[CANCER RESEARCH 52, 3011-3014, June 1, 1992]

Stimulation of Anchorage-independent Cell Growth by Endothelin in NRK 49F Cells[1]

Masatoshi Kusuhara,[2] Ken Yamaguchi, Masaru Kuranami, Ai Suzaki, Shiro Ishikawa, Hanlim Moon, Isamu Adachi, Shingo Hori, and Shunnosuke Handa

Growth Factor Division, National Cancer Center Research Institute [M. Kus, K. Y., M. Kur, A. S., S. I., H. M.] and Department of Medicine, National Cancer Center Hospital [I. A.], Tsukiji 5-1-1, Chuo-ku, Tokyo 104; and Department of Medicine [S. Ho., S. Ha.], Keio University School of Medicine, Shinanomachi 35, Shinjuku-ku, Tokyo 160, Japan

ABSTRACT

Endothelin (ET) is a vasoconstrictor peptide originally isolated from vascular endothelial cells. Recent studies have revealed that ET has many biological functions including growth factor-like activity. The present study aims to clarify whether ET-1 possesses the ability to stimulate anchorage-independent cellular growth, an indicator of factors with transforming activity.

We found that NRK 49F cells possess a large number of high-affinity ET-1 receptors; labeled ^{125}I-ET-1 binding was displaced by unlabeled ET-2 in a similar dose response, but in the case of ET-3, 100-fold more was required. Specific ^{125}I-ET-3 binding was undetectable in NRK 49F cells, indicating that ET receptors in NRK 49F cells are ET-1/ET-2 selective.

NRK 49F is a cell line which is most commonly used to assay for anchorage-independent cellular growth. Therefore, we explored whether ETs promote anchorage-independent cellular growth in this cell line. ET-1 and ET-2 stimulated NRK colony formation dose dependently in the presence of 1 nM epidermal growth factor (EGF). In contrast, ET-3 did not have colony-stimulating ability. In the presence of EGF, the maximal effect of ET-1 was approximately 90% of that of transforming growth factor-β. Moreover, in the presence of maximal stimulating concentrations of EGF and transforming growth factor-β, ET-1 additionally induced colony formation. These results indicate that ET-1 and -2 possess transforming growth factor-like activity for NRK 49F cells. Since ET-1 and -2 increased intracellular calcium levels, this ion may participate in signal transduction pathways by which ET-1 and -2 promote colony formation.

INTRODUCTION

ET-1[3] was isolated originally as a factor possessing vasoconstrictive activity from culture media conditioned by primary-cultured endothelial cells (1). Three types of ET, ET-1, ET-2, and ET-3, are now known to compose the ET family, and their production and secretion have been demonstrated in many tissues (2). Recent research has revealed that ET-1 also has a wide range of pharmacological effects in tissues other than blood vessels, such as an inotropic effect on cardiac myocytes (3), a neurotransmitter effect on the central nerve system (4), and modulation of endocrine secretions (5-8).

Several studies, including ours, demonstrated that ET-1 stimulates DNA synthesis in various types of cultured cells, including Swiss 3T3 (9, 10), vascular smooth muscle (11, 12), and renal mesangial (13). However, how ET-1 influences cellular growth is poorly understood. Our recent observations that cancer cells frequently produce and secrete ET-1 and that primary-cultured human fibroblasts possess ET-1 receptors suggest that ET-1 produced by cancer cells could act as a paracrine growth factor on mesenchymal cells (14).

In the present study, we explored whether ET-1 promotes anchorage-independent cell growth. For these experiments, NRK 49F was selected as an indicator cell line, which is most commonly used to assay for anchorage-independent cell growth (15, 16).

MATERIALS AND METHODS

Cell Culture and Materials. NRK 49F cells were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). They were grown in DMEM (Nissui Seiyaku, Tokyo, Japan) containing 5% CS (GIBCO Laboratories, Grand Island, NY), 100 μg of streptomycin/ml, and 100 units of penicillin G/ml at 37°C in a fully humidified atmosphere of 5% CO_2 in air. Confluent cells were further cultured in DMEM containing 1% CS for 24 h to minimize the effect of serum.

