Expression and Localization of Endothelin-Converting Enzyme-1 Isoforms in Human Endothelial Cells

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Endothelin-converting enzyme (ECE)-1 is a membrane-bound metalloprotease responsible for production of vasoactive endothelin (ET)-1 from inactive big ET-1. ECE-1 exists as four separate isoforms, ECE-1a, b, c, and d, which differ only in their amino-terminal regions. We investigated the expression and localization of the ECE-1 isoforms in primary human umbilical vein endothelial cells (HUVECs) and EAhy926 cells. Reverse transcriptase polymerase chain reaction showed expression of all four isoforms in both cell lines, with ECE-1d seeming, at least qualitatively, to be the predominant isoenzyme. Isoform-specific polyclonal antibodies were used to investigate isoform protein expression. ECE-1a, b, and c protein was detected in EAhy926 cells by immunoblotting; only ECE-1a and ECE-1c were detected in HUVECs. Using immunofluorescence microscopy analysis, both HUVEC and EAhy926 cells showed nuclear immunoreactivity with a monoclonal antibody recognizing all ECE-1 isoforms. The ECE-1a antibody also showed nuclear immunoreactivity in both cell lines; this seemed to colocalize with nucleolin. The ECE-1b antibody showed nuclear immunoreactivity in EAhy926 cells, but no overlap with nucleolin was seen. Intracellular immunoreactivity was seen in both cell lines using the ECE-1c antibody; this showed some colocalization with concanavalin A (an endoplasmic reticulum marker). von Willebrand Factor was used as a marker for Weibel-Palade bodies in HUVECs, but no colocalization with ECE-1 was seen during this study. The data presented here sheds new light on the localization of ECE-1a, b, and c in cultured human endothelial cells, which may further understanding of the ET system and aid design of therapeutic ECE inhibitors. Exp Biol Med 231:718–722, 2006

Key words: endothelin-converting enzyme (ECE); isoform; localization; endothelial cells

Introduction

Endothelin-converting enzyme (ECE)-1 is a member of the M13 family of metalloproteases, and hydrolyzes inactive big endothelin (ET)-1 to vasoactive ET-1 (1). Although ECE-1 seems to be the physiologic enzyme responsible for this hydrolysis, a homolog, ECE-2, has also been identified (2). ET-1 is implicated in numerous disease states, including hypertension and atherosclerosis, renal disease, and many cancers, for example, prostate cancer (3, 4). In humans, ECE-1 can exist as one of four separate isoforms, ECE-1a, b, c, and d. These isoforms are generated from a single gene through alternative promoters and differ only in their amino-terminal cytoplasmic domains (5). The subcellular localization of these isoforms has been the subject of numerous investigations. Previously, it was shown that ECE-1a and ECE-1c were localized to the plasma membrane in Chinese hamster ovary (CHO) cells, whereas ECE-1b was localized intracellularly and colocalized with a trans-Golgi network marker (6). Subsequently, in transfected AtT-20 neuroendocrine cells, ECE-1b was localized to late endosomes and multivesicular bodies, and ECE-1d to recycling endosomes (7). Recent research has shown the presence of both ECE-1a and ET-1 in the nucleus of transfected CHO cells and microvascular endothelial cells (8). In addition, ECE-1 has been detected in endothelial cell–specific storage granules, known as Weibel-Palade bodies (9, 10). Weibel-Palade bodies contain the plasma glycoprotein von Willebrand factor (vWF), responsible for platelet adhesion and thrombus formation (11).

There is evidence that the amino-terminal sequence of ECE-1 is involved in intracellular targeting. ECE-1b contains two di-leucine–based motifs (LL and LV), which may be responsible for its intracellular localization; whereas ECE-1c expresses only one such motif (LV), which may explain its moderate cell-surface expression (12). ECE-1a does not contain di-leucine–based motifs, possibly explaining its plasma membrane expression. Muller *et al*. recently showed that ECE-1 isoforms can form heterodimers, and that the ECE-1b targeting signal is the dominant signal (7). Interaction of ECE-1b with ECE-1a or ECE-1c resulted in

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decreased plasma membrane expression, reduced extracellular activity, and increased expression of ECE-1a and ECE-1c in late endosomes or multivesicular bodies. ECE-1b contains a specific di-leucine motif ($L^{12}L^{13}$) that may be involved in targeting to the degradation pathway (7).

