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# ENDOTHELIN IN HUMAN PLASMA AND CULTURE MEDIUM OF AORTIC ENDOTHELIAL CELLS —— DETECTION AND CHARACTERIZATION WITH RADIOIMMUNOASSAY USING MONOCLONAL ANTIBODY

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**SUMMARY:** We have developed monoclonal (KY-ET-1-I) and polyclonal (ET-F5) antibodies against endothelin-1 (ET-1) and established sensitive radioimmunoassays (RIAs) with different specificities. The RIA with KY-ET-1-1 detected ET-1, ET-2 and ET-3, while the RIA with ET-F5 recognized ET-3 very weakly. Using these RIAs, we have investigated the concentration and molecular forms of ET-1-like immunoreactivity (-LI) in culture medium of bovine aortic endothelial cells and human plasma. Culture medium of endothelial cells contained two major components compatible with big ET and ET-1. ET-1-LI was also detected in human plasma. ET-1-LI in human plasma consisted of apparent two components, the small molecular form emerging at the position of ET-1 and the large form with the peak eluting at the preceding fraction of the elution position of big ET. The concentration of the small form of ET in human plasma was about 5 pg/ml. 

Endothelin (ET) is a novel 21-amino acid peptide purified from culture medium of porcine aortic endothelial cells (1). Preproendothelin mRNA is expressed in not only cultured endothelial cells but porcine aortic intima in vivo. The intravenous administration of ET induces the sustained elevation of arterial pressure with initial hypotension in anesthetized rats. It has been also reported that endothelin is a potent coronary vasoconstrictor in vivo. These observations suggest that endothelin is involved in the control of cardiovascular homeostasis. Recently, Yanagisawa et al. reported the presence of three endothelin-related genes in the human genome, and three products were designated ET-1, ET-2 and ET-3 (1).

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Abbreviations: ET, endothelin; RIA, radioimmunoassay; -LI, -like immunoreactivity; GPC, gel permeation chromatography.

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In the present study, we have developed monoclonal and polyclonal antibodies against ET-1 and established two kinds of radioimmunoassay (RIA) for ET; one detects ET-1, ET-2 and ET-3, and the other recognizes ET-3 very weakly. Using these RIAs, we have investigated the concentration and molecular forms of ET-1-like immunoreactivity (-LI) in human plasma and in culture medium of bovine aortic endothelial cells.

## MATERIALS AND METHODS

Peptide: ET-1, ET-2 and ET-3 were purchased from Peptide Institute Inc. (Minoh, Japan). Human big ET (human ET[53-90]) and a common carboxy-terminal fragment of ET (ET[17-21]) were synthesized by the solid phase method.

Immunization: Adult female BALB/c mice were immunized with the conjugate containing 10 μg of ET-1 emulsified in complete Freund's adjuvant as previously described (3). They were boosted every 3 weeks and bled 10-14 days after each booster injection. Three out of 10 mice gave a positive antibody response (ET-F5, ET-F9, ET-F10).

Preparation of monoclonal antibody: Two of the 3 mice were further boosted. Fusion of spleen cells with a non-producing mouse myeloma cell line, X63-Ag8.653, was carried out in the ratio of 6:1 with 50 % polyethylene glycol (PEG 4000; Merck, Darmstadt, West Germany) according to the method described previously (4,5). Hybridoma selection, cloning and ascitic preparation were performed as reported elsewhere (4,5).

Characterization of monoclonal antibody: Isotyping of the monoclonal antibody was performed by Ouchterlony technique (4,5). Binding affinity was analyzed by constructing a Scatchard plot of ET-1 with RIA described below.

Radioimmunoassay (RIA): The assay buffer was 0.1 M phosphate buffer, pH 7.0, containing 0.5 % gelatin (Merck), 1 mM $Na_2EDTA$, 0.2 mM cystine, 0.1 % Triton X-100 and 0.01 % merthiolate. The RIA mixture consisted of 50-μl standard ET-1 or sample, 50-μl antibody and 100-μl assay buffer. After the incubation for 24 hr at 4°C, $^{125}$I-ET-1 (Amersham, England) (3,000 cpm) was added and the mixture was incubated for another 24 hr. Bound and free ligands were separated with 250-μl assay buffer and 1-ml suspension of dextran-coated charcoal consisting of 400 mg of Norit SX Plus (N.V. Norit-Vereeninging, Holland) and 40 mg of Dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.05 M phosphate buffer (pH 7.4).

