Impaired proliferation of pancreatic beta cells, by reduced placental growth factor in pre-eclampsia, as a cause for gestational diabetes mellitus

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Received 23 August 2014; revision accepted 29 September 2014

Abstract

Objectives: Reduced increase in serum placental growth factor (PLGF) levels frequently occurs in patients with pre-eclampsia (PE) and thus has been used as a predictive factor for developing PE. However, it has remained elusive how shortage of PLGF could affect pancreatic endocrine homeostasis and function in pregnancy to lead to development of gestational diabetes mellitus (GDM).

Materials and methods: We used L-NAME injection in mice, as a model of human PE, in which PLGF levels were significantly reduced.

Results: We not only confirmed reduced serum PLGF levels in patients with PE but also detected strong correlation of serum PLGF levels and presence of GDM. We found that growth of beta cell mass during pregnancy was significantly impaired by L-NAME injection, as a result of reduced beta cell proliferation. This may explain the higher risk of developing GDM in patients with PE. Moreover, provision of exogenous PLGF in L-NAME-treated pregnant mice significantly rescued beta cell proliferation, with subsequent increase in beta cell mass, suggesting that shortage in PLGF may be responsible for impaired beta cell growth and higher occurrence of GDM in patients with PE.

Conclusions: Our study highlighted a pivotal role for PLGF in prevention and treatment of GDM in patients with PE.

Introduction

Pre-eclampsia (PE) and gestational diabetes mellitus (GDM) are two common complications of gestation, and affect more than 10% of human pregnancies worldwide (1–5). Being two leading causes of maternal death, and major contributors to maternal and perinatal morbidity, PE and GDM have been shown to affect function of both maternal and foetal endothelium, leading to endothelial dysfunction and increasing future risk of developing cardiovascular disease (1–5). PE and GDM share many risk factors such as obesity, elevated blood pressure, dyslipidaemia, insulin resistance and hyperglycaemia (1–5). Moreover, GDM is associated with increased incidence of PE, while PE occurs more often in patients with GDM (1–5). Nevertheless, the exact pathogenesis of PE and GDM as well as their inter-relationship has not up to now, been fully elucidated.

Chronic inhibition of nitric oxide synthase, by treating pregnant animals with *N*-omega-nitro-L-arginine methyl ester (L-NAME) is an accepted method of PE induction and the experimental outcome is similar to human PE (6–9). Animals subjected to chronic inhibition of this enzyme develop a PE-like syndrome that exhibits various pathologies including hypertension, proteinuria, renal damage, intrauterine growth restriction and tumour necrosis factor alpha (TNF- α) production, which have all been implicated in the pathophysiology of PE (10–14).

Placental growth factor (PLGF) is a member of the vascular endothelial growth factor (VEGF) family, which play a pivotal role in vasculogenesis and angiogenesis (15). Although involvement of PLGF has been shown in pathological angiogenesis (16,17), prominent PLGF expression is largely restricted to the placenta under normal physiological conditions (18). Previous studies have shown that maternal serum PLGF levels are relatively low over 5–15 weeks of gestation and increase dramatically afterwards. Serum PLGF

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levels peak at approximately 26–30 weeks and then significantly reduce (19,20). As amniotic fluid PLGF levels follow a similar pattern, it suggests that PLGF may enter both foetal and maternal circulations to influence vessel dynamics, in both systems. Of note, PLGF levels are significantly low in PE, which is characterized by poor placental vascularization (19,20). However, the exact effect of reduced PLGF on maternal tissue and organs has not been adequately studied. It is noteworthy that VEGF-A, another member of the VEGF family, has an established role in development and maintenance of pancreatic beta cells (21–25), while the role of PLGF in this setting has not previously been demonstrated.

Diabetes mellitus is a metabolic disease resulting from dysfunction and/or loss of pancreatic insulin-secreting beta cells, and is characterized by chronic hyperglycaemia (26). Adequate functional beta cell mass is critical for maintaining appropriate glucose-stimulated insulin release in response to metabolic need, and changes in metabolic need require adaption of beta cell mass, for example, during pregnancy (27). Although most previous studies have demonstrated that beta cell replication is the predominant mechanism for post-natal beta cell population expansion (28–33), specially during pregnancy (32,34–40), there have also been reports suggesting occurrence of beta cell neogenesis by other means (41,42). As evaluation of beta cell mass in PE/GDM patients is hard to be performed, corresponding studies on experimental animals are very informative, have been largely lacking.

