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Endothelin-1 Gene Expression and Biosynthesis in Human Endometrial HEC-1A Cancer Cells[1]

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ABSTRACT

In this study, evidence was obtained that endothelin-1 (ET-1) is produced by an established endometrial cancer (HEC-1A) cell line. PreproET-1 mRNA is present in HEC-1A cells, and immunoreactive endothelin is secreted into the medium of these cells maintained in culture. Cycloheximide treatment of these cells caused superinduction of preproET-1 mRNA. Transforming growth factor-β acts in these cells to increase the levels of preproET-1 mRNA. This effect of transforming growth factor-β on preproET-1 mRNA accumulation was accompanied by an increase in the amount of immunoreactive endothelin secreted into the culture medium. ET-1, added to the culture medium, did not act as a mitogen in HEC-1A cells. We speculate that ET-1 (which is known to stimulate fibroblast proliferation) produced by endometrial adenocarcinoma cells may participate in the angiogenic process that occurs during the establishment of this carcinoma *in vivo*.

INTRODUCTION

Previously, we found that ET-1[3] is synthesized and secreted into the medium of human endometrial stromal cells and glandular epithelium in culture (1). In addition, preproET-1 mRNA is present in human endometrial tissue (1). In this study, we evaluated the potential for ET-1 synthesis in an established human endometrial cancer (HEC-1A) cell line. We also evaluated the effects of ET-1 (alone or in combination with various growth factors) on DNA synthesis in these cells.

In addition to endometrial glandular epithelium (1), other epithelial cells, *i.e.*, amnion (2, 3) and breast (4), as well as several human tumor cell lines with epithelium-like morphology, *viz.*, laryngeal, lung, hepatocellular, pancreatic, mammary, and cervical (5–7), produce ET-1. Although the biological significance of ET-1 production by tumor cells is not known, it has been suggested that ET-1 may act in these tumors as an autocrine or paracrine growth factor (6, 7). ET-1 receptors are present in two ET-1-producing human epithelial carcinoma cell lines established from human cervical (HeLa) and laryngeal (HEp-2) carcinomas, and ET-1 acts to stimulate proliferation of these cells in a concentration-dependent manner (7). There also is evidence that ET-1 acts as a mitogen for mesenchymal cell types such as Swiss 3T3 fibroblasts (8, 9) and vascular smooth muscle cells (10). Some investigators have speculated that ET-1 produced by carcinoma cells lacking ET-1 receptors, *e.g.*, pancreatic carcinoma cells, serves to promote growth of mesenchymal cells contiguous with the carcinoma (6).

In this study, we found that preproET-1 mRNA is expressed constitutively in HEC-1A cells in culture, that immunoreactive ET is secreted into the medium of these cells, and that preproET-1 mRNA levels and immunoreactive ET production are increased by treatment with TGF-β. ET-1 does not act as a mitogen in HEC-1A cells when added to the culture medium alone or together with any one of a number of growth factors.

MATERIALS AND METHODS

Maintenance of HEC-1A Cells. HEC-1A cells (11) were obtained from the American Type Culture Collection. The cells were maintained in McCoys 5a medium (modified) that contained 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (25 mM), sodium bicarbonate (1.4%, w/v), antibiotics-antimycotics, (1%, v/v), and fetal bovine serum (10%, v/v) in plastic flasks (75 cm^2). The cells were passed by standard methods of trypsinization into 24-well or 100-mm-diameter plastic plates; media were changed at 24 h and, thereafter, at 2-day intervals. The cells were allowed to replicate to confluence (attained in 3–4 days), after which experiments were conducted.

Confluent HEC-1A cells were treated with test agents that included TGF-β (1 ng/ml), EGF (15 ng/ml), or cycloheximide (2 μM) for various times. At the end of the treatment time, the culture media were collected and frozen at −70°C (for quantification of ET by radioimmunoassay); cells were used for quantification of total protein (12) or for isolation of RNA.