The aim of this study, therefore, was to determine gene and protein expression and subcellular localization of the ECE-1 isoforms in two human endothelial cell lines. This may be valuable in offering further insight into the complex ET system.

Materials and Methods

Materials. cDNA encoding human ECE-1b was kindly provided by Dr. O. Valdenaire (INSERM U460, Paris, France). Full-length human ECE-1a, c, and d DNA was amplified from EAhy926 RNA by one-step reverse transcriptase (RT) polymerase chain reaction (PCR) using an isoform-specific primer incorporating a *Not*I site and a common reverse primer containing an *Xba*I site. PCR products were inserted into pcDNA3 using the *Not*I and *Xba*I sites.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were obtained from TCS Cellworks (Buckingham, United Kingdom) and grown on 0.1% gelatin–coated flasks in supplemented endothelial cell growth medium (PromoCell, Heidelberg, Germany). EAhy926 cells were a kind gift from Dr. C. J. Edgell (University of North Carolina, Chapel Hill, NC) and grown in DMEM supplemented with 10% fetal bovine serum and 100 μ*M* hypoxanthine, 0.4 μ*M* aminopterin, and 16 μ*M* thymidine.

RT-PCR. Total RNA was isolated from cells using the Qiagen RNeasy kit according to the manufacturer's instructions. One microgram of RNA was subjected to one-step RT-PCR using the GeneSys RT-PCR kit (GeneSys Ltd., Sussex, United Kingdom). Isoform-specific primers were used as follows. ECE-1a sense: 5′-TGATGCCTCTCCAGGGCCTG-3′, antisense: 5′-GAAGAAGTCATGGCAGGGGTC-3′; ECE-1b sense: 5′-CCCTGCTGTCGGCGCTGGGG-3′, antisense: 5′-TGTGGGGTCCATGGAGCTCAAG-3′; ECE-1c sense: 5′-CGGAGCACGCGAGCTATGATG-3′, antisense: same as ECE-1a; ECE-1d sense: 5′-TGCATTTGGCCTTGCAGATGTC-3′, antisense: same as ECE-1b. ET_A receptor (R) sense: 5′-AGCTTCCTGGTTACCACTCATCAA-3′, antisense: 5′-TCAACATCTCACAAGTCATGAG-3′; ET_BR sense: 5′-CGAGCTGTTGCTTCTTGGAGTAG, antisense: 5′-ACGGAAGTTGTCATATCCGTGATC; ET-1 sense: 5′-CTCCTCCTTGATGGACAAGG-3′, antisense: 5′-CTTGATGCTGTTGCTGATGG-3′. GAPDH (used as a control) sense: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, antisense 5′-CATGTGGGCCATGAGGTCCACCAC-3′.

Antibodies. The ECE-1 monoclonal antibody was kindly provided by Dr. K. Tanzawa (Sankyo Research Laboratories, Tokyo, Japan). Polyclonal antibodies to ECE-1a, ECE-1b, and ECE-1c were targeted to specific amino-terminal sequences (13). The monoclonal antibody to nucleolin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal antibody to nucleoporin 62 was obtained from BD Biosciences (San Jose, CA). The polyclonal antibody to vWF was purchased from DakoCytomation (Cambridgeshire, United Kingdom). Fluorescent-conjugated secondary antibodies were obtained from Molecular Probes Inc. (Eugene, OR).

