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Genetic transfer of endothelin converting enzyme activity to CHO-K1 cells: detection of positive cells by reverse hemolytic plaque assay

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Abstract We have established a novel method of molecular cloning of endothelin converting enzyme, a key enzyme in the production of a potent vasoconstrictor endothelin-1, by modification of the reverse hemolytic plaque assay. Also, we demonstrated that a cell line, CHO-K1, showed no detectable activity of endothelin converting enzyme. This cell line was transfected with a cDNA library of bovine endothelial cells. The modified reverse hemolytic plaque assay was shown to detect even a single CHO-K1 cell that was changed to produce mature ET-1 by transfection. Thus, this novel method is suggested to be useful for the molecular cloning of other secreted antigens and their processing enzyme.

Key words: Endothelin-1; Endothelin converting enzyme; Reverse hemolytic plaque assay

1. Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictive 21-amino-acid peptide produced by vascular endothelial cells. Endothelin converting enzyme (ECE) is a key enzyme in the synthesis of ET-1. It converts an intermediate form of endothelin-1, big endothelin-1 (big ET-1), to the mature form [1], by cleaving the Trp^{21}–Val^{22} bond of big ET-1. This substrate specificity of ECE seems to be unique for this enzyme. The conversion is essential in the pressor response to big ET-1, because the vasocontractile activity of ET-1 is higher than that of big ET-1 by two orders of magnitude [3], and phosphoramidon, an inhibitor of ECE [4–7], blocked the vasocontractile activity of big ET-1. This result also suggests an involvement of ECE in the regulation of vascular tonus. Involvement of ET-1 in the pressor response is suggested by the fact that an antagonist of the ET receptor effectively decreases the mean arterial pressure of sodium-depleted squirrel monkeys [8]. Accordingly, an expectation that the inhibitors of ECE block the production of ET-1 and subsequently depress the ET-dependent regulation of vascular tonus has prompted us to investigate the properties of ECE. The determination of the structure of ECE is an inevitable step for the elucidation of the role of ECE.

To elucidate this mechanism, isolation of the cDNA clone for ECE is an essential step. We devised a novel method of detection of a single cell that produces mature ET-1. We found also that CHO-K1 cells can produce big ET-1, but not mature ET-1, because of the lack of phosphoramidon-sensitive ECE activity. Then, the CHO-K1 cells were transfected with the cDNA library of endothelial cells. We successfully detected a transfected single CHO-K1 cell that produced mature ET-1 by this new method.

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Abbreviations: ET-1, endothelin-1; big ET-1, big endothelin-1; ECE, endothelin converting enzyme.

2. Materials and methods

2.1. Cell culture

Bovine aortic endothelial cells (BAE) were harvested by gently scraping the inner wall of fresh thoracic aortas with a cover glass. The isolated BAE were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui) supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO_2. The CHO-K1 cells were maintained in Ham's F12 medium (Nissui), supplemented with 10% fetal calf serum (FCS) at 37°C under the same conditions as described above.

2.2. Enzyme immunoassay

ET-1 and big ET-1 were measured by the sandwich enzyme immunoassay as described previously [9,10].

2.3. Measurement of ECE activity

A confluent culture of BAE or CHO-K1 in 10-cm dishes was washed twice with phosphate-buffered saline. Cells were harvested with a rubber scraper in 5 ml of 10 mM Tris-HCl (pH 7.7). Then, the cells were homogenized by a polytron homogenizer at the maximum speed. The membrane fraction was prepared and solubilized in 100 μl of 10 mM Tris-HCl (pH 7.7), 0.1% (w/v) Lubrol PX as described previously [11]. One μl of solubilized membrane preparation was incubated with 50 pmol of big ET-1 in 100 μl of buffer solution containing 0.1 M Tris-HCl (pH 7.0) with or without 0.1 mM phosphoramidon for 1 h at 37°C. Enzyme reactions were stopped by the addition of 100 μl of 0.1 M EDTA. Mature ET-1 generated was measured by enzyme immunoassay [9].

2.4. DEAE dextran transfection

Subconfluent monolayers of CHO-K1 grown in 6-well culture dishes were transfected with plasmid by a DEAE-dextran-transfection method with chloroquine treatment [12]. Two days after the transfection, the culture media were collected for measurement of concentration of ET-1 and big ET-1, and the cells were dispersed by treatment with 0.25% trypsin, and used for hemolytic assay as described below.

