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Expression of placenta growth factor mRNA in the rat placenta during mid-late pregnancy

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The placenta is an essential organ that synthesizes several growth and angiogenic factors for its own growth as well as fetal development. It is known that the placenta growth factor (PlGF) is a member of the vascular endothelial growth factor family and is critical for placental growth and fetal development. However, there is little information regarding the expression pattern and cellular localization of PlGF mRNA in rat placenta during pregnancy. The aim of this study was to define the distribution of PlGF mRNA in rat placenta at various gestations. RT-PCR analysis showed that the expression level of PlGF mRNA increased as gestation advanced. Using in situ hybridization histochemistry, positive cells of PlGF mRNA were detected in chorionic villi. PlGF mRNA was expressed in the trophoblast cells and stroma cells surrounding the blood vessels within chorionic villi on day 13 and 15. Also, positive signals of PlGF mRNA were strongly detected in stroma cells of chorionic villi on day 17, 19, and 21. In particular, the density and number of positive signals of PlGF mRNA was significantly increased as gestation advanced. The expression pattern of PlGF mRNA in rat placenta during pregnancy demonstrates that PlGF plays a functional role for placental growth and fetal development during mid-late pregnancy.

Key words: placenta, placenta growth factor, rat

Introduction

The placenta is a vital organ for both fetal development and the maintenance of pregnancy. It is the biosynthetic site of growth hormones and several growth factors, basic fibroblast growth factor and insulin like growth factor, which are responsible for placental growth as well as fetal development [4,7,20,22]. The placenta requires neovascularization for successful placentation. It is known that angiogenesis and vascular transformation are important processes for the normal development of placenta [8]. It has also been shown that the placenta produces vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF), which are essential for placental growth and fetal development [6,23].

It is well accepted that VEGF is a major regulator of blood vessel growth and induces vascular permeability [13]. VEGF contributes to the development and growth of the endothelium and is potentially an important regulator of angiogenesis, particularly during extensive tissue growth [9, 11]. Another member of the VEGF family, PlGF, promotes in vitro proliferation of endothelial cells [17]. PlGF is a polypeptide growth factor that shares a 53% amino acid sequence homology with the platelet derived growth factor domain of VEGF. PlGF produces at least three isoforms: PlGF-1, PlGF-2, and PlGF-3, which are derived from the same gene via alternate splicing [3,18]. PlGF was formerly known as a potent angiogenic growth factor capable of inducing the proliferation, migration, and activation of endothelial cells [17]. Unlike VEGF, it was reported that abundant expression of PlGF is restricted to the placenta [18]. It is now known to distribute in many nonplacental cells, including cells of the microvasculature during pathological angiogenesis such as tumors and wound healing cells [10,16].

In a human term placenta, PlGF is expressed in villous trophoblasts and vascular endothelium [23]. The distribution of PlGF in these cells demonstrates that PlGF plays an important endocrinological and nutritional role, and contributes to the regulation of placental function. However, the expression pattern and cellular localization of PlGF mRNA in rat placenta during pregnancy is still unknown.

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The present study was performed to determine the distribution of PlGF mRNA in rat placenta at various gestations.

Materials and Methods

Animals and tissue preparation

Adult female Sprague-Dawley rats (weighting 250-300 g, Gyeongsang National University, Laboratory Animal Breeding Center, Jinju, Korea) were maintained under the contions of controlled temperature (25°C) and lighting (14L:10D) and allowed free access to food and water. Day 0 of pregnacy was determined by appearance of vaginal sperm or a copulatory plug in the morning following overnight exposure to males. Rats (6 rats per group) were killed in the morning of the pregnant days 13 to 21 with 2-day intervals to remove placentas from uteri. The placentas were then quickly frozen in liquid nitrogen for RNA extration. For *in situ* hybridization studies, animals (3 rats per group) were perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) through the left cardiac ventricle. Placentas were fixed and cryoprotected with 20% sucrose phosphate buffer for 24 hr. Sections of 15 μm thickness were prepared on the probe-on plus-charged slides (Fisher Scientific, USA), and stored at −70°C until use.

