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Production of Endothelin in Human Cancer Cell Lines[1]

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ABSTRACT

Endothelin (ET)-1 is a vasoconstrictor peptide derived from endothelial cells and now known to be a local regulator of vascular tonus. Recent studies, however, have revealed that ET-1 functions also as growth factor in various cells. By using a specific ET-1 radioimmunoassay, immunoreactive (IR) ET-1, ranging from 4.2 to 150 pM (minimum detectable amount, 4.0 pM), was detected in 13 of 42 human cancer cell lines. The frequencies of IR-ET-1 production and its concentrations were high in mammary, pancreatic, and colon carcinoma cell lines. IR-ET-1 produced by cancer cells possessed the same molecular size as synthetic ET-1 and also had ET-1-like biological activity. Moreover, Northern blot analysis revealed bands corresponding to ET-1 mRNA in cancer cell lines, indicating that IR-ET-1 produced by cancer cells is a product of the ET-1 gene. Since ET-1 in the spent media is present in a sufficient amount to stimulate cellular growth, we sought ET-1 receptors in four pancreatic carcinoma cell lines and human skin fibroblasts. No ET-1 receptors were detected in the pancreatic carcinoma cell lines. However, human skin fibroblasts possessed a large number of ET-1 receptors. This finding raises the possibility that ET-1 produced by cancer cells plays a modulatory role in the growth of stromal cells surrounding cancer cells.

INTRODUCTION

ET[3] is a factor with vasoconstrictive activity originally isolated from cultured media of porcine vascular endothelial cells (1). Current evidence suggests that this peptide regulates blood pressure by modulating vascular tonus. However, we demonstrated that ET possessed a novel biological activity which stimulates DNA synthesis in Swiss 3T3 fibroblasts at the same concentration as that required for vasoconstriction of vessels (2). ET was also revealed to promote cellular growth in vascular smooth muscle cells (3, 4) and renal mesangial cells (5). Recent observations by Inoue *et al.* (6) indicated that the human genome had two other DNA sequences hybridizable to synthetic oligonucleotides probe encoding human ET. They termed these two putative peptides ET-2 and ET-3, while renaming the originally isolated human ET as ET-1. The physiological roles of ET-2 and ET-3 were not yet elucidated.

In the present study, we attempted to develop a specific radioimmunoassay for ET-1. By using this method, we examined whether cancer cells have the capacity to produce ET-1, although ET-1 is now believed to be a specific product of vascular endothelial cells. Since cancer cells were found to produce a large amount of ET-1, we further investigated the presence of ET-1 receptors in cancer cells as well as fibroblasts for testing the possibility whether ET-1 was an autocrine or paracrine growth factor.

MATERIALS AND METHODS

Materials. Synthetic human ET-1, big ET-1, ET-2, ET-3, and GRP were purchased from Peptide Institute (Osaka, Japan); ANP, AVP, CGRP, ω-conotoxin GVIA, NPY, somatostatin, substance P, and VIP were from Peninsula Laboratories, Inc. (Belmont, CA); EGF was from Earth Chemical Co., Ltd. (Hyogo, Japan); porcine thyroglobulin was from Sigma Chemical Co. (St. Louis, MO); BSA (Cohn Fraction V) was from Daiichi Pure Chemicals (Osaka, Japan); ODS cartridge (Sep-Pak C_{18}) was from Waters Associates (Milford, MA); Sephadex G-50 superfine was from Pharmacia (Uppsala, Sweden); $Na^{125}I$ was from New England Nuclear (Boston, MA); and ^{125}I-ET-1 with a specific activity of 74 TBq/mmol from Amersham International (Amersham, Buckinghamshire, United Kingdom); 28S and 18S rRNA (calf liver) was from Pharmacia.

