Expression and Oxygen Regulation of Endocrine Gland-Derived Vascular Endothelial Growth Factor/Prokineticin-1 and Its Receptors in Human Placenta during Early Pregnancy

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Angiogenesis is a key process of dynamic tissue remodeling occurring during placentation. Compelling evidence indicates that vascular endothelial growth factor (VEGF) is an important mediator of placental angiogenesis and appears to be deregulated in preeclampsia. Recently a new angiogenic factor, endocrine gland-derived VEGF (EG-VEGF), also known as prokineticin 1 (PK1), has been identified, and its expression was shown to be restricted to endocrine glands, including the placenta. In this study we investigated the pattern of expression of EG-VEGF, its related factor Bv8/PK2, and their common receptors, PKR1 and PKR2, in human placenta during the first trimester of pregnancy. We also examined EG-VEGF and PKR1 regulation by oxygen tension in isolated trophoblast cells (TCs). Our results show that EG-VEGF, but not Bv8/PK2, is expressed in human placenta. EG-VEGF is mainly localized to the syncytiotrophoblast layer with the highest expression detected between the 8th and 10th wk of gestation. EG-VEGF expression within placental villi is different from that of VEGF, which is mainly localized in the cytotrophoblast and extravillous trophoblast cells. In TCs, PKR1 mRNA is about 80 times more abundant than PKR2 mRNA. Both EG-VEGF and PKR1 mRNAs appear to be regulated by hypoxia. These findings suggest that EG-VEGF has a direct effect on TCs via its receptor PKR1 and is likely to play an important role in human placentation. The expression pattern of EG-VEGF, its regulation by oxygen tension, and its complementary localization to that of VEGF suggest that this new factor might also be deregulated in preeclampsia. (Endocrinology 147: 1675–1684, 2006)
angiogenesis and angiopoietins being involved in the latter stages of fetal blood vessel maturation (8). Besides its mitogenic action on endothelial cells, VEGF has also been reported to stimulate the proliferation of the choriocarcinoma cell line BeWo (9) and extravillous trophoblasts (10). Low VEGF levels or increased production of VEGF antagonists such as the soluble form of its receptor-1 (VEGF-R1/flt-1), have been proposed as possible mediators of preclampsia (11–14). Although VEGF and angiopoietins are selectively acting on any type of endothelial cell, they are ubiquitously expressed.

The existence of organ-specific angiogenic factors has been postulated for many years (15–18) but only recently received confirmation when such a factor, named endothelial gland-derived vascular endothelial growth factor (EG-VEGF), was characterized and sequenced (19). This new factor was found to be expressed in testis, adrenal gland, ovary, and placenta (19). In addition, its angiogenic action appeared to be restricted to endothelial cells derived from endocrine tissues (19). In endothelial cells isolated from steroidogenic tissues, EG-VEGF was shown to promote proliferation, survival, and chemotaxis (19, 20). Very interestingly, in vivo delivery of adenoviruses encoding EG-VEGF resulted in endocrine tissue-specific angiogenesis (21). EG-VEGF is a member of a class of proteins that also includes Bv8, a frog peptide purified from the skin secretion of the yellow-bellied toad, *Bombina variegata* (19), also known as prokineticin (PK)-2. Human EG-VEGF and Bv8 proteins present 83% identity, exert the same functions (21), and share the same G protein-coupled receptors (PKRs), termed PKR1 and PKR2 (22).

EG-VEGF expression in the placenta was briefly described in the initial report by LeCouter *et al.* (19). Since then, no further characterization of its cellular localization and expression during pregnancy has been reported. The specificity of EG-VEGF expression in endocrine glands and its similarity of action with VEGF suggested to us that this new factor might be important in human placentation. The aims of this study were therefore to examine the expression of EG-VEGF, Bv8, PKR1, and PKR2 in human placentas during the first trimester of pregnancy, determine their sites of expression within the chorionic villi, and examine the effect of oxygen tension on their expression levels in isolated human trophoblast cells.

