Localization of rat endothelin-converting enzyme to vascular endothelial cells and some secretory cells

Masaaki TAKAHASHI,* Kuniaki FUKUDA,* Kohei SHIMADA,* Kay BARNES,† Anthony J. TURNER,† Masahiro IKEDA,‡ Hiroyuki KOIKE,‡ Yumiko YAMAMOTO* and Kazuhiko TANZAWA*§

*Biological Research Laboratories and ‡Pharmacology & Molecular Biology Research Laboratories, Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan, and †Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Endothelin is a potent vasoconstrictive peptide that is produced by vascular endothelial cells; it is formed from its precursor, big endothelin, by endothelin-converting enzyme (ECE). In this work, ECE was studied using specific monoclonal antibodies. In immunoblotting, ECE was estimated to be a 300 kDa protein on SDS/PAGE under non-reducing conditions, and 130 kDa under reducing conditions. Cross-linking experiments revealed that ECE is composed of two disulphide-linked subunits. Localization of ECE was studied at the cellular and subcellular levels in various rat tissues and cells. High-level expression of ECE was observed in membrane fractions of simian virus 40-transformed rat endothelial cells by immunoblotting, but the immunoreactive band was absent from aortic smooth muscle cells and cytosolic fractions of endothelial cells. In immunohistochemical analysis,

ECE was found to be localized in the endothelial cells of the aorta, lung, kidney, liver and heart. Confocal immunofluorescent microscopy showed that most of the ECE in endothelial cells and cells transfected with ECE cDNA was clustered along the plasma membrane. Intact COS or CHO cells transfected with ECE cDNA rapidly and efficiently cleaved big endothelin-1 added to the culture medium. Thus endothelial cells express ECE on the plasma membrane and the active site of the enzyme faces outside the cells, i.e. it is an ectoenzyme. Other than endothelial cells, ECE was also present in some secretory cells. The enzyme was abundant in the adrenal gland, and localized in chromaffin cells. ECE was also highly condensed in pancreatic islet β cells. It is concluded that ECE and endothelin may be involved in the regulated secretion of hormones.

INTRODUCTION

Endothelin-1 (ET-1) is a 21-amino-acid vasoconstrictive peptide originally isolated from the supernatant of cultured porcine aortic endothelial cells [1]. The three endothelin isopeptides (ET-1, -2 and -3) are first produced as precursors of approx. 200 residues, corresponding to preproETs [2]. Intermediates termed big ETs are cleaved from preproETs by dibasic pair-specific endopeptidases [1], and then mature ETs are generated by proteolytic cleavage between Trp²¹ and Val/Ile²² by the endothelin-converting enzyme (ECE) [3,4]. The biological activity of big ET-1 is negligible [5], indicating that ECE is a key enzyme in the biosynthetic pathway of ETs.

Purification of ECE from rat lung [6] and porcine aortic endothelium [7] revealed that it is a phosphoramidon-sensitive, membrane-bound neutral metalloproteinase with a subunit molecular mass of 130 kDa. The purified rat lung enzyme specifically cleaves the bond between Trp²¹ and Val²², and quantitatively converts big ET-1 into ET-1. The purified lung ECE preferentially cleaves big ET-1 rather than big ET-2 and big ET-3; the ratio of cleavage rates is 4:1:2. Porcine aortic endothelium ECE shares properties with rat lung ECE, but it exhibits a much greater preference for big ET-1 than for other big ET isopeptides. The physiological relevance of phosphoramidon-sensitive ECE has also been confirmed by experiments *in vivo*. Intravenously administered big ET-1 is converted into ET-1, and phosphoramidon consistently blocks this conversion event [8,9]. The recent molecular cloning of rat [10], bovine [11,12] and human [13,14] ECE cDNAs revealed that the enzyme is a highly

glycosylated protein consisting of 10 possible N-linked glycosylation sites, a zinc-binding domain and a single membrane-spanning region. It has structural and sequence identity to neutral endopeptidase-24.11 (NEP) and to Kell blood group protein, which is a putative neutral endopeptidase. The ECE cloned from endothelial cells is specific for big ET-1, consistent with the fact that endothelial cells produce only ET-1 [10].