In the current study, we not only confirmed reduced serum PLGF levels in patients with PE but also detected significant correlation of serum PLGF levels and development of GDM. In a mouse PE model with significantly reduced serum PLGF levels, we detected significant impairment of growth of beta cell mass by reduced beta cell proliferation, which was efficiently rescued by application of exogenous PLGF.

Materials and methods

Human subjects

Pregnant women with PE, when admitted were identified at the Department of Gynecology and Obstetrics of Shengjing Hospital affiliated to China Medical University. Subjects were diagnosed with severe PE, on the basis of presence of high blood pressure (no less than 160/110 mmHg) and presence of proteinuria (no less than 3.5 g in a 24-h period) or random urinary protein/creatinine ratio higher than 0.3. They had no previous history of hypertension. Normotensive pregnant women

were characterized by uncomplicated pregnancies with normal-term deliveries.

Mouse handling

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Shengjing Hospital affiliated to China Medical University (Animal Welfare Assurance). Surgery was performed under ketamine/xylazine anaesthesia, according to the Principles of Laboratory Care, supervised by a qualified veterinarian. Female Balb/C mice (Jackson, Bar Harbor, ME, USA) pregnant at 12 weeks of age, were used in the current study. Five mice were analysed in each experimental condition. For quantification of beta cell proliferation, 1 mg/ml BrdU (bromodeoxyuridine; Sigma, St. Louis, MO, USA) was added into either 1% sucrose mouse drinking water from day 9 gestation to day 17, as previously described (43–45).

The L-NAME PE model

Inhibition of nitric oxide synthase with *N*-omega-nitro-L-arginine methyl ester (L-NAME) was used as experimental model of human PE. Animals subjected to chronic inhibition of L-NAME during pregnancy have been shown to develop a PE-like syndrome with PE-associated pathology (6–14). Here, we performed daily subcutaneous injection to the pregnant mice, of L-NAME at 200 mg/kg body weight, between day 9 gestation and day 17. Control mice received the same volume of phosphate-buffered saline (PBS).

PLGF pump implantation

For provision of exogenous PLGF in the PE model, 1 mg recombinant human PLGF (Sigma) was dissolved in PBS in mini-osmotic pumps (Alzet, Cupertino, CA, USA), which were then implanted subcutaneously to the mice, on day 9 gestation. Mini-pumps containing PBS only, were implanted into control mice.

Blood glucose and blood pressure measurement

Blood glucose was measured after a 3-h fasting period at 10 am. Blood pressure was measured in the tail artery at different times along the pregnancy. Measurements were performed using a computerized, non-invasive tail-cuff acquisition system (CODA System, Kent Scientific Corporation, Torrington, CT, USA). The CODA system utilizes volume-pressure recording technology to detect changes in tail volume that correspond to systolic and diastolic pressures, and calculates mean arterial pressure

of each measurement cycle (46). We repeated all measurements three times and took the mean for each value. We found very little variation, which confirmed reproducibility of the method.

Quantification of PLGF, albumin, creatinine and total urine protein

Concentration of human or mouse serum PLGF was determined by corresponding ELISA kit (R&D, Los Angeles, CA, USA). Concentration of mouse urine albumin was determined by ELISA kit (Abcam, Cambridge, MA, USA). ELISAs were performed according to the manufacturer's instructions. Urine creatinine was quantified using a Creatinine Companion kit (Exocell, Philadelphia, PA, USA), based on Jaffe' reaction of alkaline picrate with creatinine. Total protein from 24-h urine collections was quantification by sulphosalicylic acid precipitation testing.

Immunohistochemistry

After sacrifice, mouse kidney and pancreas were dissected out and fixed in 4% paraformaldehyde for 10 h, then cryo-protected in 25% sucrose overnight. Samples were then sectioned at 6 μ m. H&E staining was performed on renal samples, double immunostaining for insulin and BrdU was performed on samples of pancreas. Primary antibodies were guinea pig polyclonal anti-insulin and rat polyclonal anti-BrdU (Abcam). Antigen retrieval by incubation of slides in 1 mol/l HCl at room temperature for 45 min was performed for BrdU staining. Secondary antibodies were Cy3- and Cy2-conjugated antibodies for corresponding species (Jackson Labs). 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei.

