**INTRODUCTION**

Pregnancy-induced hypertension (PIH) usually develops during the second half of the pregnancy and accompanies proteinuria and edema. PIH is estimated to affect 7 to 10% of all pregnancies in the United States (1), making common complications associated with feto-maternal death and morbidity.

While the etiology of PIH remains unclear, it is known to be associated with endothelial damage and dysfunction (2). There are also an increased concentration of vasoconstrictive agents such as endothelin (3), increased capillary permeability (4), and the endothelial damage is also associated with neutrophil (5).

An impaired trophoblastic invasion of the maternal placental bed is considered to be the initiator of endothelial damage in PIH (6). This defect might result in poor fetoplacental perfusion along with a release of factors into the maternal circulation, leading to a widespread endothelial damage and the resulting clinical symptoms of PIH (7). This placental dysfunction could be mediated by the factors present in the maternal circulation as a result of impaired trophoblastic invasion (6). Angiogenic growth factors are also thought to play a substantial role in the placentation, and can be strong endothelial cell activators (8).

Recently, placenta growth factor (PIGF), a dimeric secreted factor that shares close amino acid homology (53% identity) to vascular endothelial growth factor (VEGF), was known as a potent angiogenic growth factor capable of inducing the proliferation, migration, and activation of endothelial cells (9). Unlike VEGF, abundant expression of PIGF is restricted to the placenta (10). Alternative splicing of PIGF mRNA produces at least three polypeptides, PIGF-1, PIGF-2 and PIGF-3, which have different secretory patterns, heparin-binding affinities and dimerization properties (10, 11).

Ideal placental development requires an adequate and organized interaction of vascular growth factors and their receptors, including VEGF and PIGF; both VEGF and PIGF act through their common tyrosine kinase receptors (12). The expression of VEGF and KDR (PIGF-1/VEGFR-2) is strongest during early gestation and decrease as pregnancy advances (13-15) while PIGF and Flt-1 (PIGF-1/VEGFR-1) increase towards term (16, 17). In the present study, we examined the expression patterns and localization of PIGFs and their recep-

---

**Key Words**: Growth Factors; Receptors, Growth Factor; Placenta; Human; Pre-Eclampsia
tors in the normal and PIH human placentas at term to get insight into their relation to PIH.

MATERIALS AND METHODS

Tissue collection and preparation for in situ hybridization

Placentas used for analyses were collected from 8 normal [37 (4 cases), 38 (2 cases), 39 wk (2 cases)] and 5 PIH [36 (2 cases), 37, 38, 39 wk] mothers at caesarean section delivery in Gyeongsang National University Hospital (GNUH). The Human Subject Research Committee of GNUH approved these human tissues for the present study. The parameters to define PIH were systolic, and diastolic blood pressures above 140 and 90 mmHg, respectively, in at least two consecutive measurement, and proteinuria of >0.3 g/24 hr, in women after 20-week of gestation. The placentas (approximate weight: 100 mg) were snap frozen in liquid nitrogen immediately after collection for total RNA extraction. For in situ hybridization, the placentas were dissected on ice, cut into cubes fit into slide glass, and washed with phosphate buffered saline (PBS, pH 7.0). And then those tissues were post-fixed by a 48 hr-immersion in 4% neutrally buffered paraformaldehyde and cryoprotected by immersion in 20% sucrose in PBS for 24 hr. The tissues were frozen using OCT compound (Sakura Finetek, CA, U.S.A.), and 12 μm sections were cut with a cryostat (Leica, Heidelberg, Germany) and thaw-mounted on gelatin-coated slides, dried and stored at -70 °C until use.

Hematoxylin and eosin staining

The frozen sections were washed with tap water for 5 min, immersed in hematoxylin for 2 min and checked for complete staining in tap water. Eosin staining was carried out for 3 min. Sections were dehydrated through a graded series of alcohol (70 to 100% ethanol, 3 min each), cleared in xylene, cover-slipped and observed with a light microscope.