Immunofluorescence Microscopy. Cells were grown to confluence on coverslips and fixed with prewarmed 10% formalin in neutral buffered saline (Sigma, Poole, United Kingdom) for 5 mins, permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 mins, and fixed again for 5 mins. After washing with PBS, cells were incubated in NH_4Cl solution for 10 mins and washed again. Cells were incubated with 5% blocking serum for 30 mins. Primary antibodies (1:50 anti–ECE-1, 1:100 anti–ECE-1a, 1:50 anti–ECE-1b, or 1:100 anti–ECE-1c) were applied for 2 hrs at room temperature. Cells were incubated with fluorescence-conjugated secondary antibodies for 30 mins at room temperature. Slides were examined at high resolution using the DeltaVision Optical Restoration Microscopy System (Applied Precision Inc., Issaquah, WA). Negative controls (excluding primary antibody) showed no nonspecific immunoreactivity (results not shown).

Western Blot Analysis. Total cell lysates were made using buffer with 50 m*M* Tris-HCl, pH 8.0; 150 m*M* NaCl; 0.02% NaN_3; 0.1% sodium dodecyl sulfate (SDS); 1% Nonidet P40; and 0.5% sodium deoxycholate. Samples were analyzed on a 6% SDS–polyacrylamide gel electrophoresis (PAGE) gel and transferred to polyvinylidene difluoride membrane. Primary antibodies (1:500 anti–ECE-1, 1:200 anti–ECE-1a, 1:200 anti–ECE-1b, or 1:500 anti–ECE-1c) were applied overnight. Secondary antibodies were applied for 1 hr, and specific bands were detected using an enhanced chemiluminescence system (Amersham).

Results

ECE-1 Gene Expression. This investigation was carried out in primary HUVECs and a transformed human endothelial cell line (EAhy926). Previous investigations have shown that both cell lines express mRNA for ECE-1a, b, c, and d (5, 14). In addition, these cell lines express mRNA for ET-1 and ET_BR, but not for ET_AR. Figure 1 shows the expression of the ET system in both cell lines. Qualitatively, ECE-1d seemed to be the predominant isoenzyme in both cell lines, followed by ECE-1c, ECE-1a, and, finally, ECE-1b.

ECE-1 Protein Expression. Western blotting was used to investigate protein expression and revealed that both cell lines express ECE-1 protein, but EAhy926 cells express approximately 1.4-fold more ECE-1 than HUVECs (Fig. 2).

Isoform-specific antibodies were used for immunoblotting. Figure 2 shows that HUVECs express ECE-1a and c protein, whereas EAhy926 cells express ECE-1a, b, and c. By densitometry, EAhy926 cells express approximately 8-

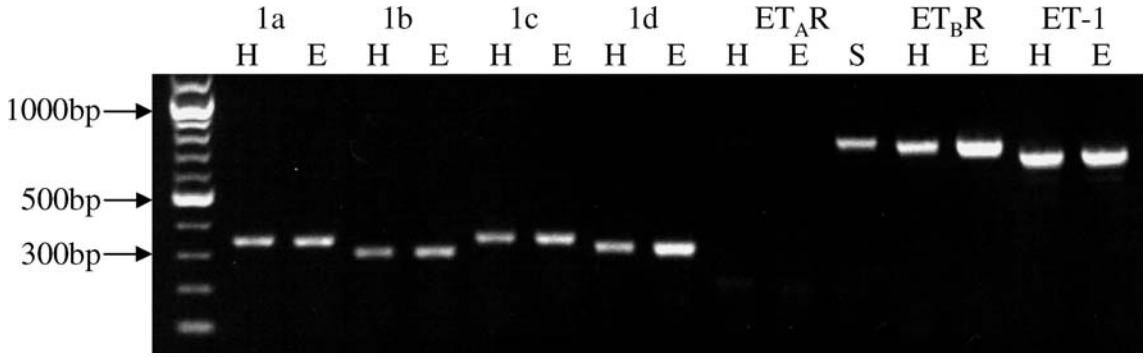

Figure 1. Expression of ET system mRNA in endothelial cell lines. One-step RT-PCR was performed on 1 μg of total RNA using isoform-specific primers and PCR products analyzed on a 1% agarose gel. H, HUVEC; E, EAhy926; S, saphenous vein smooth-muscle cell; 1a, ECE-1a; 1b, ECE-1b; 1c, ECE-1c; 1d, ECE-1d, ET_AR endothelin A receptor, ET_BR endothelin B receptor, endothelin-1.