Culture of bovine aortic endothelial cells: Bovine aortic endothelial cells were dispersed with collagenase and cultured as previously reported (6). The culture medium was changed to the serum-free medium (GIT, Nihon Pharmaceutical Co. Ltd., Tokyo, Japan) 2 hr before the sampling.

Subjects and blood sampling: Eight normal male volunteers, aged 26-40 years old, were studied. Blood samplings were performed from the antecubital vein at a recumbent position after an overnight fast.

Extraction of endothelin from plasma: Extraction of ET from plasma was carried out using polystyrene beads coated with the monoclonal antibody. The monoclonal antibody was purified using a Protein A affinity column (Affi-Gel Protein A MAPS II Kit, Bio-Rad Laboratories, Richmond, CA). Polystyrene beads (diameter 6.5 mm, Ichiko, Komaki, Japan) were immersed in 0.05 M phosphate buffer (pH 7.5) for 24 hr at 4°C containing 0.01 % purified monoclonal antibody, 0.9 % sodium chloride and 0.05 % sodium azide.

The extraction mixture consisting of 1-ml plasma and 1-ml phosphate buffer (0.1 M, pH 7.0) containing 0.1 % gelatin, 1 mM EDTA, 0.2 mM cystine and aprotinin (2,000 KIU/ml) and the antibody-coated bead were incubated at 4°C for 24 hr with gentle agitation. The bead was washed with 0.05 M phosphate buffer and then, was heated in 300 µl of 0.1 M phosphate buffer (pH 7.0) containing 0.5 % gelatin at 85°C for 30 min. The heated extract was directly used for the RIA. Recoveries of 25 pg and 50 pg of ET-1 added to 1-ml plasma were 52 % and 55 %, respectively. Recovery of 50 pg of big ET added to 1-ml plasma was 48 %.

Gel permeation chromatography (GPC): GPC was performed on a Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (0.7 x 50 cm) and eluted with the assay buffer as a solvent. The flow rate was 3 ml/hr and the fraction volume was 0.5 ml. The column was calibrated with blue dextran (Vo), γ-human atrial natriuretic peptide (γ-hANP) (13K), human big ET (4K), α-hANP (3K), ET-1, ET-2, ET-3, and $^{125}I$ (for salt peak). The medium (200 µl) was directly applied to the column. The extract from 30-ml human plasma was used in the analysis of plasma.

## RESULTS

### Preparation and characterization of monoclonal antibody

ET-antibody-producing cells were recognized in two of the wells. One clone which produced antibody with the highest titer was selected for expansion and characterization. The obtained monoclonal antibody (KY-ET-1-I) belonged to the $IgG_1$ subclass. Analysis by a Scatchard plot revealed a high affinity for ET-1 with a Ka of $7.0 \times 10^{10}$ $M^{-1}$.

### RIA for ET-1

Ascites (KY-ET-1-I) and antiserum (ET-F5) were used as antibodies in RIAs at the final dilution of $3.0 \times 10^7$ : 1 and $1.5 \times 10^4$ : 1, respectively. The standard curves in RIAs using KY-ET-1-I and ET-F5 are shown in Figs. 1a and 1b, respectively. In both RIAs, the value of 50 % inhibitory concentration was 6 pg/tube and the minimal detectable amount was 0.5 pg/tube. Although the RIA with ET-F5 recognized