Northern blot analysis

Total RNA was extracted from placenta, using acid guanidium thiocyanate-phenol chloroform method, and quantified by UV spectrophotometer. Twenty micrograms of total RNA was subjected to electrophoresis in a 1.2% agarose-formaldehyde gel and then capillary-transferred to a nylon membranes (Nytran, pore size: 0.45 μm, Schleicher & Schuell, Keene, NK). The partial cDNAs of human PIGF-1/-2 were obtained from human placenta using RT-PCR and cloning. Then we confirmed PIGF cDNAs after sequence analysis and used as template for probe synthesis. Probes were labeled by random priming with [32P]-dCTP, and purified with a Sephadex G-50 nick column (Pharmacia Biotech, Upppsula, Sweden). Hybridization was carried out for 16-18 hr at 42 °C in 20 mL of hybridization buffer [50% Deionized Formamide, 5 ×SSPE (20 ×SSPE: 3 M NaCl, 0.2 M NaH2PO4, and 20 mM EDTA, pH 7.4), 5 ×Denhardt’s solution (1 ×Denhardts solution, 0.02% polyvinyl-pyrolidone, 0.02% Ficoll, 0.02% BSA), 0.1% SDS, 1 mg/mL heated-denatured salmon sperm DNA]. Membranes were washed in 2 ×SSPE with 0.1% SDS at room temperature for 10 min twice and in 0.1 × SSPE with 0.1% SDS at 42 °C for 10 min. The membranes were exposed to radiography film (Fuji RX & Fuji Co, Tokyo, Japan) for 3 days at -70 °C.

In situ hybridization

The 35S-UTP-labeled probes were generated by in vitro transcription from partial cDNA clone amplified from PIH placentas. The probes were purified with Sephadex G-50 nick column and radioactivity was measured on a β-counter. Prehybridization was carried out for 1 hr at 37 °C in prehybridization buffer [50% Deionized Formamide, 0.25 M NaCl, 1 × Denhardts solution, 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 10% Dextran Sulfate, yeast tRNA (250 μg/mL), 50 mM DTT]. Hybridization of the slides with antisense 35S-UTP-labeled RNA transcripts (7 ×105 cpm per section) in prehybridization buffer was performed at 60 °C for 20 hr. Sections were washed in 4 ×SSC, treated with 20 μg/mL of RNase A, washed in 2 ×SSC, and followed with a further wash in 0.1 × SSC containing 1.0 M DTT at room temperature. Sections were dehydrated through a graded series of alcohol (50% to 100% ethanol) and air dried at room temperature, then autoradiographed for 7 days at -70 °C using Amersham β-max hyperfilm (Amersham, NJ, U.S.A.). Slides were then dipped in NTB2 autoradiographic emulsion (Kodak, NY, U.S.A.) and exposed in the dark at 4 °C for 14 days, and developed with D-19 developer, fixed with rapid fixer (both from Kodak, NY, U.S.A.), counter-stained with cresyl violet, and mounted.

RT-PCR analysis of PIGFR-1 and PIGFR-2 mRNAs

Complementary DNA was synthesized in a mixture consisting of 1 μg total RNA from each placenta, 200 unit of RNaseH Muloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, U.S.A.) in 20 μL of the RT reaction mixture (150 pmol of oligo d(T), 40 units of RNase inhibitor, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 1 mM dNTPs [N=A, T, G, and C], and 10 mM DTT) at 37 °C for 1 hr, followed by 5 min at 95 °C. After heat inactivation, cDNA (total 20 μL) was stored at -20 °C until PCR.