ET Radioimmunoassays. Immunoreactive ET was quantified using radioimmunoassay kits [ET-1, -2 ^{125}I assay system (RPA.535) and ET 1–21-specific ^{125}I assay system (RPA.555)] purchased from Amersham Corp. (Arlington Heights, IL). In the RPA.535 assay system, the antiserum recognizes the NH_2-terminal portion of the endothelin molecule; thus, ET-1, ET-2, and big ET-1 (100, 204, and 38% cross-reactivities, respectively) are immunoreactive in the RPA.535 assay. In the RPA.555 assay system, the antiserum recognizes the COOH-terminal portion of the 21-amino acid endothelin molecules; thus, ET-1, ET-2, and ET-3 (100, 144, and 52% cross-reactivities, respectively) are immunoreactive in the RPA.555 assay. Culture medium (corresponding to that of samples being assayed and with or without fetal bovine serum) was used as the assay diluent. ET in aliquots (100 μl) of culture media was quantified by direct radioimmunoassay. The sensitivity of the RPA.535 assay was approximately 2 fmol ET-1/assay tube; the intraassay coefficient of variation was 9.5%. The sensitivity of the RPA.555 assay was 0.5–1 fmol ET-1/tube, and the intraassay coefficient of variation was 11.5%.

Preparation of Total RNA and Northern Analysis. Northern analyses of preproET-1 mRNA in preparations of total RNA were conducted as described previously (3). Briefly, a complementary DNA (1.2 kilobases) probe with a sequence that corresponds to that reported for preproET-1 by Itoh *et al.* (13) was used. Total RNA was extracted from HEC-1A cells by the guanidinium isothiocyanate-cesium chloride method (14), size fractionated by electrophoresis on formaldehyde (1%)-agarose gels (5 or 10 μg/lane), and transferred electrophoretically to Zeta-probe membrane (Bio-Rad). Prehybridization, hybridization, washing, and autoradiography of the membranes was conducted as described previously (3). The presence of equal amounts of total RNA in each lane was verified by visualization of 28S and 18S rRNA subunits and by analysis of 18S rRNA using a specific oligonucleotide probe [5′-CTT-CCT-CTA-GAT-AGT-CAA-GTT-CGA-CCG-TCT-3′ (15)] end labeled with [γ-^{32}P]ATP.

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[3] The abbreviations used are: ET-1, endothelin-1; ET, endothelin; TGF-β, transforming growth factor-β; EGF, epidermal growth factor; irET-1,2 (NH_2-term), immunoreactive material detected by use of the RPA.535 radioimmunoassay; irET-1,2,3 (COOH-term), immunoreactive material detected by use of the RPA.555 radioimmunoassay; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; IL-1α, interleukin-1α.

[^{3}H]Thymidine Incorporation. After incubation for 24 h in serum-free medium, confluent HEC-1A cells (in 24-well plates) were treated with serum-free medium that contained no additions, EGF (15 ng/ml), PDGF (10 ng/ml), TGF-β (1 ng/ml), FGF (10 nM), IL-1α (10 units/ml), or insulin (bovine pancreas, 2 μM). Twenty-two h later, [^{3}H] thymidine (3 μCi; specific activity, 71 Ci/mmol) was added to each well. After 3 h, the media were discarded and the cells were sonicated in water. An aliquot (10% in replicates of 3 or 4) of the sonicate from each well was transferred to a filter paper (Whatman 3 MM) disc; DNA (and protein) was precipitated by mixing the discs with cold trichloroacetic acid (20%, w/v) for 20 min. Thereafter, the discs were washed with trichloroacetic acid (5%), ethyl alcohol, and acetone successively and then dried under air. Radioactivity on each disc was quantified.

Materials. Human recombinant EGF, basic FGF, and IL-1α as well as TGF-β, purified from human platelets, were purchased from Collaborative Research. PDGF, purified from human or porcine platelets, was from R&D Systems. Insulin (bovine pancreas) was from Grand Island Biochemical. ET-1_{1-21} was purchased from Peptides International.