Total RNA extration and Reverse transcription PCR analysis

Total RNA from tissues was extracted by the acid guanidium thiocyanate phenol choroform method [5]. Complemantary DNAs were prepared from 500 ng of total RNA using oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA). The reaction components were incubated at 22°C for 10min and at 37°C for 70 min, heated to 95°C for 5 min, and flash cooled to 4°C. The oligonucleotide primers used for the amplification of PlGF cDNA were 5'-ATGCCGCTCATGA GGGCTG-3' and 5'-CTTCATCTTCTCCCACAGAG-3'. The RT product samples were subjected to 30 cycles of amplification in a Perkin-Elmer PCR Thermal Cycler (Perkin Elmer, USA) with denaturation at 94°C for 30 sec, primer annealy at 63°C for 30 sec, and primer extension at 72°C for 15 min. PCR products were electrophoresed in a 1.2% agarose gel and were stained with ethidium bromide and photographed with Polaroid Type 667 instant film (Hertfordshire, UK). Beta-actin was used as an internal control for procedual variation. For quantification, the intensity of PCR bands was measured densitometrically and analyzed using SigmaGel (version 1.0; Jandel Scientic Software, USA) software. Our results are the mean of five independent experiments and expressed as mean ± S.E.M.

In situ Hybridization Histochemistry

All solutions were made with sterile water, and glassware was autoclaved to prevent contamination by RNase. *In situ* hybridization histochemistry was carried out as described by Angerer *et al.* [1]. The slides were dried, washed with 0.1 M PBS, treated with proteinase K, TE buffer, and acetylation solution. The sections were covered with prehybridization buffer containing 50% deionized formamide and incubated at 37°C for 1 hr. After removal of the prehybridization buffer, the slides were covered with the mixture containing the prehybridization buffer, 50 μg/ml yeast tRNA (Sigma, USA), 10 mM dithiothreitol (Sigma, USA), and ^{35}S-labeled PlGF cRNA probe. The slides were covered with cover glasses and incubated at 60°C for 24 hr. ^{35}S-UTP labeled probe was prepared using *in vitro* transcription kit (Promega, USA). Antisense and sense cRNA probes were purified with a Sephadex G-50 nick column (Pharmacia Biotech, Sweden) and eluted with SET buffer containing 0.1% SDS, 1 mM EDTA, 10 mM Tris, and 10 mM DTT. Tissue slides were posthybridized in a posthybridization buffer. Following a wash in 4 × SSC for 30 min, the sections were then treated with ribonuclease A (50 μg/ml) at 37°C for 10 min, washed twice in 2 × SSC and 1 × SSC, transferred to a washing buffer containing 1 × SSC at 65°C for 30 mins, and dehydrated in alcohol solutions with ascending concentrations. The slides were exposed to β-max autoradiography X-ray film (Amersham, Sweden) for 4 days in light-tight cassettes at −70°C. They were dipped into NTB2 emulsion (1 : 1 dilution; Eastman Kodak, USA), exposed at 4°C for 2 weeks, developed in Kodak D19 developer (1 : 1 dilution; Eastman Kodak, USA) at 15°C, and counterstained with hematoxylin. The slides were observed under a dark and a bright field microscope, and then photographed.

Data analysis

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

Results

We evaluated the expression and distribution of PlGF mRNA in rat placenta at various gestations. RT-PCR analysis revealed the up-regulation of PlGF mRNA in rat placenta during mid-late pregnancy. The expression level of PlGF mRNA increased as gestation advanced (Figs. 1A and 1B). In particular, the expression of PlGF was markedly increased on day 19 and 21 during late pregnancy.

In situ hybridization histochemistry indicated that the expression of PlGF mRNA was detected in trophoblast cells and stroma cells of chorionic villi within the labyrinth zone. Positive signals of PlGF mRNA were specifically observed in trophoblast cells and stroma cells of the surrounding blood vessels within the chorionic villi on day 13 (Figs. 2A

