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EXPRESSION OF THE ENDOTHELIN-CONVERTING ENZYME GENE IN HUMAN TISSUES

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Summary: Based on the cDNA sequence of the human and bovine endothelin converting enzyme (ECE-1) we have developed a novel reverse transcription polymerase chain reaction to investigate the tissue expression of this gene. We were able to specifically detect the gene mRNA starting from very limited amount of tissue in all human as well as bovine tissues examined. Thus, our results confirm a widespread expression of the ECE-1 gene in human tissues, in keeping with the findings in other species, and suggest a major biological role of ECE-1. © 1995 Academic Press, Inc.

Endothelin (ET) -1, the most potent vasoconstrictor known as yet identified by Yanagisawa et al. (1), is the prototype of a family of peptides with multiple biological actions. Its participation in numerous diseases, including arterial hypertension, coronary artery disease, pulmonary hypertension, acute renal failure and subarachnoid haemorrhage, has been suggested (see for a review, 2). More recently, a crucial role of ET-1 in the normal development of tissues derived from the first branchial arch as well as of the Auerbach and Meissner intestinal plexus has been shown (3,4). In humans the conversion of the inactive 38 aminoacid residues precursor pro-ET-1 (big endothelin-1) to the active 21 aminoacid residues peptide is brought about by the endothelin-converting enzyme (ECE-1), a neutral phosphoramidon-sensitive, membrane-bound metalloprotease, that cleaves big-ET-1 at Trp-21-Val-22, intracellularly (5-7). Recently the ECE-1 has been characterised biochemically from rat lung microsomes (8,9), porcine and bovine endothelial cells and tissues (10,11) and human umbilical veins (12). Furthermore, the cDNA of ECE-1 has been identified, cloned and functionally expressed from rat (13,14), bovine (11) and human endothelial cells (15). Although this allowed to investigate ECE-1 gene expression in rat and bovine tissues (11,13,14), only limited data are available in humans as yet (15). Thus, based on the sequence of human ECE-1 cDNA in this study we have developed a reverse transcription polymerase chain reaction (RT-PCR) method to investigate the expression of the ECE-1

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gene in different human tissues starting from very limited amounts of tissue from biopsy and surgical specimen.

MATERIALS AND METHODS

Collection of tissues. Human tissues were collected from consenting patients undergoing surgery and snap frozen in liquid nitrogen in the operating room; they were thereafter maintained at - 195 °C until used for RNA extraction. Bovine tissues, obtained at a local slaughterhouse, were collected immediately after excision and frozen with an identical procedure. Total RNA was extracted starting from 10-250 mg of tissue after homogenisation by the guanidium isothiocyanate methods. After isolation, RNA was checked for integrity by gel electrophoresis and UV absorbance as reported (16); concentrations of total RNA were then calculated by spectrophotometric measurements at 260 nm wavelength.

Polymerase chain reaction. For use in the polymerase chain reaction, total RNA was reversely transcribed to cDNA with the Random Hexamers (2.5 μM) and 2.5 U of cloned Moloney Murine Leukemia Virus reverse transcriptase (M-MLV-RT; GeneAmp RNA PCR Core Kit, Perkin Elmer, Norwalk, Ct, USA), as previously reported in detail (16). After incubation at 42 °C for 15 min, temperature was raised to 99 ° C for 5 min and then reaction mixture tubes were quickly chilled on ice. For amplification of the resulting cDNA, 20 μl of the reverse transcription mixture were used. The sample volume was increased to 100 μl with a solution containing 50 mM KCl, 10 mM Tris (pH 8.3), 2 mM $MgCl_2$, and 0.2 μM of up- and downstream primers as well as 2.5 U of Taq polymerase (AmpliTaq DNA Polymerase, Perkin Elmer/Cetus). The thermal profile used in a Delphi Thermal Cycler (Oracle Biosystem) included a denaturation step at 94 °C for 1 min, annealing at 59 ° C for 1 min and extension step at 72 °C for 1 min for a total of 40 cycles. An additional extension step at the temperature of 72 °C for 7 min was then carried out. Based on the sequence of human and bovine ECE-1 cDNA (15) the following two primers were designed, using the software OLIGO (OLIGO 4.1, Medprobe, Oslo, Norway) licensed to our institution. The 5' (upstream) primer consisted of 20 bp (494-513: AGC GTG AGC GAG GCA GAG AG) and the 3' (downstream) primer of 20 bp (1041-1060: GGG GTA GAA GAT GGT GTT GA). To rule out the possibility of genomic DNA amplification, in some experiments the PCR was performed without prior reverse transcription of the RNA. As a positive control, amplification of a 838 bp fragment of the human β-actin gene was carried out in parallel, as already reported (16).

Restriction analysis of the amplification product. The amplification products were digested for 120 min at 37 ° C with AvaII, AccI, EcoRI, FokI, HpaII, MboII, PstI and PvuII, according to the specifications of the manufacturer (Boheringer Mannheim, Milano, Italy).

RESULTS AND DISCUSSION

Tissue distribution of ECE-1 mRNA. The RT-PCR allowed the detection of ECE-1 mRNA in several human tissues as shown in Figure 1. Stronger bands of amplification product were seen in the normal adrenal gland, pheochromocytoma tissue, renal cortex, parathyroid gland and lung. However, evident bands were seen also in a number of arterial wall specimen including abdominal aorta, common carotid artery and gastric artery.