RESULTS

Regulation of PreproET-1 mRNA Levels and ET-1 Synthesis in HEC-1A Cells. The level of preproET-1 mRNA in HEC-1A cells was increased after 4 h treatment with TGF-β (1 ng/ml) or cycloheximide (2 μM); the effect of these agents when added together was greater than that of either agent alone (Fig. 1). There was no detectable increase in the level of preproET-1 mRNA in HEC-1A cells treated with EGF (15 ng/ml) alone for 4 h; the level of preproET-1 mRNA was increased somewhat in response to treatment with EGF together with cycloheximide (2 μM), but the increase was no greater than that effected by cycloheximide alone (Fig. 1).

Immunoreactive ET was secreted into the culture medium of HEC-1A cells; the amount of irET-1,2 (NH_2-term) that accumulated during a 6-h incubation period was greater (1.4- to 3.2-fold) than that of irET-1,2,3 (COOH-term) (Table 1). EGF had little or no effect on the amount of irET-1,2,3 (COOH-term) or irET-1,2 (NH_2-term) that accumulated in the medium at 6, 12, and 24 h. In cells treated with TGF-β, the levels of irET-1,2 (NH_2-term) and irET-1,2,3 (COOH-term) were increased at 6, 12, and 24 h. At 24 h, the levels of irET-1,2 (NH_2-term) and irET-1,2,3 (COOH-term) in media of cells treated with TGF-β were 2- and 3-fold greater, respectively, than those in controls (Table 1).

Effect of ET-1 on DNA Synthesis by HEC-1A Cells. DNA synthesis in confluent HEC-1A cells was not increased by treatment for 25 h with ET-1 (10 nM), EGF (15 ng/ml), PDGF (10 ng/ml), TGF-β (1 ng/ml), FGF (10 nM), or IL-1α (10 units/ml) or by treatment with ET-1 together with any one of these other agents (Fig. 2). Insulin did stimulate DNA synthesis in HEC-1A cells by 2-fold; but this effect of insulin was not modified (either increased or decreased) in the presence of ET-1 (10 nM) (Fig. 2).