2.5. Preparation of red blood cells coupled with protein A

Human red blood cells (RBCs) were coupled with staphylococcal protein A (Pearce) using $CrCl_3$ as described previously [13,14]. Briefly, 0.5 mg of protein A and 1 mg of $CrCl_3 \cdot 6H_2O$ were mixed in 11 ml of saline. Then, the mixture was added with 1 ml packed volume of RBCs prewashed with saline. The RBSs were incubated at 30°C for 1 h, and then washed once with 5 ml of saline and twice with 5 ml of Ham's F12/0.3% BSA. The RBCs were suspended in 10 ml of Ham's F12 containing 0.3% BSA.

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2.6. Reverse hemolytic plaque assay

Before the following assay, the cells were stained, if necessary, with 5 μg/ml of FAST DiI (Molecular Probe) according to the manufacturer's instructions. The cells were mixed with the RBCs coupled with protein A in Ham's F12/0.3% BSA containing 1 mg/ml of ascorbic acid, and seeded on 10 cm dishes precoated with 15 μg/ml of poly-L-lysine (M_r 150,000–300,000). The cells were incubated for 45 min at 37°C, and then washed with 5 ml of Ham's F12/0.3% BSA. Then, the culture media were changed to the media with anti ET-1 monoclonal antibody (final concentration of 5 μg/ml), guinea pig serum (final 1 : 100 dilution, Gibco) as a source of complement and ascorbic acid (1 mg/ml). The cells were further incubated at 37°C for 8 h, and observed under a microscope. We recognized a positive cell that was located in a hemolytic plaque.

2.7. Introduction of a stop codon for the production of mature ET-1 (ET-stop)

The *Eco*RI fragment of human ppET-1 cDNA was subcloned into a mammalian expression vector pME18sf– after digestion with *Bst*XI and blunting. Using the primer (5'-GGA CAT CAT TTG GTG AAA CAC TCC CGA G-3'), mutagenesis was carried out by Kunkel's method [15] to substitute codon GTC coding Val^{22} of big ET-1 with TGA coding with stop codon. Sequence of the mutant was confirmed by sequencing.

