FULL PAPER *Anatomy*

Streptozotocin-Induced Diabetes Decreases Placenta Growth Factor (PlGF) Levels in Rat Placenta

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ABSTRACT. The placenta produces several growth factors, including placenta growth factor (PlGF), which are essential for placenta growth and fetal growth. Diabetic pregnancy induces the abnormal placental growth and fetal development. This study investigated whether diabetes in pregnant rats induces changes in PlGF expression in the placenta. Diabetes was induced by a single intravenous injection of streptozotocin (35 mg/kg body weight) on day 0 of pregnancy, blood and tissue samples were collected on day 20 of pregnancy. In the diabetic group, maternal body weight and fetal weight significantly decreased compared to controls. RT-PCR and Western blot analyses showed that expression of PlGF was significantly decreased in placenta by streptozotocin treatment. Immunohistochemical study showed that the positive signal of PlGF in trophoblast cells was decreased in the diabetic group compared to controls. These findings demonstrate the decline of PlGF in the placenta in diabetic pregnancy.

KEY WORDS: diabetes, placenta, PlGF.

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The placenta is a critical organ for both fetal development and the maintenance of pregnancy. Growth of the placenta is regulated by several growth factors, including placental growth factor (PlGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor-2 [1, 14, 15]. These growth factors are responsible for fetal development as well as placenta growth [5, 9, 20, 22]. PlGF is a polypeptide growth factor that shares a 53% amino acid sequence homology with the platelet-derived growth factor domain of VEGF [17]. Unlike VEGF, abundant expression of PlGF is restricted to the placenta [18]. PlGF was formerly known as a potent angiogenic growth factor capable of inducing the proliferation, migration, and activation of endothelial cells [17]. PlGF plays an important endocrinological and nutritional role, and contributes to the regulation of placental function.

Gestational diabetes is one of the most common complications of pregnancy, and is associated with both abnormal placental growth and fetal development [3, 16, 19]. Severely diabetic pregnancy can induce abortion and prematurity [3, 19]. Recently, apoptotic cell death was increased in the placenta of pregnant women with gestational diabetes [21]. PlGF acts as an anti-apoptotic factor for trophoblast *in vitro* [11]. Therefore, we propose that diabetic pregnancy induces change in PlGF expression and this dysregulation may affect placental development. However, little data is available on the expression of PlGF in the placenta tissue in diabetic pregnancy. Therefore, the present study was performed to provide this information.

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MATERIALS AND METHODS

Experimental animals: Female Sprague-Dawley rats (200–220 g, *n*=30) were purchased from Samtako Co. (Laboratory Animal Breeding Center, Korea), randomly divided into 2 groups, control group and diabetic group (*n*=15 per group). Animals were maintained under controlled temperature (25°C) and lighting (14/10 light/dark cycle), and were allowed to have free access to food and water. Animals were treated in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (U.S.A.). Diabetes was induced by a single intravenous injection of streptozotocin (STZ, Sigma, St. Louis, MO, U.S.A.) in 0.1 M citrate buffer (pH 4.0) at a dose of 35 mg/kg body weight on day 0 of pregnancy. Control animals received an equal volume of citrate buffer. Onset of pregnancy was determined by vaginal smear inspection after overnight mating. The day when a positive smear was found was designed day 0 of pregnancy. On day 3 of pregnancy, diabetes was confirmed by measurement of blood glucose concentrations and defined as a blood glucose above 350 mg/d*l*. Maternal blood glucose was also determined on day 20 of pregnancy to confirm the diabetic condition. On day 20, animals were weighed and anesthetized with ethyl ether, maternal blood was collected by heart puncture between 0900–1200 hr, after which a cesarean section was performed. Placenta and fetus were removed and rapidly weighed. And then, placentas were frozen in liquid nitrogen or immersed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 12 hr.