Cell Culture. Two endothelial cell lines, FBHE (fetal bovine heart endothelial cell) and CPAE (calf pulmonary artery endothelial cell) were purchased from the American Type Culture Collection (Rockville, MD) (7). The 42 human cancer cell lines examined were 4 mammary carcinomas (MCF-7, BT-20 ZR-75-1, and ZR-75-30), 6 pancreatic carcinomas (PANC-1, ASPC-1, BxPC-3, FA-6, PSN-1, and MIA PaCa-2), 4 colon carcinomas (HT-29, COLO 201, COLO 320, and KM12L4), 8 lung carcinomas (5 non-small cell lung carcinomas, A-549, PC-9, PC-14, Lu-65, and Lu-99, 3 small cell lung carcinomas, Lu-134, Lu-135, and NCI-H69), 4 gastric carcinomas (KATO-III, MKN-28, MKN-45, and MKN-74), 2 hepatocellular carcinomas (Alexander and Li-7), 4 melanomas (SEKI, A-375, G-361, and C32TG), an acute promyelocytic leukemia (HL-60), 2 adult T-cell leukemias (HUT 102 and MT-1), 2 chronic myelocytic leukemias (KOPM-28 and K-562) and 5 Burkitt's lymphomas (Daudi, Ramos, Jiyoye, Namalwa, and CA46). PSN-1, Lu-65, Lu-99, Lu-134, Lu-135, Li-7, and SEKI were established at the National Cancer Center Research Institute (Tokyo, Japan) (8–12). FA-6 was kindly provided from Dr. N. Nagata (National Defense Medical College, Saitama, Japan) (13). PC-9 and PC-14 were kindly provided by Dr. Y. Hayata (Tokyo Medical College, Tokyo, Japan) (14). NCI-H69 was kindly provided by Dr. A. F. Gazdar (National Cancer Institute, Bethesda, MD) (15). KM12L4 was kindly provided by Dr. K. Morikawa (Hokkaido University School of Medicine) (16). KOPM-28 was kindly provided by Dr. S. Nakazawa (National Saitama Hospital, Saitama, Japan) (17). HUT 102 was kindly provided by Dr. A. F. Gazdar and Dr. R. C. Gallo (National Cancer Institute) (18). MT-1 was kindly provided by Dr. I. Miyoshi (Kochi Medical College, Kochi, Japan) (19). A-375, G-361, C32TG, A-549, KATO-III, MCF-7, BT-20, ZR-75-1, ZR-75-30, HT-29, PANC-1, ASPC-1, BxPC-3, MIA PaCa-2, HL-60, K-562, Ramos, Jiyoye, Namalwa, and CA46 were purchased from the American Type Culture Collection (7). MKN-28, MKN-45, MKN-74, Alexander, COLO 201, COLO 320, and Daudi were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan) (20). All of these cell lines were maintained at 37°C under 5% CO_2:95% air in 75-cm^2 plastic tissue culture flasks by using the original medium described in the references or the catalogues. The culture media for all these cell lines were supplemented with 10% FCS. When the cells had grown to 90% confluence, the spent media of these cell lines were collected and analyzed.

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[3] The abbreviations used are: ET, endothelin; RIA, radioimmunoassay; ODS, octadecylsilyl silica; FCS, fetal calf serum; BSA, bovine serum albumin; ANP, atrial natriuretic peptide; AVP, arginine vasopressin; CGRP, calcitonin gene-related peptide; EGF, epidermal growth factor; GRP, gastrin-releasing peptide; NPY, neuropeptide Y; VIP, vasoactive intestinal peptide; IR, immunoreactive; FBHE, fetal bovine heart endothelial cell; CPAE, calf pulmonary artery endothelial cell.

Monolayer cultures of human skin fibroblasts were derived from explants of elbow skin obtained from 3 healthy male volunteers (29–32 years old) by cutting with a razor (21). Skin fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. In this study 20–30 population-doubling levels of skin fibroblasts were used.

Extraction Method. The spent medium (20 ml) was applied to an activated ODS cartridge. The material retained on the ODS cartridge was eluted with 3 ml of 80% acetonitrile in 0.1% trifluoroacetic acid. The eluates were lyophilized and reconstituted to 0.4 ml with assay buffer (see below). As control, the same volume of fresh medium (newly prepared culture medium supplemented with 10% FCS) for each human cancer cell line was extracted in the same manner. Furthermore, to determine the recovery rate of the extraction method, synthetic ET-1 (80 fmol or 8 pmol) was added to fresh medium (20 ml) and extracted.

RIA. Synthetic ET-1 was conjugated to porcine thyroglobulin by carbodiimide condensation (22). The conjugate was emulsified with an equal volume of complete Freund's adjuvant and used for immunization. Ten guinea pigs were immunized and one of the anitsera (GP-ET-1-8-2) was used at a final dilution of 1:7000 in this study. The assay was performed in 0.1 M phosphate buffer, pH 7.4, containing 0.1 M NaCl, 0.06% (v/v) monoethanolamine, 1% (w/v) disodium EDTA, 0.9% (w/v) tetrasodium EDTA and 0.1% BSA. Monoethanolamine was used to minimize the adsorption of peptides to the test tubes. Synthetic ET-1 was used as the assay standard and the results were expressed as mol equivalent of ET-1. The total incubation volume was 0.7 ml, and the incubation was carried out for 24 h at 4°C. After addition of properly diluted goat anti-guinea pig γ-globulin antibody (Antibodies Inc., Davis, CA), assay tubes were further incubated for 24 h at 4°C. For separating the bound from free ^{125}I-ET-1, assay tubes were centrifuged at 1500 × *g* for 30 min at 4°C and decanted, and the precipitant was counted by gamma counter. Cross-reaction with other peptides including big ET-1, ET-2, ET-3, ANP, AVP, CGRP, ω-conotoxin GVIA, EGF, GRP, NPY, somatostatin, substance P, and VIP was examined.

