Expression of endothelin precursor genes in human trophoblast in culture

Brigitte Robert, André Malassiné, Christelle Bourgeois, Thérèse-Marie Mignot, Laurent Cronier, Françoise Ferré and Paulette Duc-Goiran

INSERM U.361, Université René Descartes, Maternité Baudelocque, Paris, France; CNRS URA 1869, UFR, Poitiers, France


We have shown previously the presence of immunoreactive endothelin in cultured trophoblastic cells from human term placenta as well as in the trophoblast-conditioned medium. To confirm whether or not the differentiated syncytiotrophoblast is a site for endothelin synthesis, we investigated, by reverse transcription and polymerase chain reaction, the expression of the three preproendothelin genes in 3-day cultured trophoblast. While no endothelin-2 precursor mRNA was detected, preproendothelin-1 mRNA was found to be expressed by the trophoblast. The endothelin-3 precursor gene was also expressed, but at low level and it was detected only after Southern blotting and oligonucleotide hybridization. The ability of trophoblast in culture to express the endothelin precursor genes supports the idea that, in human term placenta, villous syncytiotrophoblast that lines the intervillous space containing maternal blood acts as an endothelial layer.

B Robert, U.361 INSERM, Maternité Baudelocque, 123 Bld de Port-Royal, 75014 Paris, France

Endothelins (ETs) consist of a group of three isopeptides, ET-1, ET-2 and ET-3. Initially, ET-1 was described in vascular endothelial cells and was identified as a potent vaso-active agent. This peptide was found subsequently in various tissues and was reported to have many effects on both vascular and non-vascular target sites, including endocrine organs. Southern blot analysis of human genomic DNA indicates that three separate genes encode the three ET isopeptides (1). Cloning of cDNAs encoding human preproET-1 (2), preproET-2 (3) and preproET-3 (4, 5) led to the elucidation of the amino acid sequence of the ET precursors (preproETs). These precursor proteins are post-translationally processed into biologically inactive intermediate forms (bigETs) and finally into mature peptides (ETs).

In normal pregnancy, immunoreactive endothelin (irET) levels, reported in umbilical cord plasma and amniotic fluid, were higher than maternal plasma ET concentrations (6). In the human term placenta, ET-1, big ET-1, ET-3 and a very small amount of ET-2 were identified (7). Endothelin was detected in situ by immunocytochemistry in the syncytiotrophoblast layer of placental villi, as well as in the endothelium of feto-placental vessels and in the extravillous cytotrophoblast of the basal and chorionic plates (8). Similarly, when trophoblast was cultured, irET was detected by immunocytochemistry; the medium also contained irET that had been released (8, 9). To ascertain the potential of human trophoblast to express the three ET precursor genes, we investigated preproET-specific mRNA expression, using reverse transcription-polymerase chain reaction (RT-PCR) in cultured syncytiotrophoblast from human term placenta.

Materials and methods

Placenta

Human placenta were obtained aseptically, immediately after elective caesarean section from healthy mothers, in the 39th week of pregnancy. The caesarean section, carried out before the onset of labour, was performed because of earlier diagnosed cephalopelvic disproportion.

Trophoblast cell culture

Cytotrophoblastic cells were isolated according to Kliman et al. (10), with slight modifications (8). Briefly, after trypsin-DNase digestions, followed by Percoll gradient purification, the isolated cells were transferred to culture dishes coated with the monoclonal antibody W6-32 HL (Sera Lab, Crawley, UK) directed against HLA-A, -B and -C monomorphic antigens. After 15 min at 37°C, non-adherent villous cytotrophoblasts known to be negative HLA class I cells, were recovered by gently rocking the dishes. Cell viability, estimated by Trypan blue exclusion, was 90%. The cells were plated (1 x 10^6 cells/ml) in Dulbecco’s modified Eagle’s medium containing 25 mmol/l glucose,
20 mmol/l HEPES, 100 µg/ml streptomycin, 100 IU/ml penicillin and 15% fetal calf serum. They were incubated in humidified 95% air and 5% carbon dioxide at 37°C for 3 days. Cell culture was characterized by transmission and scanning electron microscopy, immunohistochemistry for α-hCG, β-hCG and cytokeratin and by β-hCG and progesterone secretion. After 3 days, cell culture mainly consisted of multinucleated syncytiotrophoblasts. Cells were collected and frozen at −80°C.

**Myometrium**

Samples of human myometrium were taken from term pregnant patients during elective caesarean section. This study was approved by the Consultative Committee of Persons Involved in Biomedical Research (CCPPRB) of Paris-Cochin.