Synthetic ET-1, -2, and -3 were purchased from the Peptide Institute (Osaka, Japan); recombinant TGF-β and human EGF from Earth Chemical Co., Ltd. (Hyogo, Japan); bovine serum albumin (Cohn fraction V) from Dai-ichi Pure Chemicals Co., Ltd. (Osaka, Japan); ^{125}I-ET-1 (specific activity, 74 TBq/mmol) and ^{125}I-ET-3 (specific activity, 74 TBq/mmol) from Amersham International (Buckinghamshire, United Kingdom); and Fura-2 acetoxymethyl ester from Molecular Probes, Inc. (Eugene, OR).

Binding Assay. The ET-1 binding assay was performed as previously described, with slight modification (17). NRK 49F cells were grown to confluence in a 48-well plate and further cultured for 24 h in DMEM containing 1% CS. Hanks' balanced salt solution containing 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) and 0.1% bovine serum albumin (pH 7.4) was used as assay buffer. After these cells were washed, they were incubated at 37°C for 60 min with 0.12 nM ^{125}I-ET-1 plus various concentrations of unlabeled ET-1, -2, and -3. They were washed three times and solubilized with 1 N NaOH. Cell-bound radioactivity was determined in a gamma spectrometer. Cell numbers were estimated by counting other wells which were treated in the same manner as those of the binding studies. Specific binding was calculated as total binding minus binding in the presence of 1 μM unlabeled ET-1.

Since specific ^{125}I-ET-3 binding could not be detected under the above conditions, the binding assay was done by using a larger number of NRK 49F cells. NRK 49F cells in 60-mm dishes were incubated with 0.3 nM ^{125}I-ET-3 in the presence or absence of a 5000-fold excess of unlabeled ET-3.

Soft Agar Colony Formation Assay. To assess anchorage-independent cell growth, a soft agar colony formation assay using NRK 49F cells was performed as previously described, with slight modification (18). NRK 49F cells (20,000/dish) were suspended in 0.9 ml of culture medium containing 0.3% agar and spread over a prehardened feeder layer comprising 0.9 ml of the same medium, but containing 0.5% agar, in a 35-mm Petri dish. Test materials dissolved in 0.2 ml of culture medium were placed on the top of the prehardened agar layers. After 14 days of incubation at 37°C in a humidified CO_2 incubator, colonies

Received 6/19/91; accepted 3/20/92.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This investigation was supported in part by a research grant from the Princess Takamatsu Cancer Research Fund, by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy of Cancer Control, and by Grants-in-Aid for Cancer Research (1-5 and 2-9) from the Ministry of Health and Welfare.

[2] To whom requests for reprints should be addressed, at Growth Factor Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104, Japan.

[3] The abbreviations used are: ET, endothelin; DMEM, Dulbecco's modified Eagle medium; CS, calf serum; TGF, transforming growth factor; EGF, epidermal growth factor; $[Ca^{2+}]_i$, intracellular calcium ion concentration; K_d, dissociation constant.

with a diameter ≧70 μm were counted with a colony counter (Oriental Instruments Ltd., Tokyo, Japan).

In this study, we used EGF at a concentration of 1 nM and TGF-β at a concentration of 0.12 nM. We observed maximal stimulation of NRK colony formation with these concentrations (data not shown).

Measurement of $[Ca^{2+}]_i$. Most experiments were performed with NRK 49F cells attached to round glass coverslips. $[Ca^{2+}]_i$ was measured by using Fura-2 as previously described (19). In brief, cells were grown on round glass coverslips (13.5-mm diameter) until confluence, and the coverslips were washed with DMEM and further incubated for 24 h in DMEM containing 1% CS. They were then incubated for 40 min at 37°C in buffer solution (Hanks' balanced salt solution with 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), pH 7.4) containing 5 μM Fura-2 acetoxymethyl ester. The cells were placed diagonally in a standard 1-cm^2 quartz glass cuvette.

