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Endothelin-1 is an Autocrine/Paracrine Growth Factor for Human Cancer Cell Lines

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Abstract
We studied whether a novel vasoconstrictor peptide, endothelin-1 (ET-1), is synthesized by and released from human carcinomacell lines, and whether ET-1 stimulates proliferation of these tumor cells. ET-1-like immunoreactivity was released from both HeLa and HEp-2 cells as a function of time. Reverse-phase HPLC of the conditioned media from HeLa cells revealed a major peak coeluting with standard ET-1. Northern blot analysis demonstrated the expression of mRNA for ET-1 precursor in both tumor cell lines. Both cell lines contained a single class of specific binding sites for ET-1. ET-1 dose-dependently induced increases in cytosolic free Ca2+ concentration in fura-2-loaded tumor cells, whose effect was completely abolished by chelating extracellular Ca2+ or by Ca2+-channel blocker. ET-1 stimulated proliferation of the quiescent cell lines in a dose-dependent manner, whose effect was inhibited by Ca2+-channel blocker. Polyclonal antibody for ET-1 inhibited proliferation of these cell lines, whereas nonimmune serum had no effect. These results demonstrate that ET-1 is synthesized by and released from human epithelial carcinoma cell lines, and that exogenous and endogenous ET-1 stimulates proliferation of the cells possibly through Ca2+ influx, suggesting its role as an autocrine/paracrine growth factor for certain tumor cells. (J. Clin. Invest. 1991. 87:1867-1871.) Key words: radioimmunoassay • receptor • cytosolic free Ca2+ concentration • cell proliferation • Northern blot analysis

Introduction
Endothelin-1 (ET-1),1 originally characterized from the supernatant of cultured porcine endothelial cells, is a potent vasoconstrictor/pressor peptide (1). Subsequent cDNA cloning of human genomic library revealed three isopeptides, termed ET-1, ET-2, and ET-3 (1, 2). Specific receptors for ET-1 are distributed not only in cardiovascular system, but also in a wide variety of tissues (3), suggesting its diverse physiological functions. Recently, it has been reported that ET-1 stimulates proliferation of rat vascular smooth muscle cells (4), fibroblasts (5), and glomerular mesangial cells (6), and the expression of protooncogenes (c-myc, c-fos) in these cells, suggesting its potential role as a growth factor. At present, no information is yet available whether ET-1 is produced by carcinoma cells and acts by themselves. In the present study, we demonstrate that two epithelial carcinoma cell lines derived from human cervix (HeLa) and larynx (HEp-2) express mRNA for ET-1 precursor, and release ET-1 into medium, and further show that both tumor cells possess specific ET-1 receptors through which ET-1 stimulates cell proliferation possibly via influx of extracellular Ca2+.

Methods

Cell culture. HeLa cells (American Type Culture Collection, Rockville, MD) were cultured in MEM supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS), and HEp-2 cells (ATCC) in Eagle's MEM with Earle's balanced salt solution and 10% FBS at 37°C in a 95% room air-5% CO2 humidified incubator. Medium was changed every 2-3 d. After reaching confluency, cells were replaced with a serum-free MEM for 2 d. The conditioned media were pooled and stored at -40°C until processed for extraction. Cell number was measured by Coulter Counter Model ZM (Coulter Electronics, Inc., Hialeah, FL).

Extraction of ET-1. For chromatographic analysis, the pooled conditioned media (100 ml) were acidified with 0.1% trifluoroacetic acid (TFA), and the supernatant applied to Spe C18 cartridge (J. T. Baker Chemical Co., Phillipsburg, NJ) and eluted with 2 ml 60% acetonitrile/0.1% TFA, as reported previously (7). The eluates were evaporated to dryness and subjected to reverse-phase HPLC. The recovery of synthetic ET-1 during the extraction procedure was 77%.

Radioimmunoassay (RIA). ET-1-like immunoreactivity (LI) was determined by specific RIA for ET-1 as reported (7). The antibody used in the present RIA mainly recognizes the COOH-terminal Trp27 residue of ET-1, and cross-reacts fully with ET-2 and ET-3, but not with big ET-1, or other polypeptide hormones. The final dilution of antiserum was 1:150,000. The bound ligands were separated from the free ones by the double antibody method. The sensitivity of ET-1 RIA was 1.0 fmol/tube, and the 50% intercept was 14 fmol/tube. The intra- and interassay variations were 3.2 and 8.6%, respectively.

