RAPID COMMUNICATION Jpn. J. Cancer Res. 80, 302–305, April 1989

Endothelin Potentiates Growth Factor-stimulated DNA Synthesis in Swiss 3T3 Cells

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A murine cell line, Swiss 3T3, is commonly used for the study of cellular growth. The present study revealed that this cell line possesses high-affinity receptors for endothelin, a vasoactive peptide derived from vascular endothelial cells. In this cell line, endothelin potentiated DNA synthesis stimulated by platelet-derived growth factor, basic fibroblast growth factor or insulin. The endothelin concentration required for potentiating DNA synthesis in this cell line is almost the same as that reported previously in endothelin-induced vasoconstriction. Since endothelin increased intracellular calcium levels, this ion may participate in the signal transduction pathways by which endothelin induces potentiation of DNA synthesis.

Key words: Endothelin — Swiss 3T3 cells — Growth factor — Intracellular calcium — Receptor

Factors produced by vascular endothelial cells regulate vasodilation[1)] and vasoconstriction[2)] by acting on vascular smooth muscle cells. Endothelin, one of these factors with vasoconstrictive activity, has recently been isolated from the spent medium of cultured endothelial cells of porcine vessels.[3,4)] Recently, Komuro *et al.* reported a novel biological effect of endothelin to stimulate DNA synthesis in vascular smooth muscle cells,[5)] but the dose required for stimulation of DNA synthesis is about 10 to 100 times greater than that required for vasoconstriction.[6,7)] We report here the presence of specific binding sites for endothelin in a murine cell line, Swiss 3T3, that is commonly used for the study of cellular growth. In this cell line, it was found that a low dose of endothelin potentiated DNA synthesis stimulated by three growth factors, i.e., platelet-derived growth factor, basic fibroblast growth factor and insulin.

Two murine cell lines, Swiss 3T3 and NIH 3T3, were examined for the presence of specific binding sites. These cell lines were provided by the Japanese Cancer Research Resources Bank (Tokyo). Saturable specific binding of labeled endothelin was detected in Swiss 3T3 cells (Fig. 1), but not in NIH 3T3 cells (data not shown). Scatchard plot analysis[8)] revealed that the dissociation constant (*Kd*) of the specific binding in Swiss 3T3 cells was 5.0×10^{-10} *M* and the number of binding sites was 1.0×10^{5} per cell. Endothelin receptors have been detected in primary-cultured rat vascular smooth muscle cells.[9)] Comparison with these data revealed that the *Kd* of the specific binding in Swiss 3T3 cells is almost the same as that in smooth muscle cells, while the number of specific binding sites is greater. Swiss 3T3 cells possess receptors for other growth factors and bioactive peptides including platelet-derived growth factor,[10)] epidermal growth factor,[11)] fibroblast growth factor[12)] and gastrin-releasing peptide.[13)] In comparison with these receptors, the number of binding sites for endothelin was higher, suggesting that endothelin may induce some important biological effects in this cell line.

The effect of endothelin alone and together with other growth factors was investigated on DNA synthesis in Swiss 3T3 cells. Endothelin alone did not stimulate DNA synthesis in quiescent Swiss 3T3 cells (Fig. 2A). In contrast, a recombinant platelet-derived growth factor of a B chain homodimer (PDGF c-*sis*), (Amersham, Buckinghamshire), stimulated DNA synthesis in the concentration range from 3.3×10^{-12} to 3.3×10^{-10} *M* in a dose-dependent manner. When endothelin and PDGF c-*sis* were added simultaneously, marked potentiation of DNA synthesis was observed. This synergistic effect of endothelin in stimulating DNA synthesis was elicited at endothelin concentrations of 1.0×10^{-10} *M* or greater; endothelin at 1.0×10^{-8} *M* elicited about a 4-fold potentiation of DNA synthesis compared with PDGF c-*sis* alone. DNA synthesis stimulated by recombinant basic fibroblast growth factor (bFGF) (Amersham) or insulin (Sigma Chemical Co., St. Louis, MO) was also potentiated by addition of endothelin (Fig. 2B and 2C). The EC_{50} of endothelin (concentration required for the half-maximal effect) for potentiating growth factor-