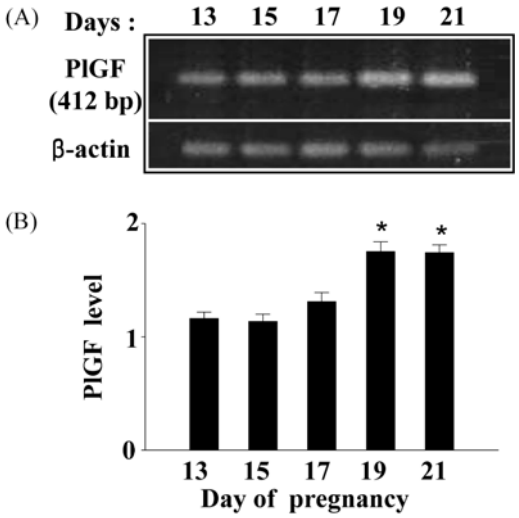

Fig. 1. RT-PCR analysis of PlGF from rat placental extracts during mid-late pregnancy. (A) The expression of PlGF was increased at the late stage of pregnancy. (B) Densitometric analysis of PlGF mRNA levels are represented as an arbitrary unit (A.U.) that is normalized to β-actin. *$p < 0.05$ (vs. control).

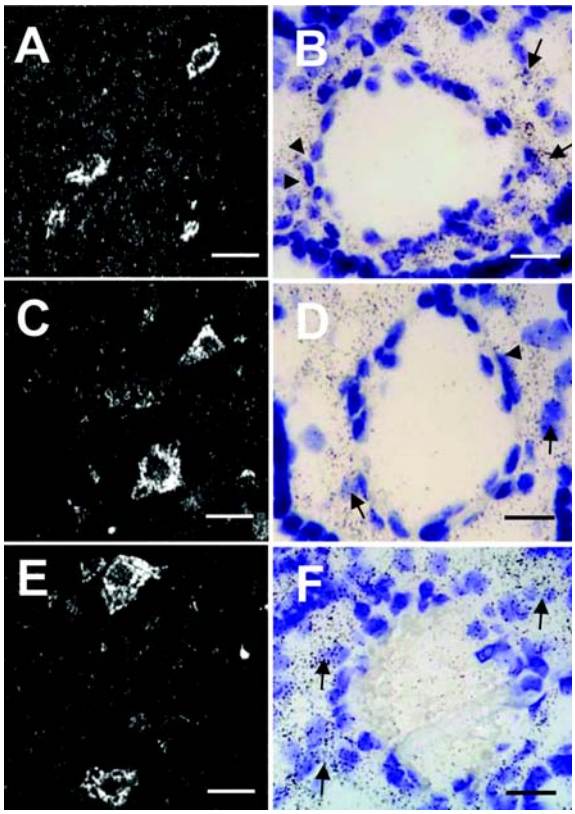

Fig. 2. Localization of PlGF mRNA in rat placenta on day 13 (A and B), 15 (C and D), and 17 (E and F). Dark-field (A, C, and E) and bright-field (B, D, and F) microphotographs of PlGF mRNA. Positive signals of PlGF mRNA were detected in trophoblast cells (arrowheads) and stroma cells (arrows) of chorionic villi within the labyrinth zone. Scale bar: A, C, and E, 200 μm; B, D, and F, 25 μm.

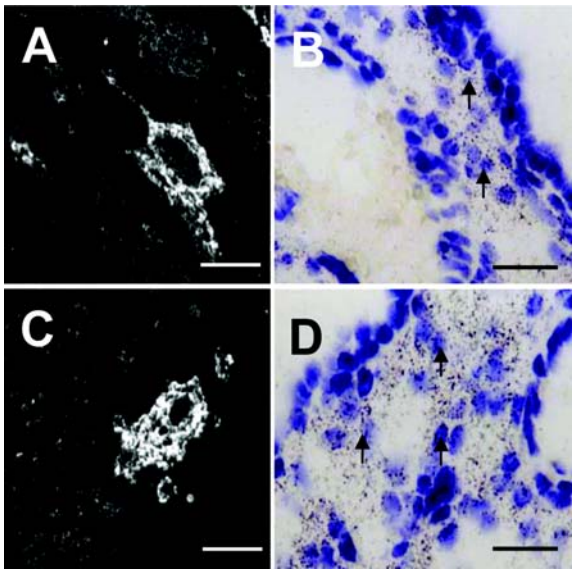

Fig. 3. Expression of PlGF mRNA in rat placenta on day 19 (A and B) and 21 (C and D) by *in situ* hybridization histochemistry. Positive cells of PlGF mRNA were detected in stroma cells within the chorionic villi. Arrows indicate the positive signals. Scale bar: A and C, 200 μm; B and D, 25 μm.

and 2B). Also, positive cells of PlGF mRNA appeared in trophoblast cells and stroma cells within chorionic villi on day 15 (Figs. 2C and 2D). On day 17, the expression of PlGF mRNA was significant in stroma cells within chorionic villi (Figs. 2E and 2F). Positive signals of PlGF mRNA were strongly expressed in stroma cells within the chorionc villi on day 19 and 21 (Figs. 3A, 3B, 3C, and 3D). In particular, the density and number of positive signals of PlGF mRNA were significantly increased as gestation advanced.