Quantification of beta cell proliferation and beta cell mass

Five mice of each experimental condition, were measured. Insulin staining was used to identify beta cells. Quantification of BrdU⁺ beta cells in all beta cells was based on five sections 120 μ m apart from each other. More than 1000 beta cells were counted for each animal.

Quantification of beta cell mass was performed as previously described (31). Briefly, pancreata were weighed then fixed in 4% paraformaldehyde for 6 h. They were then cryo-protected in 30% sucrose overnight to allow for their longitudinal sections (head to tail) to be obtained. Sections at 120 μ m intervals from whole pancreas were immunostained for insulin and analysed

using Image J software (NIH, Bethesda, MA, USA). Relative cross-sectional area of beta cells was determined by quantification of cross-sectional area occupied by beta cells divided by total tissue cross-sectional area. Beta cell mass per pancreas was calculated as product of relative cross-sectional area of beta cells per total tissue, and weight of the pancreas. Beta cell mass was calculated by examining pancreata from five animals for each group.

Isolation of non-islet pancreatic fractions

Mouse pancreas was first perfused with 0.125 mg/ml LiberaseTL (Roche, Nutley, NJ, USA) from the common bile duct, and was then incubated in 0.125 mg/ml LiberaseTL in a 37 °C shaker at 200 rpm for 45 min. Histopaque1077 and Histopaque1119 (Sigma) were mixed 9:33 (vol:vol) to generate Histopaque1110. Combined centrifugation in Histopaque1100 and hand-picking was then performed to remove islets to obtain islet-deprived pancreatic fractions.

Quantitative real time PCR (RT-qPCR)

RNA was extracted from non-islet pancreatic fractions, with Trizol (Invitrogen, St. Louis, MO, USA) and used for cDNA synthesis. Quantitative PCR was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). Ngn3 primers are as follows: forward: 5'-tctcaagcatctcgctcttc-3' and reverse: 5'-acagcaagggtaccgatgaga-3'. GAPDH primers: forward: 5'-gtgttcctaccccaatgtgt-3' and reverse: 5'-attgtcattaccaggaaatgagctt-3'. Values of Ngn3 were normalized against GAPDH, and compared to control (=1).

Statistical analysis

All values are depicted as mean $\pm$ SD from at least five individuals per category, considered significant if $P < 0.05$. All data were statistically analysed using one-way ANOVA with Bonferroni correction.

Results

Reduced serum PLGF levels detected in pregnant women with PE

First, we examined serum PLGF levels of pregnant women with PE, and compared them to PLGF levels of pregnant women without PE. Data showed significantly higher levels of serum PLGF in pregnant women with PE, consistent with previous reports (Fig. 1).

Figure 1. Reduced serum placental growth factor (PLGF) levels detected in pregnant women with pre-eclampsia (PE). Serum PLGF levels in pregnant women with PE ($n = 26$) were compared to serum PLGF levels in pregnant women without PE ($n = 28$). Data shown as mean $\pm$ SD and statistically analysed using one-way ANOVA with Bonferroni correction. * $P < 0.05$.

Table 1. Tertile distributions of serum PLGF levels grouped by presence of GDM, and difference in development of GDM with each PLGF tertile

PLGF tertile	No GDM ($n = 324$)	GDM ($n = 26$)	P value
T1 (0–200 pg/ml)	42 (66.7%)	21 (33.3%)	PLGF T1 versus T2: $P < 0.001$ PLGF T1 versus T3: $P < 0.001$
T2 (200–400 pg/ml)	68 (93.2%)	5 (6.8%)	
T3 (>400 pg/ml)	214 (100%)	0 (0%)	

Values are expressed as number (ratio to total). Fisher exact test was used to compare different proportions.

PLGF, placental growth factor; GDM, gestational diabetes mellitus.

Correlation of serum PLGF level and development of GDM

Next, we examined correlation of serum PLGF level and development of GDM, where strong correlation was detected (Table 1, $P < 0.001$), suggesting that reduced serum PLGF seemed to induce GDM.

Establishment and validation of the L-NAME PE model

Inhibition of nitric oxide synthase with *N*-omega-nitro-L-arginine methyl ester (L-NAME) was used as experimental model of human PE (6–14). We gave daily subcutaneous injections of L-NAME to the pregnant mice, between days

9 and 17 of gestation. Control mice received injections of the same volume of PBS. We found a significant increase in blood pressure of mice that received L-NAME, compared to controls (Fig. 2a), and robust development of significant proteinuria (Fig. 2b–c). We also examined renal histology by H&E staining, and detected extensive renal damage in samples from mice injected with L-NAME. The majority of glomeruli were of normal to smaller size with endothelial swelling, causing narrowing and total obliteration or occlusion of glomerular capillary spaces – endotheliosis (Fig. 2d). These data suggest that L-NAME induced characteristics closely resembling those of human PE, and after L-NAME injection to pregnant rats (9). Moreover, we analysed serum PLGF levels in the L-NAME-treated pregnant mice, which were significantly reduced (Fig. 2e).

