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One of the endothelin gene family, endothelin 3 gene, is expressed in the placenta

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A cDNA encoding human endothelin 3 (ET-3) precursor was cloned from a cDNA library from the placenta, and its nucleotide and deduced amino acid sequences were determined. This ET-3 cDNA was found to contain 2.3 kb pairs and encode prepro-ET-3 protein consisting of 224 amino acid residues. The putative big-ET-3 seems to consist of 42 amino acid residues. Two of the intron insertion sites were determined with information from nucleotide sequences of the cloned genomic ET-3 gene. This is the first direct evidence that the ET-3 gene is transcribed and expressed in the placenta.

Human endothelin 3; cDNA cloning; Gene expression; (Human placenta, COS-7 cells)

1. INTRODUCTION

A novel vasoconstrictor peptide, endothelin, was purified from the culture medium of porcine aortic endothelial cells and its cDNA was cloned [1]. We have already cloned human cDNA encoding prepro-endothelin from the cDNA library of the placenta and identified the 21 amino acid sequences of human and porcine endothelin [2]. Genomic DNA studies on endothelin have revealed that there is an endothelin gene family comprising 3 types of endothelin, i.e. endothelin 1, 2 and 3 (ET-1, -2 and -3) [3]. ET-1, the first to be found, exists in many organs, such as the lung, kidney and brain, and has various biological functions including vasoconstriction [4]. As for ET-2 and ET-3, the functions except for vasoconstriction have not been fully examined and the structures of their precursors, which are needed to understand the process of biosynthesis have not been elucidated. The presence of ET-2 and ET-3 in vivo was not proven until precise tissue distribution studies of ETs could be done with the combination of a highly sensitive enzyme immunoassay (EIA) of ETs and reverse phase high performance liquid chromatography (RP-HPLC) [5].

Particularly interesting is the distribution in rats of immunoreactive ET-3, which is abundant in the brain and intestine, and seems to be a brain-gut peptide [5]. The precise functions of this peptide remain unknown.

In the present study, we found that ET-3 also exists in the placenta, like ET-1, the presence of which was confirmed with ET-1-specific EIA. We tried to find direct evidence for ET-3 gene expression by cloning the cDNA encoding the precursor of ET-3 from the cDNA library of human placenta and succeeded in elucidating its structure. Based on structural comparison between the precursor of ET-1 and ET-3, the common processing steps of ETs from the precursors could be explained.

Here we present the first report on the nucleotide and the predicted amino acid sequences of human prepro-ET-3.

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes, T4 DNA ligase and *Xho*I linker, were obtained from New England Biolabs (Beverly, MA, USA) and Nippon Gene (Tokyo, Japan). Exonuclease III and VII were from Takara Shuzo (Kyoto, Japan) and Bethesda Research Labs. [α-^{32}P]dCTP was obtained from Amersham Co. DNA modification, ligation, and transformation were carried out as described by Maniatis et al. [6].

2.2. *Enzyme immunoassay of endothelins and RP-HPLC*

Preparation of the tissue extracts of the rat placenta (1.9 g wet weight) and their partial characterization with RP-HPLC (TSK ODS-80 column, 4.6 × 250 mm, Tosoh, Japan) were conducted as described elsewhere [5]. Rat placentas were obtained from 18-day pregnant Sprague-Dawley (SD) rats after bleeding under anesthesia. Enzyme immunoassay for ET-1(ET-2) and ET-3 was done under the protocol described elsewhere [7].

2.3. *Cloning and sequencing analysis of endothelin 3 cDNA*

A human placenta cDNA library constructed in λgt11 was obtained from Clontech Laboratories Inc. The cDNA library containing about 1.8×10^6 clones was screened with ^{32}P-labeled 650 bp genomic ET-3 DNA fragment previously cloned (unpublished results) and synthetic 33mer oligonucleotides corresponding to the 11 amino acids located upstream of the N-terminal amino acid (Cys) of ET-3 predicted from

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the cloned genomic ET-3 DNA [3]. Plaque hybridization was conducted at 58°C for 16 h and washed with 2 × SSC (saline sodium citrate solution; 1 × SSC = sodium chloride, 8.765 g/l, sodium citrate, 4.41 g/l) buffer at 56°C [8]. The cloned cDNA from the hybridization positive phage was subcloned into the plasmid pUC118/119. Then various clones containing the 3'- or 5'-deleted plasmid were used to prepare single stranded DNAs for DNA sequencing by the dideoxynucleotide chain termination method with [α-^{32}P]dCTP [9,10]. The nucleotide sequences were also determined with an automatic DNA sequencer, ABI Model 370 A (Applied Biosystems Inc.) as described previously [11].