Discussion

We showed the expression and distribution of PlGF mRNA in rat placenta at various gestations. Vuorela *et al.* [23] demonstrated that PlGF was expressed in villous trophoblast and vascular endothelium in human placenta at term. However, there is little information regarding the expression pattern and distribution of PlGF mRNA in rat placenta during pregnancy. We confirmed the existence of PlGF in rat placenta at various gestations. RT-PCR analysis and in situ hybridization histochemistry showed that the expression of PlGF mRNA was increased as gestation progressed, and it was strongly detected in trophoblast cells and stroma cells within chrionic villi.

Angiogenesis and vascular transformation are important processes for the normal development of the placenta and fetus [8]. The previous studies reported that the angiogenic growth factors VEGF and PlGF exist in placenta and act as important factors for placental development and fetal

growth [6,23]. Furthermore, Ni and colleagues [19] reported that VEGF mRNA was detected in rat placenta; approximately five times more was seen in late pregnant tissue than in mid pregnant tissue. Like the expression pattern of VEGF, the expression of PlGF mRNA in rat placenta increased as gestation advanced. In particular, the expression level of PlGF significantly increased in late pregnancy on day 19 and 21. The high expression of PlGF in chorionic villi demonstrates that PlGF acts as a tropic factor in chorionic villi and promotes the development of the fetus.

It is known that the placenta produces several growth factors including an insulin-like growth factor and a basic fibroblast growth factor. It also produces VEGF and PlGF, which are essential for placental growth and fetal development [6,23]. Previous studies demonstrated the expression of PlGF in the trophoblast cells of human placenta [23]. Athanassiades *et al.* [2] showed that exogenous PlGF stimulates the proliferation of first trimester extravillous trophoblasts. Thus, PlGF may contribute to successful placentation by regulating trophoblast apoptosis and function during gestation [2]. In this study, data showed that PlGF was strongly expressed in trophoblast cells and stroma cells of chorionic villi during the mid gestation period. However, the expression of PlGF mRNA was significantly increased in whole stroma cells during the late gestation period. VEGF was expressed in stroma cells within villi in human placenta [21]. Localization of VEGF in stroma cells demonstrates that VEGF plays an important role in the physiological growth and function of the vascular system in the villous stroma. Also, VEGF has been known to act directly on vascular endothelial cells by promoting cell proliferation and permeability [13]. Our data on the localization of PlGF in stroma cells of chorionic villi coincides with the expression of VEGF in these cells.

We reported the cellular distribution of pituitary adenylate cyclase activating polypeptide (PACAP) and its receptor in human and rat placenta [14,15]. It is well accepted that PACAP acts as a growth factor in various cells, and stimulates VEGF release [12]. Our previous studies showed that PACAP and its receptor mRNAs were expressed in stroma cells of stem villi and terminal villi [14,15]. Also, the expression of these genes was significantly increased during late pregnancy. Our data demonstrated that PACAP plays an important role for placental growth and fetal development. In this study, PlGF was strongly expressed in stroma cells of chorionic villi within a labyrinth zone during mid-late pregnancy. The expression level of these genes increased as gestation advanced. Vascular growth in the placenta and fetus required tissue growth during the mid-late pregnancy. Generally, the labyrinth zone carries out the exchanges of substances between maternal and fetal body through both maternal and fetal circulation. The expression of PlGF in chorionic villi can demonstrate that PlGF contributes to the development and growth of the fetus. Thus, we demonstrated that PlGF contributes to the placental growth and fetal development during the mid-late pregnancy period. In conclusion, our findings can suggest that PlGF may have a functional role in a rat placenta for the maintenance of pregnancy.

Acknowledgments

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