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Synthesis of the vasoconstrictor peptide endothelin in kidney cells

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The expression plasmid containing human prepro-endothelin cDNA was constructed and introduced into COS-7 cells. Mature endothelin, consisting of 21 amino acid residues, was secreted into the culture medium of the transfected cells and was also synthesized by non-transfected COS-7 cells. Normal kidney cells derived from other species also synthesized and secreted endothelins. Partial characterization of endothelins produced by kidney cells suggested the existence of new types of endothelin. This is the first report of the vasoconstrictor peptide endothelin being synthesized in kidney cells.

Preproendothelin; Gene expression; (COS-7 cell, Kidney cell, Human)

1. INTRODUCTION

The vasoconstrictor peptide endothelin has been obtained from the culture medium of porcine aortic endothelial cells and its structure, the nucleotide sequence of the cloned cDNA, determined [1]. We have also succeeded in cloning the human prepro-endothelin cDNA, and identified the amino acid sequences of human and porcine endothelin [2]. Recently, the amino acid sequences of two other types of human endothelin were deduced by sequencing of the cloned human genomic DNAs and termed endothelin 2 and 3 (ET-2 and -3) [3]. However, these types of endothelin have not yet been detected in any cells, tissues, or organs. Also, the precise mechanisms of processing endothelin from prepro-endothelin, consisting of 212 amino acids, remain unknown. We thus attempted to express the cDNA encoding the human prepro-endothelin in mammalian cells in order to determine the processing steps, and found that matured, processed endothelin 1 (ET-1) was secreted in the culture medium of COS-7 cells. We also observed that normal kidney cells from other species synthesized and secreted endothelins. We report here on the synthesis and secretion of endothelin by kidney cells and their partial characterization.

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Abbreviation: ET-1 (2,3), endothelin 1 (2,3)

2. MATERIALS AND METHODS

2.1. *Materials*

DEAE-Dextran (M_r 500000, n = 0.5) was obtained from Pharmacia; Sep-Pak C18 cartridge from Waters Associates (MA); Dulbecco's and Earle's minimum essential medium (DMEM, EMEM) from Flow Labs, fetal calf serum (FCS) from Microbiological Co.; restriction enzymes from Takara Shuzo Co.; and T_4 DNA ligase and *Bgl*II linkers from New England Biolabs.

2.2. *Cells and cell culture*

COS-7 cells and other kidney cell lines were obtained from the American Tissue Culture Collection (ATCC) and cultured in DMEM or EMEM containing 10% FCS, glutamine (0.3 mg/ml), penicillin (50 U/ml), and streptomycin (0.05 mg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37°C.

2.3. *Construction of expression plasmid and cell transformation*

DNA modification, ligation and transformation were carried out as described by Maniatis et al. [4]. The plasmid with human endothelin cDNA, pHET 4-3, and expression plasmid for animal cells, pTB551, were used.

Plasmid pHET 4-3 was digested with the restriction enzyme *Eco*RI and a 1.17 kb cDNA fragment was obtained. After T_4

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DNA polymerase reaction to fill the single-stranded portion, the *BglII* linker d(GAAGATCTTC) was ligated and the *BglII* fragment containing the coding region of prepro-endothelin was inserted into the *BglII* sites of pTB551 to obtain pTS6003 as shown in fig.1. DNA transfection was performed with DEAE-dextran as in [5].

2.4. *High-performance liquid chromatography (HPLC) of the culture medium*

The culture medium of COS-7 cells was recovered 5 days after confluence, acidified with acetic acid to a final concentration of 3% and concentrated using the Sep-Pak C18 cartridge as described [6]. The eluted material in 4 ml acetic acid/ethanol/H_2O (4:86:10) was dried under a stream of N_2 and resolved with 250 μl of 60% acetonitrile containing 0.05% trifluoroacetic acid (TFA). The preparation was then applied to a TSK ODS-80 column (4.6 × 250 mm, Tosoh, Japan). During elution, the concentration of acetonitrile containing 0.05% TFA was elevated linearly from 0 to 27% over 5 min, from 27 to 43.5% over 20 min, and finally from 43.5 to 60% over 5 min at a flow rate of 1 ml/min. Each separated preparation was lyophilized and assayed for endothelin by sandwich-EIA.

2.5. *Enzyme immunoassay (EIA)*

The culture media of the kidney cells and the preparations separated by HPLC were assayed for endothelin as described elsewhere [6].