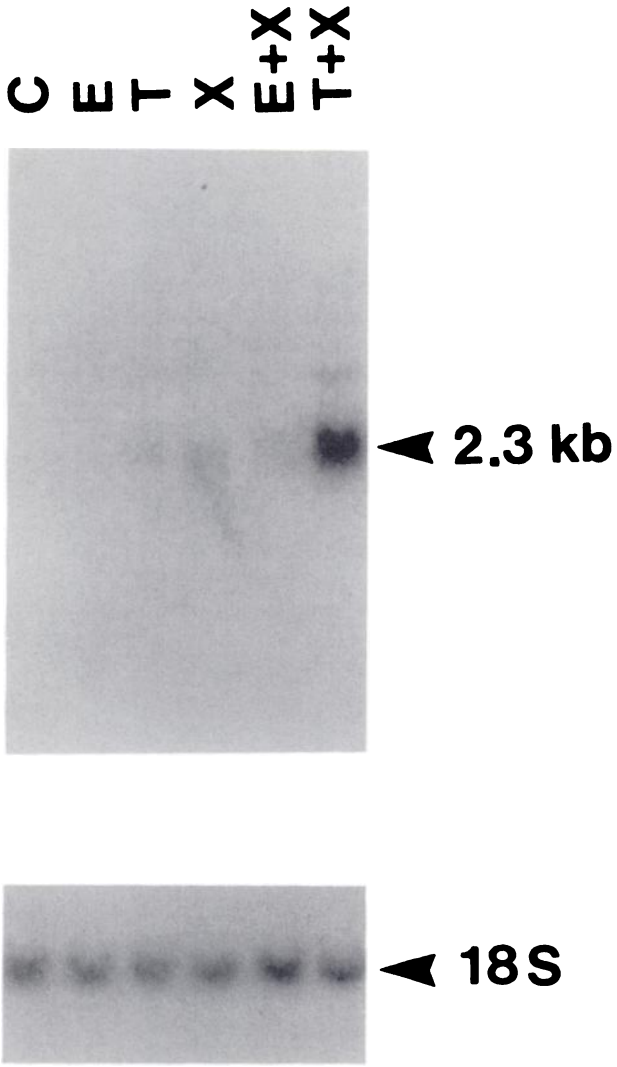

Fig. 1. PreproET-1 mRNA levels in HEC-1A cells in culture. HEC-1A cells were treated for 4 h with TGF-β (1 ng/ml) in serum-free culture medium that contained no additions (control, *C*), EGF (15 ng/ml, *E*), TGF-β (1 ng/ml, *T*), cycloheximide (2 μM, *X*), EGF plus cycloheximide (*E* + *X*), or TGF-β plus cycloheximide (*T* + *X*). Thereafter, total RNA (5 μg/lane) was prepared, blotted, and probed for preproET-1 mRNA and 18S rRNA. At longer exposure times, preproET-1 mRNA is detectable in control cells. kb, kilobase.

DISCUSSION

Previously, we found that ET-1 is synthesized in human endometrium and in human endometrial stromal cells and glandular epithelium in culture (1). In the present study, we demonstrated that a human endometrial cancer line, HEC-1A, also produces ET-1. PreproET-1 mRNA is demonstrable, and the cells synthesize and release immunoreactive ET into the medium. The level of preproET-1 mRNA in HEC-1A cells is increased in response to treatment with TGF-β. A similar effect of TGF-β on the levels of prepro-ET-1 mRNA and ET-1 production has been demonstrated in other cells, *e.g.*, endothelial cells (16), amnion cells (2), breast epithelial cells (4), and HepG-2 cells (5). It is likely that TGF-β stimulates preproET-1 gene transcription by way of the nuclear factor-1-binding element in the 5′-flanking region of the human preproET-1 gene (17).

Treatment of HEC-1A cells with the protein synthesis inhibitor, cycloheximide, led to a marked increase in the levels of preproET-1 mRNA. Superinducibility (in response to inhibitors of protein synthesis) is characteristic of mRNA species, such as preproET-1 (17) and tumor necrosis factor-α (18), with AUUUA-rich motifs in the 3′ region. These regions are believed to target the mRNA for specific ribonuclease-mediated degradation and may contribute to modifications in translatability of the mRNA (19). This likely is an important mechanism for the regulation of tissue levels of a number of transiently synthesized, potent bioactive peptides.

The increase in preproET-1 mRNA in HEC-1A cells in

Table 1 *Production of immunoreactive endothelin by HEC-1A cells*

Confluent HEC-1A cells in monolayer culture were incubated in the absence or presence of EGF or TGF-β for various times. Thereafter, immunoreactive ET in the culture media was quantified by use of radioimmunoassays as described in "Materials and Methods." Data are presented as means ± SEM for replicates of 4.

Treatment	Time of incubation (h)	irET-1,2 (NH_2-term) (pg/mg protein)	irET-1,2,3 (COOH-term) (pg/mg protein)
Control	6	35 ± 1	20 ± 1
	12	50 ± 4	18 ± 1
	24	53 ± 6	21 ± 0.7
EGF (15 ng/ml)	6	40 ± 2	20 ± 0.6
	12	60 ± 1	19 ± 0.3
	24	51 ± 3	26 ± 4
TGF-β (1 ng/ml)	6	49 ± 2[a]	33 ± 2[a]
	12	67 ± 1[a]	47 ± 3[a]
	24	109 ± 6[a]	65 ± 2[a]

[a] $P < 0.03$ compared with respective controls.

