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Differential Expression of VEGF, EG-VEGF, and VEGF Receptors in Human Placentas from Normal and Pre-eclamptic Pregnancies

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Abstract

Vascular Endothelial Growth Factor (VEGF) is a potent regulator of placental vascular function. Endothelial dysfunction is a key factor associated with pre-eclampsia. In this study, we examined expression of VEGF, endocrine-gland-derived VEGF (EG-VEGF), and VEGF receptors (VEGF receptor-1 [VEGFR-1] and -2 [VEGFR-2], and neuropilin-1 [NP-1] and -2 [NP-2]) in human placentas from women with normal and pre-eclamptic (PE) pregnancies using quantitative or semi-quantitative PCR. We found that total VEGF mRNA expression was increased 2.8 fold ($p < 0.05$), along with increases in mRNA expression of VEGF121, 165, and 189 ($p < 0.05$; 1.7, 1.9, and 1.8 fold, respectively) in PE vs normal placentas. Expression of VEGFR-1 mRNA, but not EG-VEGF and the other three VEGF receptors studied, was elevated ($p < 0.05$) 2.7 fold in PE vs normal placentas. Protein expression of VEGF and its four receptors was determined using Western blot analysis. For VEGF, two major isoforms (VEGF165 and 189) were detected. For VEGFR-1, VEGFR-2, NP-1, and NP-2, one major band was observed at 180, 235, 130 and 130 kD, respectively. All of these bands were corresponding to their positive controls. Of these five proteins studied, only VEGFR-1 levels were increased ($p < 0.05$; 1.7 fold) in PE placentas. The expression of VEGF and the four VEGF receptors was confirmed using immunohistochemistry. They were primarily present in syncytiotrophoblasts and endothelial cells of villous capillaries and large vessels. Thus, together with the previous reports that VEGFR-1 mediates trophoblast function and inhibits VEGF-induced angiogenesis and endothelium-dependent vasodilation, these data suggest that the increased VEGFR-1 expression may alter VEGF-mediated function on trophoblast and endothelial cells in PE placentas.

Keywords

VEGF isoforms; VEGF receptors; Pre-eclampsia; Placenta

INTRODUTION

Abnormal vascular growth and impaired endothelial function in placentas are associated with abnormal pregnancy conditions such as pre-eclampsia. Pre-eclampsia, a life threatening

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complication of pregnancy, is characterized by the onset of high blood pressure and proteinuria. Pre-eclampsia occurs in about 7–10 % of all pregnancies (1), accounting for almost 15% of pregnancy-associated deaths and one of the major causes of iatrogenic prematurity among newborns (2). The pathogenesis of pre-eclampsia is thought to act at three levels, defective placentation, placental ischemia, and endothelial cell dysfunction. Of these, endothelial dysfunction is considered to be a key factor associated with pre-eclampsia (3).

VEGF is a critical regulator for vascular growth and function. Targeted disruption of a single allele of VEGF gene in mouse causes impaired cardiovascular development, leading to embryonic death (4, 5). Human VEGF has at least six isoforms of 121, 145, 165, 183, 189, and 206 amino acids. These VEGF isoforms differ in their bioavailability and biological activities (6). For example, VEGF121 does not bind heparin and is freely released from producing cells. VEGF165 is partially soluble with a significant fraction remaining bound to the cell surface and the extracellular matrix. Of these VEGF isoforms, VEGF121, 165, and 189 are widely expressed, while VEGF145, 183, and 206 expression is found in a few tissues (7–9). Moreover, VEGF165 is more potent than VEGF121 in stimulating endothelial cell proliferation (10). Thus, the expression of multiple VEGF isoforms in the same tissue and different biochemical properties of individual VEGF isoforms imply that they have distinct roles in regulating vascular development and function. This is supported by the fact that mouse embryos which express only VEGF120 survive to term although they exhibit severe vascular defects (11). Additionally, expression of each VEGF isoform varies temporally and spatially in association with vascular formation, differentiation, and function in the developing mouse tissues (12,13).

Endocrine gland-derived (EG)-VEGF is a newly identified angiogenic factor which is able to stimulate proliferation, migration and fenestration in capillary endothelial cells selectively derived from endocrine glands (14). Expression of human EG-VEGF messenger RNA is restricted to the steroidogenic glands including placenta, and is induced by hypoxia (14). However, to date, the data are lacking to compare EG-VEGF expression in human placentas from normal and pre-eclamptic (PE) pregnancies.

VEGF actions are mediated via binding to its receptors which include VEGF receptor–1 (VEGFR-1/Flt1) and –2 (VEGFR-2/Flk1/KDR). VEGFR-2 is the major signal transducer of VEGF in endothelial cells, and mediates most known VEGF's bioactivities (i.e. cell proliferation, migration, and permeability) (15). In contrast, VEGFR-1 has been implicated in the inhibition of VEGF-dependent endothelial function (16,17). Both these receptors, however, are critical for regulating vasculogenesis and angiogenesis, since null mutation of either of these receptors in the mouse results in abnormal vascular formation and development, leading to impaired vascular function and embryonic death (18,19).