Endothelial cells possess relatively high ECE activity. ECE-like activities have also been reported in several other cell types, including smooth muscle cells, the cytosolic fractions of endothelial cells and polymorphonuclear leucocytes [15–19]. It is unclear, however, whether or not the same molecule is responsible for these activities, and the physiological relevance of each enzymic activity has not been examined. Although ECE mRNA is distributed in various tissues [10,11], localization of ECE in these tissues has not been confirmed to date, and the precise mechanism of ET production remains to be clarified. Analysis of the conversion of big ET-1 into ET-1 by ECE *in vivo* suggests that the conversion takes place on the surface of vascular endothelial cells in the blood flow [8,9]. On the other hand, studies on the conversion activity of CHO cells expressing ECE or ECE/preproET-1 indicate that big ET-1 is mainly converted intracellularly through the constitutive secretory pathway [11]. We have raised monoclonal antibodies (mAbs) which specifically recognize rat ECE [10,14]. We have now studied the expression of ECE in various rat tissues by immunohistochemical analysis. We also describe the subcellular localization of ECE in endothelial cells and in cells expressing ECE in order to define the mechanisms of production of ETs.

Abbreviations used: ET, endothelin; ECE, endothelin-converting enzyme; HUVEC, human umbilical vein endothelial cells; mAb, monoclonal antibody; NEP, neutral endopeptidase-24.11; TRLEC-03 cell, simian-virus-40-transformed rat lung vascular endothelial cell.

§ To whom correspondence should be addressed.

EXPERIMENTAL

Cell culture

Simian virus 40-transformed rat lung vascular endothelial cells (TRLEC-03 cells) were kindly supplied by Dr. S. Tsurufuji (Institute of Cytosignal Research, Tokyo, Japan), and were cultured on a collagen-coated flask (Corning) in RPMI-1640 medium containing 10% (v/v) fetal calf serum. TRLEC-03 cells express ECE, and produce ET-1 in a phosphoramidon-sensitive manner (M. Takahashi and Y. Yamamoto, unpublished work). Rat aortic smooth muscle cells [20] and COS-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. CHO-K1 cells were cultured in α -minimal essential medium containing 10% (v/v) fetal calf serum. Human umbilical vein endothelial cells (HUVEC) were purchased from Kurabo and cultured as described [21]. All cells were grown in a humidified incubator in a 5% CO₂/95% air atmosphere at 37°C. All media were purchased from Gibco BRL and were supplemented with penicillin/streptomycin.

Antibodies

Two anti-ECE mAbs, AEC27-121 (IgG1) and AEC32-236 (IgG1), were produced by conventional procedures [10,14]. Hybridoma cultures were screened with an ELISA, and were further characterized by immunoblotting. mAb AEC27-121 was specific to rat ECE when tested by immunoblotting. The other mAb, AEC32-236, recognized rat, human, porcine, rabbit and bovine ECEs by immunoblotting. The mAbs were purified from ascites fluid by precipitation with ammonium sulphate and Protein G-Sepharose column chromatography.

Expression of rat ECE in COS-1 cells and CHO-K1 cells

The cDNA for rat endothelial ECE was obtained as described previously [10]. The full-length rat ECE cDNA and the 5'-deletion construct lacking the 5' region encoding the 98 amino acid residues of the N-terminus, including the putative transmembrane domain (Δ 98 ECE cDNA), were subcloned into a pCDL-SR α 296 expression vector [22]. For transient expression, the constructs were transfected into COS-1 cells by electroporation as described previously [10]. For constitutive expression, the full-length ECE cDNA was co-transfected with pE4WT carrying the dihydrofolate reductase gene into CHO-K1 cells by the calcium phosphate method [23]. The monoclonal cell line expressing ECE was chosen by immunoblot analysis.

For measurements of cellular ECE activity in intact cells, COS cells transfected with rat ECE cDNA were plated at 8×10^4 cells per well in 24-well tissue culture plates, and cultured for 60 h. CHO/ECE cells (2×10^5 cells/well) were cultured in 24-well tissue culture plates for 24 h. Human big ET-1, -2 or -3 (Peptide Institute Inc., Osaka, Japan) was incubated with the cells in 0.5 ml of the culture media for 1–4 h at 37°C, and the ET-1, -2 or -3 thus formed was determined by sandwich ELISA (Immuno-Biological Lab. Co.) [24].

Electrophoresis and immunoblotting of ECE

Rat lung ECE was purified as described previously [6]. In some experiments the purified enzyme was deglycosylated with glycopeptidase F (Takara) before electrophoresis [10]. ECE was analysed by 4–20% gradient SDS/PAGE [25] or 2–15% native PAGE [26]. Gels were stained with silver reagents (Daiichi-kagaku) or analysed by immunoblotting.