fold more ECE-1c than ECE-1a, with barely detectable ECE-1b levels. HUVECs express approximately 13-fold more ECE-1c than ECE-1a, with no detectable ECE-1b. ECE-1d was not tested for due to lack of a specific antibody. A 75-kDa band was also detected in both cell lines using the ECE-1a antibody; Jafri *et al*. described this as a degradation of ECE-1 (8). CHO lysates were also analyzed, but showed no immunoreactivity to any antibody (data not shown).

ECE-1 Subcellular Localization. Immunofluorescence microscopy analysis was used to study ECE-1 localization. The monoclonal antibody recognizing all ECE isoforms showed nuclear and cytoplasmic immunoreactivity in both HUVECs and EAhy926 cells (Fig. 3).

Isoform-specific antibodies were used to determine which isoform was present in the nucleus. Both cell lines showed identical immunoreactivity patterns; results shown are for EAhy926 cells (Fig. 4). Specific markers were also used to determine subcellular localizations. The ECE-1a antibody showed nuclear immunoreactivity in both HUVECs and EAhy926 cells, and seemed to colocalize with nucleolin (a nucleolar protein). ECE-1b nuclear immunoreactivity was also seen in EAhy926 cells, but no colocalization with nucleolin or nucleoporin 62 (a nuclear pore complex protein) was observed. The ECE-1c antibody showed nuclear and perinuclear immunoreactivity in both cell lines, and some colocalization with concanavalin A (an endoplasmic reticulum marker) was seen. No colocalization with a trans-Golgi network marker (TGN46) or tubulin was seen (data not shown). Nuclear fractionation was also used to confirm the presence of nuclear ECE-1. The ECE-1 monoclonal antibody detected a band of 125-kDa in the nuclear fraction of EAhy926 cells; a band of the same size was also seen using the ECE-1a antibody (data not shown).

Double Labeling of ECE-1 and vWF. Colocalization of ECE-1 with von Willebrand Factor (vWF) was also studied. vWF is a plasma glycoprotein stored in Weibel-Palade bodies (endothelial cell-specific storage granules; Ref. 11). As such, vWF is commonly used as an endothelial cell marker (15). It has previously been reported that both ET-1 and ECE-1 are stored in Weibel-Palade bodies (16, 17). HUVECs and EAhy926 cells were, therefore, double-labeled with antibodies to vWF and ECE-1. EAhy926 cells express vWF but show few Weibel-Palade bodies, therefore, HUVECs were used for this experiment. HUVECs show typical Weibel-Palade body immunoreactivity, but no colocalization with ECE-1 was seen (Fig. 5).

Discussion

This study presents new data on the subcellular localizations of the ECE-1 isoforms. ECE-1a was shown to localize to the nucleolus in both HUVEC and EAhy926 endothelial cells; this is a novel finding, to our knowledge, and may represent a unique role for ECE. The nucleolus of the cell is the site of ribosomal RNA synthesis and