The PCR reaction mixture (50 μL) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 25 mM MgCl2, 2 units of Taq polymerase, 0.2 mM dNTPs, 4 μL of each placental cDNA, and 10 pmol of each specific PCR primer for
PlGFR-1 (upstream: 5'-TGTCAATGAAACCCCAGA-3', downstream: 5'-TTTCTTCCCACAGTCCCAAC -3') and PlGFR-2 (upstream: 5'-TGTATGTCCCACCCCAGATT-3', downstream: 5'-ACATTTGCCGCTTGGATAAC-3') were added. The PCR reaction mixture was subjected to 30 cycles of amplification using a PCR system with denaturation at 94°C for 1 min, primer-annealing at 55°C for 1 min, and primer-extension at 72°C for 2 min. PCR products (10 μL) were subjected to electrophoresis in a 1.2% agarose gel in TAE buffer, visualized by UV illumination after ethidium bromide staining, and photographed using Polaroid 667 film (Hertfordshire, U.K.). β-actin was used as an internal control for procedural variation. The intensity of PCR bands was measured densitometrically and analyzed using SigmaGel (version 1.0, Jandel Scientific Software) software.

Statistics

RT-PCR signals were normalized to their β-actin signals. One-way analysis of variance was determined using Graphpad Instat Software (Version 1.15). A p value <0.05 was deemed statistically significant; data are expressed as mean ±SEM.

RESULTS

H & E staining in the normal and PIH placentas

Representative hematoxylin and eosin-stained sections of the normal and PIH placentas were shown in Fig. 1. In the PIH placenta, specific histologic changes were seen compared to control; the syncytial membranes were continuously expanded and the number of syncytial knots was increased in the PIH placental villi.

Expression of PIGF-1 and PIGF-2 mRNAs in the normal and PIH placentas

Northern blot analyses were conducted to evaluate differences in the abundance of PIGF-1 and PIGF-2 mRNA in normal and PIH placentas. Both PIGF-1 and PIGF-2 mRNAs were less abundant in PIH placenta compared to normal (Fig. 2); of two genes, the PIGF-2 was markedly decreased (Fig. 2B).
Localization of PlGF-1 and PlGF-2 mRNAs in the normal and PIH placentas by in situ hybridization

The PlGF-1 (data not shown) and PlGF-2 mRNAs were localized in the vasculosyncytial membrane and the villous stroma of placental villi in the normal (Fig. 3A) and PIH placenta (Fig. 3B). While no significant difference was detected in the expression of PlGF-1 mRNA between normal (Fig. 3A) and PIH placentas (Fig. 3B), the expression of PlGF-2 mRNA was decreased in the PIH placenta (Fig. 3C) compared to the normal one (Fig. 3B). (original magnification: ×2.1). PlGF-1, placenta growth factor -1; PlGF-2, placenta growth factor -2. Scale bar in A, B= 50 μm.

Expression of PIGF-1 and PIGFR-2 mRNAs in the normal and PIH placentas

The mRNAs encoding PIGF-1 and PIGFR-2 were detected by RT-PCR in both the normal and PIH placentas. While the expression of PIGF-1 was significantly higher in the PIH placentas compared to the normal one (p<0.05) (Fig. 4A, B), that of PIGFR-2 showed no significant difference (Fig. 4C, D). Relative densities were normalized by β-actin signals.

DISCUSSION

Although many theories have been proposed to explain the development of PIH, most of previous reports have not provided a clear picture. What is known, to date, is that PIH originates in the placenta and involves vasospasm of blood vessels in both mother and the placenta causing damage to the interior lining of blood vessels; its association with endothelial damage and dysfunction has been reported (2).

PIGFs, angiogenic growth factors, and their receptors are essential for placental vascular development (12). In this study, we examined the expression level of PIGF-1 and PIGFR-2 mRNAs in both the normal and PIH placentas at term and found a decreased mRNA level of PIGF-2 in PIH placentas by Northern blots and in situ hybridization. This result agrees well with previous reports; serum PIGF level was significantly lower in PIH as compared to normal pregnancies (18, 19);
the transcription of PIGF-2 mRNA in the placenta and decidua from PIHs decreased significantly (20).

Biological activities of PIGF-2 are mediated by the PIGFR-1; endothelial cells, Hoffbauer cells and trophoblasts, with extravillous trophoblast showing particularly high expression (23). In normal pregnancies, fetal trophoblasts invade the maternal decidua and remodel the spiral arteries, converting them to lower resistant vessels. In pregnancies complicated by pre-eclampsia, this trophoblastic cell invasion is inadequate, resulting in poor placental perfusion and fetal hypoxia (24, 25). The increased syncytial knots in the PIH placenta (Fig. 1) compared to normal one implicate an inadequate invasion of trophoblastic cells and this may be caused by a lower level of PIGF-2.