Various rat tissues and cells were homogenized in 10 vol. of homogenization buffer (20 mM Tris/HCl, pH 7.5, 5 mM MgCl₂,

0.1 mM PMSF, 20 μ M pepstatin A and 20 μ M leupeptin) using a Polytron homogenizer at setting 7 for 90 s. The homogenates were centrifuged at 100000 *g* for 45 min. The membranes were washed three times by resuspension in 10 vol. of homogenization buffer and re-centrifugation after homogenization using a Teflon/glass homogenizer. The membrane proteins and soluble cytosolic proteins thus obtained were used for immunoblotting. Proteins were separated on SDS/PAGE (4–20% gels) and transferred to poly(vinylidene difluoride) membranes (Millipore). The blots were blocked in 5% (w/v) dried milk in PBS for 30 min, incubated with 10 μ g/ml mAb AEC27-121, washed in 0.1% Tween 20 in PBS and incubated with 2000-fold diluted peroxidase-conjugated rabbit anti-mouse IgG (Amersham Corp). Both primary and secondary antibodies were diluted by 0.1% Tween 20 in PBS, and incubations with antibodies were carried out at room temperature for 1 h. The immunoreactive bands were visualized with the ECL detection system (Amersham Corp). In control experiments, the primary antibody was replaced by mouse IgG1 (Chemicon International Inc.) or normal mouse ascites (Seikagaku Kogyo), which did not stain ECE.

Cross-linking of ECE in rat lung microsomal fractions

Rat lung microsomal fractions were prepared as described previously [6]. The fraction was washed three times with 50 mM sodium phosphate, pH 8.0, containing 0.15 M NaCl, and then resuspended in the same buffer at a protein concentration of 4 mg/ml. Aliquots (0.2 ml) of the suspension were treated with various concentrations of cross-linkers. The reactions were allowed to proceed for 30 min at room temperature and then quenched by addition of 40 μ l of 0.25 M Tris/HCl, pH 7.5. After centrifugation, the membrane pellets were boiled in SDS sample buffer [25]. The samples were analysed by immunoblotting on SDS/PAGE (2–15% gels).

Immunohistochemistry

Various tissues removed from male Sprague-Dawley rats (body weight 150 g) were fixed in 10 mM NaIO₄, 75 mM lysine, 2% paraformaldehyde and 37.5 mM sodium phosphate, pH 6.2, for 12 h at 4°C, and embedded in paraffin. Sections (4 μ m thick) were laid on poly(L-lysine)-coated glass slides. Dewaxed sections were immersed in methanol containing 3% H₂O₂ for 15 min to quench any endogenous peroxidase activity in order to block non-specific antibody binding. The sections were preincubated with 1% normal goat serum in PBS for 30 min at room temperature, and then in a moist chamber for 60 min at room temperature with 40 μ g/ml mAb AEC27-121. Immunostaining was performed by the biotin/streptavidin-complex method using Stravigen (BioGenex Corp). After incubation, the sections were treated with diaminobenzidine/HCl for 10 min and counterstained with haematoxylin. In control preparations the primary antibody was replaced by normal mouse ascites, which did not stain any tissue tested.

Indirect immunofluorescence

Cells were grown on untreated or collagen-coated glass coverslips for 20 h as described above. All subsequent processing was performed at room temperature. The cells were fixed with 3% formaldehyde in PBS for 15 min, quenched with 0.1 M glycine in PBS and incubated in 0.1% saponin in PBS for 10 min for permeabilized samples. The samples were blocked with normal 10% goat serum in PBS for 1 h, incubated with 40 μ g/ml mAb AEC27-121 or AEC32-236 for 1 h, washed in PBS, and then incubated with fluorescein isothiocyanate-conjugated goat anti-

mouse IgG (Jackson) for 1 h. In all cases the antibodies were diluted in 10% goat serum in PBS. The coverslips were mounted in a 1:1 (v/v) mixture of PBS/glycerol containing 2% 1,4-diazabicyclo[2.2.2]octane to prevent photobleaching. Samples were viewed under a Bio-Rad MRC-600 confocal fluorescence microscope and photographed using an HC1500 film processor (Toshiba). In control preparations, the primary antibody was replaced by mouse IgG1 (Chemicon) or normal mouse ascites (Seikagaku Kogyo), which did not stain the cells.