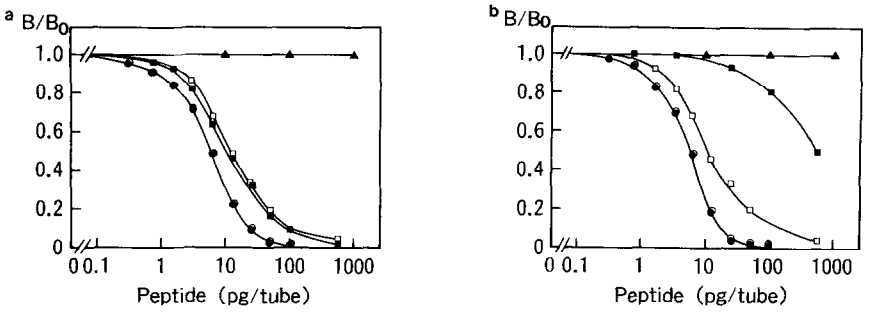


Fig. 1. Typical standard curves of ET-1 and cross-reactivities with other related peptides in RIAs with KY-ET-1-I (Fig. 1a) and with ET-F5 (Fig. 1b). ET-1 (closed circle), ET-2 (open circle), ET-3 (closed square), human big ET (open square), and ET[17-21] (closed triangle) were studied.

ET-3 very weakly (cross-reactivity: 2 %), the RIA with KY-ET-1-I shows cross-reactivity of 60 % with ET-3. Cross-reactivities with ET-2 and human big ET were 100 % on a molar basis in each RIA. These RIAs did not recognize the common carboxy-terminal fragment of ET, ET[17-21] (Fig. 1).

ET-1-LI in culture medium of bovine aortic endothelial cells

ET-1-LI was detected in culture medium of bovine aortic endothelial cells. The serial dilution curve of the medium was parallel to the standard curve in each RIA. The secretory rate of ET from endothelial cells was 0.45 ng/hr/$10^6$ cells during 2-5 hr after the beginning of serum-free maintenance of endothelial cells. ET-1-LI in the medium consisted of two major and one minor components in GPC (Fig. 2). The two major peaks were emerged at the elution positions of synthetic human big ET and ET-1, respectively. ET-1-LI in the two peaks determined by the RIA with KY-ET-1-I was identical to that determined by the RIA with ET-F5. The minor peak was eluted at the position of the larger molecule than big ET.

ET-1-LI in human plasma

ET-1-LI was detected in the extract of human plasma. Serial dilution curves of plasma extracts were parallel to the standard curve in each RIA. Plasma ET-1-LI levels in normal healthy volunteers (n=8) are shown in Table 1. The plasma ET-1-LI level was 19.1 ± 1.1 pg/ml in the RIA with KY-ET-1-I and 9.9 ± 0.5 pg/ml in the RIA with ET-F5.

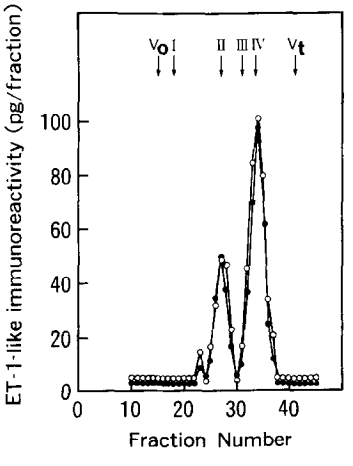


Fig. 2. A GPC profile of culture medium of bovine aortic endothelial cells performed on a Sephadex G-50 (0.7 x 50 cm) column. Arrows I, II, III and IV donate the elution positions of γ-human atrial natriuretic peptide (ANP) (13K), human big ET (4K), α-human ANP (3K) and ET-1 (2.5K), respectively. Closed and open circles indicate immunoreactivities determined by RIAs with KY-ET-1-I and with ET-F5, respectively.

Table 1
Plasma level of ET-1-like immunoreactivity in healthy man determined by two different RIAs

| Subject | Age (yr) | Sex | ET-1-LI (pg/ml) | |
|---|---|---|---|---|
| | | | KY-ET-1-I | ET-F5 |
| 1 K.N. | 40 | M | 24.4 | 12.2 |
| 2 H.I. | 31 | M | 21.4 | 10.4 |
| 3 Y.S. | 32 | M | 19.1 | 9.6 |
| 4 K.H. | 30 | M | 19.1 | 8.7 |
| 5 G.S. | 31 | M | 19.0 | 11.3 |
| 6 M.J. | 30 | M | 18.6 | 9.3 |
| 7 Y.I. | 31 | M | 14.6 | 8.7 |
| 8 O.N. | 26 | M | 17.0 | 9.4 |
| mean ± SE | 31.4 ± 1.5 | | 19.1 ± 1.1 | 9.9 ± 0.5 |