Neuropilin-1 (NP-1) and –2 (NP-2) are two VEGF receptors which bind to VEGF165, but not VEGF121 (20–22). Unlike VEGFR-1 and –2, neither NP-1 nor NP-2 has a tyrosine kinase domain and therefore can not induce cellular responses by themselves alone. However, both NP-1 and NP-2 are required for normal vascular development since disruption of NP-1 or NP-2 gene in mouse results in abnormal vascular formation and development, leading to embryonic death (23). Moreover, a ternary complex of VEGF165, VEGFR-2, and NP-1 potentiates VEGF165 binding to VEGFR-2 and therefore enhances VEGF-induced angiogenesis (20,24).

The data on VEGF expression in normal and PE pregnancies are controversial. The majority of these studies compared serum VEGF levels in the maternal circulation, reporting either decreased or increased levels in normal vs PE pregnancy, largely depending on

methodologies used (25). Similarly, placental VEGF mRNA levels have also been reported to be either decreased (26), increased (27), or unchanged (28) in normal vs PE pregnancy. To date, little is known about protein expression of VEGF and VEGF receptors in placentas from normal and PE pregnancies. Thus, to determine whether expression of individual VEGF isoforms and VEGF receptors in placentas is associated with pre-eclampsia, in this study, we have examined mRNA and protein expression of VEGF and its four receptors (VEGFR-1, VEGFR-2, NP-1 and NP-2) as well as EG-VEGF mRNA expression in placental tissues from normal and PE pregnancies.

Materials and Methods

Tissue collection

Placentas were obtained immediately after delivery from women with normal and PE pregnancies. PE was defined according to standard criteria: onset of hypertension during late pregnancy with systolic and diastolic blood pressure > 140/90 mmHg on at least two occasions and urinary protein > 2+ on dipstick or > 0.3 g/24 h. Collection of placentas was approved by the Institutional Review Board, University of Wisconsin-Madison, and followed the recommended guidelines for using human subjects. Placental villi from beneath the chorionic and basal plates were quickly dissected (approximately 10 g each), snap frozen in liquid nitrogen, and stored at −80° C. Additional placental tissues were fixed overnight at 4° C in 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS) for immunohistochemical analysis.

Total RNA Purification

Total RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The placental tissue (~800 mg/placenta) was homogenized using a rotor-stator homogenizer (PawerGen700, Fisher Scientific, Hampton, NH) until the samples is uniformly homogeneous. The concentration of RNA was measured using a spectrophotometer (DU7400, Beckman, Fullerton, CA) and RNA quality was confirmed on agarose gels.

Real Time PCR Analysis

A total RNA sample (2 μg/sample), 20 pmol of oligo deoxythymidine (dT) primer, 2 μl of deoxynucleotide triphosphates (dNTP) mix (5 mM each), and 1 μl of reverse transcriptase (Omniscript Reverse Transcriptase, Qiagen) were used in 20 μl scale to generate cDNA.

The primers (Table 1) were designed for human VEGF, EG-VEGF, VEGFR-1, VEGFR-2, NP-1, and NP-2 using Primer Express 2.0 (Applied Biosystems, Foster City, CA). The primer set for VEGF was designed to detect all VEGF isoforms. The specificity of these primers was first analyzed by RT-PCR and the each PCR product was confirmed by sequencing.

Real Time PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green using Taqman7700 sequence detection system (PerkinElmer Life Sciences, Boston, MA) as described (29). 10 μl of SYBR Green PCR Master Mix (AppliedBiosytem), 4 μl of cDNA described above, and 2 pmol each of primers were used for Real Time PCR in a final volume of 20 μl. The reaction was carried out at 95° C, 15 sec and 60° C, 1 min for 40 cycles after denatured at 95° C for 10 min. For each sample, PCR reactions were performed in duplicate or triplicate. The cDNA levels were determined using the standard curve of cycle thresholds. The standards used for the standard curve were purified PCR products made from one normal placenta sample. All data for each cDNA examined were

within its corresponding standard curve. Data obtained were then normalized to β-actin cDNA.

Semi-quantitative RT-PCR for VEGF Isoforms

Since the real time PCR cannot detect individual VEGF isoforms, mRNA expression of three VEGF isoforms was quantified by RT-PCR, using a specific Human VEGF 3 Isoforms and Beta-Actin Genes Dual-PCR Kit (Maxim Biotech, San Francisco, CA) according to the manufacturer's instructions. The estimated sizes of RT-PCR products for β-actin, VEGF189, VEGF165, and VEGF121 were 474, 306, 234, and 104 bp, respectively. Total RNA (2 μg/sample) was used and data were normalized to β-actin.