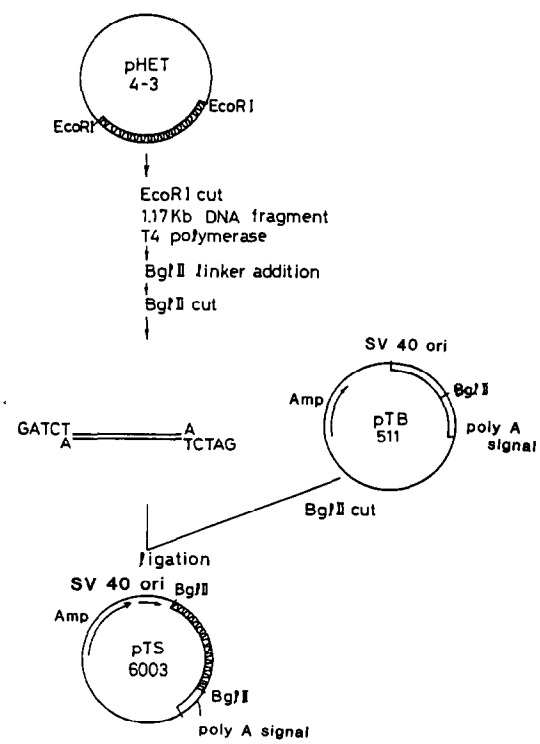

Fig.1. Construction of human endothelin expression plasmid of animal cells. The *BglII* fragment coding for human prepro-endothelin was inserted into the *BglII* sites of pTB551 to obtain pTS6003.

3. RESULTS AND DISCUSSION

3.1. *Synthesis and secretion of endothelin by COS-7 cells*

The sensitivity of the EIA that we developed for endothelin is very high; for example, 2 pg/ml of endothelin can be detected in this [1-21] assay system. Thus, this method is useful for detecting endothelin without the need to concentrate preparations. With this assay system, we confirmed that COS-7 cells transfected with the expression plasmid containing human prepro-endothelin cDNA could synthesize and secrete mature endothelin in the culture medium at about 650 pg/ml after 96 h culture (to appear elsewhere).

These transfection experiments showed that non-transfected COS-7 cells secreted immunoreactive endothelin into the culture medium. The fact that endothelin, which was first found in the culture medium of endothelial cells of the vein, was also

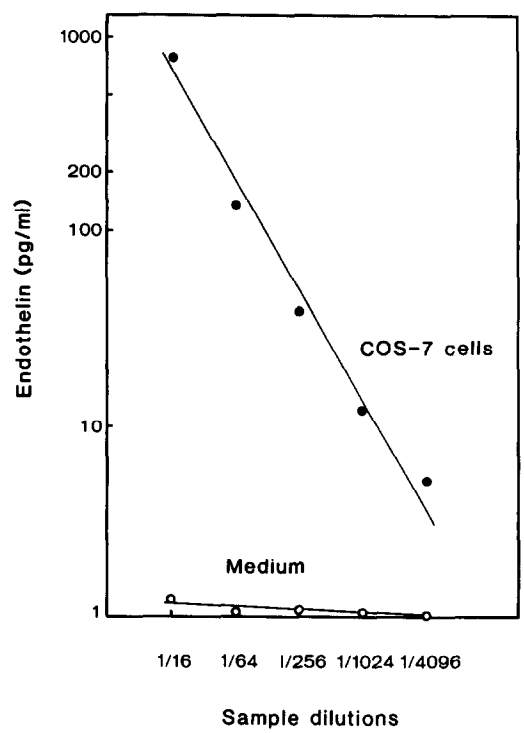

Fig.2. Titration of endothelin in concentrated culture medium of COS-7 cells. Culture medium (10 ml obtained 96 h after the confluent state of COS-7 cells) was concentrated to 0.3 ml with Sep-Pak and diluted with buffer for sandwich EIA and assayed. Values are means (n = 2). (●—●) Cultured medium of COS-7 cells, (○—○) medium.