Reverse-phase HPLC. The extract of the conditioned media from HeLa cells was loaded on a column (0.45 × 25 cm, 5 µm, C18, Nucleosil, Macherey-Nagel, Düren, FRG) eluted with a linear gradient (15-60%) of acetonitrile in 0.09% TFA for 60 min at a flow rate of 1 ml/min. After evaporation, each eluate was subjected to ET-1 RIA. The recovery of standard ET-1 was 96%.
Northern blot analysis. Total RNA from confluent cells was extracted with LiCl-urea and subjected to poly(A)+ RNA selection. Poly(A)+ RNA (10 μg) was fractionated in a formaldehyde/1% agarose gel electrophoresis and transferred to a GeneScreen Plus membrane (DuPont Co., Wilmington, DE). The probe was 3′ noncoding exon from cloned human preproET-1 gene (1), labeled with [32P]dCTP (sp act, 3,000 Ci/mm; Amersham International, Amersham, UK) by the random-primed labeling method, and was incubated at 42°C for 16 h with membranes in hybridization buffer containing 1 M NaCl/50% (vol/vol) formamide/1% SDS/250 μg/ml of salmon sperm DNA. The membranes were washed with 0.3 M NaCl/30 mM sodium citrate/1% SDS at 60°C, and autoradiographed on a Kodak XAR-1 film with an intensifying screen at ~80°C for 8–16 h (8).

Binding experiments. Binding experiments were performed essentially in the same manner as previously described (9). Confluent cells (106 cells) were washed twice with HBSS containing 0.1% BSA, and incubated at 37°C for 2 h with 1.3 × 10−11 M [125I]-ET-1 (sp act, 2,000 Ci/mm; Amersham International, Amersham, UK) in the absence and presence of various concentrations of unlabeled ET-1 (Peptide Institute, Osaka, Japan). The cells were then washed twice with ice-cold HBSS, solubilized with 1 N NaOH at 37°C, and the cell-bound radioactivity was measured. Specific binding was calculated as total binding minus nonspecific binding in the presence of excess unlabeled ET-1. The apparent dissociation constant (Kd) and maximal binding capacity (Bmax) were calculated by Scatchard analysis of binding data.

Determination of intracellular Ca2+ concentration ([Ca2+]i). Confluent HeLa and HEp-2 cells which had been deprived of FBS for 48 h were dispersed with 0.25% trypsin and 0.02% EDTA, and incubated with 5 μM fura-2 acetoxyxymethylene (Dojin Chemical, Kumamoto, Japan) at 37°C for 20 min in HBSS. Suspended fura-2-loaded cells were washed, and incubated for 20 min in physiological salt solution (130 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, and 20 mM Hepes, pH 7.4), containing 0.5% BSA and 10 mM glucose to allow for intracellular dye cleavage. Fluorescence of fura-2-loaded suspended cells (5 × 106 cells/ml) was measured at 37°C using continuous rapid alternating excitation from dual monochromators (340 and 380 nm), and emission at 505 nm (CAF-100, Japan Spectroscopic Co. Ltd., Tokyo) as reported (9). Fluorescent measurement was converted to [Ca2+]i, by determining the maximal fluorescence (Rmax) with 10% Triton-X-100, followed by the minimal fluorescence (Rmin) with 15 mM EGTA, pH 10.5. The following formula was used: 

\[ [\text{Ca}^{2+}]_i = \frac{K_d (R_{\text{max}} - R_{\text{min}})}{R_{\text{obs}} - R} \times \frac{380_{\text{nm}}}{340_{\text{nm}}} \]

assuming that the Kd for the fura-2:Ca2+ complex is 224 nM at 37°C (10).

Cell proliferation. Cells were seeded into 12-well cluster dishes (2.9 × 104 cells/well). After 24 h, the cells were washed, and replaced with 2 ml serum-free MEM with or without ET-1 and incubated for the indicated times. After completion, the cells were trypsinized, and the cell number was measured by Coulter Counter. To determine whether the antibody for ET-1 used for RIA affects the cell proliferation, tumor cells which had been replaced with fresh MEM containing 0.2% FBS (HeLa cells) or 3% FBS (HEp-2 cells) were incubated in the absence and presence of various dilutions (1:1,000–1:1,000,000) of rabbit ET-1 antisem and nonimmune rabbit serum (1:1,000) as control. After 72 h, cell number was determined.