RESULTS

Structural analysis of rat ECE using mAb AEC27-121

As demonstrated by immunoblot analysis, mAb AEC27-121 specifically recognized the purified rat lung ECE, the enzyme in rat lung membrane fractions and the cloned endothelial ECE expressed in COS cells or CHO cells (Figure 1). While the molecular mass of the purified ECE was 130 kDa, the enzyme in COS cells appeared as a broad band, and the enzyme in CHO cells showed a slightly higher molecular mass than the purified ECE. Since ECE is a highly glycosylated protein with 10 possible N-linked glycosylation sites, this difference may be explained by differences in glycosylation. The epitope for mAb AEC27-121 did not include the carbohydrate chain portion of ECE, as deglycosylated ECE was recognized by the mAb. The deletion construct of ECE ($\Delta 98$ ECE) lacking the N-terminus (including the transmembrane domain) was expressed in COS cells. $\Delta 98$ ECE was recognized by mAb AEC27-121, revealing that the epitope for mAb AEC27-121 is localized in the C-terminal domain containing the catalytic site and the zinc-binding site.

When purified ECE was denatured in 1% SDS solution in the absence of a reducing agent and analysed by SDS/PAGE, the enzyme appeared as a 300 kDa protein. Similar high-molecular-mass ECE bands were obtained when membrane fractions of rat lung, COS/ECE cells and CHO/ECE cells were analysed by

immunoblotting under non-reducing conditions. In native PAGE the molecular mass of purified ECE was also approx. 300 kDa (Figure 2a). These results suggest that native ECE forms a disulphide-linked homodimer. This possibility was examined by cross-linking of ECE in lung microsomes (Figure 2b). Two protein bands (I and II) were seen in samples cross-linked with ethylene glycol bis(succinimidyl succinate) by immunoblot analysis. Calibration of these bands indicated that the molecular masses of bands I and II were 130 kDa and 300 kDa respectively. Thus bands I and II correspond to the monomeric and dimeric forms of ECE respectively. The dimeric form was the major product when the concentration of the cross-linker was above 1 mM. No other cross-linked products with higher molecular masses were observed at any cross-linker concentration used. Similar results were obtained with other cross-linkers such as

Figure 2 Homodimeric structure of ECE

(a) Purified ECE (100 ng) was subjected to native PAGE (2–15% gels), and stained with silver reagents. The arrow indicates the position of the native ECE. Positions of molecular mass markers are shown on the left. (b) Cross-linking of rat lung microsomes. Microsomal membrane suspensions (4 mg/ml) were cross-linked with ethylene glycol bis(succinimidyl succinate). The numbers indicate the concentration of the cross-linker. The cross-linked products (20 μ g) were analysed by immunoblotting. I and II are the positions of the monomeric and dimeric forms of ECE respectively. Positions of molecular mass markers are shown on the right.

Figure 1 SDS/PAGE and immunoblots using mAb AEC27-121 to purified rat lung ECE and cells transfected with rat ECE cDNA

(a) Lane 1, 100 ng of purified rat lung ECE stained with silver reagents; lanes 2–4, immunoblots of 10 ng of purified rat lung ECE (lanes 2 and 4) and deglycosylated ECE (lane 3). (b) Immunoblots of 30 μ g of rat lung whole membranes (lanes 1 and 6), 3 μ g homogenates of COS cells transfected with the control vector (lane 2), the full-length ECE cDNA (lane 3 and 7) or $\Delta 98$ ECE cDNA (lane 4), and 10 μ g homogenates of the CHO cells transfected with the full-length ECE cDNA (lane 5 and 8). The samples were boiled in SDS gel sample buffer in the presence (+) or absence (−) of β -mercaptoethanol (β -ME) and were subjected to SDS/PAGE (4–20% gels). Immunoreactive bands were visualized using mAb AEC27-121. Positions of molecular mass markers are shown (kDa).

Figure 3 Immunoblot analysis of expression of ECE in rat tissues and cells

Samples of 30 μ g of protein were subjected to SDS/PAGE (4–20% gels) and analysed by immunoblotting using mAb AEC27-121. (a) Whole membrane fractions of rat tissues; (b) membrane fractions (M) and cytosolic fractions (C) of TRLEC-03 cells or cultured rat aortic smooth muscle cells (SMC).

Figure 4 For legend see facing page.

Figure 5 For legend see facing page.

disuccinimidyl tartrate and ethylene glycol bis(sulphosuccinimidyl succinate).