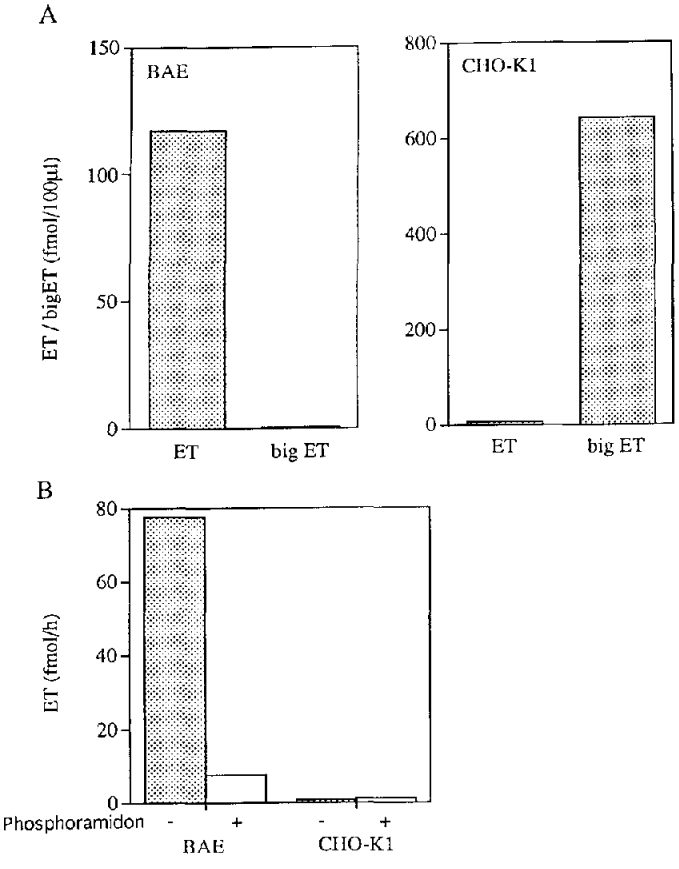

Fig. 1. ECE activities in BAE and CHO-K1 cells. (A) Secretion of ET-1 or big ET-1 from BAE and CHO-K1. Both cells were grown until they become confluent and cultured for 2 days. Ir-ET-1 and ir-big ET-1 in conditioned medium of BAE and CHO/big were measured by a sandwich enzyme immunoassay (see text). (B) Effect of phosphoramidon on ECE activity in BAE and CHO-K1 cell. One μl of membrane fraction from each cell, which was dissolved in 10 mM Tris-HCl (pH 7.4) containing 0.1% (v/v) Lubrol PX, was incubated with 50 pmol human big ET-1 with or without 0.1 mM phosphoramidon for 1 h. The generated mature ET-1 was measured by the sandwich enzyme immunoassay.

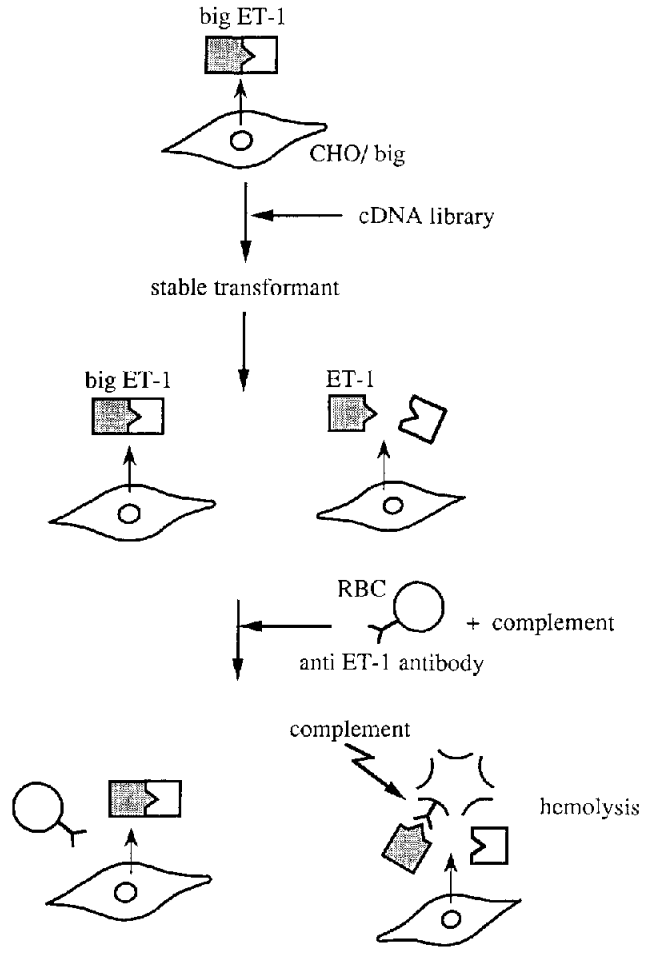

Fig. 2. Schematic representation of the method for screening of a cDNA library by the reverse hemolytic plaque assay (see text).

2.8. Development of a stable transformant that expresses ppET-1 (CHO/big)

Thirty % confulent CHO-K1 cells were transfected by the calcium-phosphate transfection method [16] with 1 μg of the plasmid of the ppET-1 cDNA subcloned in pME18sf– as described. Stable transformants were selected in Ham's F12/10% FCS containing 1 mg/ml of G418 (Gibco).

2.9. Screening of cDNA library

A BAE cDNA library composed of 7×10^5 independent clones was prepared with the pME18sf– vector (kind gift from Dr. K. Maruyama). Ten μg of amplified cDNA library and 0.2 μg of pSV2bsr were co-transfected to CHO/big by the calcium-phosphate transfection method [16]. Stable transformants were selected in Ham's F12/10% FCS containing 2 μg/ml of blasticidin S (Kaken Pharmaceutical Co.). Colonies were dispersed by 0.25% trypsin and used for the hemolytic assay as described above.