**Ribonucleic acid preparation and reverse transcription**

Total RNA was extracted from trophoblast cells by the Chomczynski and Sacchi method (11), and from myometrial samples according to Chirgwin et al. (12). Reverse transcription was performed, using random hexanucleotides (600 pmol/l) as primers, on 2 µg and 5 µg of total RNA in 50 mmol/l TRIS/HCl (pH 8.3) at 37°C, 3 mmol/l MgCl2, 75 mmol/l KCl, 1 mmol/l dithiothreitol, 0.5 mmol/l each of dATP, dCTP, dGTP and dTTP, 20U of rRNasin (Promega) and 200U of Mo-MLV reverse transcriptase (Life technologies) in a final volume of 25 µl at 37°C for 60 min. Then, RT products were incubated at 95°C for 5 min.

**Polymerase chain reaction**

The resulting cDNAs (5 or 10 µl) were subject to amplification in a final volume of 50 or 100 µl containing forward and reverse external oligonucleotide primers (0.4 µmol/l each), 20 mmol/l TRIS/HCl (pH 8.3), 50 mmol/l KCl, 1–6 mmol/l MgCl2, 0.4 mmol/l each of dATP, dCTP, dGTP and dTTP and 1 U of Taq polymerase (Life Technologies) for typically 32 cycles in a DNA thermal cycler (PHC-3 Dri-Black Cycler, Techné). Human preproET-1 (13), preproET-2, and preproET-3 forward and reverse external oligonucleotides were designed to localize in separate exons and in homologous regions (Table 1). The human β2-microglobulin amplifier set was from Clonetech. The specificity of each amplification product was checked by gel electrophoresis analysis of the predicted length, cleavage by a restriction enzyme specific to each preproET cDNA sequence and hybridization with a reverse internal oligonucleotide positioned between the primer pairs. The same RT products from trophoblastic RNAs from three term placentae were used to amplify a preproET-1, ET-2 and ET-3 DNA fragment and also a β2-microglobulin DNA fragment.

**Southern blot analysis**

One-tenth to one-third of each PCR reaction was electrophoresed through a 3% NuSieve GTG agarose gel (FMC). The DNA bands were visualized by ethidium bromide staining. For hybridization detection of the PCR products, gels were treated in 0.25 mol/l HCl for 2 × 10 min, 1.5 mol/l NaCl and 0.5 mol/l NaOH for 15 and 30 min, 1.5 mol/l NaCl, 0.5 mol/l TRIS-HCl (pH 7.2) and 1 mmol/l EDTA for 2 × 15 min and finally in 10 × SSC (saline and sodium citrate buffer: 0.15 mol/l sodium chloride and 0.015 mol/l sodium citrate, pH 7.0). Then gels were transferred to Hybrond-N+ membranes (Amersham) in 20 × SSC. Membranes were prehybridized for 14 h in hybridization solution (5 × SSC, 5 × Denhardt’s (1% solution of bovine serum albumin, Ficoll and polyvinyl pyrrolidone), 0.5% (w/v) sodium dodecyl sulphate, 10 mmol/l ethylene diamine tetraacetic acid and 100 mg/l salmon sperm deoxy-ribonucleic acid). An internal oligonucleotide was used as a hybridization probe after labelling with [γ-32P]dATP (>5000 Ci/mmole, Amersham) using T4 polynucleotide kinase, and used at a final concentration of 1 × 106 counts·min⁻¹·ml⁻¹ hybridization solution. Hybridization was carried out for 6 h at a temperature specific to each oligonucleotide. Membranes were washed in 2 × SSC and then exposed to...
X-ray film, with intensifying screens, at −80°C. The duration of exposure varied from 1 to 24 h.

**Linearity with number of cycles**

To show the linearity of preproET-1 and preproET-3 amplification versus that of the β2-microglobulin gene, we prepared ten reactions with the number of cycles ranging from 12 to 40. For ET-1 the range was 18–30: for ET-3 it was 22–40 and for β2-microglobulin it was 12 to 28. The PCR products at different numbers of cycles were analysed by gel electrophoresis, Southern blot and hybridization with specific probes. Autoradiograms were analysed by scanning densitometry using a Beckman DU-640 spectrophotometer and the area under the curve was calculated for each peak.

**Sequencing**

After electrophoresis of the PCR reaction and ethidium bromide staining, the resulting DNA band was excised from the gel, treated with phenol/chloroform and ethanol-precipitated. The sequencing method used was that of Sanger et al. (14), as modified by Tabor and Richardson (15) using a T7 sequencing kit (Pharmacia). The process was slightly changed: the DNA denaturation was effected using heat (10 min at 100°C) and quick cooling in dry ice.