An experiment in which cells were suspended in extracellular Ca^{2+}-free buffer was performed by the method described previously (9), since NRK 49F cells easily detach from round glass coverslips under this experimental condition. Fura-2 was loaded on NRK 49F cells in a 75-cm^2 flask in the manner described above. Cells were harvested, resuspended, and transferred to a 0.3-cm^2 cuvette in the spectrofluorometer.

Fluorescence of Fura-2-loaded cells attached to the coverslip or suspended in the buffer was measured with a spectrofluorometer (CAF-100, Japan Spectroscopic Co., Ltd., Tokyo, Japan) with excitation at 340 and 380 nm and emission at 500 nm. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz *et al.* (20).

Statistical Methods. Statistical significance was evaluated by Student's *t* test or analysis of variance with $P < 0.01$ regarded as significant.

RESULTS

Specific Binding of ^{125}I-ET-1 to NRK 49F Cells. Specific ^{125}I-ET-1 binding detected in NRK 49F cells was displaced in a dose-dependent manner by unlabeled ET-1 (Fig. 1*A*). Labeled ^{125}I-ET-1 binding was also displaced by unlabeled ET-2 in a similar dose response, but in the case of ET-3, 100-fold more was required (Fig. 1*A*). Scatchard plot analysis (21) of ET-1 binding revealed a K_d of 0.3 nM and 9.4×10^4 binding sites/cell for specific binding in NRK 49F cells (Fig. 1*B*). Labeled ET-1 binding to NRK 49F cells was not inhibited by 10 nM TGF-β and 100 nM EGF (Fig. 1*A*).

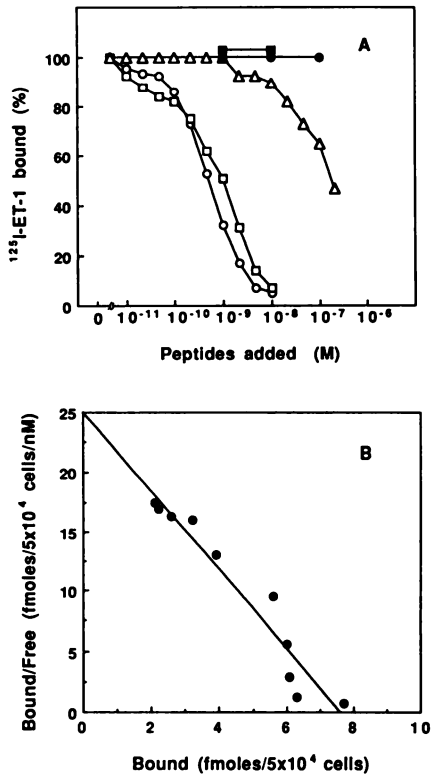

Fig. 1. ^{125}I-ET-1 binding to NRK 49F cells. *A*, displacement by unlabeled ETs. ○, ET-1; □, ET-2; △, ET-3; ■, TGF-β; ●, EGF. *B*, Scatchard plot analysis. *Point*, mean of duplicate determinations.

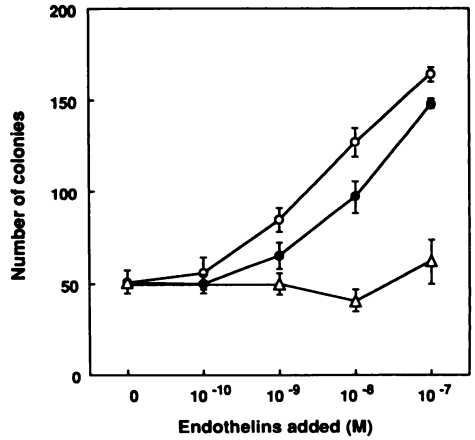

Fig. 2. The effect of increasing doses of ETs on colony formation by NRK 49F cells. ○, ET-1; ●, ET-2; △, ET-3. In all cases the assay was performed in the presence of 1 nM EGF. *Point*, mean (*bar*, ±SEM) of triplicate determinations.