Western blot Analysis

Placental tissues were homogenized and lyzsed by sonication in buffer (50 mM HEPES, 0.1 M NaCl, 10 mM EDTA, 4mM sodium pyrophosphate, 10mM sodium fluoride, 2 mM sodium orthovanadate (pH 7.5); 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin). After centrifugation, protein concentrations of the supernatant were determined with BSA (fraction V; Sigma, St. Louis, MO) as standards.

Because the weak positive signal and intensive background were detected in the preliminary study, placental VEGF, NP-1, and NP-2 proteins were first enriched using immunoprecipitation, followed by Western blot analysis. Briefly, protein samples (2 mg for VEGF, and 1 mg for NP-1 and NP-2) were pre-cleaned by mixing the samples with 0.25 μg of rabbit control IgG (Vector Laboratories, Burlingame, CA) and 10 μl protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4° C with end-over-end rotation. The supernatants were obtained and incubated with the polyclonal rabbit antibody (5 μg) against human VEGF (protein A-agarose conjugated), NP-1, or NP-2 (Santa Cruz Biotechnology) at 4° C for 2 h. For NP-1 and NP-2, the mixture of proteins and antibodies was subsequently incubated with 30 μl protein A-agarose at 4° C overnight. The immunoprecipitates were collected by centrifugation.

Western blot analysis was conducted as described (30,31). The protein samples (for VEGFR-1 and VEGFR-2) or immunoprecipitates (for VEGF, NP-1, and NP-2) were heat-denatured (95°C, 5 min) in Laemmli buffer, separated on pre-casted SDS-PAGE gels (4–15% gradient, Bio-Rad, Hercules, CA), and electrically transferred to polyvinylidine difluoride (PVDF) membranes. The membranes were immunoblotted with a mouse monoclonal antibody against human VEGF (1:100), VEGFR-2 (1:500), NP-1 (1:100), or NP-2 (1:200) or with a rabbit anti-human VEGFR-1 antibody (1:500). All antibodies used were purchased from Santa Cruz Biotechnology. Proteins were visualized using ECL reagents, followed by exposure to chemiluminescence films. The immunoreactive signals were analyzed by densitometry. The membranes used for detecting VEGFR-1 and VEGFR-2 were reprobed with a GAPDH antibody (Research Diagnostics, Flanders, NJ). Recombinant human VEGF121 and 165 (R&D system, Minneapolis, MN) were used as positive controls for VEGF. Human umbilical vein endothelial cells (HUVEC; CAMBREX, East Rutherford, NJ) were used as positive controls for the VEGF receptors.

Immunolocalization

Immunolocalization of VEGF, VEGFR-1, VEGFR-2, NP-1, and NP-2 in the placental tissues from normal (n = 5) and PE (n = 5) pregnancies was visualized by indirect detection via the avidin:biotinylated-peroxidase complex method with 3-amino-9-ethylcarbazole (AEC) as the chromogen (30, 31; Vector Laboratories). Briefly, tissue sections were heated in sodium citrate buffer (0.1M, pH 6.0) in microwave for 5 mins. Endogenous peroxidase in tissue sections was quenched with 3% H_2O_2 in methanol for 15 min. The sections were

stained briefly with Harris' hematoxylin. After washing with PBS containing 0.3% Triton X-100, nonspecific binding was blocked with 1% normal goat serum for 20 min. The tissue sections were incubated with a rabbit anti-VEGF as we described (31), VEGFR-1, VEGFR-2, NP-1 or NP-2 antibody (4 μg/ml; Santa Cruz Biotechnology) for 1 hr. Controls consisted of replacing the primary antibody with preimmune rabbit IgG (Vector Laboratories) at the same concentration as the primary antibody.

Statistical analysis

The data were analyzed by the Student's t-test. Results were considered significant at $p < 0.05$.

Results

The mRNA expression of total VEGF, EG-VEGF and the four VEGF receptors (VEGFR-1, VEGFR-2, NP-1, and NP-2) in human placentas was first confirmed using the RT-PCR analysis (Fig. 1). One band for each mRNA studied was observed at the estimated size as shown in Table 1 and these PCR products were confirmed by sequencing, indicating the specificity of each primer set. The mRNA levels of total VEGF, EG-VEGF and the four VEGF receptors quantified using real-time PCR are shown in Figure 2. The mRNA levels of total VEGF and VEGFR-1 were increased 2.8 and 2.7 fold ($p < 0.05$) respectively in PE vs normal placentas. No significant difference in mRNA levels of EG-VEGF and the other three VEGF receptors was observed between PE and normal placentas. Among these genes studied, VEGFR-1 was the most abundant (1/8 of β-actin), followed by VEGFR-2 (1/19), NP1 (1/66), VEGF (1/186), EG-VEGF (1/473), and NP-2 (1/631) in normal placentas.