**Results**

**Expression of preproET-1 by cultured trophoblast**

The expression of preproET-1 mRNA was investigated in trophoblast cultured for 3 days. After RT and PCR amplification of the resulting cDNAs from trophoblast using external oligonucleotides specific for preproET-1 sequence, a product of correct predicted size (442 bp) was obtained, as revealed by ethidium bromide staining after agarose gel electrophoresis (Fig. 1A, lane 3) as well as in positive control endothelial cells (Fig. 1A, lane 1). The resulting DNA fragment from the cultured trophoblast was digested with restriction endonuclease Nhe I, which has no site in the preproET-2 and ET-3 sequences. After Southern blot and hybridization with an internal ³²P-labelled preproET-1-specific oligonucleotide, the undigested 442-bp (Fig. 1B, lanes 4 and 6) and the 291-bp Nhe I-treated (Fig. 1B, lane 7) amplification products were labelled, thus providing confirmation of the preproET-1 sequence. No amplification product was observed when reverse transcriptase was omitted (Fig. 1B, lane 5).

**Expression of preproET-2**

The same trophoblast cDNAs were amplified with the preproET-2 oligonucleotide primers. Ribonucleic acid from human pregnant myometrium was used as a positive control. After electrophoresis analysis and ethidium bromide staining, the predicted 232-bp product was obtained only from the myometrium (data not shown). To confirm the absence of the preproET-2 mRNA expression in trophoblast. Southern blot of RT-PCR products was hybridized with a ³²P-labelled preproET-2-specific internal oligonucleotide. The labelled amplification product of the correct size was obtained only from the myometrium (Fig. 2A, lane 4), while no amplification product could be detected with trophoblast (Fig. 2A, lanes 1–3). Further, no amplification product was detected even after a longer period (48 h) of X-ray film exposure (data not shown). To control the quality of trophoblast RNAs and efficiency of the RT step in the three trophoblast samples, we verified the amplification of a DNA fragment of β2-microglobulin, a gene normally expressed in all somatic cells (Fig. 2B, lanes 1–3). Specificity for myometrium RT-PCR products was confirmed by restriction endonuclease Nsi I digestion specific to the preproET-2 fragment sequence and hybridization of the digested product (168 bp) with the preproET-2 internal oligonucleotide (Fig. 2A, lane 5). Additionally, to exclude the possibility that there was still a non-specific product measured in the ET-2 control, we sequenced the amplification product obtained from the myometrium. The resulting sequence of this fragment was exactly the same as that described by Bloch et al. (3).
Expression of preproET-3 gene

Expression of preproET-3 mRNA was investigated in cultured syncytiotrophoblast and in a positive control, the normal human pituitary gland. After 32 cycles of amplification, agarose gel electrophoresis and ethidium bromide staining, a DNA fragment of 479 bp in length, as predicted, was obtained from pituitary gland while no amplification product could be detected with the cultured trophoblast (data not shown). However, after Southern blot of RT-PCR products and hybridization with a $^{32}$P-labelled preproET-3-specific internal oligonucleotide, a labelled amplification product of the correct size was obtained from cultured syncytiotrophoblast (Fig. 3A, lane 1 and Fig. 3B, lane 3) as well as from the pituitary gland (Fig. 3B, lane 5). No DNA fragment could be detected in trophoblast if the reverse transcriptase was omitted (Fig. 3A, lane 2). Restriction endonuclease Xma III, the site of which is specific to the preproET-3 DNA fragment sequence, was used to verify the PCR products. The Xma III-digested 231-bp fragment from trophoblast and from pituitary gland was also labelled, as shown in Fig. 3B lanes 4 and 6, respectively. As a control, RNA from a monoblastoid...
cell line, U937, was reverse transcribed and amplified in the same conditions. The PCR products at 32, 36 and 40 cycles were analysed by Southern blot and hybridization. No signal was obtained after a 3-day X-ray exposure (data not shown).

Comparison of expression levels of preproET-1 and preproET-3 versus β2-microglobulin

To compare the expression of preproET-3 to that of preproET-1, the same RT products (5 µl) from a cultured trophoblast RNA sample (2 µg) were subject to amplification for preproET-1, preproET-3 and the housekeeping gene, β2-microglobulin, at varying numbers of cycles. The linearity of signals obtained in each amplification can be demonstrated in a range of cycles depending on the expression level of each transcript. The maximal optical density level was reached at 26 cycles for β2-microglobulin, 30 cycles for preproET-1 and 40 cycles for preproET-3. The optical density equivalent to half of the maximal response (OD50) was reached at 20.5, 25.5 and 34 cycles, respectively (Fig. 4). Comparable results were obtained with two RNA samples.