SE = standard error

Fig. 3 shows the representative GPC profile of ET-1-LI in human plasma. ET-1-LI in human plasma consisted of apparent two major components. The peak with the small molecular size was located at the elution position of synthetic ET-1. The other peak was eluted at the preceding fraction (6K) of the position of human big ET (4K). There existed ET-1-LI at the elution position of big ET. ET-1-LI with the small form was 5 pg/ml and corresponded to 25.8 ± 1.5 % of ET-1-LI in the RIA with KY-ET-1-I but 36.7 ± 2.4 % in the RIA with ET-F5. There was no significant difference in ET-1-LI of the small molecule between two RIAs, while ET-1-LI in the large molecule determined by the RIA with ET-F5 was about 70 % of that determined by the RIA with KY-ET-1-I.

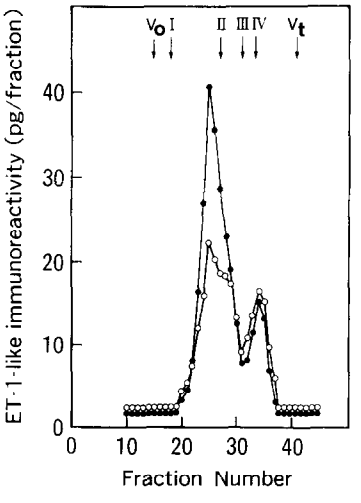


Fig. 3. A representative GPC profile of the human plasma extract performed on a Sephadex G-50 (0.7 x 50 cm) column. Arrows and circles are the same as those in Fig. 2.

## DISCUSSION

We have developed monoclonal (KY-ET-1-I) and polyclonal (ET-F5) antibodies against ET-1. Using these antibodies, we established two RIAs with different specificities.

The GPC profile of ET-1-LI in culture medium of bovine aortic endothelial cells revealed the presence of two peaks eluting at the positions of synthetic big ET and ET-1. The GPC profile in the RIA with KY-ET-1-I was almost identical with that in RIA with ET-F5. These findings indicate that endothelial cells secrete big ET as well as the small molecular form of ET. Because of no significant difference in ET-1-LI levels of small molecular form measured by two RIAs with and without significant corss-reactivity with ET-3, endothelial cells produce, if any, little ET-3. In addition, since there is no evidence that the ET-2 gene is expressed in endothelial cells (2), the small form of ET in the culture medium is considered to be mainly ET-1. This observation on big ET and ET-1 in culture medium of aortic endothelial cells is consistent with the hypothesis proposed previously (1).

The present study demonstrates the existence of ET-1-LI in human plasma. This observation raises the possibility that ET is a circulating hormone as well as a local hormone. ET-1-LI in human plasma was also examined by GPC coupled with the RIAs. The small molecular form of ET in human plasma was eluted at the position of ET-1. There was no difference in the ET-1-LI of the small form between two RIAs. It is possible, therefore, that the major component of the small form of ET in human plasma is ET-1 like in the culture medium of endothelial cells. Taking the plasma ET-1-LI level and the GPC profile into account, the plasma concentration of the small form of ET, or ET-1, is about 5 pg/ml.

The elution position of the large form of ET in human plasma was clearly different from that in the medium of endothelial cells. The peak was observed at the preceding fraction (6K) of big ET (4K). ET-1-LI was also seen at the position of big ET. These observations suggest that the large form of ET-1-LI in human plasma consists of, at least, big ET and another component with a larger molecular weight. Since 1-ng ET-1 added to 30-ml plasma was eluted at the position of ET-1 after the same extraction process (data not shown), it is unlikely that large forms of ET-1-LI in human plasma are artifacts due to aggregation of ET-1. When examined by the RIA with ET-F5, the large form of ET-1-LI was about 70 % of that determined by the RIA with KY-ET-1-I. This finding explains the difference in the plasma ET-1-LI

level between two RIAs (19.9 ± 1.1 pg/ml vs 9.9 ± 0.5 pg/ml). Further studies are necessary to elucidate the precise nature and source of the large form of ET in human plasma.

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