synthesized in the kidney cells suggests that there may be other endothelin-producing organs or tissues. To characterize the immunoreactive endothelins produced by non-transfected COS-7 cells, the culture media from both plasmid pTS6003-transfected and non-transfected COS-7 cells were subjected to adsorption experiments with anti-whole endothelin antibodies (AwETN40 antibody) coupled with Sepharose 4B. The immunoreactive endothelins were completely adsorbed on beads coated with AwETN40 antibodies (not shown). These results suggested that the immunoreactive endothelin produced by COS-7 cells had the same antigenic determinant as the expression product of plasmid pTS6003. To confirm further the secretion of immunoreactive endothelin from COS-7 cells and to eliminate possible contamination of endothelin from the FCS used in cell culture, the culture medium of COS-7 cells and medium containing 10% FCS were concentrated with Sep-Pak, diluted 1/4 serially and assayed. As shown in fig.2, immunoreactive endothelin was clearly detected in the concentrated culture medium of COS-7 cells and the contents decreased with sample dilution. No immunoreactive endothelin was detected in the medium for the COS-7 cells. This result suggested that non-transfected COS-7 cells secreted the endothelin in the cultured medium.

3.2. *Synthesis of immunoreactive endothelin in other kidney cells*

As the COS-7 cell is one of the SV40 transformed cells, we examined other normal kidney cells to determine whether they synthesize endothelin. Some kidney cell lines from monkey, dog, rabbit, rat and hamster were cultured in Falcon dishes with EMEM or DMEM and culture media from these cells were used for EIA of endothelin. As expected, and shown in table 1, monkey kidney CV-1 cells, which are the original normal kidney cells from African green monkey, and Vero cells synthesized immunoreactive endothelin. MDCK, RK-13, NRK-52 and BHK-21 cells also synthesized immunoreactive endothelin. The quantities of endothelin produced in these kidney cell lines were about 1/100–1/10th of that of porcine aortic endothelial cells (PAE) used as a control cell line, however the endothelin was clearly produced not only in endothelial cells but also in kidney cells. Support for these results came from the finding that primary cultured cells of rat kidney also secreted immunoreactive endothelin (not shown). We have also detected endothelins in tissue extracts of kidney, lung and brain of rat (to be published).

Table 1

Synthesis of immunoreactive endothelin by cultured kidney cell lines

Cell lines	Total amount of endothelin (pg)	pg endothelin produced/10^6 cells per 2 days
Monkey		
COS-7	4860 ± 620[b]	1310 ± 150
CV-1	5100 ± 690	2460 ± 440
Vero	625 ± 40	554 ± 25
Dog		
MDCK	3560 ± 930	1115 ± 180
Rabbit		
RK-13	1440[c]	240
Rat		
NRK-52E	1580	530
Hamster		
BHK-21	540	110
Pig AE[a]	17550	14630

[a] Porcine aortic endothelial cells prepared by the method of Neich et al. [10]
[b] Mean ± SD (n = 3)
[c] Mean (n = 2)
Kidney cells from monkey, dog, rat, rabbit, and hamster were cultured in 5 ml DMEM or EMEM supplemented with 10% FCS. 2 days after reaching confluence for each cell line, culture media were harvested and used for EIA of endothelin. Cell numbers were counted with a hemocytometer

3.3. *Properties of immunoreactive endothelin produced by COS-7 cells*

To characterize the immunoreactive endothelin produced by COS-7 cells, culture medium was concentrated and subjected to HPLC as described above. The control, chemically synthesized endothelins ET-1 and ET-2 were also subjected to HPLC and their elution profiles compared. As shown in fig.3, immunoreactive endothelin eluted in fractions 23–24 and 25–26. As the capacity of HPLC for separating small peptides such as endothelin is believed to be adequate, this broad pattern suggested the existence of other immunoreactive materials without mature endothelin. When