Immunoblot analysis of ECE expression in rat tissues and cells

The tissue distribution of ECE was assessed by immunoblot analysis using mAb AEC27-121 (Figure 3a). High-level expression of ECE was observed in the membrane fractions of lung and adrenal gland. The membrane fractions of liver and intestine showed weak expression, and the other tissues contained ECE at extremely low levels. Immunoreactive bands were not detected in the cytosolic fractions from any of these tissues. Among the cellular fractions tested, an immunoreactive band was present in the membrane fractions of TRLEC-03 cells, but absent from the cytosolic fractions of TRLEC-03 cells and aortic smooth muscle cells (Figure 3b). Since ET-1 production in endothelial cells is stimulated by various procontractile factors [27], we examined whether or not ECE was involved in this increase in ET production. However, stimulation by thrombin, phorbol esters, platelet-derived growth factor, transforming growth factor- β or calcium ionophore did not increase the amount of ECE in TRLEC-03 cells on immunoblot analysis (results not shown).

Expression of ECE in endothelial cells

Localization of ECE in the aorta abdominalis and lung was studied with mAb AEC27-121 and a histochemical reaction with conjugated peroxidase (Figure 4), since immunoblot analysis suggested high-level expression of ECE in these tissues. The aorta abdominalis showed strong expression of ECE in the

endothelium, while no immunoreaction was detected in smooth muscle cell layers (Figure 4a). In lung, immunoreactivity was observed in the endothelial cells lining the lumen of the arteries (Figure 4b). ECE was also recognized in small veins and bronchial arteries (Figures 4c and 4d). No significant staining was noted in capillaries, alveolar walls or bronchial epithelial cells.

Various functions of ET-1 have been reported in kidney, liver and heart [28]. Therefore localization of ECE was also examined in these tissues where expression of ECE is low, as shown by immunoblot analysis. Immunoreactivity was found only in the endothelial cells in the sections from these tissues (Figure 5). In kidney, the endothelial cells of an interlobular artery were stained by mAb AEC27-121. No staining was observed in the glomeruli, proximal convoluted tubules or distal convoluted tubules. In liver, ECE was found in an interlobular artery and a portal vein, while an interlobular duct and a sinusoidal wall were negative. In heart, endothelial cells were stained by mAb AEC27-121, but myocardial cells were not stained.

Subcellular localization of ECE in endothelial cells and cells transfected with rat ECE cDNA

In order to analyse the kinetics of ECE in living cells, we examined whether intact cells expressing ECE can convert big ET-1 that is exogenously added to the culture medium. For this purpose, CHO/ECE and COS/ECE cells were employed for the following reasons. Although intact endothelial cells were reported to cleave big ET-1 that was exogenously added to the culture medium [29], the level of expression of ECE in endothelial cells is too low to characterize its activity. In addition, endothelial

Figure 6 Conversion of big ETs into ETs by intact COS/ECE cells

(a) Time-dependent conversion of 1 μ M big ET-1 (●), big ET-2 (○) or big ET-3 (▲) by COS/ECE cells. COS cells transfected with rat ECE cDNA were plated at 8×10^4 cells per well in 24-well tissue culture plates, and cultured for 60 h. Human big ET-1, -2 or -3 was added to the culture media, and then the amounts of ETs produced in the media were determined by sandwich ELISA (see the Experimental section). (b) Various concentrations of big ET-1 (●), big ET-2 (○) or big ET-3 (▲) were added to the culture medium. After 4 h of incubation at 37 °C, ETs produced in the medium were determined. The results are means of two experiments.

Figure 4 Immunohistochemical localization of ECE in aorta abdominalis (a) and lung (b–d)

(a) Immunolabelling was not observed over the smooth muscle layer (S). Endothelium exhibited immunoreaction (arrow). (b–d) In lung, immunoreactivity was observed in the endothelial cells of arteries (arrowheads) and veins (arrows). No significant staining was noted in capillaries (small arrowheads), alveolar walls (A) or bronchial epithelial cells (E). Bar = 20 μ m.

Figure 5 Immunohistochemical studies of kidney (a), liver (b) and heart (c)

(a) Endothelial cells of an interlobular artery (arrow) were stained by mAb AEC27-121. No staining was observed in glomeruli (G), proximal convoluted tubules (P) or distal convoluted tubules (D). (b) ECE was found in an interlobular artery (arrowhead) and portal vein (arrow), but not in an interlobular duct (I) or sinusoidal wall (S). (c) The endothelial cells were stained by the mAb (arrowheads), but myocardial cells (M) were not stained. Bar = 20 μ m.