2.4. *Construction of expression plasmid and cell transformation*

Plasmid pHET-3(P) was digested with restriction enzyme, *Eco*RI, and a 2.3 kb cDNA fragment was obtained. After filling the single-stranded portion with the T4 DNA polymerase reaction, the *Xho*I linker, 5'-pd(CCTCGAGG)-3' was ligated and the *Xho*I fragment containing the whole coding region of ET-3 precursor was inserted into the *Xho*I site of pSVL [12] to obtain plasmid pTS 6009. DNA transfection to COS-7 cells was performed as described previously [13].

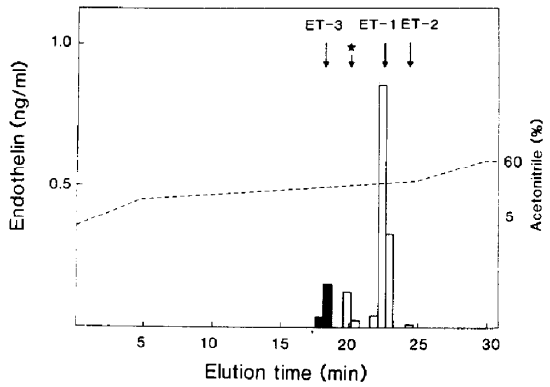

Fig.1. RP-HPLC profiles of the extract of the rat placenta. Each fraction was concentrated by lyophilization and subjected to EIA specific for ET-3 (solid bar) and ET-1 and for ET-2 (open bar). The star marked position is the Met-sulfoxide form of ET-1.

3. RESULTS AND DISCUSSION

3.1. *Detection of immunoreactive ET-3 in the rat placenta*

The extracts were assayed with ET-1- (ET-2-) and ET-3-specific enzyme immunoassays as described previously.

The sensitivity of EIA for ET-1 (ET-2) and ET-3 is 0.2 pg/well, which is enough to detect ET-1 (ET-2) or ET-3 in the blood or for the preparation of tissue extracts. The crossreactivity of the ET-3 EIA was less than 0.1% with other ETs [14,15]. The RP-HPLC profiles of immunoreactive ET-1, ET-2 and ET-3 are shown in fig.1, with arrows indicating the fractions where the chemically synthesized ET-1, ET-2 and ET-3 were eluted. The results showed that the immunoreative ET-3 was clearly separated from ET-1. ET-2, and the content of ET-3 in the rat placenta was estimated as one-tenth that of ET-1. ET-2 was present in only a small amount. In the case of human placenta, the content of ET-3 was estimated as 4.2 ± 1.2 pg/g wet tissue (n = 6) and about 20-fold ET-1 was detected (unpublished results). As we could clone the ET-1 cDNA from a cDNA library of human placenta, the existence of ET-1 in the placenta was confirmed here by EIA. Precise studies on the tissue distribution of ETs, especially ET-3 have been reported elsewhere [5].

Our observations motivated us to clone the human ET-3 cDNA encoding the whole precursor from a cDNA library derived from human placenta and to try to explain how the processing steps of ET-3 might occur from the big-ET-3 form, if exists in the precursor protein.