**Materials and Methods**

**Tissue collection**

First-trimester human placentas from 4 to 12 wk of gestation, corresponding to 6–14 wk amenorrhea were obtained from elective terminations of pregnancies. The sampling was as follows [4 wk of gestation (n = 3); 5 wk of gestation (n = 3); 6 wk of gestation (n = 3); 7 wk of gestation (n = 4); 8 wk of gestation (n = 4); 9 wk of gestation (n = 4); 10 wk of gestation (n = 4); 11 wk of gestation (n = 3), and 12 wk of gestation (n = 3)]. Shortly after collection, tissue was snap frozen in dry ice and stored at −80°C for protein and RNA extraction, fixed in paraformaldehyde at room temperature (for immunohistochemistry), or placed in ice-cold Hanks’ balanced salt solution (Ca2+/Mg2+/HBBSS) and transported to the laboratory for *in vitro* primary culture. A total of eight placentas from 7 to 10 wk of gestation were used for primary culture.

Collection and processing of human placentas was approved by the University Hospital Ethics Committee, and informed consent was obtained from each patient. Mouse testis was also collected and used as positive control in this study. Animal surgery was conducted following both institutional and European Community guidelines for the use of experimental animals.

**Immunohistochemistry**

Placental tissues collected at 4–12 wk of gestation were fixed for 24 h at 4°C in 4% (vol/vol) paraformaldehyde, embedded in paraffin, and cut into 5-μm sections as described previously (23). To control tissue integrity and select the most representative sections, every 10th section was stained with hematoxylin and eosin. Adjacent sections were stained using specific antibodies and the avidin-biotin immunoperoxidase detection method. Endogenous peroxidase activity was quenched by pre-treatment with 3% (vol/vol) hydrogen peroxide in methanol for 30 min. Tissue sections were then washed in PBS and incubated with normal goat serum (10%) that served as a blocking agent for nonspecific binding. Immunoreactive EG-VEGF or Bv8 was detected using rabbit polyclonal antibodies raised against the following peptides: human EG-VEGF peptide 84–96, LLCSRFDPGKYRC; and a mixture of Bv8-derived peptides 26–40, DDAVITGACDKDSCQ, and 94–108, CLRSTSNRFICLAQK. The immunization was performed for our laboratory by CovaLab (Lyon, France). For immunohistochemical detection, anti-EG-VEGF and anti-Bv8 antibodies were incubated with the tissue sections for 18 h at 4°C and used at final concentrations of 0.33 and 0.023 μg/ml, respectively. The tissue sections were subsequently washed three times with PBS and incubated with biotinylated goat antirabbit IgG (1:250 dilution in blocking solution; Sigma Aldrich, Saint-Quentin Fallavier, France) for 1 h at 4°C. After three PBS washes, the slides were incubated with an avidin-biotin complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 1 h. After a final PBS wash, the immunoreactive proteins were visualized after the addition of 3,3′-diaminobenzidine (Dako, Trappes, France) for 2 min and then counterstained with hematoxylin. Control sections were treated with anti-EG-VEGF and anti-Bv8 antibodies that had been preabsorbed overnight at 4°C with the appropriate antigen peptides or preimmune serum.

**Western blotting analysis**

Frozen placental samples were homogenized on ice for 1 min in radioimmunoprecipitation assay lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin] as previously described (24). The homogenates were centrifuged (15,000 × g at 4°C) for 15 min, and the supernatants were collected. Proteins were also extracted from cultured trophoblast cells using the same procedure. Protein concentrations were determined using the Bradford assay. Because of the weakness of the signal in the preliminary experiments, a specific protocol was set up to detect EG-VEGF protein. One hundred micrograms placental protein and 70 μg protein extracted from trophoblast cells were electropheretically separated on 15% acrylamide gel and electrically transferred onto 0.25-μm polyvinyliden difluoride membranes (Millipore, Bedford, MA). Appropriate transfer was confirmed by protein staining with Ponceau S (Sigma-Aldrich). The blots were washed with PBS–Tween 20 0.1% (PBS–T) and incubated overnight in blocking solution (5% skimmed milk in PBS–T). Subsequently membranes were incubated with a rabbit antibody against EG-VEGF (0.48 μg/ml) (CovaLab) for 2 h. Blots were then rinsed three times with PBS–T and incubated with biotinylated goat antirabbit IgG (450 ng/ml, 1:2000, dilution in blocking solution; Dako) for 30 min. After three PBS–T washes, the membrane was incubated with a peroxidase-conjugated extravidin (1:2000 dilution in blocking solution; Sigma Aldrich) for 30 min. Blots were washed six times with PBS–T, and the antibody-antigen complex was detected using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). The membranes were then exposed to radiographic films (Kodak Scientific Imaging Products, Rochester, NY).