Gel Permeation Chromatography. Extracts prepared from the spent media of two endothelial cell lines (FBHE and CPAE), 3 pancreatic carcinoma cell lines (PANC-1, ASPC-1, and BxPC-3), a colon carcinoma cell line (HT-29), and a mammary carcinoma cell line (MCF-7) were chromatographed on a Sephadex G-50 superfine column (1.0 × 45 cm) which was equilibrated and eluted with 1 M acetic acid. The extracts were eluted at a rate of 6 ml/h from the column by the fraction collector-pump control system (23). Successive fractions of 0.8 ml each were collected, lyophilized, and reconstituted in the assay buffer. The column was calibrated with ET-1, big ET-1, ^{125}I-BSA and Na^{125}I.

Biological Activity of Partially Purified IR-ET-1 Produced by Cancer Cells. Biological activity was estimated by isometric contraction of rat thoracic aortic strips in a 10-ml organ bath (1).

Serum free-spent medium of PANC-1 (1500 ml) was extracted by the method described above. The extract was chromatographed on a Sephadex G-50 superfine column. Fractions corresponding to IR-ET-1 were pooled, measured for total IR-ET-1 content, and then tested in the bioassay.

Northern Blot Analysis. Five cell lines were examined for expression of ET-1 mRNA. These were an endothelial cell line (FBHE), a colon carcinoma cell line (HT-29), and 3 pancreatic carcinoma cell lines (PANC-1, ASPC-1, and MIA PaCa-2). Polyadenylated RNA extraction, gel electrophoresis, and Northern blot hybridization were performed by the previously described method (24). For detecting ET-1 mRNA, synthetic oligodeoxyribonucleotides 5′-CCA AAT GAT GTC CAG GTG GCA GAA GTA GAC ACA CTC TTT ATC CAT CAG GGA CGA GCA GGA GCA-3′, containing the coding sequence for Cys^{1}-Trp^{21} of the ET-1 (25), are used as the probe. With the aim of determining the integrity of tissue polyadenylated RNA extracted, the expressed of human β-actin mRNA was examined by the previously described method (26).

Binding of ^{125}I-ET-1. Binding assay of ^{125}I-ET-1 in 4 pancreatic carcinoma cell lines (PANC-1, ASPC-1, BxPC-3, and FA-6) producing IR-ET-1 and 3 primary cultured human skin fibroblasts was studied. Confluent cells in a 35-mm dish were incubated with ^{125}I-ET-1 (50 pM) in 0.5 ml Hanks' balanced salt solution containing 0.1% BSA at 37°C for 60 min. They were washed and solubilized with 1 M NaOH, and then the cell-bound radioactivity was determined. Specific binding was calculated by subtracting nonspecific binding determined by experiments performed in the presence of a 100-fold excess of unlabeled ET-1. In two primary cultured human skin fibroblasts expressing specific ET-1 binding sites, Scatchard plot analysis (27) were performed by the previously described method (2).

RESULTS

RIA for ET-1. The amounts of ET-1 which inhibited the labeled antigen binding by 10 and 50% were 1.5 and 20 fmol/tube, respectively (Fig. 1). The variation coefficients of inter- and intraassay at 20 fmol/tube were 9.6% (n = 8) and 7.0% (n = 10), respectively. Cross-reaction with other peptides was determined with comparison of the amounts of peptide to inhibit the labeled antigen binding to antiserum by 50%. When the cross-reactivity of ET-1 was taken to be 100%, this value of big ET-1 was 150%; however, ET-2 and ET-3 did not cross-react significantly (0.014 and 0.005%, respectively). These results suggest that this antiserum is highly specific for ET-1 and big ET-1. Other peptides including ANP, AVP, CGRP, ω-conotoxin GVIA, EGF, GRP, NPY, somatostatin, substance P, and VIP did not show any significant cross-reactivity (less than 0.005%).

IR-ET-1 in Spent Media. When 20 ml of fresh medium containing 80 fmol or 8 pmol of ET-1 were extracted, the recovery rates were 74 ± 11.5 and 73 ± 9.5% (mean ± SD, n = 5), respectively. IR-ET-1 was not detected in any extract prepared from fresh medium. The levels of IR-ET-1 in two spent media prepared from FBHE and CPAE were 2.9 and 3.0 nM, respectively. As shown in Fig. 2, IR-ET-1 was detected in 13 spent media of 42 cancer cell lines (31%) and the concentrations ranged from 4.2 to 150 pM. These 13 ET-producing cancer cell lines were all 4 mammary carcinoma cell lines (MCF-7, BT-20, ZR-75-1, and ZR-75-30), 5 of 6 pancreatic carcinoma cell lines (PANC-1, ASPC-1, BxPC-3, FA-6, and PSN-1), 2 of 4 colon carcinoma cell lines (HT29 and COLO 320), 1 of 4 gastric carcinoma cell lines (KATO-III), and 1 giant cell carcinoma of 8 lung carcinoma cell lines (Lu-65). The dose-response curves of the extracts with IR-ET-1 activity were parallel to that of