Impaired growth of beta cell mass in L-NAME injected mice

We then aimed to discover whether reduced PLGF in PE affected growth of beta cell mass during pregnancy, as a cause for GDM. Analyses of beta cell mass on day 17 gestation (G17: 9 days after first L-NAME injection) showed significant reduction of beta cell mass, suggesting that in these animals, beta cell mass failed to expand in response to pregnancy-related metabolic need (Fig. 3a).

Impaired growth of beta cell mass by reduced beta cell proliferation

Although most previous studies have demonstrated that beta cell replication is the predominant mechanism for post-natal beta cell growth during pregnancy (32,34–40), there have also been reports suggesting occurrence of beta cell neogenesis (41,42). Thus, we examined possible changes in beta cell proliferation and beta cell neogenesis in the L-NAME-treated mice. Continuous provision of BrdU in drinking water from G9 to G17 allowed continuous labelling of proliferating beta cells over this period. We found significant reduction in BrdU-incorporated beta cells in mice that received L-NAME injections, suggesting that impaired growth of beta cell mass was substantially due to fall in beta cell proliferation (Fig. 3b,c).

To discover whether beta cell neogenesis could also be involved, we examined islet-deprived pancreatic cells of Ngn3 expression. Ngn3 is a marker of beta-cell neogenesis (47), expressed at low level in mature beta cells (32,48–51). Purity of islet-deprived fractions was assured by low levels of insulin in these cells (Fig. 3d, less than 2% of levels in the islet fraction). We detected no significant changes in Ngn3 levels in islet-deprived

Figure 2. L-NAME is a pre-eclampsia model with reduced placental growth factor levels. (a–d) Subcutaneous injection of L-NAME to pregnant mice was performed daily between day 9 (G9) and day 17 (G17) gestation. Control mice received the same volume of phosphate-buffered saline (PBS) injection. (a) Systolic blood pressure was measured, showing a significant increase in pregnant mice receiving L-NAME injection, compared to control. (b, c) Urine albumin and creatinine ratio (b), or 24-h urine protein (c), was quantified at G17, showing development of significant proteinuria in L-NAME-treated pregnant mice. (d) Renal histology by H&E staining, showing extensive renal damage in samples from pregnant mice injected with L-NAME. The majority of glomeruli show normal to small glomeruli with endothelial swelling, causing narrowing and total obliteration or occlusion of the glomerular capillary spaces – endotheliosis. At least 100 glomeruli were analysed per mouse and five mice per group were analysed. (e) Placental growth factor levels in L-NAME-treated pregnant mice were significantly lower than controls. Data are shown as mean $\pm$ SD and statistically analysed using one-way ANOVA with Bonferroni correction. * $P < 0.05$. $n = 5$. Scale bar is 50 μm .

pancreatic cells from pregnant mice, from the onset of pregnancy, regardless of L-NAME injection, compared to non-pregnant mice, suggesting that beta cell neogenesis was not involved either in pregnancy itself, nor specifically in L-NAME-treated pregnant mice (Fig. 3e).

Exogenous PLGF rescued beta cell proliferation in L-NAME-treated pregnant mice

Our data demonstrated that expansion of beta cell mass reduced significantly during pregnancy in the PE model with reduced PLGF. However, whether this reduced growth directly resulted from attenuated PLGF was not clear. Thus, we provided L-NAME-treated mice with exogenous PLGF as a subcutaneous implant pump, at G9 when L-NAME was injected for the first time (Fig. 4a). Serum concentration of PLGF in PLGF-implanted L-NAME-treated mice reached levels of control mice without L-NAME treatment (Fig. 4b). We found that provision of exogenous PLGF in L-NAME-treated pregnant mice significantly rescued beta cell proliferation (Fig. 4c), and subsequently increase in beta cell mass (Fig. 4d). This indicates that shortage of PLGF was responsible for impaired beta cell expansion

and higher occurrence of GDM in patients with PE. The model is summarized in Fig. 4e.