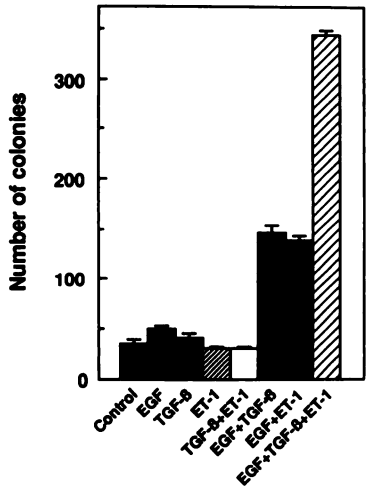

Fig. 3. Comparison of colony-forming activity among ET-1 (100 nM), EGF (1 nM), and TGF-β (0.12 nM). *Column*, mean (*bar*, ±SEM) of triplicate determinations.

Specific ^{125}I-ET-3 binding was undetectable in NRK 49F cells (<1% of the total ^{125}I-ET-3 added).

Effect of ETs on Anchorage-independent Cell Growth. ET-1 alone did not induce anchorage-independent cell growth of NRK 49F cells as assessed by soft agar colony formation. In the presence of EGF, ET-1 induced colony formation by NRK 49F cells dose dependently (Fig. 2). This phenomenon was elicited at an ET-1 concentration of 1 nM. ET-2 at a concentration ≧1 nM also stimulated colony formation in NRK 49F cells in the presence of 1 nM EGF. On the other hand, ET-3 up to 100 nM did not have a significant effect on colony formation by NRK 49F cells.

One nM EGF, 0.12 nM TGF-β, and 100 nM ET-1 did not induce significant additional colony formation (Fig. 3). Both TGF-β and ET-1 induced colony formation in the presence of EGF. In contrast, the combination of ET-1 and TGF-β induced no significant effect. ET-1 induced colony formation even in

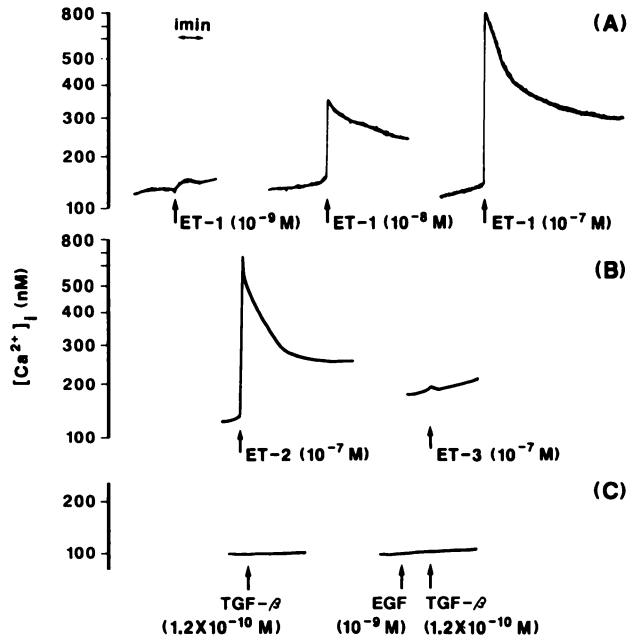

Fig. 4. Effect of ETs, TGF-β, and EGF on $[Ca^{2+}]_i$ in NRK 49F cells. *Arrows*, test materials added. *A*, dose-dependent effect of ET-1 on $[Ca^{2+}]_i$; *B*, effect of ET-2 and ET-3 on $[Ca^{2+}]_i$; *C*, effect of TGF-β (0.12 nM) in the absence and presence of EGF (1 nM). The tracings presented are typical of five studies.

the presence of both EGF and TGF-β (Fig. 3).

