cultured rat intestinal epithelial cells can synthesize and release ET-1 and that IL-2 significantly stimulates ET-1 production by these cells. In addition, we found that intestinal epithelial cells showed no ability to produce ET-3. ET production is not limited to vascular endothelial cells but has been demonstrated in a wide variety of other cell types. Expression of ET-1 mRNA has been reported
in vascular smooth muscle cells, myocytes, bronchial epithelial cells, mesangium cells, astrocytes, and macrophages. On the other hand, although ET-3 mRNA has been found in tissue from the brain, spleen, adrenal gland, and small intestine, ET-3 production has not been demonstrated in cultured cell lines (2), a negative finding consistent with our own results.

Several factors have been shown to promote synthesis and release of ET-1 in experiments using vascular endothelial cells. These include such growth factors as TGF-β, cytokines such as IL-1 or IL-6, thrombin, vasopressin, and angiotensin II, as well as mechanical shear stress and hypoxia (II, 29). However, factors regulating ET-1 release in other cell types have not been investigated extensively. Nonendothelial cells are regulated not only by the same stimulatory factors as endothelial cells, but also by cell-specific ones (27).

Fig. 8. Effect of IL-2 on DNA synthesis by IEC-6 (A) and IEC-18 cells (B) as assessed by bromodeoxyuridine uptake. DNA synthesis in both cell lines was determined at 24 h in serum-free media. Rate of proliferation was expressed as %increase of control values without IL-2. Recombinant rat IL-2 was added to the subconfluent monolayers at the concentration of 10 U/ml, and the culture continued up to 24 h. In some experiments, ET-1 at the concentration of 10 U/ml, and the culture continued up to 24 h. Moreover, the effect on DNA synthesis by IL-2 was also determined at 24 h when these cells were cultured in media containing 5% FCS (FCS(+)1). Values are expressed as means ± SE of 6 experiments.

*P < 0.05 vs. controls. #P < 0.05 vs. IL-2(+)1.

Fig. 9. Effect of ET-receptor antagonists on IEC-6 and IEC-18 cell proliferation. BQ-123 or bosentan was added at the concentration of 10 μM to the culture media containing 5% FCS, and cell proliferation was determined at 24 h by the MTT assay. *P < 0.05 vs. control.
umbilical veins, high concentrations of ET-1 and ET-3 failed to further increase cellular proliferation, describing a bell-shaped dose-response curve. One explanation for these effects could be that the ET_A receptor may inhibit cell proliferation. Our results suggest a dual effect of ET-1 on cell proliferation. At low concentrations, ET-1 exerted a growth-promoting effect on intestinal epithelial cells under serum-free unstimulated conditions. In contrast, higher ET-1 concentrations inhibited cell growth. Moreover, under conditions of cell growth stimulated by IL-2, ET-1 also was shown to attenuate cell growth. Because IL-2 demonstrated an ability to enhance ET-1 release from cultured intestinal epithelial cells, ET-1 is considered a factor that modulates cell growth of intestinal epithelial cell in combination with this cytokine. Moreover, inhibition of DNA synthesis in intestinal epithelial cells by administration of IL-2 in FCS-containing media suggests that at high concentrations ET-1 may participate, at least in part, in this growth inhibition.

Two cell populations in the liver cell, Ito cells and sinusoidal endothelial cells, are known to secrete ET-1. Mallet et al. (20) have shown that binding of ET-1 to ET_B receptors causes potent growth inhibition of human Ito cells, in contrast to other cell types. Tamamori et al. (40) have reported that ET-3 induces hypertrophy of neonatal rat cardiac myocytes and that the ET_B receptor mRNA is upregulated in these hypertrophied cells. Tamamori et al. (40) also suggested that the ET_B receptor in cardiac myocytes may contribute in part to the hypertrophy-promoting action of ET-3. In the present study, however, either BQ-123 or bosentan produced a selective and concentration-dependent stimulation of intestinal epithelial cells; this suggests that the growth-modulating effect of ET-1 on intestinal epithe-

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