The intensities of immunoreactive bands were measured by scanning the photographic film and analyzing the image on a desktop computer (Scion Image software (version 4.02; Scion Corp., Frederick, MD)). The mean pixel density for each band was analyzed to obtain relative OD units for EG-VEGF protein. To standardize for sample loading, the blots were stripped in [6.25 mM Tris-HCl (pH 6.6), 10 mM β-mercaptoethanol, 2% sodium dodecyl sulfate] and reprobed with an anti-Gβ
Isolation and treatment of trophoblasts

Placental cytotrophoblasts were isolated from first-trimester human placentas (7–10 wk of gestation, n = 14) and cultured using a combination of two techniques described by Kliman et al. (25) and Tarrade et al. (26). Briefly, the tissue was thoroughly washed in 50 ml cold sterile Ca2+–Mg2+-HBS until the supernatant was nearly free of blood. Areas rich in chorionic villi were selected and were minced into small pieces between scalpels. Tissue was incubated in HBSS containing 0.125% trypsin (Sigma), 4.2 mM MgSO4, 25 mM HEPES, and 50,000 U/ml DNase IV (Sigma) for 35 min at 37 C without agitation to remove extravillous trophoblast cells. After tissue sedimentation, the supernatant was discarded and the chorionic villi were submitted again to sequential 30 min trypsin (0.25%) and DNase (0.2 mg/ml) digestions, as previously described (25, 27). The dispersed placental cells were filtered through 100-μm nylon gauze and loaded onto a discontinuous Percoll gradient (5–70% in 5% steps of 3 ml each) and then centrifuged at 1200 × g for 20 min at room temperature to separate the different cell types. Cytotrophoblast cells that sedimented between the density markers of 1.049 and 1.060 g/ml were collected and washed with DMEM. Isolated cells were then incubated with anti-CD9 antibodies and subjected to negative immunomagnetic separation using MiniMacs columns (Miltenyi Biotec, Paris, France). This allowed the elimination of CD9-positive placental mesenchymal cells, placental macrophages, and blood monocytes. Briefly, 10 μl of anti-CD9 antibody (Biosource International, Camarillo, CA) were added to 107 freshly isolated cells and the mixture was incubated for 40 min on ice with gentle agitation. Twenty microliters of goat antirat IgG microbeads (Miltenyi Biotec) were mixed with the cell suspension, and mixture was kept at 4 C for 15 min under gentle rotation.

The unabsorbed cell fraction containing trophoblasts was collected, washed three times, and seeded at a density of 106 cells/ml in DMEM supplemented with 2 mM glutamine, 10% fetal bovine serum, 25 mM HEPES, 100 IU penicillin, and 10 μg/ml streptomycin. Approximately 4–5 × 105 cells were obtained per gram of chorionic villi. The dispersed trophoblasts were cultured for 24 h at 37 C in 5% CO2–95% air to allow attachment. The cells were then divided into two groups: half were incubated under standard tissue culture conditions (95% air–5% CO2) for 24 h. Trophoblast cells were also treated with hypoxia-mimicking drugs such as deferoxamine (DFO, 200 μg/ml) and cobalt chloride (CoCl2, 200 μM) for 24 h. In some experiments trophoblast cells were treated by 50 μg/ml of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a potent RNA polymerase inhibitor, or 10 μg/ml cycloheximide, a translation inhibitor. Purity of the cell preparation was assessed at the end of the experiment by immunofluorescent staining for cytokeratin, an epithelial cell lineage marker (Dako). In addition, several control reactions were routinely run in parallel including RT-PCR run in the absence of reverse transcriptase to confirm the absence of genomic DNA contamination and reverse transcription reactions without RNA to check for reagent contamination. The PCR conditions were: step 1, 94 C for 1 min; step 2, 25–35 cycles consisting of 95 C for 15 sec, temperature indicated in Table 1 for 5 sec, and 72 C for 10 sec. VEGF primers were designed to amplify the four human VEGF isoforms. The results were normalized to 18S rRNA expression levels. To assess linearity and efficiency of PCR amplification, standard curves for all transcripts were generated by using serial dilutions of cDNA. For standard-curve generation, we used cDNAs from human placenta or untreated trophoblast cells. A melt curve analysis was carried out on the products of amplification reaction to ascertain the melting temperature of the product. The RealQuant analysis software (Roche Diagnostics) was used to quantify levels of expression.