Figure 7 Subcellular localization of ECE by confocal laser immunofluorescence analysis using mAb AEC27-121 (a–f) or AEC32-236 (g)

CHO/ECE cells (a, b), COS/ECE cells (c–e), TRLEC-03 cells (f) and HUVEC (g) were fixed in 3% formaldehyde for surface-staining (a) or fixed and permeabilized for both cell-surface and intracellular staining (b–g). In all cases, plasma membranes were heavily stained by the mAbs. However, in a few permeabilized ECE/CHO and ECE/COS cells, staining was also seen intracellularly (b, e; arrows). Bar = 10 μ m.

cells (either cells in primary culture or TRLEC-03 cells) produce ET-1 endogenously, which may interfere with the experimental results. As shown in Figure 6, the added big ET-1 was efficiently converted into ET-1 by COS/ECE cells in a time-dependent fashion. At a substrate concentration of 1 μ M, 36% of the added big ET-1 was converted into ET-1 (Figure 6a). The conversion rate by CHO/ECE cells was 18% under similar experimental conditions (results not shown). The conversion by both cell types was specific for big ET-1, and the relative conversion ratio for big ET-1, -2, and -3 was approx. 10:1:1 over the whole concentration range examined (Figure 6b). It is unlikely that a large peptide such as big ET-1 can be easily incorporated into the cells, cleaved inside and then secreted in a short period of time. The present results suggest, therefore, that at least a part of ECE is localized to the cell surface as an ectoenzyme, with its catalytic sites facing outside the cells.

Next, the subcellular localization of ECE was studied by confocal laser immunofluorescent micrography (Figure 7). In non-permeabilized CHO/ECE cells, immunoreactivity was observed on the plasma membrane (Figure 7a), while CHO cells were not stained (results not shown). Since the epitope of mAb AEC27-121 is in the C-terminal region containing the catalytic site, recognition of ECE in the non-permeabilized cells by the mAb also supports the idea that the catalytic domain of the plasmalemmal ECE is facing outside the cells, as suggested from

the conversion experiments. ECE seems to cluster at the cell surface, as staining with mAb AEC27-121 revealed many large patches scattered throughout the cells. The clustering pattern of ECE was very unstable, and fixation of the cells by methanol diffused or disrupted the stained patches. In addition, the structure was easily detached from the cell surface by Triton X-100 treatment, resulting in the complete loss of immunoreactivity in the cells. For further subcellular localization studies, therefore, cells were fixed with formaldehyde, and were then permeabilized by saponin. The staining pattern of the permeabilized cells was the same as that of the non-permeabilized cells (Figure 7b), i.e. large patches were scattered throughout the cells. Analysis in different focal planes revealed that the stained patches were at or near the plasma membrane. Less than 10% of the stained cells, however, showed intracellular accumulation of ECE. Staining was seen on top of the nucleus, where the Golgi complex is often located in these cells (Figure 7b, arrow).

The staining pattern of COS/ECE cells transiently expressing rat ECE was similar to that of CHO/ECE cells. The plasmalemmal clusters were observed in most of the stained cells (Figure 7c). In other cells expressing relatively high amounts of ECE, the plasma membrane was diffusely and densely stained and the clustered structure was unclear (Figure 7d). In some of these cells, intracellular granules were also heavily stained (Figure 7e). ECE is a highly glycosylated protein, and the carbohydrate

chains are attached to the peptide backbone in the Golgi complex. As CHO/ECE and COS/ECE cells express large amounts of ECE, it is presumed that ECE was localized in the Golgi complex in some cells actively producing the enzyme. In TRLEC-03 cells, whose expression of ECE was much lower than that of CHO/ECE cells, only the scattered patches on the plasma membrane were stained by mAb AEC27-121, with no immunoreactivity in intracellular organelles even in the permeabilized condition (Figure 7f). Staining of primary cultured HUVEC by another mAb, AEC32-236, which recognizes human ECE, resulted in the same staining pattern as that of TRLEC-03 cells with mAb AEC27-121 (Figure 7g).

Expression of ECE in other tissues

As mentioned above, ECE is highly concentrated in the adrenal gland, as shown by immunoblotting. ECE mRNA has also been detected in the adrenal gland [10,11]. Therefore the localization of ECE in the adrenal gland was studied using mAb AEC27-121. Chromaffin cells of the adrenal medulla were diffusely stained by the immunohistochemical reaction, but the adrenal cortex was not stained (Figure 8a). We then examined whether or not other secretory cells expressed ECE. Among the cells tested, pancreatic islets showed strong immunoreactivity while acinar cells were not stained (Figure 8b). The ECE-positive cells were found in most pancreatic islet cells, indicating that they were insulin-containing β cells (Figure 8c). It should be noted that immunoreactivity was not observed in thyroid gland or brain.

It has been reported that testes also contain high levels of ECE mRNA [11]. Therefore a section from testis was stained by mAb AEC27-121. Immunoreactivity was found in interstitial cells, but the seminiferous tubules were not stained (Figure 8d).