3.2. *Cloning and sequencing of ET-3 cDNA*

In the first hybridization screening test with the ^{32}P-labeled 650 bp probe, 26 positive clones were obtained. As this probe had consensus sequences encoding 14 amino acid residues, Asp^{8}–Trp^{21}, of endothelins, in the second and third screening ^{32}P-labeled ET-3-specific 33-mer oligonucleotide was used. Finally, one positive clone named λHET-3(P) (human endothelin 3 cDNA derived from the placenta) was obtained. The purified phage DNA of λHET-3(P) was treated with *Eco*RI and the cDNA insert was subcloned into a plasmid pUC 118 named pHET-3(P). The partial restriction enzyme map and the sequencing strategy are shown in fig.2. The total sequenced nucleotides of pHET-3(P) cDNA were 2299 bp, which encode the prepro-ET-3 protein consisting of 224 amino acid residues (fig.3). The molecular mass of the prepro-ET-3 was calculated as 23946 Da. The amino acid sequence of the mature ET-3, Cys^{97}–Trp^{117}, is boxed in the figure. Like the big-ET-1, which is known to exist as an intermediate form in the post-translational processing of prepro-ET-1 [15,16], a similar big form, big-ET-3 was found in prepro-ET-3. Big-ET-3 was found to consist of 42 amino acid residues (Cys^{97}–Gly^{138}) and cleaving probably occurs at the dibasic amino acid pairs Arg^{95}–Arg^{96} and Lys^{139}–Arg^{140}. The N-terminal 16 amino acids of prepro-ET-3 may be the signal sequence, which can be predicted by the method of von Heijne [16]. In the nucleotide sequences encoding this prepro-ET-3, there are 24 bp direct repeats correspon-

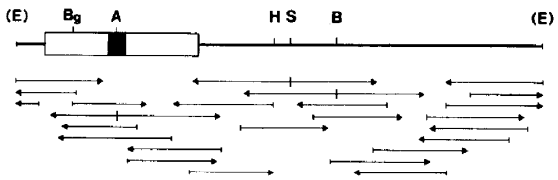

Fig.2. Restriction enzyme map and sequencing strategy for the cDNA insert in the plasmid pHET-3(P). The coding nucleotide sequences for prepro-ET-3 and mature ET-3 are indicated by open and closed boxes. The arrows indicate the direction of sequencing and location and the length of the sequenced regions. Representative restriction enzyme sites: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; S, *Sph*I; and E, linker *Eco*RI.

ding to 8 amino acid residues of Glu^{48}-Thr-Val-Ala-Gly-Pro-Gly-Glu^{55} and Glu^{56}–Glu^{63}. These sequences were also found in the cloned genomic ET-3 DNA.

The splicing acceptor site, the 5′-end of the exon containing mature ET-3, was revealed to be GA, the nucleotide and the amino acid number is 162–163, the

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   1  CGAACCCCCACAGCTGGAGGGCGAGGCCAGCTGTACCCGGCCCCAGTGCCCTTTCGCGGCCACAAGCGGCCGTCCTCCT   79

  80  GGTCCGGTGCTCCGGCGCCTGATCTAGGTTC ATG GAG CCG GGG CTG TGG CTC CTT TTC GGG CTC ACA   146
   1                                  Met Glu Pro Gly Leu Trp Leu Leu Phe Gly Leu Thr    12

 147  GTG ACC TCC GCC GCA GGA TTC GTG CCT TGC TCC CAG TCT GGG GAT GCT GGC AGG CGC GGC   206
  13  Val Thr Ser Ala Ala Gly Phe Val Pro Cys Ser Gln Ser Gly Asp Ala Gly Arg Arg Gly    32

 207  GTG TCC CAG GCC CCC ACT GCA GCC AGA TCT GAG GGG GAC TGT GAA GAG ACT GTG GCT GGC   266
  33  Val Ser Gln Ala Pro Thr Ala Ala Arg Ser Glu Gly Asp Cys Glu Glu Thr Val Ala Gly    52

 267  CCT GGC GAG GAG ACT GTG GCT GGC CCT GGC GAG GGG ACT GTG GCC CCG ACA GCA CTG CAG   326
  53  Pro Gly Glu Glu Thr Val Ala Gly Pro Gly Glu Gly Thr Val Ala Pro Thr Ala Leu Gln    72

 327  GGT CCA AGC CCT GGA AGC CCT GGG CAG GAG CAG GCG GCC GAG GGG GCC CCT GAG CAC CAC   386
  73  Gly Pro Ser Pro Gly Ser Pro Gly Gln Glu Gln Ala Ala Glu Gly Ala Pro Glu His His    92

 387  CGA TCC AGG CGC TGC ACG TGC TTC ACC TAC AAG GAC AAG GAG TGT GTC TAC TAT TGC CAC   446
  93  Arg Ser Arg Arg Cys Thr Cys Phe Thr Tyr Lys Asp Lys Glu Cys Val Tyr Tyr Cys His   112