Semiquantitative PCR

Semiquantitative PCR was used to examine the effect of oxygen tension on EG-VEGF and PKR1 expression in isolated trophoblast cells. Before PCR, quantities of cDNA samples were adjusted to yield equal amplification. Ribosomal 18S was used as the internal reference. Specific oligonucleotide primers for EG-VEGF and PKR1 were designed using MacVector software (Table 1). PCRs were performed in a final volume of 25 μl containing appropriate quantities of cDNAs in 1 × PCR buffer, 1.5 mM MgCl2, 200 μM deoxynucleotide triphosphates, and 400 mM each primer, 0.5 U Taq polymerase (Q. Biogene, Illkirch, France). In addition, several control reactions were routinely run in parallel including RT-PCR run in the absence of reverse transcriptase to confirm the absence of genomic DNA contamination and reverse transcription reactions without RNA to check for reagent contamination. The PCR conditions were: step 1, 94 C for 1 min; step 2, 25–35 cycles at hybridization temperature indicated in Table 1; and step 3, 72 C for 5 min. To ensure semiquantitative results in the RT-PCR assays, the number of PCR cycles was selected to be in the linear range of amplification. PCR products were visualized after electrophoresis on 1.5 to 2% gels by ethidium bromide staining.

Statistical analysis

All data are expressed as mean ± se. Statistical comparisons were made using one-way ANOVA test and Student’s t test. Calculations were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA).

RNA isolation and RT-PCR analysis

Total RNA was extracted from placental tissue and isolated trophoblasts after 48 h of cell culture. The extraction was done in 4 M guanidium isothiocyanate buffer using a rapid RNA isolation system (RNAgent; Promega, Charbonnières, France). The integrity of extracted RNA was analyzed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Reverse transcription was performed on 1 μg total RNA with Superscript II-RNaseH reverse transcriptase (Invitrogen, Cergy Pontoise, France) under conditions recommended by the manufacturer.

Real-time PCR analysis

EG-VEGF, Bv8, PKR1, PKR2, VEGF mRNA, and 18S rRNA expression was quantified by real-time RT-PCR using a Light Cycler apparatus (Roche Diagnostics, Meylan, France). The PCR was performed using the primers shown in Table 1 and SYBR green PCR core reagents (Light Cycler-FastStart Master SYBR Green I, Roche Diagnostics) according to the manufacturer’s instructions. For negative controls, we used a complete DNA amplification mix in which the target cDNA template was replaced by water. PCR conditions were: step 1, 94 C for 10 min; step 2, 45 cycles consisting of 95 C for 15 sec, temperature indicated in Table 1 for 5 sec, and 72 C for 10 sec. VEGF primers were designed to amplify the four human VEGF isoforms. The results were normalized to 18S rRNA expression levels. To assess linearity and efficiency of PCR amplification, standard curves for all transcripts were generated by using serial dilutions of cDNA. For standard-curve generation, we used cDNAs from human placenta or untreated trophoblast cells. A melt curve analysis was carried out on the products of amplification reaction to ascertain the melting temperature of the product. The RealQuant analysis software (Roche Diagnostics) was used to quantify levels of expression.

Semiquantitative RT

Semiquantitative PCR was used to examine the effect of oxygen tension on EG-VEGF and PKR1 expression in isolated trophoblast cells. Before PCR, quantities of cDNA samples were adjusted to yield equal amplification. Ribosomal 18S was used as the internal reference. Specific oligonucleotide primers for EG-VEGF and PKR1 were designed using MacVector software (Table 1). PCRs were performed in a final volume of 25 μl containing appropriate quantities of cDNAs in 1 × PCR buffer, 1.5 mM MgCl2, 200 μM deoxynucleotide triphosphates, and 400 mM each primer, 0.5 U Taq polymerase (Q. Biogene, Illkirch, France). In addition, several control reactions were routinely run in parallel including RT-PCR run in the absence of reverse transcriptase to confirm the absence of genomic DNA contamination and reverse transcription reactions without RNA to check for reagent contamination. The PCR conditions were: step 1, 94 C for 1 min; step 2, 25–35 cycles at hybridization temperature indicated in Table 1; and step 3, 72 C for 5 min. To ensure semiquantitative results in the RT-PCR assays, the number of PCR cycles was selected to be in the linear range of amplification. PCR products were visualized after electrophoresis on 1.5 to 2% gels by ethidium bromide staining.