DISCUSSION

In this study we have characterized ECE using specific anti-ECE mAbs. Earlier estimations of the molecular mass of ECE were controversial. The predicted molecular mass of ECE is 86 kDa based on the cDNA sequence [10,11], and SDS/PAGE analysis of purified ECE indicated that it is a 120–130 kDa protein [6,7]. On the other hand, other workers have suggested that the native molecular mass of ECE is 250–350 kDa [13,30–32]. This discrepancy can be explained by the dimeric structure of ECE under native conditions. The present results demonstrate that ECE is composed of two subunits covalently bound through cysteine residues. Several membrane peptidases have been reported to be dimeric proteins [4], but covalent association of subunits is relatively uncommon among them except for meprin and membrane dipeptidase [33,34]. NEP and ECE are highly conserved proteins over their whole sequence. In particular, 10 of the 14 cysteine residues in ECE are conserved in NEP, suggesting that the tertiary structure of ECE is similar to that of NEP [10,11]. It was also reported that NEP is dimeric in kidney [35], but the electrophoretic mobility of NEP is the same under either reducing or non-reducing conditions. It is strongly suggested that both ECE and NEP have homodimeric structures, but that only ECE dimerizes through one or more of the four cysteine residues that are not conserved in NEP.

We have demonstrated the expression of ECE in several cells and tissues isolated from rats. ECE was highly enriched in endothelial cells of aorta, lung, kidney, liver and heart. On RNA blot analysis, ECE was present in various tissues in which no immunoreactive band was detected [10,11]. These small amounts of ECE RNA are likely to be derived from vascular endothelial

cells in each tissue, because immunohistochemical analysis revealed that ECE is only localized in vascular endothelial cells in these tissues. It has been reported that ECE is also present in vascular smooth muscle cells and the cytosolic fractions of endothelial cells. The reported activities in these fractions, however, were much lower than those in the endothelial-membrane-bound fractions, and were not sensitive to phosphoramidon in some cases [15–18]. The present study revealed an absence of immunoreactive ECE in smooth muscle cells. The epitope of mAb AEC27-121 is in the C-terminal region of the enzyme containing the catalytic site and zinc-binding site. The reported cytosolic activity in endothelial cells, therefore, cannot be derived from the same gene as produces the cloned cDNA by an alternative splicing event which deletes the transmembrane domain of ECE. It is concluded that the main site of conversion of big ET-1 into ET-1 in the vascular system is in the membrane fraction of endothelial cells.

The present immunofluorescence studies and cellular conversion assays showed that ECE is localized on the plasma membrane of endothelial cells, and that the catalytic domain of the plasmalemmal ECE faces outside of the cells. Several lines of evidence from other laboratories also support the idea that big ET-1 is mainly converted on the surface of endothelial cells. First, big ET-1, ET-1 and the C-terminal fragment of big ET-1 have all been detected in the culture medium of endothelial cells and plasma, and the amount of the C-terminal fragment is almost equal to that of ET-1 [36,37]. Secondly, intravenously administered big ET-1 is converted into ET-1, and the hypertensive effects of big ET-1 can be abolished by phosphoramidon [8,9]. Thirdly, NEP, which has a significant structural similarity to ECE over its whole sequence, also degrades peptide substrates on the surface of the plasma membrane [38,39]. From these observations, it can be postulated that ECE is present in blood vessels with access to circulating big ET-1. However, it is possible that big ET-1 is converted during secretion through the plasma membrane rather than after secretion into the circulation. In immunofluorescence analysis, ECE was found to form large clusters on the surface of the cells. Clustering is characteristic of ECE, and has not been observed for other plasmalemmal proteinases. If big ET-1 is secreted near to or through the ECE clusters from endothelial cells, it could be converted into ET-1 by this process. This system would allow effective concentration and efficient conversion of big ET-1 produced by the endothelial cells, in spite of the fact that the $V_{\max}$ value of the purified ECE and the amount of ECE expressed in endothelial cells are rather low to cleave exogenous big ET-1 efficiently *per se* [3,6]. It has been reported that the conversion of big ET-1 by ECE mainly occurs intracellularly from analyses of the differences in conversion activity of CHO cells expressing only ECE or both preproET-1 and ECE [11]; namely, the cell surface conversion of exogenous big ET-1 by CHO/ECE cells was much less efficient than the apparent intracellular conversion of endogenous big ET-1 by CHO cells co-transfected with ECE and preproET-1. Our hypothesis can explain these observations, as the experiments of Xu et al. [11] cannot distinguish the conversion at the plasma membrane through the secretion of big ET-1 from true intracellular conversion.