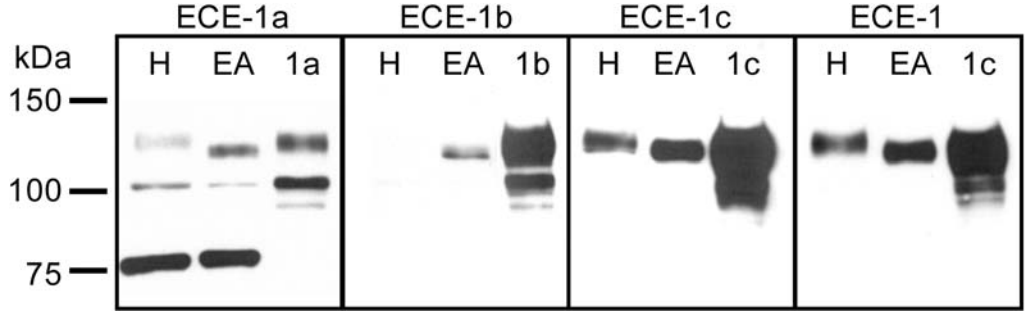

Figure 2. ECE-1 isoform expression in human endothelial cells. Samples were loaded on a 6% SDS-PAGE gel and analyzed using isoform-specific antibodies (ECE-1a, b, or c) or an ECE-1 monoclonal antibody recognizing all isoforms. ECE-1a and ECE-1b immunoblots contain 40 μg HUVEC and EAhy926 lysates; ECE-1c and monoclonal blots contain 20 μg lysates. Positive-control lysates were loaded at 10 μg per gel.

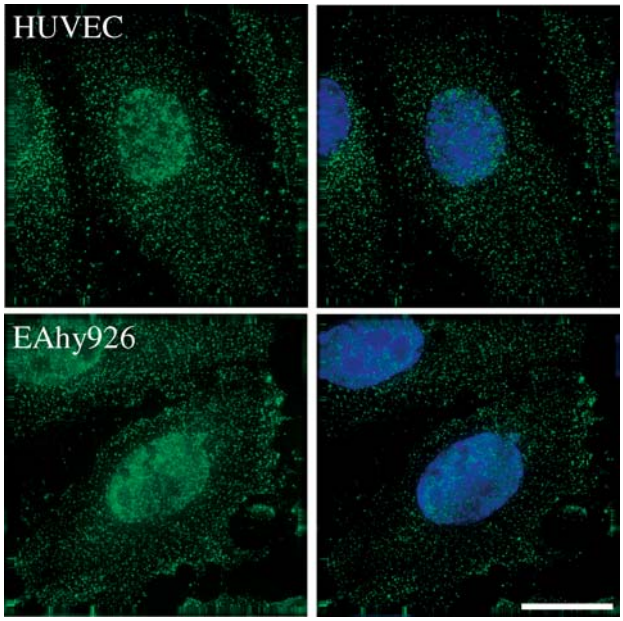

Figure 3. ECE-1 localization in HUVECs and EAhy926 endothelial cells. Cells were grown to confluence on coverslips and fixed for immunofluorescence microscopy analysis. ECE-1 was detected with a monoclonal antibody to ECE-1 that recognizes all isoforms; cells were counterstained with the nuclear stain, 4′,6-diamidino-2-phenyl indole (DAPI). Scale bar, 25 μm. (Color figures are available in the on-line version.)

biogenesis of ribosomal subunits (18). The nucleolus also sequesters regulatory complexes and is involved in regulation of cell cycle; this may highlight a novel role for ECE-1a in the cell cycle. A nuclear localization of ECE-1b was also seen in EAhy926 cells; this is previously unreported.

The existence of nuclear ECE-1 raises several important questions. As yet, it has not been determined whether nuclear ECE is functionally active, although functional ET-Rs on the nuclei of cardiac ventricular myocytes have been identified (19). This raises the possibility of a second site of activation of ET-Rs that may be part of a rapid autocrine response. The mechanism of targeting ECE-1 to the nucleus is also unknown; none of the isoforms contain a recognized nuclear localization signal, implying involvement of a chaperone(s).

Previous work has shown that ECE-1a and ECE-1c were localized to the plasma membrane in CHO cells, whereas ECE-1b colocalized with a trans-Golgi network marker (6). In this study, no plasma membrane immunoreactivity was seen with either ECE-1a or ECE-1c; similarly, no colocalization with the trans-Golgi network was seen for ECE-1b. This may be caused by differences in protein targeting between ECE-transfected cells and endogenously expressing cells. The ECE-1 isoforms may interact with specific binding partners or chaperones, which direct their subcellular localization, but such interactions might not take place in cells that do not express endogenous ECE, such as CHO cells. In addition, CHO cells may not express the chaperones involved in these interactions.