We revealed higher level of PIGFR-1 in the PIH placenta compared to normal one using RT-PCR and this was also consistent with other reports (19, 26); PIGFR-1, but not the other receptors, showed increased expression in the placental syncytiotrophoblasts among 50% of patients with severe pre-eclampsia compared to normal ones (19). Helske et al. (19) suggested that the increased PIGFR-1 expression is probably not a specific change of characteristic for pre-eclampsia, but is possibly associated with abnormal placental function and hypoxia. It was also revealed that trophoblastic cells express both insoluble PIGFR-1 and soluble PIGFR-1, and the soluble form PIGFR-1 have an antagonistic regulation function of its ligands (27). Therefore, the inadequate development of choriocarcinoma villi after mid gestation in PIH placenta may be possibly due to the lower level of PIGF mRNA and higher level of PIGFR-1 mRNA.

In the human placenta, VEGF, PIGF and their receptors are differentially expressed throughout gestation: VEGF and PIGFR-2 are most intense during early gestation and decline as pregnancy advances (13-15) while PIGF and PIGFR-1 increase towards term (16, 17). Correlation of these growth-factor effects and their expression patterns throughout gestation with a development of a villous angioarchitecture (28, 29) suggest that VEGF and PIGFR-2 are involved in the first two trimesters of pregnancy in the establishment of the richly branched capillary beds of the mesenchymal and immature intermediate villi while PIGF and PIGFR-1 are more likely to be involved in the formation of the long, poorly branched, terminal capillary loops in the last trimester (30).

Although PIGF itself does not significantly stimulate angiogenesis in vivo and in vitro, this growth factor modulates the angiogenic activity of VEGF by forming PIGF/VEGF heterodimers (31). Therefore, the amount of VEGF is also important in complicated placenta etiology. However, recent reports provide conflicting conclusions regarding the expression of VEGF in pre-eclamptic pregnancies; at the RNA level, VEGF expression was found to decrease in single biopsy samples of pre-eclamptic placenta as compared to biopsies from normal placentas (32); VEGF protein levels in serum of pre-
eclamptic women were found to be elevated in some studies (33, 34) while others have reported decreased levels (35), relative to normal pregnant women; PlGF homodimers are themselves angiogenic (36) and may also function to enhance the activity of suboptimal concentrations of VEGF (21). Furthermore, naturally occurring VEGF/PlGF heterodimers maintain high-affinity receptor binding and mitogenic activity on HUVE cells (37). While VEGF binds with high affinity to both PlGFR-2 and PlGFR-1, PlGF exhibits high affinity only to PlGFR-1 (21). Therefore, it is suggested that the main cause of delicate placental complications is PlGF rather than VEGF considering its amount consistency in complicated pregnancy placenta and its specificity to PlGFR-1. Especially, the adequate PlGF-2 mRNA expression level in placenta seems to be essential for normal placenta morphometric development in late pregnancy.

ACKNOWLEDGEMENT

This work was supported by a grant of the Brain Korea 21 Project, Ministry of Education in Korea, and the Korea Health 21 R & D Project (02-PJ1-PG3-20708-0010), Ministry of Health and Welfare, Republic of Korea.