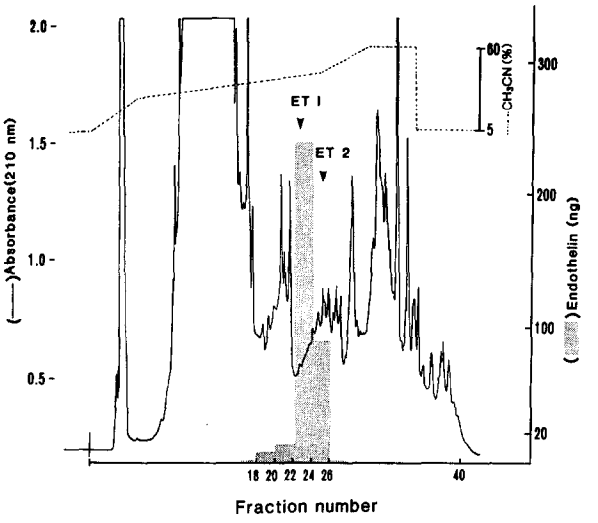

Fig.3. HPLC elution profile of immunoreactive endothelin from concentrated culture medium of COS-7 cells. Culture medium of COS-7 cells (1.3 l) was concentrated as described in Section 2 and 1.5 ml of the resolved preparation in 60% CH_3CN containing 0.05% TFA was applied to a TSK DS-80 column, eluted and then dried. A 1/40 volume from each fraction was used for EIA of endothelin. The immunoreactive endothelin contents were calculated as the original volume.

the material eluting in fractions 23–24 was rechromatographed on a TSK ODS-80 TM column with 34% CH_3CN containing 0.05% TFA, two main peaks appeared at fractions 3 and 6, respectively (fig.4A). Fractions 25–26 in fig.3 were rechromatographed under the above conditions, resulting in the major peak appearing in fraction 6 (fig.4B) which was the same as the second peak that appeared on rechromatography in fig.4A. The peak fraction, nos 23–24 in fig.3, coincided with the position of chemically synthesized ET-1, however, immunoreactive endothelin in fractions 25–26 in fig.3 and fraction 6 in fig.4B were completely different from ET-1, although close to the position of ET-2. These experimental results suggested that COS-7 cells synthesized the vasoconstrictor peptide endothelin and secreted it into the culture medium. The mature form of the polypeptide comprised 21 amino acid residues. Another immunoreactive endothelin which might be of a different type was recently reported by others [3]. These results suggest that endothelin, which was first observed in endothelial cells, might be produced in other organs such as kidney or lung. We

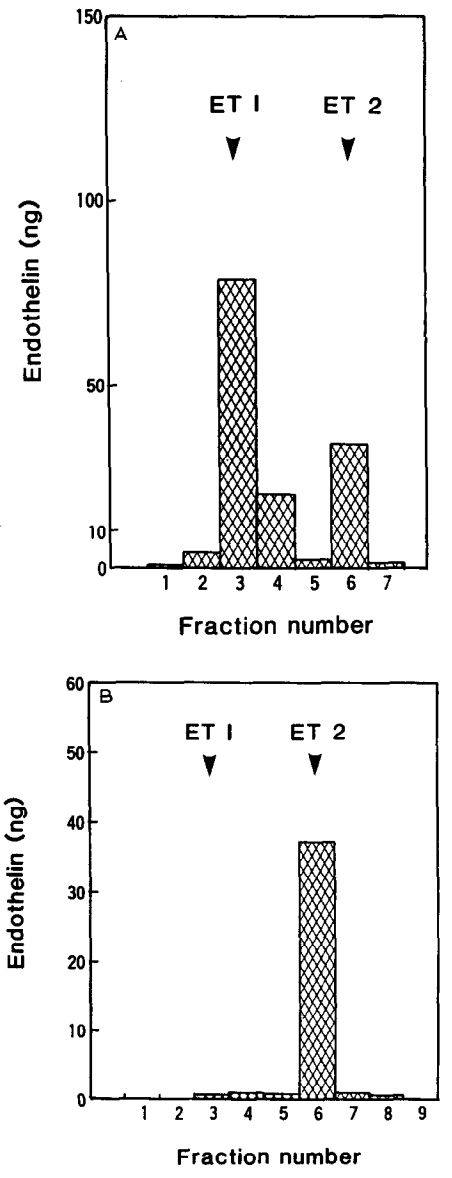

Fig.4. Second HPLC elution profiles of the immunoreactive endothelin. The preparations in fractions 23–24 and 25–26 in fig.3 were rechromatographed on HPLC and eluted with non-gradient solvent (34% CH_3CN containing 0.05% TFA), dried and assayed. (A) Rechromatographed fractions 23–24, (B) fractions 25–26.

have already found that immunoreactive endothelin was produced in primary cultured cells from rat lung. Endothelin has also been reported to inhibit the release of renin from juxtaglomerular cells in the kidney [8] and to induce contraction of the

trachea in guinea pig lung [9]. We therefore conclude that endothelin seems to play important physiological roles locally in the kidney, lung, and other organs with vasoconstrictor and other activities.

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