ECE-1 has also been detected in the Weibel-Palade

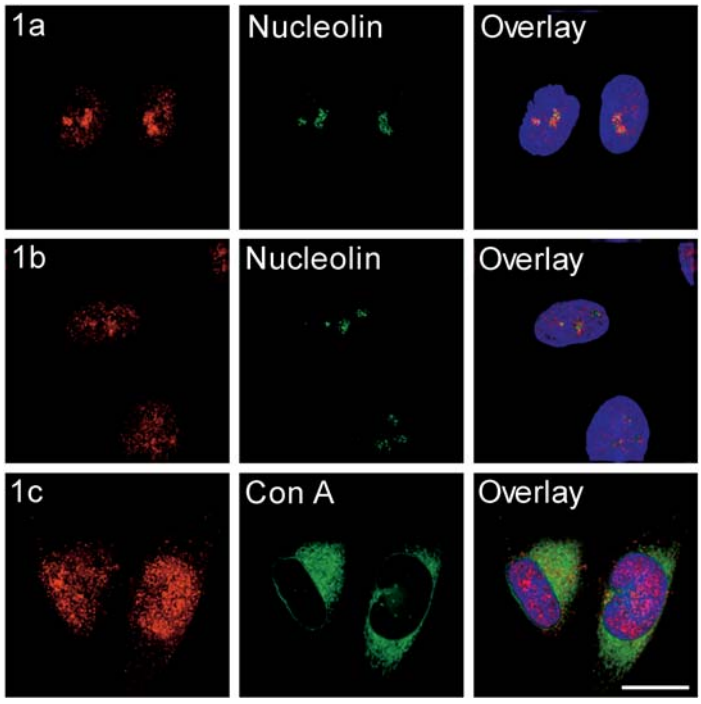

Figure 4. Localization of ECE-1 isoforms in EAhy926 cells. Cells were grown to confluence and fixed for immunofluorescence microscopy. ECE-1 isoforms were detected with specific antibodies targeted against the amino-terminal regions; organelle markers were also used. Cells were counterstained with DAPI. Scale bar, 25 μm. (Color figures are available in the on-line version.)

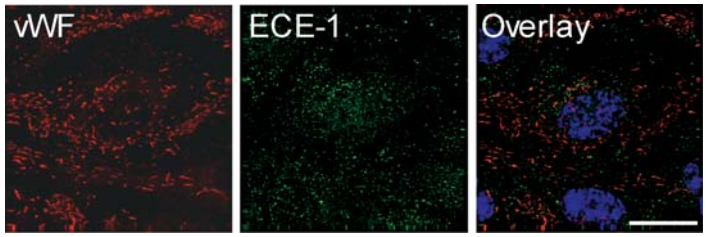

Figure 5. Localization of ECE-1 and vWF in HUVECs. Cells were grown to confluence on gelatin-coated coverslips and fixed. ECE-1 was detected with a monoclonal antibody recognizing all ECE isoforms; Weibel-Palade bodies were detected using an antibody to vWF. Cells were counterstained with DAPI. Scale bar, 25 μm. (Color figures available in the on-line version.)

body storage granules in HUVECs (9, 10). No such localization was seen during this study. However, the cells were cultured in slightly different media, which may have an effect on ECE-1 protein localization. This is an important consideration for future localization studies.

Although differing in only a short region of their sequence, the ECE-1 isoforms clearly show different expression patterns and subcellular localizations. These differences may be useful in providing further insight into the complex ET system, and may be of use for developing therapeutic inhibitors. Currently, several specific ECE inhibitors are being tested *in vitro* (20, 21), but no isoform-specific inhibitors are available. Further investigations are needed to determine the function of the four isoforms because their different expression patterns and subcellular localizations may represent contrasting roles within the cell. Inhibition of all four isoforms may, therefore, not be desirable. Inhibitor molecules could be targeted more effectively to specific ECE isoforms once the subcellular localizations were conclusively determined.

To summarize, we studied the gene and protein expression and subcellular localization of the ECE-1 isoforms in human endothelial cells. A novel nuclear localization of ECE-1b and a nucleolar localization for ECE-1a in human endothelial cells was demonstrated.

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