Clustering of membrane proteins, e.g. the acetylcholine receptor, is important for their physiological function, and anchor proteins are generally necessary for protein clustering [40]. Identification of anchor proteins for ECE will be informative in the elucidation of the physiological significance of ECE clustering and in understanding the secretory pathway of ET-1. It is possible that caveolae may be a site of ET processing. Caveolae are plasma membrane invaginations that can close off to form

Figure 8 Expression of ECE in adrenal gland (a), pancreas (b, c) and testis (d)

Sections were incubated with mAb AEC27-121. (a) Chromaffin cells of the adrenal medulla (M) showed immunoreactivity. The cortex (C) was not stained. Bar = 20 μm. (b) Immunoreactivity was observed in pancreatic islets (arrowheads), while acinar cells (A) were not stained. Bar = 100 μm. (c) The ECE-positive cells were found in most of the pancreatic islet cells, presumably β cells. Bar = 20 μm. (d) In testis, immunoreactivity was observed in interstitial cells (arrows), while seminiferous tubules (S) were not stained. Bar = 20 μm.

non-clathrin-coated vesicles. These vesicles are extremely abundant in endothelial cells and contain high concentrations of a coat protein, caveolin [41]. It is reported that angiotensin-converting enzyme is localized to plasma membrane caveolae [42], and that ET-1 and the ET_A receptor are co-localized with caveolin [43].

ECE was also present in some endocrine cells, such as chromaffin cells in the adrenal gland and β cells in pancreatic islets, both of which release hormones through the regulated pathway. ECE and the ET produced may play a role in the regulation of hormone secretion from secretory granules. Indeed, chromaffin cells produce ET-1 [44], and the ET produced stimulates the release of catecholamines [45]. The functions of ET in pancreatic β cells have not been reported to date, although the effects of insulin on the production of ET-1 in endothelial cells are well established [46]. Nitric oxide is reported to be a key transmitter in insulin secretion from pancreatic β cells [47]. It is possible that nitric oxide and ET regulate insulin secretion in addition to vascular tone. Further characterization of ECE in β cells will be fascinating for elucidating the functions of ET, especially in diabetic conditions. In testes, ECE was present in interstitial cells but not in the seminiferous tubules. RNA blot analysis also revealed high-level expression of ECE in testes [11].

Although the functions of ET in testes have not been well established to date, a recent observation suggests that ET-1 acts as an autocrine/paracrine factor in rat testes [48]. Interstitial conversion of big ET-1 into ET-1 may participate in local control by ET in testes. Several other membrane peptidases are also present in testis, including NEP and angiotensin-converting enzyme [49,50].

While the cellular and subcellular distributions of ECE are now defined in various tissues, the present study raises several questions. ECE is present only in endothelial cells in the vascular system, and the endothelial ECE is specific to big ET-1, confirming previous observations in cell-free experiments [10]. This substrate specificity is in good accord with the fact that endothelial cells produce only ET-1. Various tissues, however, contain ET-2 and ET-3 isopeptides [2], and the purified rat lung ECE can convert big ET-3 into ET-3 [6]. The molecular identity of the ECE isoforms which cleave big ET-2 and/or big ET-3 in non-vascular systems remains unknown. If ET acts as a local regulator of the circulation, it is possible that ECE is localized at the basal (non-luminal) side of the endothelial cells and that converted ET is not released primarily into the circulation but rather is taken up by the tissues, e.g. smooth muscle, close to the conversion site. In this case, regulation of ECE activity is a key

for ET production. However, it seems unlikely that the level of expression of ECE plays a regulatory role in the increase in ET-1 production on stimulation by various procontractile factors. Other regulatory mechanisms may be present in endothelial cells or other cells. Recent molecular cloning studies of ECE have revealed that rats and humans have two types of the enzyme, presumably as a result of alternative splicing [51]. The variable region between the isoforms is restricted to the N-terminus, and our two mAbs recognize both types of ECEs [51]. It is possible that the production of the two ECEs derived from alternative splicing may be individually regulated by different mechanisms, and that heterodimers may be formed that possess different characteristics from homodimers. For further characterization of ECE, especially in pathophysiological events, i.e. cardiovascular diseases, vascular disorders and renal disease, assessment must be made of the physiological role of ECE and ET. This will also provide indications of useful therapy for conditions associated with altered production of or responsiveness to ET.

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