Discussion

Our data show for the first time that human placental syncytiotrophoblast in culture expresses preproET-1 and preproET-3 mRNAs, while no ET-2 precursor mRNA is detected. These results substantiate our previous findings concerning localization of irET in the trophoblastic layer of human term placental sections and its production by cultured trophoblast (8, 9). The expression of preproET genes in the placenta was first shown by the isolation of cDNA clones encoding preproET-1 and preproET-3 from human placental cDNA libraries (2, 5). Until now, expression of preproET genes had been studied only in the whole chorionic villous tissue and significant levels of preproET-1 mRNA were shown at term of pregnancy (16, 17). In addition, very low levels of preproET-3 mRNA were found by RNase protection assay, and no preproET-2 mRNA could be detected using this technique (17). Placental villi are comprised of trophoblast and other fetal cell populations, such as vascular endothelial and smooth-muscle cells, fibroblasts and macrophages (Hofbauer cells). Considering such cell type diversity, we investigated preproET gene expression in cultured syncytiotrophoblast.

Using the more sensitive and specific RT-PCR method, we found a high level of preproET-1 mRNA expression in 3-day cultured syncytiotrophoblasts. The ET-3 precursor gene was also expressed, but at a low level and it was detected only after Southern blotting and oligonucleotide hybridization when using 32 cycles. Obviously, this expression is weaker than that of ET-1 because eight additional cycles are required to obtain equivalent OD50 values. As described previously, an expression of the preproET-3 gene has also been reported in human anterior pituitary cells (18), which produce gonadotrophins, as does the trophoblast. Consequently, it would be interesting to investigate the relative quantitative expression of these isoform precursor mRNAs in trophoblast during pregnancy, as well as their localization by in situ hybridization. By contrast, no ET-2 precursor mRNA could be detected in syncytiotrophoblast in culture, while in the same experiment a preproET-2 amplification of a RT product from the myometrium could be obtained. The specificity of this amplification product was shown by the presence of restriction enzyme cleavage, Southern blot hybridization and sequencing. The ET-2 expression in myometrium was reported previously by RNase protection assay (17).

Two different trophoblast cell populations (cytotrophoblast and syncytiotrophoblast) develop throughout pregnancy. Proliferative cytotrophoblasts surround the stromal cores of chorionic villi. They differentiate into an overlying syncytiotrophoblast, which, in floating villi, is in direct contact with maternal blood and acts as an endothelial layer (8). At term, the trophoblast consists of an uninterrupted layer of multinucleated syncytiotrophoblast and an incomplete layer of individual cytotrophoblasts. The villous trophoblast is known to be an important production site of various biologically active substances. In addition to its steroidogenic activity, it elaborates protein and polypeptide hormones very similar to or, in some cases, identical to those produced by the hypothalamus and the pituitary gland. The syncytiotrophoblast is a polarized epithelium with the microvillous membrane facing the maternal blood space and the basal plasma membrane facing fetal circulation. Specific high-affinity binding sites for ET-1 were characterized previously on both these membranes (19, 20). In addition, ET-1 binding to trophoblast membranes is modified by gestational age and preeclampsia (21, 22). These data and our results demonstrating preproET-1 and ET-3 gene expression in trophoblast are compatible with an autocrine role of ETs in the regulation of trophoblastic functions. Indeed, ET-1 and mainly ET-3 have been shown recently to increase the secretion of progesterone by syncytiotrophoblast in culture (23). Thus, endothelins may act as modulators of steroidogenesis in trophoblastic cells, as reported in adrenocortical and gonad cells (24–26).

Because of the close proximity between trophoblast and fetal vessels, ET that originates in the trophoblast may also contribute to regulation of feto-placental circulation by a paracrine mechanism. Abundant specific high-affinity binding sites for ET-1 have been characterized on membranes of smooth muscle cells of stem villi vessels (19, 20), and it has been demonstrated that ET-1 is a potent vasoconstrictor of the human placental stem villi arteries (27).
In addition, through the potent mitogenic activity of this peptide in placental fibroblasts, it was postulated that ET-1 could regulate and support the growth of placental mesenchymal cells (16). The possible role of each ET isoform in the stimulation of trophoblast proliferation/differentiation could be a very important point to elucidate. Moreover, an essential role for ET-1 in normal fetal development was reported recently in mice (28). These data are in agreement with the expression of the preproET-1 and ET-3 genes in human fetal tissues (4).

The placenta is characterized by complex anatomical interactions between fetal and maternal tissues. Material secreted by the syncytiotrophoblast could enter directly into the intervillous space and maternal circulation. Besides autocrine action of ET in trophoblast, our data suggest an important endothelium-like role for the syncytiotrophoblast layer in production of these peptides. Moreover, in the basal plate, the close proximity between extravillous trophoblast, decidual and myometrium argues for a paracrine action of ET-1 on maternal uterine tissues. Further, the presence of ETA and ETB receptor subtypes has been shown in human myometrium (29–31). It would be interesting to evaluate the contribution of ETs produced by trophoblast toward modulation of myometrial contractility, particularly in the onset or maintenance of labour.

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