3. Results and discussion

3.1. CHO-K1 cells lack ECE activity

We have elaborated the expression cloning strategy for direct isolation of a complementary DNA for ECE. For this strategy, a key point is to find a cell that lacks ECE activity. We tested several cell lines for whether they are capable of producing mature ET-1 or not. ppET-1 cDNA was introduced into various cell lines and the concentrations of ET-1 secreted in the

culture supernatant were determined. All the tested cell lines except CHO-K1 could produce mature ET-1 even if the cell lines produced neither ET-1 nor big ET-1 before the transfection. The CHO-K1 cells, after the transfection with ppET-1 cDNA, did not secrete mature ET-1 but secreted exclusively big ET-1 (Fig. 1A).

Moreover, we could not detect phosphoramidon-sensitive ECE activity in membrane fractions of CHO-K1 homogenates, as detected in BAE (Fig. 1B). These two results indicate that CHO-K1 cells lack ECE.

3.2. Reverse hemolytic plaque assay

For the expression cloning method, sensitivity of the method for detection of the objective clone is important. Accordingly, we developed a sensitive detection method using the reverse hemolytic plaque assay. This assay is based on activation of complements mediated by an immune complex. In this assay, we used anti-ET-1 antibody that selectively recognized mature ET-1 but not big ET-1. Fig. 2 shows the schema of the expression cloning method of ECE using the reverse hemolytic assay. CHO/big was transfected by a BAE cDNA library, and the stable transformants were selected. The stable transformant cells were cultured in the presence of RBCs conjugated with protein A, anti ET-1 antibody and complement. Hemolysis was observed around the transformants that produced ET-1 in the presence of complement activated by the antibody–antigen complex.

To examine whether cells producing mature ET-1 were detected by this hemolytic assay, CHO-K1 cells were transfected with the ET-1 cDNA construct, in which the codon coding Val^{22} was replaced with stop codon. These cells produced mature ET-1 (data not shown). We could successfully detect ET-1-producing CHO-K1 cells by the hemolytic assay (Fig. 3A). On the other hand, the CHO/big cells did not form hemolytic plaques (Fig. 3B). Then, we examined whether we could discriminate ET-1-producing cells from ET-1-non-producing cells in the mixed culture of these cells. We performed the hemolytic assay with the mature ET-1-producing BAE cells and the non-producing CHO-K1 cells. As shown in Fig. 3C,D, plaques were formed around BAE but not around CHO-K1 cells. The latter CHO-K1 cell was prestained with DiI. This result indicates that discrimination of BAE from CHO-K1 by this method was easy.

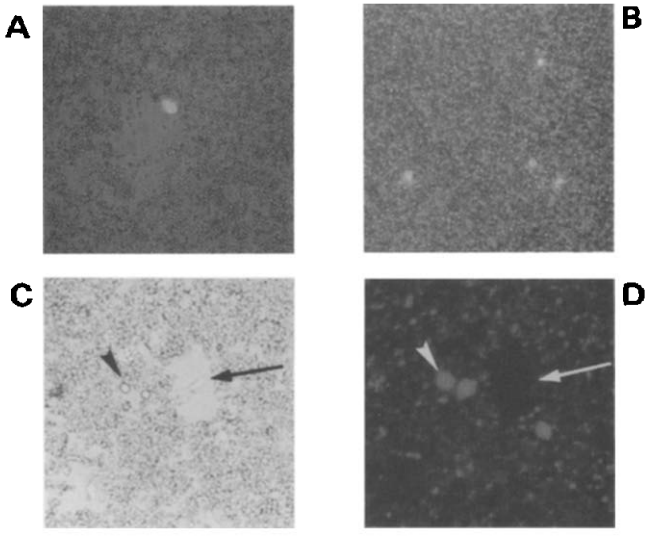

Fig. 3. Detection of single ET-1-producing cells by reverse hemolytic plaque assay. (A) Detection of a transfected CHO-K1 that produces mature ET-1. Plaques were formed around the CHO-K1 cells transfected with ET-stop (see text). (B) No plaque formation was observed around the CHO/big cells. The CHO/big cells were stained in advance with a fluorescent dye, DiI. (C,D) Detection of BAE in the mixed culture of BAE and CHO/big cells. Plaques were formed only around BAE (C, arrow) but not around CHO/big cells visualized with DiI (D, arrowhead).