Table 1. Primers used for semiquantitative (SQ) and real-time (RT) PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
<th>Temperature (C) for RT</th>
<th>Temperature (C) for SQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG-VEGF</td>
<td>CGCAGACCGTTAATAGTGGCT</td>
<td>GCCGGTCACATTCTGCGAATGTA</td>
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<td></td>
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<tr>
<td>VEGF</td>
<td>AGGTCCTCTTCTCAAGAACGG</td>
<td>TCCAGGGCCTGTCAGAAGAACAG</td>
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<tr>
<td>Bv8</td>
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<td>CATTCTCTCTGTCACACACCAAGG</td>
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<tr>
<td>PKR1</td>
<td>GCCGCGTCCGCTCAGAACGCC</td>
<td>AACAACGTTGGAAGAGAAGCGG</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>PKR2</td>
<td>CACCTCCTGTCAAGG</td>
<td>CCTTCTCCTTCACACAGATGGG</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>TTGTTGTTTTCGGAATTGACCG</td>
<td>GGCAAAAGTTCCTGCTCTGGTC</td>
<td>59</td>
<td>56</td>
</tr>
</tbody>
</table>
Results

EG-VEGF protein expression in human placenta during early pregnancy

Immunohistochemistry. Immunohistochemistry was used to define the pattern of expression of EG-VEGF and Bv8 proteins in human placenta between 4 and 12 wk of gestation. A total of 18 placentas were used (two different placental samples for each gestational age). Immunoreactive EG-VEGF (Ir-EG-VEGF) was observed from 6 to 10 wk of gestation. No staining was observed before 6 or after 10 wk of gestation. At 6 wk of gestation, expression was limited to the syncytiotrophoblast layer of the chorionic villi (Fig. 1A). At advanced gestational ages, the staining for EG-VEGF was more intense in the syncytiotrophoblast layer (7–10 wk) and became apparent in the inner cytotrophoblast layer. No staining was observed in the extravillous trophoblast layer of the anchoring villi (Fig. 1B). Ir-EG-VEGF was also found in fetal Hofbauer cells within the placental villous mesenchyme as early as 6 wk of gestation. In contrast to Ir-EG-VEGF, Bv8 immunoreactivity was barely detectable in human placenta from 4 to 12 wk of gestation. A very faint staining was observed in the syncytiotrophoblast layer between 6 and 10 wk. Because our antibody was designed to react with both human and mouse Bv8 protein, we used mouse testis as a positive control for Bv8 immunoreactivity. As expected, Bv8 immunoreactivity was detectable in primary spermatocytes and Leydig cells (Fig. 1F). To control for antibody specificity, preadsorption of both anti-EG-VEGF and anti-Bv8 antibodies with an excess of immunizing peptides and incubation with preimmune sera were used. No staining was observed in chorionic villi or mouse testis under these conditions (undersized pictures, Fig. 1, B and F).

Western blot analysis

The pattern of EG-VEGF protein expression was then analyzed by Western blot on protein extracts from placental tissue homogenates (n = 21 placentas). As shown in Fig. 2A, under our experimental conditions, EG-VEGF antibody was able to detect 20 ng recombinant human EG-VEGF, and the signal was completely abolished in the presence of an excess of antigenic peptide. Figure 2B shows a representative Western blot of EG-VEGF expression in human placenta during early pregnancy.
VEGF expression in placental extracts from 6 to 12 wk of gestation. EG-VEGF protein was detected as a major band of 12 kDa. Immunoreactivity was present since the sixth week of gestation, picked at 8 wk, and then gradually decreased. Quantification of three independent experiments revealed a significant difference in EG-VEGF expression levels between the 8th and 12th wk of gestation.