Total RNA extration and Reverse transcription PCR

analysis: Total RNA from tissues was extracted by the acid guanidium thiocyanate phenol choroform method [6]. Complemantary DNAs were prepared from 500 *n*g of total RNA using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, U.S.A.). The reaction components were incubated at 22°C for 10 min 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'-ATGCCGCT-CATGAGGGCTG-3' and 5'-CTTCATCTTCTCCCACA-GAG-3'. The RT product samples were subjected to 30 cycles of amplification in a Perkin-Elmer PCR Thermal Cycler (Perkin Elmer, Boston, MA, U.S.A.) 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. β-actin was used as an internal control for procedual variation. For quantification, the intensity of PCR bands was measured densitometrically and analyzed using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.) and SigmaPlot 4.0 (SPSS Inc., Chicago, IL, U.S.A.).

Western blot analysis: The placenta samples were snap frozen and lysed in buffer. The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's protocol. Total protein (30 μg) was applied to each lane on to 10% SDS-polyacrylamide gels. After electrophoresis, the poly-vinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.) were washed in Tris-buffered saline containing 0.1% Tween-20 and then incubated with anti-PlGF rabbit IgG (diluted 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A) as primary antibody. And the membrane was incubated with secondary antibody (1:5,000, Pierce, U.S.A.) and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscaataway, NJ, U.S.A.) according to the manufacturer's protocol was used for detection. The intensity analysis was carried out using SigmaGel 1.0.

Immunohistochemistry: For the immunohistochemical study, sections were blocked with 1% normal goat serum in PBS for 1 hr and then incubated with anti-PlGF rabbit IgG (1:100 in PBS) at 4°C for 18 hr in a humidified chamber. As a negative control, normal goat serum was used instead of anti-PlGF antiserum for antibody reaction. After washing with PBS, sections were incubated with biotin-conjugated goat anti-rabbit IgG (1:200 in PBS) for 1 hr, followed by avidin-biotin-peroxidase complex for 1 hr from a Vector ABC *Elite* kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.). Sections were washed with PBS, and incubated with diaminobenzidine tetrahydrochloride (DAB, Sigma chemical Co., St. Louis, MO, U.S.A.) solution with 0.03% hydrogen peroxidase for 3 min. Slides were counterstained with hematoxylin and observed under microscope, and then photographed.

Data analysis: All data are expressed as mean ± S.E.M. The results in each group were compared by Unpaired Student's *t*-test. The difference for comparison was considered significant at * $P<0.05$.

RESULTS

The blood glucose levels of streptozotocin-induced diabetic rats were markedly increased compared to that of control rats. The blood glucose levels were 101 ± 5 mg/d*l* and 476 ± 17 mg/d*l* in control and diabetic rats, respectively (Fig. 1A). Maternal body weights in the diabetic rats were significantly lower than those of the controls. The body weights were 379 ± 18 g and 304 ± 15 g in control and diabetic rats, respectively (Fig. 1B). The fetal weight of diabetic rats was markedly decreased compared to that of the controls, with values of 4.25 ± 0.35 g and 2.93 ± 0.41 g in control and diabetic rats, respectively (Fig. 1C). However, the placental weight did not significantly different between the control and diabetic groups, with values of 0.63 ± 0.09 g and 0.61 ± 0.08 g in control and diabetic rats, respectively (Fig. 1D).

RT-PCR analysis showed a decrease in PlGF mRNA expression in the placenta of streptozotocin-induced diabetic rats, with PlGF mRNA levels of 1.28 ± 0.04 and 0.86 ± 0.09 in control rats and diabetic rats, respectively (Fig. 2). Figure 3 showed a corresponding decrease in PlGF protein in the diabetic rats by Western blot analysis, with levels of 0.95 ± 0.03 and 0.75 ± 0.05 in control rats and diabetic rats, respectively (Fig. 3). Immunohistochemical examination showed the PlGF being expressed in trophoblast and stroma

