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Expression of Urocortin in the Extravillous Human Trophoblast at the Implantation Site

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Abstract

Urocortin (UCN) is a 40 amino acid peptide which is closely related to corticotropin-releasing hormone and binds with high affinity to both CRH type 1 and type 2 receptors. UCN is expressed in human reproductive tissues including endometrium, ovary, and placenta. This study was designed to investigate the cellular localization of UCN at the implantation site of the human blastocyst, as well as the regulation of the UCN promoter by two major intracellular signaling pathways, the cAMP/PKA and diacylglycerol/PKC pathways, in cells of placental origin. For this reason, immunohistochemistry was performed on tissue sections from paraffin-embedded human first trimester placentas and freshly isolated human invasive extravillous trophoblast cells (EVT) were analyzed for UCN expression using RT-PCR and immunofluorescence. Finally, UCN promoter activity was analyzed in the JEG3 human choriocarcinoma cell line. Immunohistochemistry revealed expression of UCN in the cytotrophoblast, the EVT and decidual cells. Both UCN mRNA and peptide were detectable in freshly isolated EVT. Finally, a human UCN promoter luciferase reporter construct transfected into JEG3 cells was significantly inducible by phorbol ester plus ionomycin, but not by phorbol ester alone or by forskolin. Collectively, the present study reports the expression of UCN in EVT and the activation of the UCN gene promoter by the diacylglycerol/PKC pathway. The functional significance of urocortin for the physiology of EVT requires further investigation.

Keywords: Implantation site; Extravillous trophoblast; Urocortin

1. Introduction

Urocortin (UCN) is a 40 amino acid peptide which is closely related to corticotropin-releasing hormone (CRH) [1,2]. The peptide has sequence homology with urotensin-1 (63%) and CRH (45%), and it binds both type 1 and type 2 CRH receptors [1]. Its affinity for the type 1 receptor is similar to that of urotensin-1 and CRH, whereas it binds the type 2 receptor with even higher affinity than CRH, suggesting that it may be the endogenous ligand for this receptor subtype. The synthetic human UCN increases the release of ACTH from dispersed rat anterior pituitary cells, an effect which is abolished by the CRF-binding protein (CRF-BP) [2].

UCN was first identified as a central nervous system-derived peptide exerting multiple effects in controlling behavior, the stress response and food ingestion [1–5]. Outside the central nervous system UCN is expressed at numerous sites, such as the immune and the cardiovascular system [6–9]. Within the female reproductive system, UCN was shown to be expressed in the ovaries [10], the myometrium [11], the endometrium [12], as well as in the human placenta and fetal membranes [13–16]. During pregnancy, UCN is present at low levels in the maternal circulation [17].

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A number of studies have reported similar expression patterns and actions for UCN and CRH in the physiology and pathophysiology of gestation, indicating a certain degree of redundancy in this system [17–20]. Recent experimental data have shown that CRH exerts an important role in early maternal tolerance toward the implanting embryo. CRH attenuates the maternal response by upregulating Fas ligand expression in extravillous trophoblast (EVT) cells, thus potentiating their ability to induce apoptosis of activated T lymphocytes bearing the Fas receptor on their surface [21]. The expression pattern and potential functional role of UCN at the implantation site remain elusive. Although a number of studies have described the expression of UCN in term placentas, no detailed analysis of UCN expression in the various trophoblast subpopulations in first trimester placentas has been performed so far.

In the present study we, therefore, examined the expression pattern of UCN at the implantation site, particularly in the invasive extravillous trophoblast (EVT). In addition, the regulation of the UCN promoter by two major intracellular signaling pathways, the cAMP/PKA and diacylglycerol/PKC pathways was examined in cells of placental origin.

2. Materials and methods

2.1. Isolation and characterization of human invasive EVT cell populations

Cultures of first trimester invasive trophoblast populations were established and characterized as previously described [22,23]. Ten placentas were obtained from healthy women after legal termination of pregnancy (5–10 weeks of gestation). Informed consent from Institutional Review Board was obtained. Briefly, the placentas were washed in sterile phosphate buffer saline (s-PBS) until the supernatant was nearly free of blood. Areas rich in chorionic villi were selected and minced between scalpel blades into small pieces. The minced tissues were washed in s-PBS and were subjected to a 10-min treatment with 0.125% trypsin and 0.2 mg/ml DNAse I (Boehringer Mannheim GmbH, Germany) per milliliter in s-PBS containing 5 mM $MgCl_2$. Cells which were released from this treatment were pooled and filtered through two layers of muslin. Trypsin was inactivated with fetal calf serum (FCS) (GIBCOBRL, Life Technologies, Paisley, UK). The filtrates were centrifuged and cell pellets were washed with s-PBS. Cells were then resuspended in 70% Percoll (Pharmacia, Uppsala, Sweden) at a density of 2×10^5 cells/ml, and put under 20 ml of 25% Percoll. Ten milliliters of s-PBS were put on top of 25% Percoll and a gradient was established by centrifuging for 20 min at 2000 rpm/min. Cells from the middle band (density 1.048–1.062 g/ml) of the gradient were pooled, washed in s-PBS and seeded at a density of 1.0×10^6 cells/ml of keratinocyte growth medium (KGM) (GIBCOBRL) supplemented with 10% FCS (GIBCOBRL).