**Differential localization of EG-VEGF and VEGF proteins in human chorionic villi during the first trimester of pregnancy**

Because both VEGF and EG-VEGF are expressed in human placenta during the first trimester of pregnancy, we sought to compare their cellular localization within chorionic villi at different gestational ages. Using immunohistochemistry, we compared EG-VEGF and VEGF protein expression in placentas from 6 to 12 wk of gestation (Fig. 3). VEGF expression was restricted to the cytotrophoblast layer in the chorionic villi (Fig. 3A). In anchoring villi, VEGF expression was also observed in extravillous trophoblasts (Fig. 3B). In contrast to VEGF, EG-VEGF was mainly localized to the syncytiotrophoblast layer (Fig. 3D). Its expression was also found in the cytotrophoblast at advanced gestational ages (Fig. 3E and F). However, no expression of EG-VEGF was ever observed in extravillous trophoblasts at all gestational ages examined (Fig. 3E). Such a differential pattern of expression for VEGF and EG-VEGF suggests possible complementary functions for the two factors in human placenta during the first trimester of pregnancy.

**EG-VEGF, Bv8, PKR1, PKR2, and VEGF mRNA expression during the first trimester of human pregnancy**

Human placenta, like other endocrine tissues, has been reported to be an important site of EG-VEGF expression (19). However, to date no study has examined the pattern of its expression during the first trimester of pregnancy when the angiogenic process is the most active. This prompted us to examine the expression pattern of EG-VEGF, Bv8, and their common receptors, PKR1 and PKR2, mRNAs in human placentas from 4 to 12 wk of gestation, using real-time RT-PCR. EG-VEGF mRNA was detected at all gestational ages examined (n = 27, but its level was strongly increased (up to 6- to 7-fold) between the 8th and 10th wk of gestation, as compared with gestational ages before and after this period (P < 0.001) (Fig. 3A). In contrast, Bv8 mRNA expression was undetectable except between the 8th and 10th wk of gestation (data not shown). PKR1 and PKR2 were also expressed during the first trimester of pregnancy. PKR1 exhibited the same pattern of expression as EG-VEGF with levels peaking between 8 and 10 wk of gestation (Fig. 4C). In contrast, PKR2 mRNA levels did not vary much until the end of the first trimester (12 wk) when it increased 3-fold (Fig. 4D).
Because VEGF is considered the most important factor controlling human placental angiogenesis during the first trimester of pregnancy (28), we sought to examine its pattern of expression in the same samples. VEGF mRNA expression did not significantly change during the first trimester. A slight increase was, however, observed by 12 wk of gestation. These results indicate that placental EG-VEGF and VEGF exhibit quite different patterns of expression during early pregnancy (Fig. 4B).

To further evaluate the relative expression of EG-VEGF and Bv8 in human placenta, we used real-time RT-PCR to measure their mRNA levels in placental tissues from 7 to 10 wk of gestation (n = 11). A similar analysis was performed on primary cultures of trophoblast cells isolated from placentas with the same gestational age (n = 8) and cultured for 48 h. In both placental tissue (Fig. 5A) and primary trophoblast cultures, EG-VEGF mRNA was 5 times more abundant than Bv8 mRNA (Fig. 5B), thereby confirming the differences observed between the two factors at their protein levels.

Expression of PKR1 and PKR2 in human placental tissue and cultured trophoblast cells during the first trimester of pregnancy

We then compared the mRNA expression levels of the EG-VEGF/Bv8 receptors PKR1 and PKR2 in both placental tissue (n = 13) and primary trophoblast culture (n = 6). The placentas used in this experiment were from 7 to 10 wk of gestation. Real-time RT-PCR was used to quantify the two receptor transcripts. Our results show that PKR1 and PKR2 transcripts are expressed at the same level in whole placental tissue (Fig. 5C), whereas PKR1 mRNA was 80 times more abundant than PKR2 mRNA in cultured trophoblast cells (Fig. 5D). These data strongly suggest that, within human placenta, there is a cellular specificity for PKR1 and PKR2 expression.

Effect of oxygen tension on EG-VEGF and PKR1 expression in human trophoblast cells