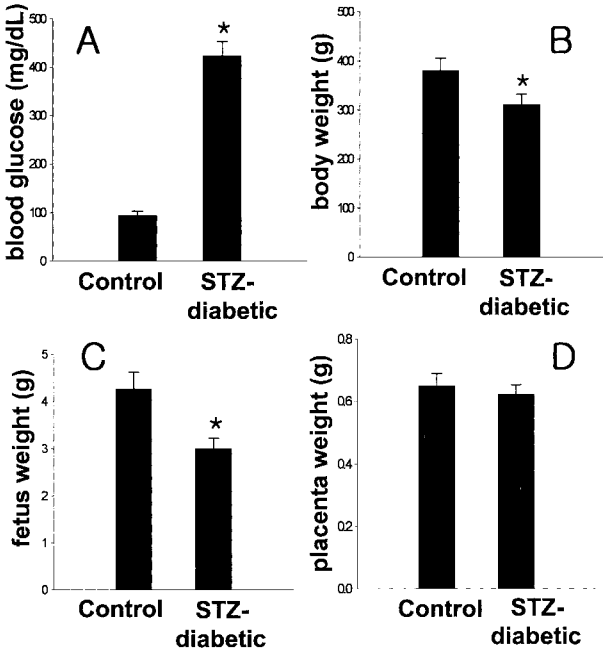

Fig. 1. Blood glucose levels (A), maternal body weight (B), fetal weight (C), and placental weight (D) of control group and streptozotocin (STZ)-induced diabetic group at the day 20 of pregnancy. All results represent the mean ± S.E.M. from 5 separate data. * $P<0.05$.

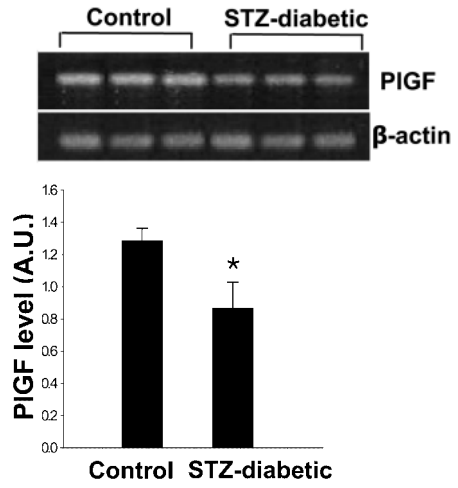

Fig. 2. RT-PCR analysis of PlGF in placenta tissues of control group and streptozotocin (STZ)-induced diabetic group at the day 20 of pregnancy. The product signals at 412 bp represent mRNA of PlGF. Each lane represents an individual experimental animal. Densitometric analysis of PlGF levels is represented as an arbitrary unit (A.U.) that was normalized to β-actin. All results represent the mean ± S.E.M. from five separate data. * P<0.05.

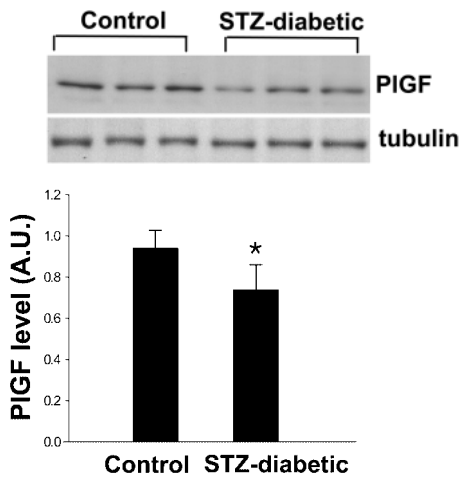

Fig. 3. Western blot analysis of PlGF in placenta tissues of control group and streptozotocin (STZ)-induced diabetic group at the day 20 of pregnancy. Each lane represents an individual experimental animal. Densitometric analysis is represented as an arbitrary unit (A.U.), normalized by α-tubulin. All results represent the mean ± S.E.M. from five separate data. * P<0.05.