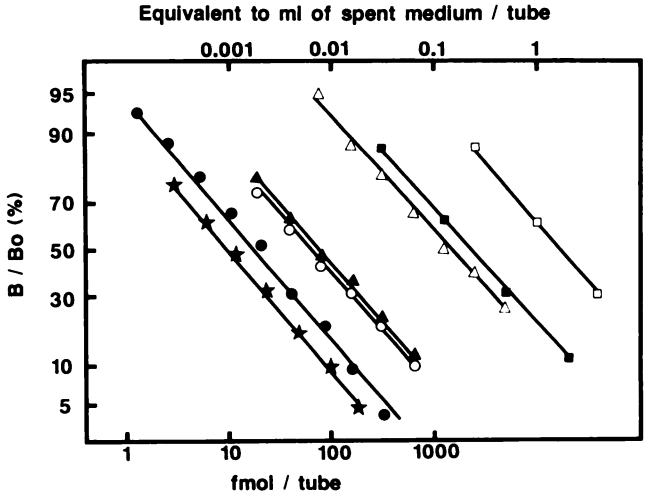

Fig. 1. Dose-response curves of ET-1, big ET-1, and the extracts prepared from spent media of several cell lines. The dose-response curves are plotted on the basis of the equivalent of ml of spent medium. The percentage of observed binding/initial binding (B/B_0) was plotted in a logit scale. ●, ET-1; ★, big ET-1; ○, CPAE; ▲, FBHE; △, PANC-1; ■, HT-29; □, BxPC-1.

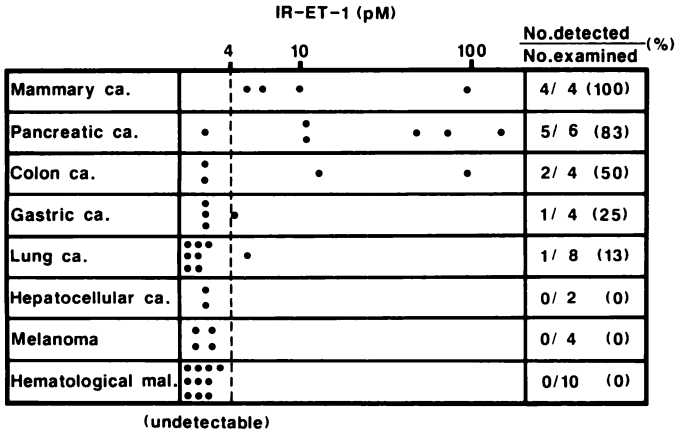

Fig. 2. Frequency of detection and the concentrations of IR-ET-1 determined by ET-1 RIA in extracts prepared from the spent media of various types of human cancer cell lines. *ca.*, carcinoma; *mal.*, malignancy.

synthetic ET-1 indicating that the active materials present in the extracts had displacement characteristics similar to those of the synthetic ET-1 (Fig. 1). It is worth noting that the frequency of IR-ET-1 production and its concentrations in mammary, pancreatic, and colon carcinomas are high when compared with other cancer cell lines.

Gel Permeation Chromatography of IR-ET-1 in Cell Lines. The gel filtration patterns of the extracts prepared from the spent media of 2 endothelial cell lines (FBHE and CPAE), 3 pancreatic carcinoma cell lines (PANC-1, ASPC-1, and BxPC-3), a colon carcinoma cell line (HT-29), and a mammary carcinoma cell line (MCF-7) are shown in Fig. 3. The major peak was eluted in a position similar to that for ET-1. In addition, a small but definite peak eluted in a position similar to big ET-1 in all extracts examined.

Biological Activity of Partially Purified IR-ET-1 Produced by Cancer Cells. The effects of synthetic ET-1 and partially purified IR-ET-1 from PANC-1 on contraction of rat aortic strips are shown in Fig. 4. IR-ET-1 partially purified from the spent medium of PANC-1 induced a slow-onset and long-lasting contraction of rat aortic strips in a dose-dependent manner, and it is difficult to wash out. This contraction pattern is similar to that of synthetic ET-1. The vasoconstrictive activity of the partially purified IR-ET-1 from PANC-1 was calculated to be approximately 40% when compared to synthetic ET-1 at the concentration of 1 nM.