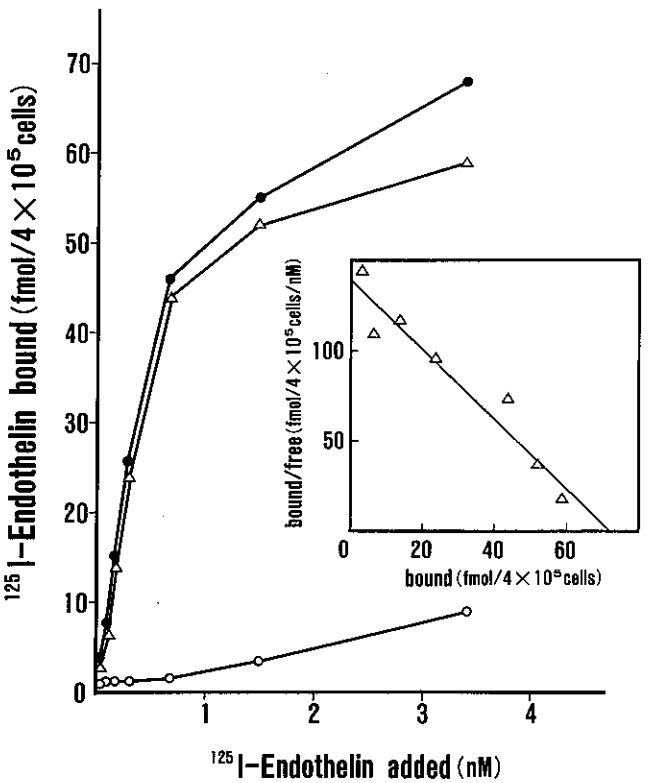

Fig. 1. Specific binding of radiolabeled endothelin to Swiss 3T3 cells and Scatchard plot analysis (inset). Synthetic endothelin (Peptide Institute Inc., Osaka) was iodinated by the chloramine T method[17] using Na[^{125}I] (New England Nuclear, Boston, MA) and used as the labeled antigen. The specific activity was 122–162 Ci/mmol. Approximately 2.0×10^5 cells were seeded into 35-mm plates and were arrested in the quiescent phase of the cell cycle by the method reported by Rozengurt *et al.*[18] After being washed, these cells were incubated with various concentrations of radiolabeled endothelin at 37°C for 60 min. They were then washed 3 times with 1 ml of ice-cold Hanks' balanced salt solution (HBSS) and solubilized with 1 *N* NaOH, and then the cell-bound radioactivity was measured. Specific binding (△) was determined in the presence (○) or absence (●) of a 100-fold excess of unlabeled endothelin.

induced DNA synthesis in Swiss 3T3 cells ranged from 1.0×10^{-10} *M* to 1.5×10^{-9} *M* (Fig. 2), which is about 20–300 times lower than that for stimulating DNA synthesis in vascular smooth muscle cells.[5] It is also worth noting that the EC_{50} of endothelin for potentiating DNA synthesis Swiss 3T3 cells is almost the same as the EC_{50} reported previously for endothelin-induced vasoconstriction.[3] These results suggest that in Swiss 3T3 cells the ability of endothelin to potentiate growth factor-induced DNA synthesis is physiologically significant, like the ability to cause vasoconstriction in smooth muscle cells.