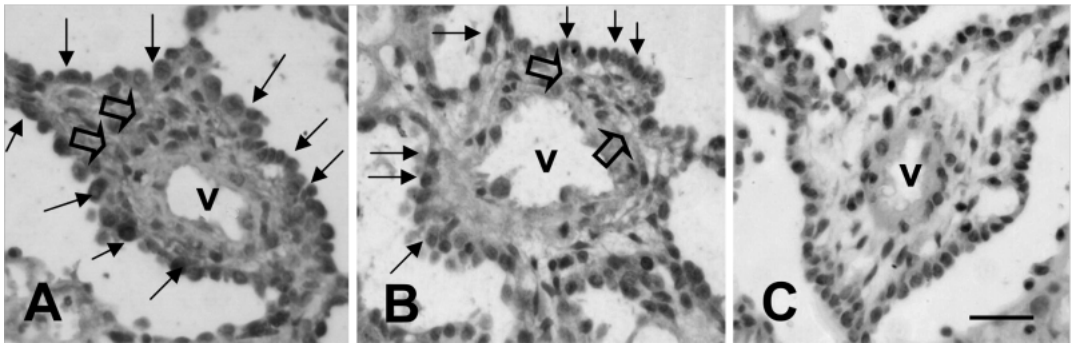

Fig. 4. Expression of PlGF in placenta tissues of control group (A, C) and streptozotocin (STZ)-induced diabetic group (B) at the day 20 of pregnancy. Positive cells were observed in trophoblast cells (close arrows) and stroma cells (open arrows) of villi within the labyrinth zone. No positive cells were detected in a negative control (C). V indicates the vessel within villi. Scale bar: 50 μm.

cells of villi within the labyrinth zone (Fig. 4A-C). PlGF signals were detected in the cytoplasm and nuclei. The PlGF signal was diminished in the diabetic group compared to the controls (Fig. 4A-B). There were no detectable signals in the negative control (Fig. 4C).

DISCUSSION

Diabetic pregnancy induces hyperglycemia and growth retardation in the fetus, and previous study showed that the body weight of fetues from diabetic rats was significantly lower than that of normal rats [12]. In this study, the body weights of these diabetic mothers were also less than normal. The present study confirmed that the body weight of fetuses was significantly decreased in diabetic pregnancy and fetal growth was retarded in streptozotocin-induced diabetic rats. However, the placental weight was only slightly decreased and not significantly different between control and diabetic groups. Although the change in the placental weight was not significant, we propose that diabetic condition decreases the PlGF level in placenta. This study focused the decreases of PlGF expression in rat placenta with diabetic condition.

Angiogenesis and vascular transformation are important processes for normal development of the placenta and fetus [10]. It is well accepted that the angiogenic growth factors VEGF and PlGF exist in placenta and act as important factors for placental development and fetal growth [8, 23]. PlGF is abundantly expressed in the trophoblast cells of pla-

centa [4, 13, 23]. We previously reported the expression and distribution of PlGF mRNA in the rat placenta at various gestational phases [7]. PlGF mRNA was expressed in the trophoblast cells and its levels significantly increased as gestation advanced [7]. Vuorela *et al.* [23] has showed that PlGF was expressed in the trophoblast cells of the human placenta, while this study focused on the expression of PlGF in placenta tissue of the streptozotocin-induced diabetic rat, where this study showed significant decreases in the expression levels of PlGF mRNA and protein.

PlGF is an angiogenic growth factor that is expressed in the trophoblast cells of the placenta [4, 13, 17]. This study confirmed by immunohistochemical method that PlGF was expressed in trophoblast cells of villi within the labyrinth zone. The PlGF signal was decreased in diabetic group compared to the control. PlGF acts as an anti-apoptotic factor for trophoblasts *in vitro* and stimulates proliferation of trophoblasts [2, 11]. PlGF contributes to successful placentation by regulating trophoblast apoptosis and function. As such, aberrant production of PlGF during pregnancy could contribute to compromised placental function. In conclusion, this finding demonstrates that streptozotocin-induced diabetic pregnancy causes a decrease of PlGF expression in the placenta.

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