Effect on $[Ca^{2+}]_i$ in NRK 49F Cells. ET-1 rapidly induced an initial transient and subsequent sustained increase in $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 4*A*). The maximal levels induced were approximately 6-fold higher than basal levels. ET-2 also induced an increase in $[Ca^{2+}]_i$ to almost the same extent. ET-3 was ineffective at a concentration of 100 nM (Fig. 4*B*). In contrast, no significant change in $[Ca^{2+}]_i$ was observed under this experimental condition when the cells were stimulated with TGF-β or TGF-β together with EGF (Fig. 4*C*).

When a Ca^{2+}-free buffer containing 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid was used, ET-1 induced an increase in $[Ca^{2+}]_i$ (Fig. 5).

DISCUSSION

The binding study demonstrated that NRK 49F cells possess ET-1 receptors. We have reported that Swiss 3T3 cells (9, 17) and human skin fibroblasts (14, 17) possess a larger number of ET-1 receptors than do vascular smooth muscle cells. The K_d value and number of specific binding sites for ET-1 in NRK 49F cells were similar to those found in vascular smooth muscle cells (22), Swiss 3T3 cells (9, 17), and human skin fibroblasts (14, 17). Recently, two types of complementary DNAs encoding ET receptor were cloned; one encodes an ET-1-/ET-2-selective receptor (ET_A receptor) (23) and the other a nonselective receptor (ET_B receptor) (24). Based on the observations that the ET receptor of NRK 49F cells showed higher affinity for ET-1 and ET-2 than for ET-3, and that ^{125}I-ET-3 did not bind to NRK 49F cells significantly, ET receptors of this cell line were considered to be mainly ET_A receptors.

Anchorage-independent NRK 49F cell growth, the ability to form colonies in soft agar, is an indicator of factors with transforming activity, such as EGF, TGF-α, and TGF-β (25, 26). We previously demonstrated that ET-1 potentiates growth factor-induced DNA synthesis in Swiss 3T3 cells, which possess many ET-1-/ET-2-selective receptors (9). The fact that NRK 49F cells possess ET-1-/ET-2-selective receptors led us to examine whether ET-1 potentiates colony formation of NRK 49F cells in soft agar. ET-1 alone or ET-1 plus TGF-β did not stimulate colony formation; in contrast, in the presence of EGF, ET-1 stimulated colony formation. This observation indicates that ET-1 can replace TGF-β in NRK 49F colony formation; under the present experimental conditions, the maximal transforming activity of ET-1 was 90% of that of TGF-β. Since ET-1 can stimulate colony formation by NRK 49F cells under conditions where they were maximally stimulated with EGF and TGF-β, the mechanism responsible for ET-1-induced colony formation seems to be different from that of TGF-β.

ET-1 and ET-2 increased $[Ca^{2+}]_i$ in NRK 49F cells. This elevation of $[Ca^{2+}]_i$ is chiefly due to mobilization from an intracellular calcium ion store. This effect is elicited at an ET-1 concentration of 1 nM; this concentration is similar to that which stimulates colony formation. Since TGF-β did not influence $[Ca^{2+}]_i$, different intracellular signal pathways may exist for ET-1 and TGF-β for their stimulation of colony formation by NRK 49F cells. In contrast, ET-3 at a concentration of 100 nM induced only slight elevation of $[Ca^{2+}]_i$. Calcium ions play an important role in cellular growth (27, 28); however, the relationship between cellular transformation and mobilization of $[Ca^{2+}]_i$ remains unclear. ET-1-induced colony formation of NRK 49F cells with an increase in $[Ca^{2+}]_i$ might be a new clue for clarifying this relationship.