Northern Blot Analysis in Cell Lines. Autoradiographs of Northern blot analysis using the probe for ET-1 mRNA are shown in Fig. 5. In all cell lines producing a large amount of IR-ET-1, the band with molecular size of 2.3 kilobases was detected. In MIA PaCa-2 which did not produce IR-ET-1, the band was not detected. When the probe for β-actin mRNA was used, a 2.0-kilobase band was detected in each cell line (data not shown).

Specific Binding of ^{125}I-ET-1 to Various Cells. As reported previously, appreciable specific binding of ^{125}I-ET-1 was observed in Swiss 3T3 fibroblasts, but no significant specific binding was found in 4 pancreatic cancer cell lines (Table 1). In contrast, a specific binding was found in 3 skin fibroblasts (Table 1). As shown in Fig. 6, Scatchard plot analysis for skin fibroblasts revealed that the K_d of the specific binding was 0.4 nM and the number of specific binding sites was 4.0×10^4/cell.

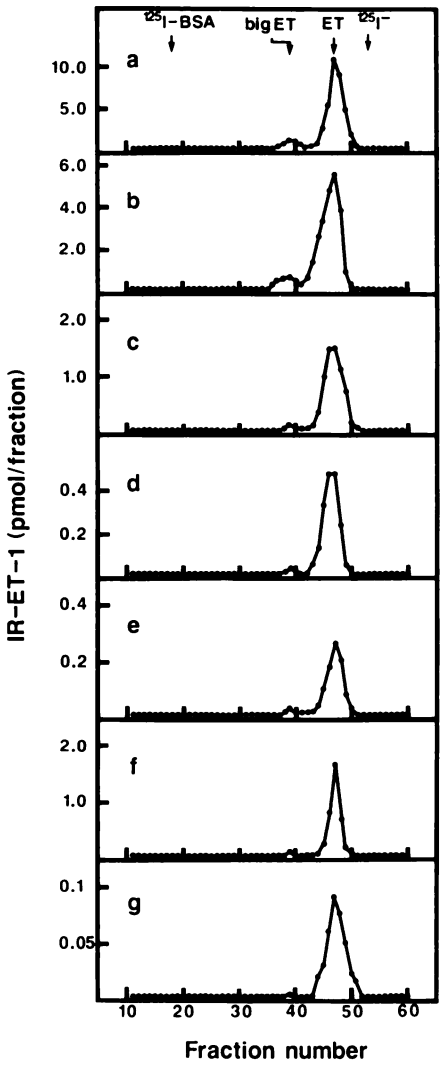

Fig. 3. Gel filtration patterns of extracts prepared from the spent media of endothelial cells and cancer cell lines measured by ET-1 RIA. *a*, FBHE; *b*, CPAE; *c*, PANC-1; *d*, ASPC-1; *e*, BxPC-1; *f*, HT-29; *g*, MCF-7. Markers are shown at the top. *big ET*, big ET-1; *ET*, ET-1.

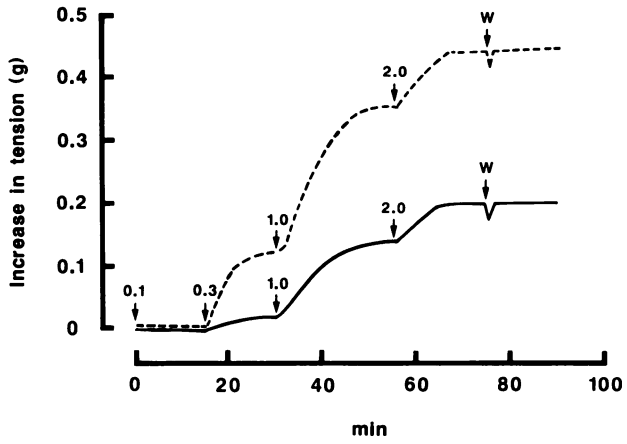

Fig. 4. Constrictive effects on rat aortic strips of synthetic ET-1 (- - - -) and IR-ET-1 (——) prepared from the conditioned medium of pancreatic carcinoma cell line, PANC-1. *Arrows* and *numerals*, time at which synthetic ET-1 and IR-ET-1 were added and the corresponding cumulative concentrations (nM), respectively; *W*, time of washout of the chamber with Krebs-Ringer solution.

DISCUSSION

In the present study, a specific RIA for ET-1 is described. When two vascular endothelial cell lines expressing ET-1 mRNA were examined by this RIA, a large amount of IR-ET-1 was detected in the extracts prepared from spent media of these cell lines.