REFERENCES

1. Granger JP, Alexander BT, Bennett WA, Khalil RA. Pathophysiology of pregnancy-induced hypertension. Am J Hypertens 2001; 14: S178-85.
2. Lyall F, Greer IA. Pre-eclampsia: a multifaceted vascular disorder of pregnancy. J Hypertens 1994; 12: 1339-45.
3. Taylor RN, Varma M, Teng NNH, Roberts JM. Women with pre-eclampsia have higher plasma endothelin levels than women with normal pregnancies. J Clin Endocrinol Metab 1997; 71: 1675-7.
4. Brown MA, Zammit VC, Lowe SA. Capillary permeability and extracellular fluid volumes in pregnancy-induced hypertension. Clin Sci (Lond) 1989; 77: 599-604.
5. Greer IA, Haddad NG, Dawes J, Johnstone FD, Calder AA. Neutrophil activation in pregnancy-induced hypertension. Br J Obstet Gynaecol 1989; 96: 978-82.
6. Pijnenborg R, Anthony J, Davey DA, Rees A, Tiltman A, Vercruysse L, Pijnenborg R, Anthony J, Davey DA, Rees A, Tiltman A, Vercruysse L. Extracellular fluid volumes in pregnancy-induced hypertension. Clin Sci (Lond) 1989; 77: 599-604.
7. Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Huchel CA, McCaughlin MK. Preeclampsia; an endothelial cell disorder. Am J Obstet Gynecol 1989; 161: 1200-4.
8. Vuorela P, Hatve T, Lymboussaki A, Kaipainen A, Joukov V, Persico MG, Altalato K, Halmesmaki EA. Expression of vascular endothelial growth factor and placental growth factor in human placenta. Biol Reprod 1997; 56: 489-94.
9. Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci USA 1991; 88: 9267-71.
10. Maglione D, Guerriero V, Viglietto G, Ferraro MG, Apriliokova O, Altalato K, DelVecchio S, Lei KJ, Chou JY, Persico MG. Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. Oncogene 1993; 8: 925-31.
11. Cao Y, Ji WR, Qi P, Rosin A, Cao Y. Placenta growth factor: identification and characterization of a novel isofrom generated by RNA alternative splicing. Biochem Biophys Res Commun 1997; 235: 493-8.
12. Regnault TR, Orbus RJ, de Vrijer B, Davidsen ML, Galan HL, Wilkening RB, Anthony RV. Placental expression of VEGF, PlGF and their receptors in a model of placental insufficiency-intrauterine growth restriction (PI-IUGR). Placenta 2002; 23: 132-44.
13. Jackson MR, Carney EW, Lye SJ, Ritchie JW. Localization of two angiogenic growth factors (PDECGF and VEGF) in human placenta throughout gestation. Placenta 1994; 15: 541-53.
14. Shiraiishi S, Nakagawa K, Kinukawa N, Nakano H, Sueishi K. Immunohistochemical localization of vascular endothelial growth factor in the human placenta. Placenta 1996; 17: 111-21.
15. Vuckovic M, Ponting J, Terman BI, Niketic V, Seif MW, Kumar S. Expression of the vascular endothelial growth factor receptor, KDR, in human placenta. J Anat 1996; 188: 361-6.
16. Crescimanno C, Marzioni D, Persico MG, Vuckovic M, Muhauser J, Castelucci M. Expression of bFGF, PlGF and their receptors in the human placenta. Placenta 1995; 16: A13.
17. Clark DE, Smith SK, Sharkey AM, Charnock-Jones DS. Localization of VEGF and expression of its receptors flt-1 and KDR in human placenta throughout pregnancy. Hum Reprod 1996; 11: 1090-8.
18. Reuvenkamp A, Velsing-Aarts FV, Poulina IE, Capello JJ, Duits AJ. Selective deficit of angiogenic growth factors characterizes pregnancies complicated by pre-eclampsia. Br J Obstet Gynaecol 1999; 106: 1019-22.
19. Helske S, Vuorela P, Carpen O, Horling C, Weich H, Halmesmaki E. Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies. Mol Hum Reprod 2001; 7: 205-10.
20. Liu W, Lin Q, Wang L. Relationship between placenta growth factor and the pathogenesis of pregnancy induced hypertension syndrome. Zhonghua Fu Chan Ke Za Zhi 2001; 36: 5-8.
21. Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem 1994; 269: 25646-54.
22. Savano A, Takahashi T, Yamaguchi S, Aonuma M, Shibuya M. Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor. Cell Growth Differ 1996; 7: 213-21.
23. Charnock-Jones DS, Sharkey AM, Boocock CA, Ahmed A, Plevin R, Ferrara N, Smith SK. Localization and activation of the receptor for vascular endothelial growth factor on human trophoblast and chorionicarcinoma cells. Biol Reprod 1994; 51: 524-30.
24. Lim KH, Zhou Y, Janapour M, McMaster M, Bass K, Chun SH, Fisher SJ. Human cytrophoblast differentiation/invasion is abnor-
25. Zhou Y, Genbacev O, Damsky CH, Fisher SJ. Oxygen regulates human cytotrophoblast differentiation and invasion: implications for endovascular invasion in normal pregnancy and in pre-eclampsia. J Reprod Immunol 1998; 39: 197-213.