To determine whether expression of individual VEGF isoforms differed in PE vs normal placentas, mRNA levels of three major VEGF isoforms (VEGF121, 165, and 189) were quantified using semi-quantitative RT-PCR (Fig 3). The overall mRNA levels of these three VEGF isoforms were increased ($p < 0.05$) 1.8 fold in PE vs normal placentas. Compared with normal pregnancy, the placental mRNA levels of three VEGF isoforms in PE were elevated ($p < 0.05$) 1.8, 1.9, and 1.7 fold, respectively for VEGF189, 165, and 121, as compared with normal placentas.

Protein expression of VEGF and its four receptors in normal and PE placental tissues was determined by Western blot analysis (Fig. 4). The VEGF antibody detected two major bands approximately at 20 and 25 kD (Fig. 4A). The former was corresponding to the molecular mass of recombinant human VEGF165, while the latter is similar to the reported molecular mass of VEGF 189 (32). There was no significant difference in protein expression of both VEGF isoforms between normal and PE placentas (Fig. 4B). For VEGFR-1, VEGFR-2, NP-1 and NP-2, one major band, corresponding to its respective control, was also identified at approximately 180, 235, 130, and 130 kD, respectively (Fig. 4A). VEGFR-1, but not the other three receptors (VEGFR-2, NP-1 and NP-2), protein levels were increased ($p < 0.05$) 1.7 fold in PE vs normal placentas (Fig. 4B).

Positive reddish staining for VEGF and its four receptors was observed primarily in syncytiotrophoblasts and endothelial cells of microvessels and large vessels in human PE (not shown) and normal placentas (Fig. 5). No apparent difference in the staining intensity for all five antigens examined was noted between PE and normal placentas. No staining was observed in the IgG controls (Fig. 5).

Discussion

Herein, we demonstrated, for the first time, that VEGFR-1 protein expression was increased in PE placentas as compared with normal placentas. We also found that mRNA levels of all three VEGF isoforms tested were increased, while protein levels of two major VEGF isoforms detected were not altered in PE placentas as compared with normal placentas. Moreover, we determined, for the first time, that there were no significant change in expression of EG-VEGF mRNA as well as VEGFR-2, NP-1, and NP-2 mRNAs and proteins between PE and normal placentas, suggesting that they may not play a critical role in PE placentas. Thus, together with the observations that VEGFR-1 mediates nitric oxide production by trophoblast cells (33) and inhibits VEGF-induced endothelial proliferation, migration, and vasodilation (16, 17, 34), these data suggest that VEGFR-1 is a key factor associated with PE pregnancy and an increased VEGFR-1 expression in PE placentas may alter VEGF-mediated function on trophoblast and endothelial cells. In addition, the increased VEGF and VEGFR-1 mRNA expression in placentas is assumed to be a potential early indicator of pre-eclampsia.

We demonstrate that mRNA of total VEGF and all three VEGF isoforms (VEGF121, 165, and 189) tested increase in PE placentas as compared with normal placentas. We also note that the ratio of the individual isoforms in the total three VEGF isoforms combined is similar between PE and normal placentas (40%, 34%, and 26% of the total three VEGF isoforms for VEGF121, 165, and 189, respectively). These data suggest that the increase in total VEGF mRNA levels in PE placentas are attributed to proportional elevations in each individual VEGF isoform. In the current study, two isoforms of VEGF proteins (VEGF165 and 189) were detected in human placentas, indicating that they are the two major VEGF isoforms expressed in human placentas. We further provided evidence showing that protein levels of VEGF165 and 189 did not increase as their mRNA expression in PE placentas. It is not clear from these data alone whether the lack of increase in protein levels of these VEGF isoforms is due to decreased translation or increased protein degradation. However, PE pregnancy is associated with increases in VEGF levels in maternal circulation and VEGF mRNA expression in placentas (27, 35, 36). Moreover, in parallel with these increases, elevations in soluble VEGFR-1 (sVEGFR-1) protein, which has a high affinity to VEGF, in the amniotic fluid and maternal circulation (25, 37, 38) and in its mRNA expressions in placentas (25) have also been reported. Therefore, it is more likely that translation of these VEGF isoforms in PE placentas may keep the same pace as their increased mRNA levels. After being released from its cells of origin or from extracellular matrix by plasmin cleavage, these VEGF could speculatively bind to sVEGFR-1 and be rapidly transported into the circulation, so contributing to the higher VEGF levels in the maternal circulation and maintaining local VEGF protein levels in PE placentas at the same levels as normal placentas.