Low oxygen tension is a key parameter that controls gene expression during the first trimester of pregnancy. Moreover, the highest level of expression of EG-VEGF and its receptor PKR1 is observed during the hypoxic period of human placentation. This observation prompted us to hypothesize that EG-VEGF and PKR1 mRNA might be regulated by oxygen tension in human trophoblast cells. To test this hypothesis, we incubated trophoblast cells isolated from 7- to 10-wk-old placentas for 24 h under either 20% O2 or 3% O2 and measured EG-VEGF and PKR1 mRNA abundance using either semiquantitative or quantitative RT-PCR. As shown in Fig. 6A, a significant increase in both EG-VEGF (160% of normoxic control) and PKR1 (200% of normoxic control) mRNA levels was observed under hypoxic conditions (3% O2). The graphs in Fig. 6, C and D, show the mean ± se increase in EG-VEGF and PKR1 mRNA levels observed in six indepen-
dent experiments. To confirm that reduced oxygen tension was responsible for the increase in EG-VEGF mRNA, hypoxia-mimicking drugs DFO and CoCl₂ were also used. The results in Fig. 6B show treatment of primary cultures of human trophoblasts for 24 h with 200 μM CoCl₂ or DFO. Both molecules resulted in a 2-fold increase in PKR1 mRNA and a 2- to 4-fold increase in EG-VEGF mRNA levels.

We then examined the effects of transcription and translation inhibitors on the hypoxic regulation of EG-VEGF expression. In the presence of the RNA polymerase inhibitor DRB (50 μg/ml), the hypoxic induction of EG-VEGF was completely abolished, whereas it was preserved in the presence of cycloheximide, a potent inhibitor of translation (Fig. 6E). These data suggest that hypoxia regulates EG-VEGF gene expression at the transcriptional level. To determine whether oxygen tension has an effect on EG-VEGF protein levels, trophoblast cells were isolated from 7- to 10-wk-old placentas and incubated for 24 h under either 20% O₂ or 3% O₂ or in the presence of hypoxia-mimicking drugs, CoCl₂ and DFO. Figure 6F shows that all three hypoxic conditions increased EG-VEGF protein. Statistical significance, however, was only reached under DFO treatment (Fig. 6G).

**Discussion**

The present study demonstrates expression of EG-VEGF and its G protein-coupled receptors PKR1 and PKR2 in human placenta during the first trimester of pregnancy and shows that EG-VEGF and VEGF have distinct spatiotemporal patterns of expression. Furthermore, we report that the expression of EG-VEGF and PKR1 peaks at a specific time during the first trimester of pregnancy (8–10 wk gestation), corresponding to the hypoxic period of placentation which precedes the establishment of the fetomaternal vascular connection. In isolated trophoblast cells, we have also demonstrated that both EG-VEGF and PKR1 are upregulated by hypoxia. Taken together the data generated by this work reveal that EG-VEGF and its receptor PKR1 may play an important role in human placenta during the hypoxic period of placentation.

To date, four studies (19, 21, 29, 30) have reported a constitutive expression of EG-VEGF in human placentas from term pregnancies, and two of them (21, 29) have shown an expression of Bv8 in the same tissue. In the present study, we examined the expression of both factors in human placentas during the first trimester of pregnancy when angiogenesis is high. Our data show that EG-VEGF is expressed at all gestational ages examined, whereas Bv8 is only barely detectable between the 8th and 10th wk of gestation. Moreover, EG-VEGF mRNA levels are five times higher than Bv8 mRNA levels during this period. This differential pattern of expression is similar to that observed in the human endometrium (31) and corpus luteum (32).

The immunolocalization experiments performed in the present study have revealed interesting patterns of distribution. The results substantiate the conclusions of the RT-PCR experiments and show that Ir-EG-VEGF is present in the syncytiotrophoblasts. The strong expression of EG-VEGF in the syncytiotrophoblast suggests that this factor is probably synthesized by this endocrine component of the placenta and that its expression is associated with the degree of trophoblast differentiation.

Given the established importance of VEGF in human placentation (33–35) and the partial similarity of its biological functions with those of EG-VEGF, we compared their pattern of expression during the first trimester of pregnancy. Interestingly, we observed that these factors were expressed in adjacent but distinct compartments. In contrast to EG-VEGF, which was localized to the syncytiotrophoblast layer, VEGF was mainly present in the cytotrophoblasts and extravillous trophoblasts. This finding is reminiscent of human ovaries, in which VEGF and EG-VEGF are rarely colocalized in the same cell type during the follicular and luteal phases (30).
Based on its specific pattern of expression and its absence of colocalization with VEGF, EG-VEGF appears to be a novel growth factor likely to play complementary biological roles to those of VEGF.