Statistical analysis. Results were expressed as mean±SEM. Statistical analysis was performed by Student’s t test for nonpaired data.

Results

Serial dilution curves generated by extract of the conditioned media of both HeLa and HEp-2 tumor cells were parallel to that of standard ET-1 in RIA (data not shown). HeLa cells under a serum-free condition released ET-1-LI as a function of time, reaching a plateau after 24 h (Fig. 1). Presence of FBS in the incubation medium potentiated the amounts of ET-1-LI released from both cells; the rates of ET-1-LI released from HeLa and HEp-2 cells under a serum-free condition were 3.53±0.44 and 22.3±4.3 fmol/24 h/106 cells (n = 4), respectively, whereas they were 220±14 and 190±26 fmol/24 h/106 cells (n = 3) in the presence of 10% FBS.

Reverse-phase HPLC profile of ET-1-LI in extract of the conditioned media from HeLa cells is shown in Fig. 2. Reverse-phase HPLC revealed two ET-1-LI components, one major component coeluting with standard ET-1 and the other having yet uncharacterized retention time.

Northern blot analysis of poly(A)+ RNA from both tumor cells using cDNA from human prepro-ET-1 as a probe is shown in Fig. 3. A single hybridization band corresponding to the size (2.3 kb) of mRNA coding for human prepro-ET-1 was demonstrated in both cells.

To characterize the specific binding sites for ET-1 in these tumor cells, binding study using [125I]-ET-1 as a radioligand was performed. Unlabeled ET-1 competitively inhibited the bind-
Nonimmune rabbit serum significantly inhibited proliferation of HEp-2 cells. (1:10,000), 75.2±6.0% (1:10,000); 37.6±2.9% (1:100,000), 7.2±5.0% (1:1,000,000). A single band with a size of 2.3 kb was observed in both tumor cells. Size markers in kilobases are indicated on the left.

The effect of ET-1 on [Ca\(^{2+}\)] was studied by measuring changes of fura-2-Ca\(^{2+}\) fluorescence (Fig. 5). The basal [Ca\(^{2+}\)] was 190.9±7.2 nM (n = 34) in HeLa cells. ET-1 induced gradual increase in [Ca\(^{2+}\)], which lasted for at least the 30-min observation period. The ET-1-induced [Ca\(^{2+}\)], increase was a dose-dependent effect; increases in [Ca\(^{2+}\)] in HeLa cells 3 min after the addition of various doses of ET-1 over the basal levels were 124.1±5.6% (10\(^{-14}\) M), 139.6±4.9% (10\(^{-13}\) M), 169.9±17.4% (10\(^{-12}\) M), 185.5±13.4% (10\(^{-11}\) M), and 216.3±9.5% (10\(^{-10}\) M) (n = 5; P < 0.01 vs. control). The increase in [Ca\(^{2+}\)], by ET-1 (10\(^{-11}\) M) was completely abolished by pretreatment with 3 mM EGTA or 10\(^{-4}\) M nifedipine. The basal [Ca\(^{2+}\)], in HEP2 cells was 124.1±4.9 nM (n = 14), and ET-1 also induced, although less effectively, [Ca\(^{2+}\)], increases which were dose-dependent: 107.0±0.5% (10\(^{-10}\) M), 109.3±1.7% (10\(^{-9}\) M), and 114.4±4.9% (10\(^{-8}\) M) over the basal levels (n = 5, P < 0.01 vs. control) (data not shown).

Under serum-free conditions, ET-1 significantly (P < 0.01) stimulated proliferation of HeLa cells in a dose-dependent manner (10\(^{-12}\)-10\(^{-9}\) M) and that of HEP-2 cells to the less extent (10\(^{-8}\)-10\(^{-7}\) M) after 72 h (Fig. 6). In HeLa cells, ET-1 as low as 10\(^{-12}\) M induced an approximate twofold increase in cell number and maximal stimulation (approximate fourfold increase) was observed at 10\(^{-12}\) M; the approximate ED\(_{50}\) was 3×10\(^{-12}\) M. The ET-1-induced proliferation of HeLa cells was attenuated by 10\(^{-8}\) M nifedipine and completely inhibited by 10\(^{-7}\) M nifedipine, whereas nicardipine (10\(^{-8}\)-10\(^{-7}\) M) added alone did not affect cell growth (data not shown). Addition of rabbit anti-ET-1 serum dose-dependently inhibited proliferation of HeLa cells [95.2±1.2% (1:1,000,000); 86.0±4.8% (1:100,000); 75.2±6.0% (1:10,000); 37.6±2.9% (1:1,000)], whereas the antiserum only at a high concentration (1:1,000) significantly inhibited proliferation of HEP-2 cells (Fig. 6). Nonimmune rabbit serum (1:1,000) had no effect on proliferation of either tumor cells. The antiserum (1:1,000) completely blocked the mitogenic effects by exogenous ET-1 (10\(^{-11}\) M, HeLa cells; 10\(^{-8}\) M, HEP-2 cells).