Our findings that VEGFR-1 mRNA levels are increased in PE placentas are consistent with the previous report (27). VEGFR-1 mRNA detected herein was a full length VEGFR-1 using the specific primers, which cannot detect sVEGFR-1. More importantly, we demonstrated, for the first time, an elevated VEGFR-1 protein expression in PE placental tissues. It is noteworthy that only VEGFR-1, out of the five proteins studied, exhibits an increase in PE vs normal placentas. These observations indicate that this increase in VEGFR-1 protein levels is specific in PE placentas and not to be due to any potential change in placental tissue or cell architecture. Moreover, we also found in the current study that in addition to endothelial cells, VEGFR-1 is also localized in trophoblast cells, confirming the previous studies (33, 39–42). Since the majority of placental cellular mass consists of trophoblast cells, the increased VEGFR-1 expression observed could be attributed largely to trophoblast cells. An important question which follows is whether and how this increased VEGFR-1 expression in trophoblast affects placental functions associated with pre-

eclampsia. Since VEGF is expressed in placental trophoblast cells as shown in the current study and other investigators (33, 39–42), VEGF is able to affects multiple trophoblast functions (i.e. invasion, differentiation, proliferation, and releasing vasorelaxants) via an autocrine loop (33, 39–42). Moreover, abnormal trophoblast invasion and differentiation are believed to be associated with pre-eclampsia (3, 42). Thus, it is plausible that the increased VEGFR-1 levels observed may substantially alter normal trophoblast function in PE placentas.

In addition to affecting their own functions, the trophoblast could also influence adjacent placental endothelial cells by producing and releasing vasoactive factors such as sVEGFR-1, nitric oxide, endothelin, and prostacyclin, possibly leading to endothelial dysfunction (3, 33, 42,43). Endothelial dysfunction has been postulated to be responsible for initiating and/or associating with many of the changes in pre-eclampsia, which is characterized by the increased endothelial-mediated vasoconstriction, increased vascular permeability, and increased endothelial-mediated platelet aggregation leading to maternal hypertension, proteinuria and thrombocytopenia (3). Moreover, although the increased VEGFR-1 expression observed could be attributed largely to trophoblast as discussed above, it is possible that a portion of this increase is derived from endothelial cells since VEGFR-1 is also expressed in endothelial cells. In this case, the increased VEGFR-1 in endothelial cells of PE placentas may interfere with VEGF mediated actions on these endothelial cells. This concept is supported by the observations that VEGF, acting through VEGFR-1, is able to inhibit VEGFR-2-mediated endothelial proliferation and migration (16, 17), and to reduce endothelial dependent uterine blood vessel relaxation in pregnancy (34).

Although the role of EG-VEGF in placental endothelial cells remained unsolved, EG-VEGF is expressed in placentas and has similar biological activity to VEGF in the endocrine glands (14). Our observation that EG-VEGF mRNA is expressed in human placentas is in agreement with the previous report (14). We also have, for the first time, provided evidence showing that there is no difference in EG-VEGF mRNA levels between normal and PE placentas. Because of the lack of a commercial available EG-VEGF antibody, we were unable to measure EG-VEGF protein expression. However, since EG-VEGF regulates angiogenesis and endothelial permeability both of which are key endothelial functions associated with pre-eclampsia, future studies are needed to investigate EG-VEGF protein expression as well as expression of its receptors in placentas.

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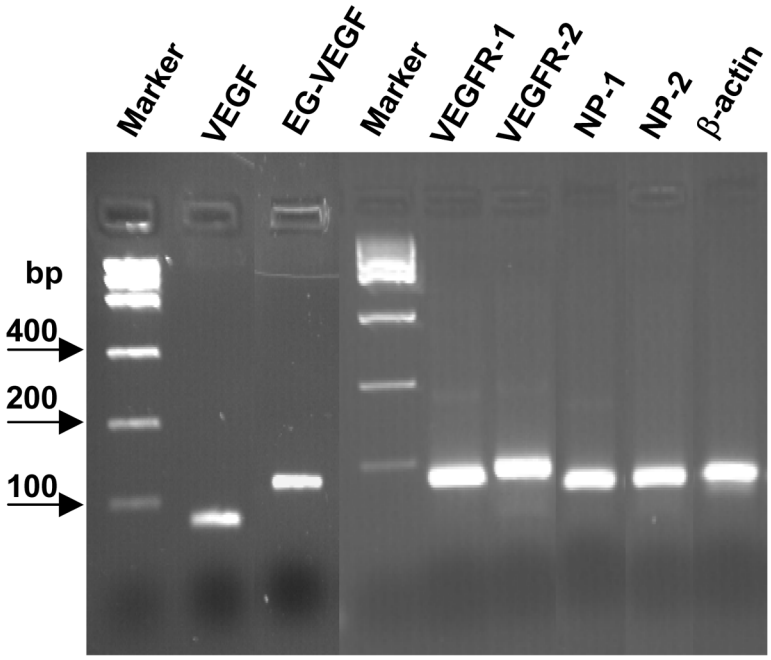

Fig. 1.