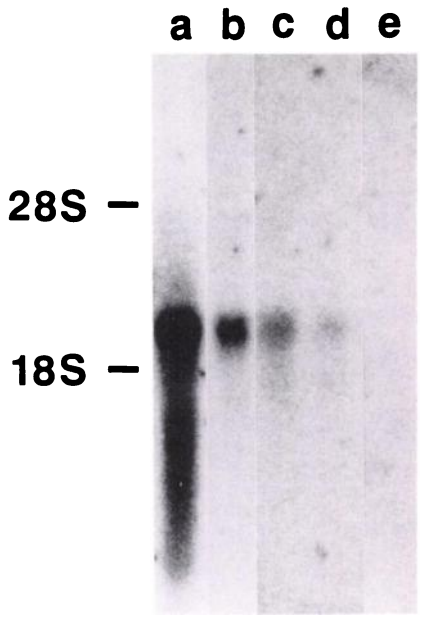

Fig. 5. Detection of ET-1 mRNA in an endothelial cell line and cancer cell lines. *a*, FBHE; *b*, HT-29; *c*, PANC-1; *d*, ASPC-1; *e*, MIA PaCa-2. Unlabeled 18S and 28S rRNAs were used as size markers.

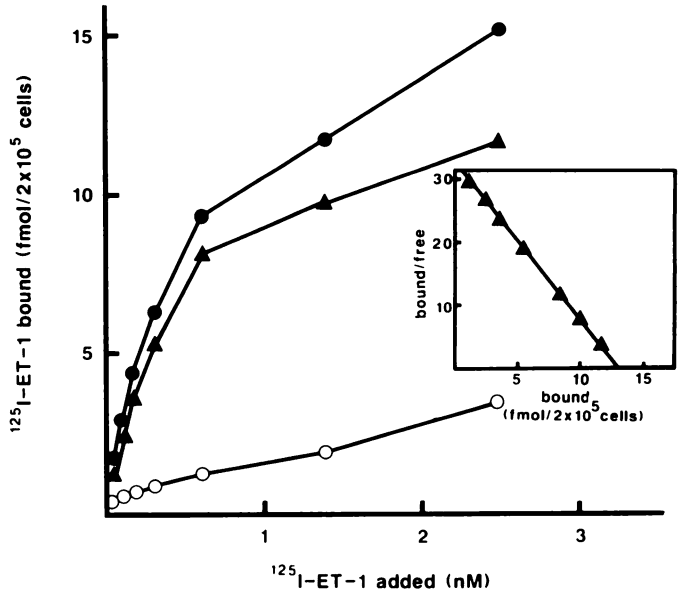

Fig. 6. Specific binding of ^{125}I-ET-1 to human skin fibroblasts and Scatchard plot analysis (*inset*). Specific binding (▲) was determined in the presence (○) or absence (●) of a 100-fold excess of unlabeled ET-1.

Table 1 *Specific binding of labeled ET-1 to cultured cells*

Cells	Specific binding (fmol/10^6 cells)
Swiss 3T3 fibroblasts	110
PANC-1 (pancreatic carcinoma)	ND[a]
ASPC-1 (pancreatic carcinoma)	ND
BxPC-3 (pancreatic carcinoma)	ND
FA-6 (pancreatic carcinoma)	ND
Human skin fibroblast	
1	66
2	46
3	41

[a] ND, not detectable.

This assay system demonstrated IR-ET-1 in 13 of 42 extracts prepared from human cancer cell lines (31%). Several points of evidence indicate that IR-ET-1 produced by cancer cells is actually ET-1: (*a*) gel filtration studies revealed that the major IR-ET-1 peak in these extracts eluted in the position corresponding to synthetic ET-1; (*b*) IR-ET-1 partially purified from the spent medium of a pancreatic carcinoma cell line had ET-1-like biological activity constricting rat aortic strips; (*c*) Northern blot analysis showed that the band with a molecular size corresponding to that of ET-1 mRNA was detected in the cancer cell lines producing a large amount of IR-ET-1. This suggests that IR-ET-1 produced by cancer cell lines was the product of a ET-1 gene. With regard to two putative peptides, ET-2 and ET-3, they did not cross-react significantly in our ET-1 RIA. Therefore, it is reasonable to conclude that IR-ET-1 in the spent media of cancer cell lines is ET-1.

The present study demonstrated a significant difference in ET-1 production according to the types of malignancies. Eleven of 14 mammary, pancreatic and colon carcinoma cell lines produced IR-ET-1. In contrast, only 2 of 18 gastric, lung, and hepatocellular carcinoma cell lines and melanoma cell lines produced IR-ET-1. Moreover, 10 cell lines of hematological malignancies did not produce IR-ET-1. Since ET-1 is the specific product of vascular endothelial cells, this phenomenon could be explained by ectopic ET-1 production by cancer cells. However, it seems that the detection frequency in several specified types of cancer cell lines were too high to be explained simply by such a mechanism. Alternatively, we speculate that the normal ductal or acinar cells may have the capacity to produce ET-1 and that cancer cells derived from these cells maintain this capacity. In this context, it will be required to know whether ET-1 exists in normal epithelial cells. At any rate, the present study suggests that some cancer cells share at least one of the properties of vascular endothelial cells, production of ET-1.