Discussion
In the present study, the apparent parallelism of serial dilution curves between the extracts of conditioned media from HeLa and HEP-2 tumor cells and standard ET-1 in ET-1 RIA as well as the time-dependent accumulation of ET-1-LI in the media strongly suggest that ET-1 and/or related peptides immunologically indistinguishable from ET-1 are released from both tumor cells. ET-1-LI release from the cells was markedly increased when cultured in the presence of FBS compared with that under a serum-free condition. This is consistent with our recent observation that the release of ET-1-LI from cultured rat
Figure 5. Representative tracings of \([Ca^{2+}]_i\) response to ET-1 in HeLa cells. Fura-2-loaded cells were challenged with various doses \((10^{-13}-10^{-10})\ M\) of ET-1 (A–C). After pretreatment with \(10^{-4}\ M\) nicardipine \((D)\) or \(3\ MM\ EGTA\ (E)\), ET-1 \((10^{-11})\ M\) was added.

Figure 6. Effects of ET-1 and rabbit anti-ET-1 serum on growth of tumor cells. \((A)\) Quiescent HeLa (●) and HEP-2 (○) cells were incubated with indicated doses of ET-1 under a serum-free condition, and cell number was measured after 72 h. Each point represents the percentage to control in the absence of ET-1 (HeLa, \(6.5\pm0.4\times10^3\) cells, \(n=11\); HEP-2, \(2.3\pm0.1\times10^5\) cells, \(n=5\)) from a single experiment; bars show SEM. *P < 0.01 vs. control. \((B)\) The cells were incubated with various dilutions \((1:1,000,000-1:1,000)\) of rabbit ET-1 antiserum (HeLa ●, HEP-2 ○) and nonimmune rabbit serum (1:1,000) (HeLa ●, HEP-2 ○) for 72 h in the presence of 0.2–3% FBS, and the cell number was measured. Each point represents the percentage to control in the absence of rabbit sera (HeLa, \(1.1\pm0.0\times10^6\) cells, \(n=8\); HEP-2, \(2.3\pm0.2\times10^5\) cells, \(n=6\)) from a single experiment; bars show SEM. *P < 0.023; **P < 0.01 vs. control.
Very recently, Kusuhara et al. have demonstrated ET-1 synthesis by other cancer cell lines and speculated a modulatory role of ET-1 in the growth of stromal cells surrounding cancer cells (16). Although they failed to detect ET-1 binding sites in only pancreatic tumor cell lines, neither the exact autocrine role of ET-1 in the tumor cell lines nor the existence of ET-1 receptors in other tumor cell lines which produce ET-1-II have been extensively studied. The far lower affinity of ET-1 receptors and the lesser effects of ET-1 on [Ca^{2+}], and cell growth in Hep-2 cells compared with those of HeLa cells in the present study might argue against the major role of ET-1 as an autocrine factor for Hep-2 cells, but for its paracrine role for the neighboring stromal cells as suggested (16). Therefore, it remains unsettled whether ET-1 production by the tumor cell lines in general acts either as an autocrine growth factor for their own cells or as a paracrine growth factor for other cells.

In conclusion, the present study demonstrates that ET-1 is synthesized by and released from human carcinoma cell lines, and that ET-1 stimulates proliferation of these cells through receptor-mediated increase in [Ca^{2+}], possibly derived from Ca^{2+} influx. Our data thus suggest that ET-1 produced by the tumor cells may function as an autocrine/paracrine growth factor for certain tumor cells.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture (01480217, 01480286, 02304055) and the Ministry of Health and Welfare (63C-1) of Japan, and a fund from Uehara Memorial Foundation.

References


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