RT-PCR analysis for VEGF, EG-VEGF, VEGFR-1, VEGFR-2, NP-1, and NP-2 in human placentas. The total RNA samples (0.5 μg/gene) from one normal placenta were used for PCR amplification. The PCR products were confirmed by sequencing and used as standards for the real-time PCR.

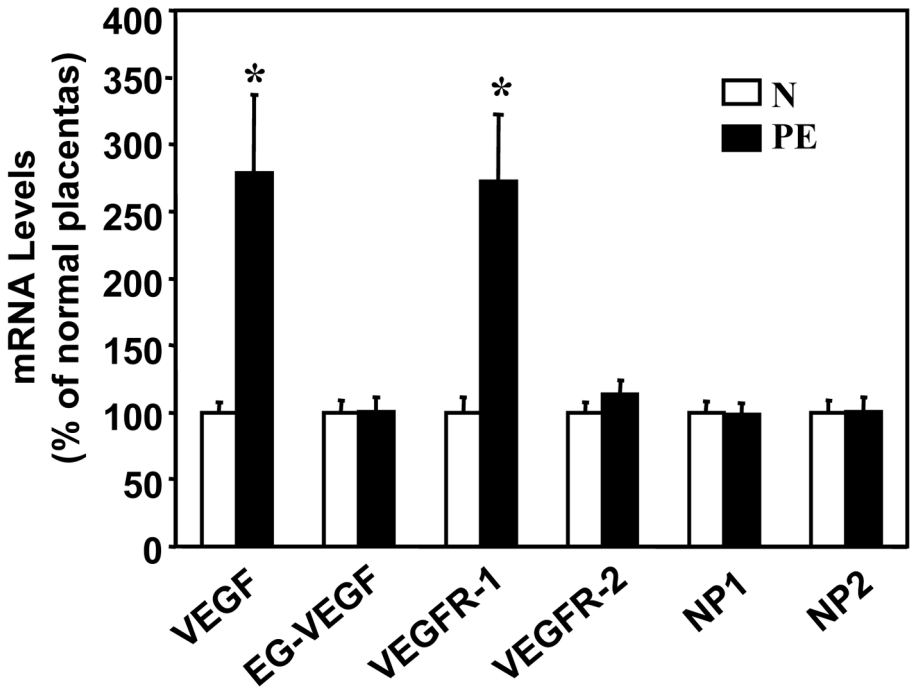

Fig. 2.

Real-time PCR analysis of the mRNA levels of VEGF, EG-VEGF, VEGFR-1, VEGFR-2, NP-1, and NP-2 in human placentas from normal and PE pregnancies. For each gene, cDNA was amplified from total RNA (2 μg/sample) of normal or PE placentas and 4 μl of cDNA per sample was used for real-time PCR. The mRNA levels were normalized to β-actin. For each sample, the real-time PCR reaction was performed in duplicate or triplicate for each mRNA. Data are expressed as means ± SE. *, differs from its corresponding N ($p < 0.05$). N: placentas from normal pregnancy (n=16), PE: placentas from preeclamptic pregnancy (n=18).