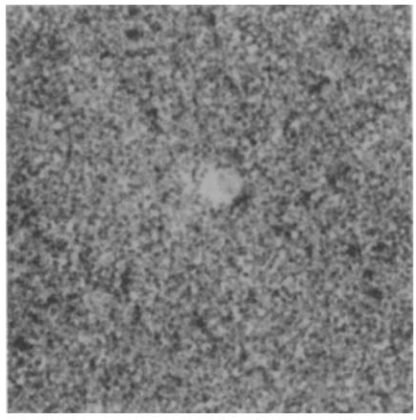

Fig. 4. Typical plaque formed around CHO/big cells after transfection with the BAE cDNA library.

3.3. Genetic transfer of ECE activity

To isolate a cDNA for ECE, we used a BAE cDNA library composed of 7×10^5 independent clones for the transfection. We carried out a hemolytic assay of the stable transformants transfected with the BAE cDNA library. We found several plaques formed around a single cell (Fig. 4). This fact implies that ECE activity was genetically transferred to CHO/big cells.

Despite numerous reports on the molecular cloning of the membrane bound antigens by the 'panning' method [12,17], no effective strategy for the cloning of a secreted antigen has been described. In the present study, we have developed an expression cloning system for the molecular cloning of secreted antigens and the enzymes that produce the secreted antigens. This novel system based on a reverse hemolytic plaque assay has the following advantages for the molecular cloning of the secreted proteins. First, identification of positive cells in mixed culture is easy under the light microscope. Secondly, the identified cells can subsequently be kept growing, since this assay is carried out on living cells. Accordingly, the resulting colony formed can be easily picked up. Our method could be applied to the cloning of other secreted antigens and their processing enzymes.

References

[1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 332, 411–415.
[2] Sawamura, T., Kumura, S., Shinmi, O., Sugita, Y., Yanagisawa, M. and Masaki, T. (1989) Biochem. Biophys. Res. Commun. 162, 1287–1294.
[3] Kimura, S., Kasuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1989) J. Cardiovasc. Pharmacol. 13 (Suppl. 5), S5–S7.

[4] Matsumura, Y., Hisaki, K., Takaoka, M. and Morimoto, S. (1990 Eur. J. Pharmacol. 185, 103–106.
[5] Fukuroda, T., Noguchi, K., Tsuchida, S., Nishikibe, M., Ikemoto, F., Okada, K. and Yano, M. (1990) Biochem. Biophys. Res. Commun. 172, 390–395.
[6] Ikegawa, R., Matsumura, Y., Tsukahara, Y., Takaoka, M. and Morimoto, S. (1990) Biochem. Biophys. Res. Commun. 171, 669–675.
[7] Sawamura, T., Kasuya, Y., Matsushita, Y., Suzuki, N., Shinmi, O., Kishi, N., Sugita, Y., Yanagisawa, M., Goto, K., Masaki, T. and Kimura, S. (1991) Biochem. Biophys. Res. Commun. 174, 779–784.
[8] Clozel, M., Breu, V., Burri, K., Cassal, J.M., Fischli, W., Gray, G.A., Hirth, G., Loeffler, B.M., Mueller, M., Neidhard, W. and Ramuz, H. (1993) Nature 365, 759–761.
[9] Suzuki, N., Matsumoto, H., Kitada, C., Masaki, T. and Fujino, M. (1989) J. Immunol. Methods 118, 245–250.
[10] Suzuki, N., Matsumoto, H., Kitada, C., Kimura, S., Miyauchi, T. and Fujino, M. (1990) J. Immunol. Methods 127, 165–170.
[11] Sawamura, T., Shinmi, O., Kishi, N., Sugita, Y., Yanagisawa, M., Goto, K., Masaki, T. and Kimura, S. (1993) Biochim. Biophys. Acta 1161, 295–302.
[12] Seed, B. and Aruffo, A. (1987) Proc. Natl. Acad. Sci. USA 84, 3365–3369.
[13] Neill, J.D. and Frawley, L.S. (1983) Endocrinology 112, 1135–1137.
[14] Smith, P.F., Frawley, L.S. and Neil, J.D. (1984) Endocrinology 115, 2484–2486.
[15] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[16] Sambrook, J., Maniatis, T. and Fritsch, E.F. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.
[17] Aruffo, A. and Seed, B. (1987) Proc. Natl. Acad. Sci. USA 84, 8573–8577.