Our previous study indicated that a ET-1 concentration greater than 100 pM is sufficient to potentiate DNA synthesis in Swiss 3T3 fibroblasts. The present study demonstrated that ET-1 concentration in the spent media ranged from 4.2 to 150 pM. These results may suggest that ET-1 concentration in the microenvironment surrounding cancer cells is sufficient to stimulate cellular growth. These findings promoted us to investigate the expression of ET-1 receptors in ET-1-producing cancer cells, but, as far as examined, they had no ET-1 receptors. Since these cancer cell lines produce appreciable amounts of ET-1, the possibility should be kept in mind that ET-1 receptors were occupied by ET-1 molecules produced by cancer cells. Then, we investigated the presence of ET-1 receptors in human skin fibroblasts following our previous findings that Swiss 3T3 fibroblasts possessed a large number of ET-1 receptors. We found that all of the fibroblasts examined in this study possessed ET-1 receptors in almost same number as those present on Swiss 3T3 fibroblasts (2) and vascular smooth muscle cells (28). Thus, it is reasonable to speculate that ET-1 plays a role stimulating cellular growth of mesenchymal cells in a paracrine fashion; these cancer cell lines were demonstrated to produce IR-ET-1 very frequently. Further studies are required to investigate the morphological and biochemical changes of fibroblasts surrounding ET-1-producing cancer cells.