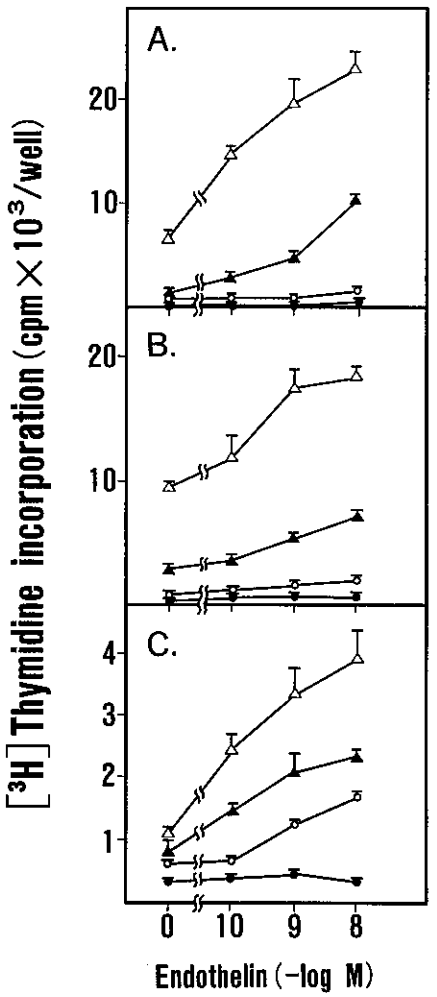

Fig. 2. Potentiation by endothelin of DNA synthesis induced by PDGF c-*sis* (A), bFGF (B) and insulin (C) in Swiss 3T3 cells. Cultured cells were seeded into 96-well multi-plates and quiescent cells were obtained by the method described by Rozengurt *et al.*[18] Quiescent cells were washed twice with Dulbecco's modified Eagle's medium containing 0.1% BSA and then incubated at 37°C in the presence of the test materials. Twenty hours later, 20 μl of [methyl-^{3}H]thymidine solution (2 μCi/ml) was added and after 4 h the cultures were trypsinized and harvested on filter paper with a semiautomatic cell harvester (Skatron, Lier, Norway). Radiolabeled thymidine incorporation into acid-insoluble fractions of DNA was determined by liquid scintillation counting. Data points are mean ± SE (bars) of triplicate determinations. (A) Synergistic effect of endothelin and PDGF c-*sis*. The concentrations of endothelin are shown in this figure. The concentrations of PDGF c-*sis* were as follows: ●, none; ○, 3.3×10^{-12} *M*; ▲, 3.3×10^{-11} *M*; △, 3.3×10^{-10} *M*. (B) Synergistic effect of endothelin and bFGF. The concentrations of bFGF were as follows: ●, none; ○, 6.3×10^{-12} *M*; ▲, 6.3×10^{-11} *M*; △, 6.3×10^{-10} *M*. (C) Synergistic effect of endothelin and insulin. The concentrations of insulin were as follows: ●, none; ○, 1.7×10^{-8} *M*; ▲, 1.7×10^{-7} *M*; △, 1.7×10^{-6} *M*.

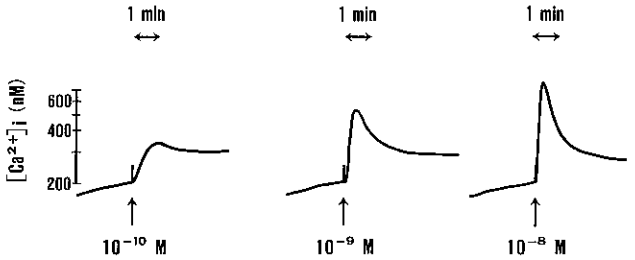

Fig. 3. Effect of endothelin on intracellular Ca^{2+} concentration in Swiss 3T3 cells. Quiescent Swiss 3T3 cells obtained by deprivation of serum in a 75-cm^2 flask were incubated at 37°C with Fura-2 tetraacetoxymethylester (Molecular Probes, Inc., Eugene, OR) at the concentration of 3 μM for 30 min. Then the cells were trypsinized, washed once with HBSS containing 10% calf serum and then washed twice with HBSS containing 20 mM HEPES, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Cells were suspended, transferred to a 0.3 cm^2 cuvette in the fluorometer and stirred continuously at 37°C. After a 5-min control period, endothelin at the indicated concentrations was added. Fluorescence was monitored by a luminescence spectrometer (CAF-100, Japan Spectroscopic Co. Ltd., Tokyo) with excitation at 340 and 380 nm and emission at 500 nm. The maximal and minimal fluorescence values were obtained by sequential addition of 0.1% Triton X-100 and 3 mM EGTA, respectively, and intracellular Ca^{2+} concentration was calculated as described by Grynkiewicz *et al.*[19] The tracings presented are typical of those obtained in 8 studies. $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

In contrast, endothelin did not potentiate growth factor-stimulated DNA synthesis in NIH 3T3 cells; these cells possess no endothelin receptors.

Increase in the intracellular level of calcium ion (Ca^{2+}) is known to be one component of the signal transduction pathway by which growth factors elicit their effects.[14] In activation of phosphoinositide turnover, an increase in intracellular Ca^{2+} as well as activation of C-kinase is observed.[15] Some growth factors increase Ca^{2+} influx through Ca^{2+} channels.[16] The present study revealed that the addition of endothelin alone at concentrations of 1.0×10^{-10} M or greater induced a significant increase in intracellular Ca^{2+} level in a dose-dependent manner (Fig. 3); also, the effective dose of endothelin for elevating intracellular Ca^{2+} is almost the same as that for potentiation of growth factor-induced DNA synthesis. Endothelin alone does not elicit the stimulation of DNA synthesis in Swiss 3T3 cells under the present experimental conditions. Therefore, it is possible to speculate that endothelin induces an increase in the intracellular Ca^{2+} level which in turn potentiates the effects of growth factors on DNA synthesis by modulating the signal transduction pathways. In the field of growth factor research, the unique mode of action of endothelin in potentiating growth factor-induced DNA synthesis in Swiss 3T3 cells may provide new insight into the role of bioactive peptides in the mechanism of cellular growth.

The authors thank the Japanese Cancer Research Resources Bank (Tokyo) for providing the cell lines. They also thank Ms. K. Otsubo and Ms. M. Ebinuma for their excellent technical assistance. This work 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 for Cancer Control, by Grants-in-Aid for Cancer Research (60S, 61-1, 61-8, 62S-1, 62-26) and by a Grant-in-Aid from the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

(Received December 23, 1988/Accepted February 20, 1989)

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