Specificity of the amplification product. The specificity of the amplification product was confirmed by three sets of evidence. First, we did not see any amplification band when PCR was performed from the same RNA samples, but without prior reverse transcription, thereby ruling out the possibility of genomic DNA amplification. Second, since the homology of human and bovine cDNA in the primers binding sequences was 100 % our primers should function equally well from either species cDNA; furthermore, the predicted size of the amplification product obtained from both bovine and human tissue should be the same. Both these predictions were confirmed by the experimental results, as shown by size fractionation electrophoresis in Figure 2. Third, the results of restriction analysis with all the eight different enzymes used gave results consistent with the published sequence of the human cDNA (Figure 2 and data not shown). Finally, we found

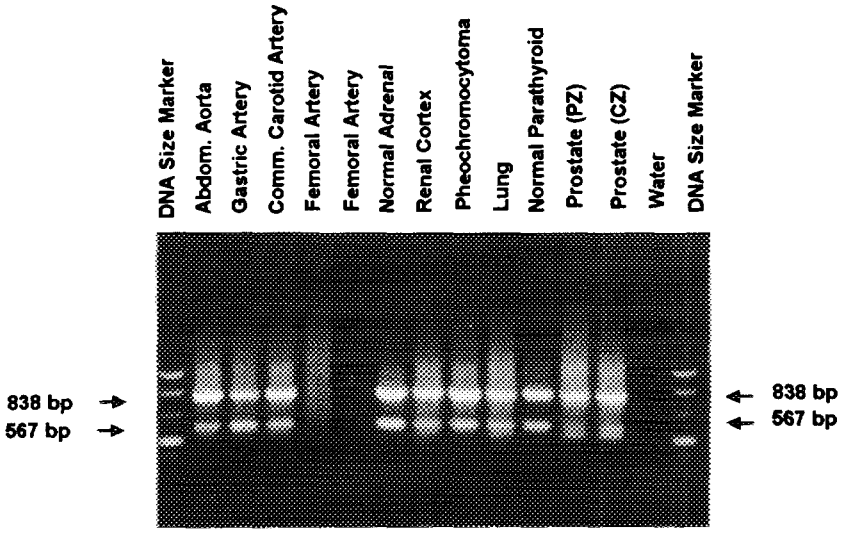

Fig. 1. Ethidium bromide stained 1.5 % agarose gel showing cDNA amplified with ECE-1 specific primers from different human tissues and cell types. Lane one was loaded with 200 ng of a size marker (PhiX174 Hae III). The amplified fragments resulted to be of the expected size, which was 567 bp. Amplification of a 838 bp fragment of the ß actin cDNA, as a positive control, is also shown.

a pattern of ECE-1 gene expression in different human tissues similar to that found in bovine specimen by Xu et al. (11).

Our results demonstrate for the first time the possibility of investigating the tissue expression of human ECE-1 by reverse transcription PCR. The data obtained confirm a widespread expression of ECE-1 in different tissues, including arterial wall of different beds, adrenal gland, renal cortex, lung, parathyroid gland and prostate, in keeping with what has been observed in other species such as rat and bovine (11,13,14). Of interest, we have previously detected with autoradiography both endothelin receptors A and B in the normal human adrenal cortex (16); we have also reported the gene expression of ET-1 and of both its receptor subtypes in the same tissue and in aldosterone-producing adenomas (17). The fact that the ECE-1 gene is coexpressed in the adrenal gland and in phechromocytoma tissue is consistent with the possibility of local synthesis of mature ET-1 and therefore of autocrine-paracrine effects of the peptide.

In this study, by using reverse transcription PCR, the tissue expression of the ECE-1 gene was investigated starting from very limited amounts of tissue, such as those available from biopsies or small surgical specimen, thereby demonstrating the possibility of using this methodology for human studies. Although a number of caveats must be considered (16), the possibility of quantifying the ECE-1 gene expression and of comparing different tissues and/or tissues with different diseases with this technique is also suggested.

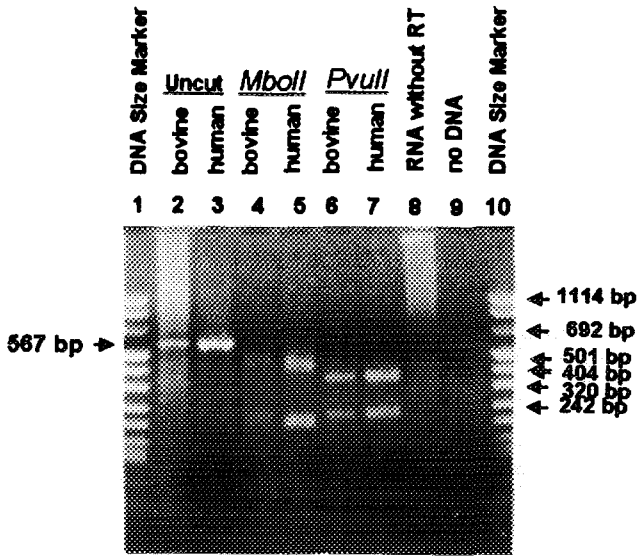

Fig. 2. Ethidium bromide stained 3 % agarose gel showing cDNA amplified with ECE-1 specific primers from bovine and human tissues. Lanes 1 and 10 were loaded with 200 ng of a size marker (PhiX174 Hae III). Lanes 2 and 3 show the identical size of the amplification product obtained from adrenal gland RNA of both species. Lanes 4-7 show the restriction pattern of both species, which was similar for MboII and identical for PvuII; in both cases it corresponded to the predicted pattern based on the published cDNAs sequence of each species (15). The specificity of the amplified fragments is further confirmed by the lack of amplification from both RNA without prior reverse transcription (lane 8) and no DNA (water, lane 9).

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