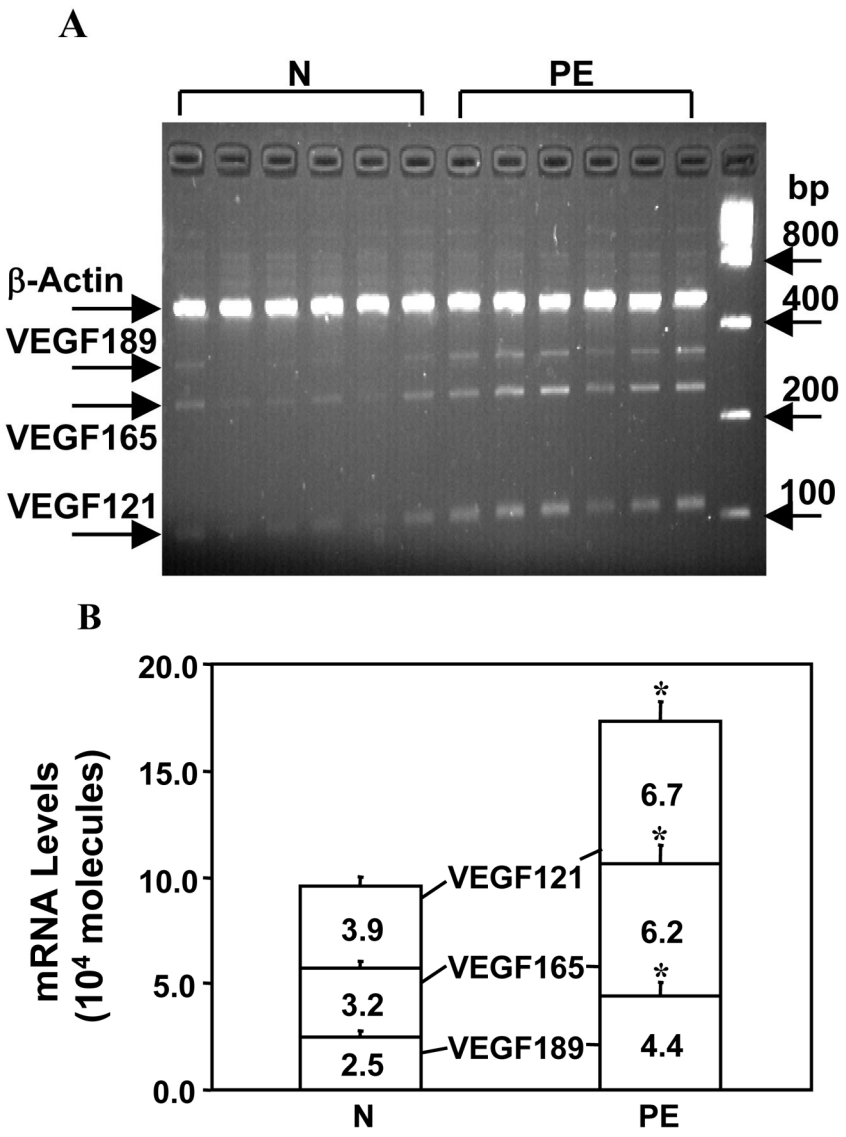

Fig. 3.

Semi-quantitative RT-PCR analysis for VEGF isoforms in human placentas from normal and PE pregnancies. The total RNA (2 μg/sample) was used for generating cDNA and PCR products were run on 4% agarose gels. (A) A representative agarose gel. The sizes of RT-PCR products are 306, 234, 104, and 474 bp for VEGF189, VEGF165, VEGF121, and β-actin, respectively. (B) mRNA levels of three VEGF isoforms. The VEGF mRNA levels were normalized to β-actin. Data are expressed as means ± SE. *, differs from its corresponding N ($p < 0.05$). N: placentas from normal pregnancy (n=16), PE: placentas from pre-eclampsia pregnancy (n=16).

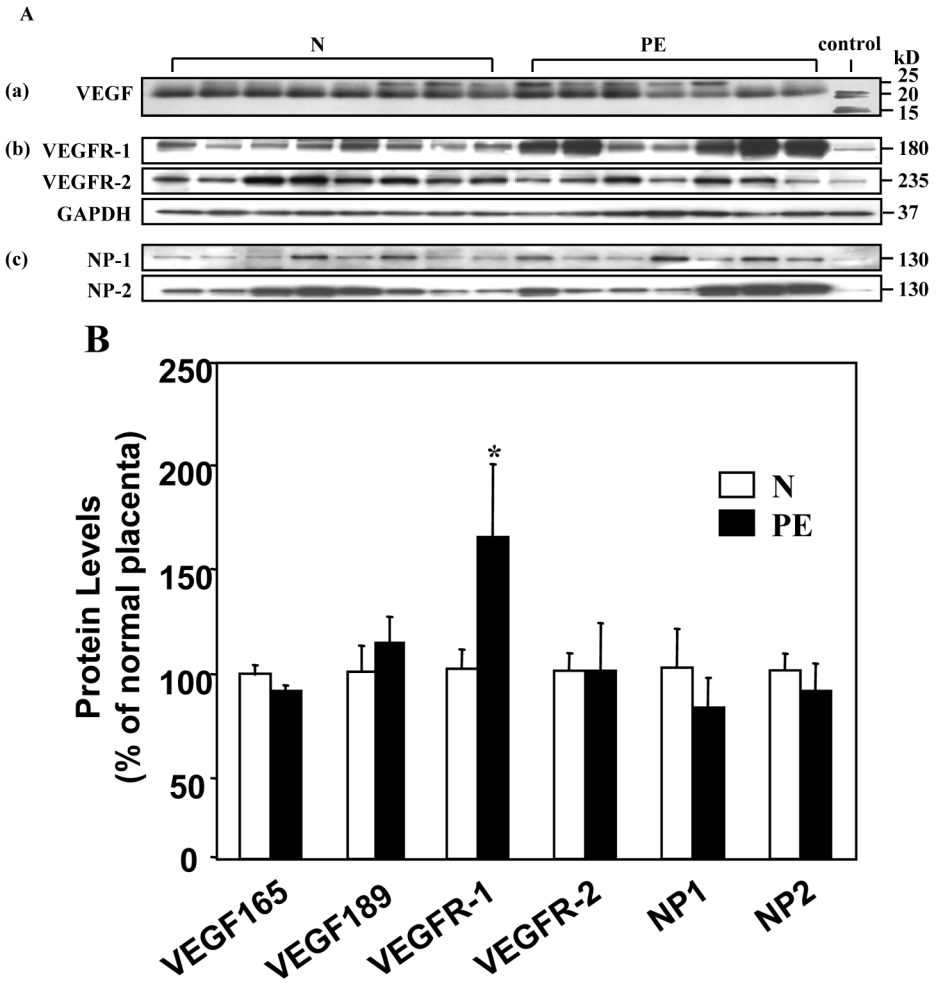

Fig. 4.