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REFERENCES

1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature (Lond.), *332:* 411–415, 1988.
2. Kusuhara, M., Yamaguchi, K., Ohnishi, A., Abe, K., Kimura, S., Oono, H., Hori, S., and Nakamura, Y. Endothelin potentiates growth factor-stimulated DNA synthesis in Swiss 3T3 cells. Jpn. J. Cancer Res., *80:* 302–305, 1989.
3. Komuro, I., Kurihara, H., Sugiyama, T., Takaku, F., and Yazaki, Y. Endothelin stimulates c-*fos* and c-*myc* expression and proliferation of vascular smooth muscle cells. FEBS Lett., *238:* 249–252, 1988.
4. Nakaki, T., Nakayama, M., Yamamoto, S., and Kato, R. Endothelin-mediated stimulation of DNA synthesis in vascular smooth muscle cells. Biochem. Biophys. Res. Commun., *158:* 880–883, 1989.
5. Simonson, M. S., Wann, S., Mene, P., Dubyak, G. R., Kester, M., Nakazato, Y., Sedor, J. R., and Dunn, M. J. Endothelin stimulates phospholipase C, Na^+/H^+ exchange, c-*fos* expression, and mitogenesis in rat mesangial cells. J. Clin. Invest., *83:* 708–712, 1989.
6. Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., and Masaki, T. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc. Natl. Acad. Sci. USA, *86:* 2863–2867, 1989.
7. Hay, R., Macy, M., Corman-Weiblatt, A., Chen, T. R., and McClintock, P. (eds.). Catalogue of Cell Lines and Hybridomas, Ed. 5. Rockville, MD: American Type Culture Collection, 1985.
8. Yamada, H., Yoshida, T., Sakamoto, H., Terada, M., and Sugimura, T. Establishment of a human pancreatic adenocarcinoma cell line (PSN-1) with amplifications of both c-*myc* and activated c-Ki-*ras* by a point mutation. Biochem. Biophys. Res. Commun., *140:* 167–173, 1986.
9. Yamada, T., Hirohashi, S., Shimosato, Y., Kodama, T., Hayashi, S., Ogura, T., Gamou, S., and Shimizu, N. Giant cell carcinomas of the lung producing colony-stimulating factor *in vitro* and *in vivo*. Jpn. J. Cancer Res., *76:* 967–976, 1985.
10. Terasaki, T., Shimosato, Y., Nakajima, T., Tsumuraya, M., Morinaga, S., Hirohashi, S., Yamaguchi, K., Kato, K., Ichinose, H., and Nagatsu, T. Changes in cell characteristics due to culture conditions in cell lines from human small cell lung cancer. Jpn. J. Clin. Oncol., *16:* 203–212, 1986.
11. Hirohashi, S., Shimosato, Y., Kameya, T., Koide, T., Mukojima, T., and Taguchi, Y. Morphological and functional aspects of human liver cell carcinomas transplanted in nude mice. *In:* T. Nomura, N. Ohsawa, N. Tamaoki, and K. Fujiwara (eds.), Proceedings of the Second International Workshop on Nude Mice, pp. 427–434. Tokyo: University of Tokyo Press, 1977.
12. Shimoyama, M. SEKI strain. *In:* S. Oboshi and H. Sugano (eds.), *In Vitro* Culture of Human Cancer Cells, pp. 208–215 (in Japanese). Tokyo: Asakura Shoten, 1975.
13. Nagata, N., Akatsu, T., Kugai, N., Yasutomo, Y., Kinoshita, T., Kosano, H., Shimauchi, T., Takatani, O., and Ueyama, Y. The tumor cells (FA-6) established from a pancreatic cancer associated with humoral hypercalcemia of malignancy: a simultaneous production of parathyroid hormone-like activity and transforming growth factor activity. Endocrinol. Jpn., *36:* 75–85, 1989.
14. Lee, Y. C., Saijo, N., Sasaki, Y., Takahashi, H., Sakurai, M., Ishihara, J., Hoshi, A., Chen, K. M., and Hamburger, A. W. Clonogenic patterns of human pulmonary adenocarcinoma cell lines (PC-9, PC-13 and PC-14) and how they influence the results of test for chemosensitivity to cisplatin in the human tumor clonogenic assay. Jpn. J. Clin. Oncol., *15:* 637–644, 1985.
15. Gazdar, A. F., Carney, D. N., Russell, E. K., Sims, H. L., Baylin, S. B., Bunn, P. A., Jr., Guccion, J. G., and Minna, J. D. Establishment of continuous, clonable cultures of small-cell carcinoma of the lung which have amine precursor uptake and decarboxylation cell properties. Cancer Res., *40:* 3502–3507, 1980.
16. Morikawa, K., Walker, S. M., Jessup, J. M., and Fidler, I. J. *In vivo* selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. Cancer Res., *48:* 1943–1948, 1988.
17. Mori, T., Nakazawa, S., Nishino, K., Sugita, K., Takane, K., Mori, M., Sagawa, K., Hayashi, Y., and Sakurai, M. Ph^1-positive CML-derived myeloid-monocytoid precursor cell line producing substance(s) that stimulates normal CFU-C. Leuk. Res., *11:* 241–249, 1987.
18. Gazdar, A. F., Carney, D. N., Bunn, P. A., Russell, E. K., Jaffe, E. S., Schechter, G. P., and Guccion, J. G. Mitogen requirements for the *in vitro* propagation of cutaneous T-cell lymphomas. Blood, *55:* 409–417, 1980.
19. Miyoshi, I., Kubonishi, I., Sumida, M., Yoshimoto, S., Hiraki, S., Tsubota, T., Kobashi, H., Lai, M., Tanaka, T., Kimura, I., Miyamoto, K., and Sato, J. Characteristics of a leukemic T-cell line derived from adult T-cell leukemia. Jpn. J. Clin. Oncol., *9* (Suppl.): 485–494, 1979.
20. Ishidate, M. (ed.). Japanese Cancer Research Resources Bank NEWSLETTER. Tokyo: Foundation for Promotion of Cancer Research, 1989.
21. Fesheny, R. I. Disaggregation of the tissue and primary culture. *In:* R. I. Fesheny (ed.), Culture of Animal Cells, Ed. 2, pp. 107–126. New York: Alan R. Liss, Inc., 1987.
22. Goodfriend, T. L., Levine, L., and Fasman, G. D. Antibodies to bradykinin and angiotensin: a use of carbodiimides in immunology. Science (Wash. DC), *144:* 1344–1346, 1964.
23. Yamaguchi, K., Abe, K., Kameya, T., Adachi, I., Taguchi, S., Otsubo, K., and Yanaihara, N. Production and molecular size heterogeneity of immunoreactive gastrin-releasing peptide in fetal and adult lungs and primary lung tumors. Cancer Res., *43:* 3932–3939, 1983.
24. Suzuki, M., Yamaguchi, K., Abe, K., Adachi, N., Nagasaki, K., Asanuma, F., Adachi, I., Kimura, S., Terada, M., Taya, Y., Matsuzaki, J., and Miki, K. Detection of gastrin-releasing peptide mRNA in small cell lung carcinomas and medullary thyroid carcinomas using synthetic oligodeoxyribonucleotide probes. Jpn. J. Clin. Oncol., *17:* 157–163, 1987.
25. Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., Fujino, M., and Masaki, T. Cloning and sequence analysis of cDNA encoding the precursor of a human endothelium-derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. FEBS Lett., *231:* 440–444, 1988.
26. Honda, S., Yamaguchi, K., Suzuki, M., Sato, Y., Adachi, I., Kimura, S., and Abe, K. Expression of parathyroid hormone-related protein mRNA in tumors obtained from patients with humoral hypercalcemia of malignancy. Jpn. J. Cancer Res., *79:* 677–681, 1988.
27. Scatchard, G. The attraction of proteins for small molecules and ions. Ann. NY Acad Sci., *51:* 660–672, 1949.
28. Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T. X., Kumagai, S., Nakajima, K., and Sakakibara, S. Cellular mechanism of action by a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. Biochem. Biophys. Res. Commun., *154:* 868–875, 1988.