 447  CTG GAC ATC ATT TGG ATC AAC ACT CCC GAA CAG ACG GTG CCC TAT GGA CTG TCC AAC TAC   506
 113  Leu Asp Ile Ile Trp Ile Asn Thr Pro Glu Gln Thr Val Pro Tyr Gly Leu Ser Asn Tyr   132

 507  AGA GGA AGC TTC CGG GGC AAG AGG TCT GCG GGG CCA CTT CCA GGG AAT CTG CAG CTC TCA   566
 133  Arg Gly Ser Phe Arg Gly Lys Arg Ser Ala Gly Pro Leu Pro Gly Asn Leu Gln Leu Ser   152

 567  CAT CGG CCA CAC TTG CGC TGC GCT TGT GTG GGG AGA TAT GAC AAG GCC TGC CTG CAC TTT   626
 153  His Arg Pro His Leu Arg Cys Ala Cys Val Gly Arg Tyr Asp Lys Ala Cys Leu His Phe   172

 627  TGC ACC CAA ACT CTG GAC GTC AGC AGA CAG GTT GAA GTC AAG GAC CAA CAA AGC AAG CAG   686
 173  Cys Thr Gln Thr Leu Asp Val Ser Arg Gln Val Glu Val Lys Asp Gln Gln Ser Lys Gln   192

 687  GCT TTA GAC CTC CAC CAT CCA AAG CTC ATG CCC GGC AGT GGA CTC GCC CTC GCT CCA TCT   746
 193  Ala Leu Asp Leu His His Pro Lys Leu Met Pro Gly Ser Gly Leu Ala Leu Ala Pro Ser   212

 747  ACC TGC CCC CGC TGC CTC TTT CAG GAA GGA GCC CCT TAG GAGGACAGGCCTGCAGCATCCTGGTCTC   813
 213  Thr Cys Pro Arg Cys Leu Phe Gln Glu Gly Ala Pro ***                               225