It has been suggested that an increase in fibronectin and collagen production induced by TGF-β is responsible for TGF-β-stimulated colony formation by NRK 49F cells (29). It is possible to speculate that ET-1/ET-2 also stimulates fibronectin and collagen synthesis. Alternatively, a possible mechanism of ET-1-/ET-2-induced colony formation is activation of protein kinase C. Phorbol esters have been shown to promote colony formation by NRK 49F cells (30). However, phorbol ester potentiates colony formation only in the presence of both EGF and TGF-β. This property is clearly different from that of ET-1 in that ET-1 can substitute for TGF-β. These results indicate that activation of protein kinase C cannot be the main mechanism of ET-1-/ET-2-induced colony formation.

Our recent study demonstrated that ET-1 is frequently pro-

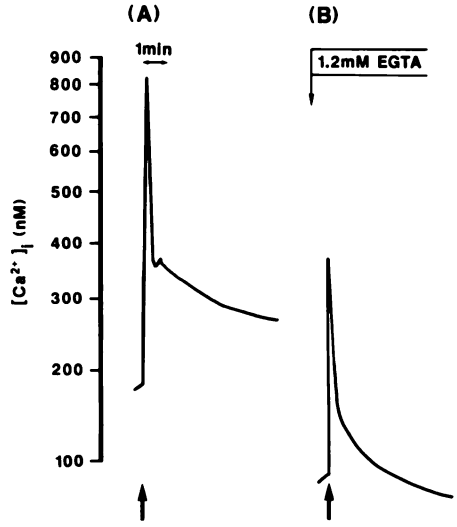

Fig. 5. Effect of extracellular Ca^{2+} on ET-1-induced Ca^{2+} mobilization. *Arrows*, addition of ET-1 (100 nM). *A*, extracellular Ca^{2+}, 1 mM; *B*, no added Ca^{2+}. The tracings presented are typical of five studies. EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

duced by human cancer cell lines (14). To investigate ET-1 as a possible autocrine or paracrine growth factor, we examined various cancer cells and fibroblasts for the presence of ET-1 receptors. Cancer cell lines producing ET-1 did not possess ET-1 receptors, but human skin fibroblasts possess large numbers of them. It is reasonable to assume that ET-1 produced by cancer cells or normal cells plays a modulatory role in the growth of stromal cells surrounding ET-1-producing cells as well as vascular endothelial cells.

In conclusion, two ETs, ET-1 and ET-2, have TGF-β-like activity in anchorage-independent NRK 49F cell growth, and ET-1-/ET-2-induced $[Ca^{2+}]_i$ may play an important role in this effect. Studies of the interaction between ET-1/ET-2 and TGF-β should provide a better understanding of how transformation proceeds along a multistep pathway.

ACKNOWLEDGMENTS

We thank Kotomi Otsubo and Mon Ebinuma for their excellent technical assistance.