EG-VEGF/PK1 and Bv8/PK2 are equally potent ligands for two structurally related G protein-coupled receptors named PKR1 and PKR2 (22, 35–37). These two receptors are expressed in gastrointestinal organs, endocrine glands, central nervous system, and many other tissues, suggesting a wide range of biological actions for EG-VEGF and Bv8 (22, 37). Different patterns of PKR1 and PKR2 expression have been observed in different cell types. For example, endothelial cells of the bovine corpus luteum appear to express both PKR1 and PKR2 (32), whereas endocrine granulosa and thecal cells preferentially express PKR1 (38). In first-trimester human placentas, our data show that PKR1 and PKR2 are expressed at the same levels, whereas in primary trophoblast culture, only PKR1 appears to be expressed. These findings clearly suggest that PKR1 and PKR2 may have different cellular localizations within the placental villi. We propose that PKR2 is likely to be present in endothelial cells of fetal blood vessels because, in other tissues, this receptor has been reported to be specifically localized to endothelial cells (21, 30, 38). The data presented herein clearly show PKR1 expression in trophoblast cells. However, it is difficult to know precisely which type of trophoblast cell expresses PKR1 because our cytotoxophoblast cell preparation contains a small amount of syncytial trophoblast cells. Because of the lack of commercially available antibodies to PKR1 and PKR2, we cannot make any firm conclusions regarding the localization...
of these receptors within the placental villi at this time. Nevertheless, the expression of PKR1 in trophoblast cells suggests a direct effect of EG-VEGF on nonendothelial cells. This finding suggests that EG-VEGF may have an important role in human placentation. Therefore, the previously characterized endocrine tissue-specific angiogenic actions of EG-VEGF (19–21) may very well be one among several other functions played by this factor in endocrine tissues.

EG-VEGF levels appeared to greatly vary during early pregnancy, progressively increasing until the 10th week of gestation and rapidly dropping afterward. These dramatic changes appear to be correlated with the hypoxic developmental period of the placenta because the junction of the fetal and maternal vascular networks is known to occur between the 10th and 12th week of gestation. This correlation is supported by the transcriptionally controlled up-regulation of EG-VEGF mRNA and concomitant increase of its protein levels that were observed in primary cultures of trophoblasts after 24 h of culture under reduced O2 tension. This regulation was not unexpected as a functional hypoxia-response element (TACGTCCGGC) able to bind the hypoxia-inducible factor-1α has been identified in the human EG-VEGF promoter (19). However, we cannot totally exclude that other factors presenting peaks in their expression during the first trimester of pregnancy may also participate in the regulation of EG-VEGF expression. Human choriogonadotropin and progesterone are two such candidates because they have been recently shown to stimulate EG-VEGF mRNA expression in human luteinized granulosa cells (32) and human endometrial tissue (31), respectively.

Regulation of placental growth factor expression by oxygen tension is well established and has been described for several angiogenic factors, including VEGF (39, 40), TGFβ3 (41), and the soluble form of VEGF receptor-1 (s-flt) (42, 43). In the present study, we also observed up-regulation of PKR1 receptor expression under hypoxic conditions. This is the first report indicating PKR1 regulation by oxygen tension. A GenBank screen of the human PKR1 promoter revealed the presence of one putative HIF-1 binding site, suggesting that the oxygen effect on PKR1 expression might occur through a pathway that involves HIF-1. This finding further supports the idea that EG-VEGF and PKR1 may play an important role in normal human placentation and perhaps in pathologies such as preeclampsia. A recent study by Chung et al. (30) reported no change in EG-VEGF expression in placentas from preeclamptic patients. In this study, changes in EG-VEGF expression have been examined during the second and third trimester of pregnancy. We now know that the origin of preeclampsia takes place during the first trimester of pregnancy and that any changes in protein expression after the establishment of the disease are considered to be consequences of the disease rather than causes of its development. Our data show that EG-VEGF expression is at its lowest levels by the end of the first trimester, suggesting that its greatest role should occur within that trimester. Moreover, given the correlation between the pattern of EG-VEGF and PKR1 expression with the hypoxic period of placental development and knowing that failure in placental angiogenesis is thought to contribute to preeclampsia development, one can speculate on the potential implication of this factor and/or its receptors in the development of preeclampsia. However, only a prospective study examining the expression of EG-VEGF and/or PKR1 during the first trimester of pregnancy in women who go on to develop preeclampsia will allow to provide an answer to this question. Future studies are also required to determine the biological activities triggered by EG-VEGF in the human placenta and identify whether dysregulation of EG-VEGF expression may result in placental pathologies.

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