Western blot analysis for VEGF, VEGFR-1, VEGFR-2, NP-1, and NP-2 in human placentas from normal and PE pregnancies. (A) A representative Western blot for VEGF, VEGFR-1, VEGFR–2, NP-1, NP–2, and GAPDH. (a) VEGF: proteins were first immunoprecipitated with a rabbit polyclonal antibody against VEGF, followed by immunobloting with a mouse-monoclonal anti-human VEGF antibody. Recombinant human VEGF165 (at 20 kD) and VEGF121 (at 18 kD) were used as positive controls. (b) VEGFR-1 and VEGFR-2: proteins were separated and detected. One major band for each protein was observed at 180 Kd and 235 Kd for VEGFR-1 and VEGFR-2 respectively, corresponding to its positive control HUVEC. The protein levels of VEGFR-1 and VEGFR-2 were normalized to GAPDH. (c) NP-1 and NP-2: proteins were first immunoprecipitated with a rabbit polyclonal NP-1 or NP-2 antibody, followed by immunobloting with a mouse monoclonal anti NP-1 or NP-2 antibody. One major band was located at ~130 Kd, corresponding to their HUVEC positive controls. (B) Protein levels of VEGF and its four receptors. *, differs from its corresponding N, $p < 0.05$. N: placentas (n=16) from normal pregnancy, PE: placentas (n=13) from pre-eclamptic pregnancy.

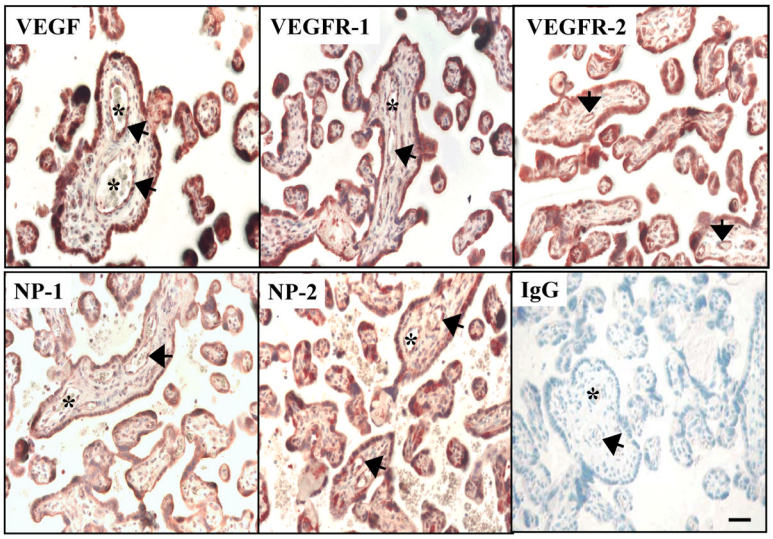

Fig 5.

Immunolocalization of VEGF, VEGFR-1, VEGFR-2, NP-1, and NP-2 in human placentas from normal pregnancy. The tissues sections were incubated either with a rabbit antibody against each indicated antigen, or preimmune rabbit IgG (4 μg/ml; IgG). *, lumen of blood vessels; arrow heads, endothelial cells. All panels are in the same magnification. Bar = 40 μm.

Table 1

Sequences of primers used for real-time RT-PCR

Transcript	Forward (5′-3′)	Reverse (5′-3′)	size	GenBank #
VEGF	TGCAGATTATGCGGATCAAACC	TGCATTCACATTTGTTGTGCTGTAG	81 bp	AB021221
EG-VEGF	ATGCTCCTCCTAGTAACTGTGTCTGACT	CTCGAAGCCACAGGCTGATG	109 bp	AF333024
VEGFR-1	CAGGCCCAGTTTCTGCCATT	TTCCAGCTCAGCGTGGTCGTA	82 bp	AF063657
VEGFR-2	CCAGCAAAAGCAGGGAGTCTGT	TGTCTGTGTCATCGGAGTGATATCC	87 bp	AF063658
NP-1	CAGAAAACACCAGGTCGAATCC	CGCGCTGTCGGTGTAAAAA	69 bp	AF016050
NP-2	GCATGGCAAAAACCACAAGGTAT	TGGAGCGTGGAGCTTGTTCA	76 bp	AF280545
β-Actin	TCCACCTTCCAGCAGATGTG	GCATTTGCGGTGGACGAT	75 bp	NM_001101