 814  GGGAGGCTTCTGTCATTGCTCACACACAGTTCAGATTTCCACCTCTTTATAGACAAGAAGTGAATTTGCCTGGGGCAGAA   893

 894  CACCCACCCAAAGAGTCCCCACTTAACAATACCCCCCCCCCACGGCAAGAATGCCCAAATCCGAATGACCCCAGTTTTCC   973

 974  TAATGAGTAAAATGATCCCAGATGTGCCCCAGAGCATGACGCCTGCAGCTCCGGTTTCATGCAGGAAATTGGTTTTGGAG  1053

1054  AGTTTTGGCAAGTTGGAAAGCCACTTACTGGCTTTTGACATGACTTCTCTTGGAGAATAAGTGGACTCCAAGCTAACTCT  1133

1134  TTGCAAATGTAAACACATGTCCATCTTGTAATAAATGCAAAATGCCCGTGCAGCAGAAGCATGCGACTTTCATATCCTTG  1213

1214  CCTAGAATAGGCTGCATGGTGTATGTCAGTGAGGGCCACGAGGCGTCGGCTTTAGACACAGATCATAGCTCTACAGGAGT  1293

1294  TTATGAATTTGAAGCTTATGGGATTTTGGCAGAGAAATTTTCAGCTGTGCTTGATACCCACCAAAAGAATGTATCTCGAA  1373

1374  AGAATGAAGGAAGAAGAAAAAAAGGATCCTTGATGTTTGTGACAAGAAAATGAGAAAGTTAGTATCTGCAATACAGAGCTT  1453

1454  GTTCCTGTTCAGTGACTGACCCTCTGTATTCTGTATAGACACCAGGCCGATACACAGTGGAGTTCCCAGGCCTTGTTTGC  1533

1534  AGGAAGCCGACTGTAAAGACAGCCCCAGCTCAAGGCTATTAGGTTGAATATTTGCTTTCATGAGTAAATGTGGATCTTTG  1613

1614  GGGAATGGCTTCAAAATAAGTCACGAACACAAATTCTTTGTAAATTATGTAAATTCCTGTTTATATAAATTGGCAACAAC  1693

1694  TTATACCGTCTGACAGTTCAAAATCTCTTTCAGCTGCGCTCTTCCCACCGAGCCGAGCTTACTGTGAGTGTGGAGATGTT  1773

1774  ATCCCACCATGTAAAGTCGCCTGCGCAGGGGAGGGCTGCCCATCTCCCCAACCCAGTCACAGAGAGATAGGAAACGGCAT  1853

1854  TTGAGTGGGTGTCCAGGGCCCCGTAGAGAGACATTTAAGATGGTGTATGACAGAGCATTGGCCTTGACCAAATGTTAAAT  1933

1934  CCTCTGTGTGTATTTCATAAGTTATTACAGGTATAAAAGTGATGACCTATCATGAGGAAATGAAAGTGGCTGATTTGCTG  2013

2014  GTAGGATTTTGTACAGTTTAGAGAAGCGATTATTTATTGTGAAACTGTTCTCCACTCCAACTCCTTTATGTGGATCTGTT  2093

2094  CAAAGTAGTCACTGTATATACGTATAGAGAGGTAGATAGGTAGGTAGATTTTAAATTGCATTCTGAATACAAACTCATAC  2173

2174  TCCTTAGAGCTTGAATTACATTTTTAAAATGCATATGTGCTGTTTGGCACCGTGGCAAGATGGTATCAGAGAGAAACCCA  2253

2254  TCAATTGCTCAAATACTCAGAAAGTACTGTCAAAAGCCTAATAAAA                                    2299
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Fig.3. Nucleotide sequence and predicted amino acid sequences of the human prepro-endothelin 3. The amino acid and nucleotide sequences of mature ET-3 are boxed. The repeated sequence of the 8 amino acid residues and the cysteine-rich, endothelin-like sequence are underlined with a single line with an arrowhead and a doubleline, respectively. Intron insertion sites are indicated by arrowheads. The cleavage site of the signal sequences is indicated by an arrow.

18th in this cloned cDNA, because the consensus sequence of (T or C) > 11 N(C or T)AG is located adjacent to this GA sequence in the cloned human genomic ET-3 DNA (unpublished results). The splicing donor site, the 3′-terminus of this exon, is GA of the 5th amino acid, Glu after the C-terminal amino acid Trp of ET-3(3). This is based on the fact that the ET-3 genomic DNA has a consensus sequence of a 5′-intron junction, GTAAGT, which was also confirmed by us, and the intron was not found in this cDNA.

It is of particular interest that endothelin like sequence, CysXCysXXXXXXXCysXXXCys, such a cysteine-rich sequence with cysteine residues at the 1, 3, 11 and 15 positions was found to be conserved in the prepro-ET-3. This amino acid sequence has been found in prepro-ET-1 at 35 or 36 amino acids downstream from the C-terminal of human and porcine ET-1(1.2). However, the meaning of this conserved sequence in both precursors of ET-1 and ET-3 remains a question.

3.3. *Expression of ET-3 in COS-7 cells*

When the ET-3 expression plasmid, pTS 6009, was introduced into the COS-7 cells, immunoreactive ET-3 was detected in the culture medium. The ET-3 content produced by COS-7 cells was 30 ± 2 pg/ml culture medium at 72 h after DNA transfection. The COS-7 cells themselves produced immunoreactive ET-1(ET-2) as described before [17]; the production of ET-3 may be inhibited in the processing from the precursor. The production of ET-3 by the COS-7 cells was not so high; however, we were able to confirm that the transcription, translation of this cloned ET-3 cDNA and the secretion of the mature form of ET-3 had proceeded.

Though endothelin was first recognized as a vasoconstrictor peptide, many other biological functions have since been found; for example, it can increase Ca^{2+} in the cells, acts as a growth factor [18], and can inhibit renin release [20].

The biological relationships among ET-1, ET-2 and ET-3 should soon be elucidated, and the significance of the expression of ET-1 and ET-3 in the placenta should be revealed. The abundance of ET-3 in the brain should serve as a key to understanding the functions of the brain. Our presentation of direct evidence of ET-3 gene expression should contribute to further understanding of endothelins and how they function.

While this manuscript was in preparation, cDNA cloning of human ET-3 from cDNA library of the hypothalamus was presented by K.D. Bloch et al. [J. Biol. Chem. (1989) 264, 18156–18161]. However, the precursor was reported to consist of 238 residues whereas the one we derived from placenta has 224 residues. Also, we found a 220 bp insertion in the 3′-noncoding region of ET-3 cDNA from the placenta.

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