26. Brockelsby J, Hayman R, Ahmed A, Warren A, Johnson I, Baker P. VEGF via VEGF receptor-1 (Flt-1) mimics pre eclamptic plasma in inhibiting uterine blood vessel relaxation in pregnancy: implications in the pathogenesis of preeclampsia. Lab Invest 1999; 79: 1101-11.

27. Clark DE, Smith SK, He Y, Day KA, Licence DR, Corps AN, Lammonglia R, Charnock-Jones DS. A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. Biol Reprod 1998; 59: 1540-8.

28. Kaufmann P, Bruns U, Leiser R, Luckhardt M, Winterhager E. The fetal vascularisation of term human placental villi. II. Intermediate and terminal villi. Anat Embryol (Berl) 1985; 173: 203-14.

29. Leiser R, Luckhardt M, Kaufmann P, Winterhager E, Bruns U. The fetal vascularisation of term human placental villi. I. Peripheral stem villi. Anat Embryol (Berl) 1985; 173: 71-80.

30. Ahmed A, Dunk C, Ahmad S, Khaliq A. Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen--a review. Placenta 2000; 21(Suppl A): S16-24.

31. Cao Y, Chen H, Zhou L, Chiang MK, Anand-Apte B, Weatherbee JA, Wang Y, Fang F, Flanagan JG, Tsang ML. Heterodimers of placenta growth factor/vascular endothelial growth factor. Endothelial activity, tumor cell expression, and high affinity binding to Flk-1/KDR. J Biol Chem 1996; 271: 3154-62.

32. Cooper JC, Sharkey AM, Charnock-Jones DS, Palmer CR, Smith SK. VEGF mRNA levels in placentae from pregnancies complicated by pre-eclampsia. Br J Obstet Gynaecol 1996; 103: 1191-6.

33. Baker PN, Krasnow J, Roberts JM, Yeot KT. Elevated serum levels of vascular endothelial growth factor in patients with preeclampsia. Obstet Gynecol 1995; 86: 815-21.

34. Sharkey AM, Cooper JC, Balmforth JR, McLaren J, Clark DE, Charnock-Jones DS, Morris NH, Smith SK. Maternal plasma levels of vascular endothelial growth factor in normotensive pregnancies and in pregnancies complicated by pre-eclampsia. Eur J Clin Invest 1996; 26: 1182-5.

35. Lyall F, Greer IA, Boswell F, Fleming R. Suppression of serum vascular endothelial growth factor immunoreactivity in normal pregnancy and in pre-eclampsia. Br J Obstet Gynaecol 1997; 104: 223-8.

36. Ziche M, Maglione D, Ribatti D, Morbidelli L, Lago CT, Battisti M, Paoletti I, Barra A, Tucci M, Parise G, Vincenti V, Granger HJ, Vigiutteo G, Persico MG. Placenta growth factor-1 is chemotactic, mitogenic, and angiogenic. Lab Invest 1997; 76: 517-31.

37. D’Salvo J, Bayne ML, Conn G, Kwock PW, Trivedi PG, Soderman DD, Palisi TM, Sullivan KA, Thomas KA. Purification and characterization of a naturally occurring vascular endothelial growth factor placenta growth factor heterodimer. J Biol Chem 1995; 270: 7717-23.