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. Mathew, W. M., Christopher, A. R., Bert, M. G., and Solomon, H. S. Endothelin: visualization of mRNAs by *in situ* hybridization provides evidence for local action. Proc. Natl. Acad. Sci. USA, *86:* 7285–7289, 1989.
3. Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K., and Masaki, T. Positive inotropic action of novel vasoconstrictor peptide endothelin on guinea pig atria. Am. J. Physiol., *255:* H970–H973, 1988.
4. Yoshizawa, T., Shinmi, O., Giaid, A., Yanagisawa, M., Gibson, S. J., Kimura, S., Uchiyama, Y., Polak, J. M., Masaki, T., and Kanazawa, I. Endothelin: a novel peptide in the posterior pituitary system. Science (Washington DC), *247:* 462–464, 1990.
5. Fukuda, Y., Hirata, Y., Yoshimi, H., Kojima, T., Kobayashi, Y., Yanagisawa, M., and Masaki, T. Endothelin is a potent secretagogue for atrial natriuretic peptide in cultured rat atrial myocytes. Biochem. Biophys. Res. Commun., *155:* 167–172, 1988.
6. Tabuchi, Y., Nakamura, M., Rakugi, H., Nagano, M., Mikami, H., and Ogihara, T. Endothelin inhibits presynaptic adrenergic neurotransmission in rat mesenteric artery. Biochem. Biophys. Res. Commun., *161:* 803–808, 1989.
7. Rakugi, H., Nakamura, M., Saito, H., Higaki, J., and Ogihara, T. Endothelin inhibits renin release from isolated rat glomeruli. Biochem. Biophys. Res. Commun., *155:* 1244–1247, 1988.
8. Cozza, E. N., Gomez-Sanchez, C. E., Foecking, M. F., and Chiou, S. Endothelin binding to cultured calf adrenal zona glomerulosa cells and stimulation of aldosterone secretion. J. Clin. Invest., *84:* 1032–1035, 1989.
9. 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.
10. Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K., and Masaki, T. A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. J. Biol. Chem., *264:* 7856–7861, 1989.
11. 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.
12. 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.
13. 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.
14. Kusuhara, M., Yamaguchi, K., Nagasaki, K., Hayashi, C., Suzaki, A., Hori, S., Handa, S., Nakamura, Y., and Abe, K. Production of endothelin in human cancer cell lines. Cancer Res., *50:* 3257–3261, 1990.
15. Rizzino, A., Ruff, E., and Rizzino, H. Induction and modulation of anchorage-independent growth by platelet-derived growth factor, fibroblast growth factor, and transforming growth factor-β. Cancer Res., *46:* 2816–2820, 1986.
16. van Zoelen, E. J. J., van Oostwaard, T. M. J., and de Laat, S. W. Transforming growth factor-β and retinoic acid modulate phenotypic transformation of normal rat kidney cells induced by epidermal growth factor and platelet-derived growth factor. J. Biol. Chem., *261:* 5003–5009, 1986.
17. Ohnishi-Suzaki, A., Yamaguchi, K., Kusuhara, M., Adachi, I., Abe, K., and Kimura, S. Comparison of biological activities of endothelin-1, -2 and -3 in murine and human fibroblast cell lines. Biochem. Biophys. Res. Commun., *166:* 608–614, 1990.
18. Kuranami, M., Yamaguchi, K., Asanuma, F., Suzuki, M., and Abe, K. The growth promoting effect of transforming growth factor-α in human breast cancer cell lines. Jpn. J. Clin. Oncol., *19:* 360–366, 1989.
19. Ohnishi, A., Yamaguchi, K., Kusuhara, M., Abe, K., and Kimura, S. Mobilization of intracellular calcium by endothelin in Swiss 3T3 cells. Biochem. Biophys. Res. Commun., *161:* 489–495, 1989.
20. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem., *260:* 3440–3450 1985.
21. Scatchard, G. The attraction of proteins for small molecules and ions. Ann. N. Y. Acad. Sci., *51:* 660–672, 1949.
22. 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.
23. Arai, H., Hori, S., Aramori, I., Ohkubo, H., and Nakanishi, S. Cloning and expression of a cDNA encoding an endothelin receptor. Nature (Lond.), *348:* 730–732, 1990.
24. Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., and Masaki, T. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature (Lond.), *348:* 732–735, 1990.
25. Shin, S., Freedman, V. H., Risser, R., and Pollack, R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth *in vitro*. Proc. Natl. Acad. Sci. USA, *72:* 4435–4439, 1975.
26. de Larco, J. E., and Todaro, G. J. Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA, *75:* 4001–4005, 1978.
27. Metcalfe, J. C., Moore, J. P., Smith, G. A., and Hesketh, T. R. Calcium and cell proliferation. Br. Med. Bull., *42:* 405–412, 1986.
28. Balk, S. D. Calcium as a regulator of the proliferation of normal, but not of transformed, chicken fibroblasts in a plasma-containing medium. Proc. Natl. Acad. Sci. USA, *68:* 271–275, 1971.
29. Ignotz, R. A., and Massague, J. Transforming growth factor β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J. Biol. Chem., *261:* 4337–4345, 1986.
30. Kraft, A. S. Effect of phorbol esters on the activity of purified transforming growth factors. Cancer Res., *46:* 1764–1767, 1986.