Cells were identified as trophoblasts by flow cytometry and immunocytochemical staining with monoclonal antibodies (mAbs) to cytokeratin (mAbs MNF116 and 35βH11, diluted 1:100, DakoCytomation, Glostrup, Denmark) and E-cadherin (mAb HECD-1, Takara Shuzo Co., Shiga, Japan), which stain only trophoblasts in the placenta [22,23]. The isolated trophoblasts were further characterized as extravillous trophoblasts by staining positive for major histocompatibility complex class I molecules (using anti-MHC mAb W6/32, diluted 1:50, DakoCytomation) and negative for hyaluronic acid (using mAb NDOG1, diluted 1:10, Serotec, Kidlington, UK), as previously described [22,23]. The cells were kept in KGM supplemented with 10% FCS and 1% Penicillin–Streptomycin (GIBCOBRL) at 5% CO_2 and 37 °C for 6–10 days prior to experiments. Percentages of cells stained positively for the above antigens before culture for 6–10 days were as follows: 97 ± 2% for cytokeratin, 97 ± 2% for E-cadherin and 96 ± 2% for MHC class I. The respective percentages after 6–10 days in culture were as follows: 92 ± 2% for cytokeratin, 92 ± 2% for E-cadherin and 91 ± 2% for MHC class I [23].

2.2. RT-PCR

Total RNA from invasive trophoblast cells was extracted using a ready to use RNA extraction kit (Macherey-Nagel) according to the manufacturer's instructions. Total RNA (1 μg) was reversely transcribed using the ThermoScript RT-PCR System (Invitrogen Life Technologies). The cDNA was amplified by PCR, which was performed in a Perkin–Elmer DNA Thermal Cycler with the following conditions: 60 s at 95 °C, 60 s at 60 °C and 60 s at 72 °C for 40 cycles. Ten microliters of the amplification product (145 bp) was separated on a 2% agarose gel and visualized by ethidium bromide staining. Human UCN primer sequences were as follows: sense primer, 5′-CAGGCGA GCGGCCGCG-3′; antisense primer, 5′-CTTGCCCACCGAGTCGAAT-3′. Human B lymphocyte (U266) and placenta homogenate total RNA were used as positive controls. Samples containing no reverse transcriptase (no-RT) were used to exclude contamination with genomic DNA or reagent contamination. DNA sequencing was performed to confirm the specificity of the obtained PCR products.

2.3. Immunohistochemistry and immunofluorescence

The tissue material was selected following histological review from the files of the Department of Gynecopathology, University Hospital Hamburg-Eppendorf. For immunohistochemistry, specimens which had been routinely fixed in 4% buffered formalin and embedded in paraffin were used. A total of 22 first trimester placentas were analyzed. Serial sections of 4–6 μm were cut from paraffin blocks and mounted on APES-coated slides, deparaffinized in xylene and rehydrated in graded alcohol to TBS (50 mM Tris, 150 mM NaCl, pH 7.4). The slides were microwaved for 4 × 5 min in 10 mM citrate, pH 6.0. After cooling down for 20 min, the slides were washed in TBS, blocked for 30 min at room temperature with normal serum (rabbit IgG, ABC kit, Vector Laboratories), diluted 1:20 in TBS and then incubated with anti-UCN serum (Phoenix Pharmaceuticals) diluted 1:100 in TBS plus 2% BSA for 1 h at room temperature. UCN immunoreactivity was detected with the alkaline phosphatase substrate kit (Vectastain; Vector Laboratories). For negative controls the primary antibody was omitted.

For double immunofluorescence, frozen sections of the selected material were cut at 5 μm and placed onto APES-coated slides, air-dried overnight, fixed in acetone for 10 min, and rehydrated in TBS. For the characterization of UCN-expressing cells in first trimester placenta tissue, cryosections were examined. Samples were fixed in 5% buffered formalin. Primary antibodies against UCN and cytokeratin-7 (Progen Biotechnik, Heidelberg, Germany) were diluted 1:200 (for anti-UCN) and to 2 μg/ml (for cytokeratin-7) with TBS, and incubated with the slides overnight at 4 °C. After washing, slides were incubated for 30 min at room temperature with FITC-labeled anti-rabbit and TRITC-labeled anti-mouse secondary antibodies (both Chemicon, Temecula, CA), diluted 1:200. The slides were finally embedded in mounting buffer containing DAPI (Chemicon), resulting in staining of the nucleus. Slides were examined with a Zeiss (Jena, Germany) Axiophot photomicroscope. Digital images were obtained with a digital-camera system (CF20DXC, KAPPA Messtechnik, Gleichen, Germany) (magnification ×400).

For indirect immunofluorescence freshly isolated EVT were grown in chamber slides. After pre-blocking, cells were incubated with anti-UCN serum (Phoenix Pharmaceuticals) diluted 1:100 in TBS plus 2% BSA for 1 h at room temperature. UCN immunoreactivity was detected after incubation with an anti-rabbit FITC-conjugated secondary antibody for 45 min in room temperature. Slides were mounted with DAPI (Chemicon), sealed with a coverslip and photographed using a Nikon Confocal microscope (magnification ×600).

2.4. UCN promoter analysis

The UCN promoter/reporter gene was constructed as follows: a 2365 bp fragment of the human UCN 5′ prime region was amplified by PCR from human genomic DNA (Boehringer, Germany). PCR was carried out with 10 pmol 5′- and 3′-primers, 0.2 mM dNTPs, 0.5 U of *Pfu* polymerase (Stratagene, Heidelberg, Germany) in a reaction volume of 50 μl. Initial denaturation was performed for 2 min at 95 °C, followed by four cycles each at 68, 66, 64, and 62 °C, and 25 cycles at 60 °C (annealing temperature). Primer sequences