response to treatment with TGF-β was accompanied by an increase in immunoreactive ET secreted into the culture medium. Immunoreactive ET was detected by use of 2 separate radioimmunoassays in which different portions of the endothelin molecules are recognized, *viz.*, the NH_2-terminus of ET-1 and ET-2 (as well as big ET-1 and big ET-2) and the COOH-terminus of ET-1, ET-2, and ET-3. The level of irET-1,2 (NH_2-term) that accumulated in the culture medium of HEC-1A cells was greater than that of irET-1,2,3 (COOH-term); this finding is suggestive that bioactive ET_{1-21} as well as big ET (*i.e.*, ET_{1-38}) is produced by these cells. The specificity of the assay that recognizes the NH_2-terminal portion of the endothelin molecules may not be limited, however, to big ET-1, big ET-2, ET-1, and ET-2; it is likely that metabolites of ET-1 (and ET-2) are recognized in this assay. ET-1, ET-2, and ET-3 are metabolized by membrane metalloendopeptidase (20, 21) also known as enkephalinase (EC 3.4.24.11). The metabolites formed include 2 chain derivatives of the 21-amino acid endothelins (cleaved between amino acids 5 and 6) that may also be hydrolyzed in the COOH-terminal region to yield 16- and 18-amino acid products (20, 21). The NH_2-terminal portion of these molecules is largely intact and, thus, these likely are immunoreactive in the assay that recognizes the NH_2 terminus; the extent of that immunoreactivity is unknown at this time. In HEC-1A cells, we found that the specific activity of membrane metalloendopeptidase is very low.[4] Specifically, the activity is 0.1–1% of that in endometrial tissue or endometrial stromal cells; the activity in endometrial glands isolated from normal endometrial tissue also is low (22). We conclude, therefore, that the formation of metabolites of ET in HEC-1A cells in significant amounts is an unlikely possibility. A portion of the immunoreactive ET produced by the HEC-1A cells, however, may be the precursor form of ET, namely, big ET.

It is possible that ET-1 is involved in endometrial adenocarcinoma growth, invasion, maintenance, or function. There was no effect of ET-1, added to the culture medium at a concentration of 10^{-8} M, on HEC-1A cell proliferation. EGF, FGF, PDGF, and TGF-β also were ineffective in promoting DNA synthesis in these cells, either alone or in combination with ET-1. DNA synthesis was increased in response to insulin, but unlike the potentiation of insulin-induced proliferation by ET-1 in Swiss 3T3 cells (9), ET-1 had no effect on insulin-stimulated DNA synthesis in HEC-1A cells.

It has been reported that adenocarcinoma of the endometrium produces an agent(s) that stimulates angiogenesis *in vitro* (23). It is conceivable that ET-1 is a participant in this process, possibly acting in concert with other angiogenic agents, *e.g.*, angiogenin or fibroblast growth factor (*cf.* Ref. 24). A principal

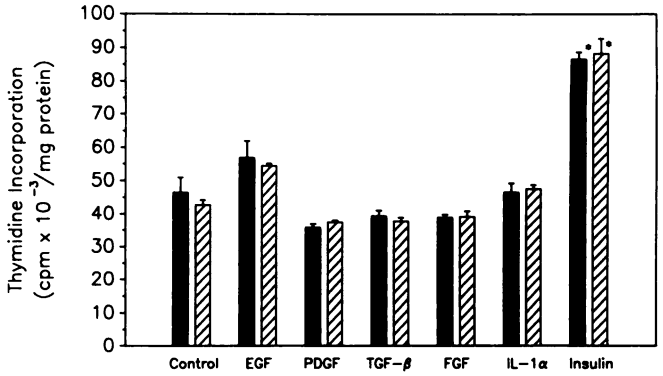

Fig. 2. Effect of ET-1 on DNA synthesis in HEC-1A cells in culture. Confluent HEC-1A cells were incubated in the absence (*solid columns*) or presence (*hatched columns*) of ET-1 (10 nM) with EGF (15 ng/ml), PDGF (10 ng/ml), TGF-β (1 ng/ml), FGF (10 nM), IL-1α (10 units/ml), and insulin (2 μM) for 25 h in serum-free medium. [^{3}H]Thymidine was added during the final 3 h of incubation, and incorporation of radioactivity into trichloroacetic acid-precipitable material was quantified as described in "Materials and Methods."

mechanism by which ET-1 acts is by increasing intracellular Ca^{2+}, which may promote cell replication and motility. Angiogenesis also can be accompanied by fibroblast migration and proliferation (25). As stated, ET-1 also is known to stimulate fibroblast proliferation *in vitro* (5, 6). It can be envisioned, therefore, that ET-1 produced by HEC-1A cells may participate in the process of angiogenesis/neovascularization that accompanies proliferation of endometrial carcinoma.

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