Discussion

Pre-eclampsia is a common obstetric complication and is a leading cause of maternal and foetal mortality. To date, the precise aetiology of PE has remained unclear, although it is generally believed that women with GDM are at increased risk of PE. On the other hand, significantly higher numbers of patients with PE suffer from GDM (19,20). This suggest presence of a causal link between the disorder of glucose metabolism and development of hypertension (52–55). In addition, clinical features associated with insulin resistance, such as obesity and degree of hyperglycaemia, increase susceptibility of pregnant women to PE (56–58). As global prevalence of GDM continuously increases and has resulted in increased numbers of pregnant women at risk of PE, better elucidation of mechanisms that underlie the inter-relationship of PE and GDM will substantially improve the pregnancy outcome, as well as prevent occurrence of future disease (1–5).

Among all the members from VEGF family, PLGF predominantly expresses in the placenta and plays a

Figure 3. L-NAME induced impaired growth of beta cell mass in pregnant mice.

(a) Beta cell mass quantification at G17 showed significant reduction in beta cell mass in L-NAME-treated pregnant mice. NP, non-pregnant mice. (b, c) Continuous provision of BrdU in drinking water from G9 to G17 allowed continuous labelling of proliferating beta cells over this period. Impaired growth of beta cell mass appeared to result from reduced beta cell proliferation, by quantification of BrdU incorporation in beta cells (b), and by representative images (c). (d) Ngn3 transcript was quantified in islet-deprived pancreatic cells from pregnant mice with or without L-NAME treatment, compared to non-pregnant mice ($=1$). Data shown as mean $\pm$ SD and statistically analysed using one-way ANOVA with Bonferroni correction. $n = 5$. $*P < 0.05$. NS, non-significant.

critical role in pregnancy. Attenuated PLGF has been shown to be associated with occurrence of PE, possibly through its effect on vessel dynamics (19,20). However, the possible involvement of PLGF in development of GDM is not clear. Moreover, although there are established roles for other VEGF family members in the development and maintenance of the pancreatic beta cells (21–25), whether PLGF also plays a role in the endocrine pancreas has not been studied before.

Here, first we confirmed reduced serum PLGF levels in pregnant women with PE; we further detected strong correlation of serum PLGF levels and presence of GDM. To study the possible causal link and underlying mechanism, we used a mouse model of human PE, L-NAME injection, which has previously been shown to cause hypertension, proteinuria, renal damage and foetal growth retardation, without affecting gestational length. This syndrome resembles PE in humans and thus has been widely used as a PE model (10–14). Moreover, we found that serum PLGF levels significantly reduced in mice treated with L-NAME. After further validation of this model by elevated blood pressure, significant proteinuria and histological changes in the kidney, we

concluded that it is an appropriate model for testing our hypothesis for a role of PLGF in PE-related GDM.

Functional beta cell mass is important for maintaining correct control of blood sugar. Previous studies have shown a significant increase in beta cell mass during pregnancy, in response to metabolic need. Thus, we first analysed whether growth of beta cell mass during pregnancy would be affected by L-NAME injection. Our results showed a significant impairment in beta cell mass expansion after L-NAME treatment. Although increases in insulin resistance in GSM have been previously reported (59), impaired growth of beta cell mass has not formerly been documented. Moreover, insulin resistance is unlikely to be involved in our model, as presence of insulin resistance normally induces compensatory increases in beta cell proliferation to increase beta cell mass, which did not occur here. Thus, we focused on changes in beta cell mass in this model.

Although most previous studies have demonstrated that beta cell replication is the predominant mechanism for post-natal beta cell expansion (28–32), specially during pregnancy (32,34–40), involvement of beta cell neogenesis has also been reported (41,42). Thus, we

Figure 4. Exogenous placental growth factor (PLGF) rescued beta cell growth in L-NAME-treated pregnant mice. (a) Exogenous PLGF in subcutaneous implant pumps was given to L-NAME-treated mice on G9. (b, c) Provision of exogenous PLGF in L-NAME-treated pregnant mice significantly rescued beta cell proliferation (b), and subsequent increase in beta cell mass (c). (d) Schematic of the model. During pregnancy, PLGF not only regulates maternal and foetal vessel dynamics but also promotes beta cell proliferation to increase beta cell mass to meet increases in metabolic need. Data shown as mean $\pm$ SD and statistically analysed using one-way ANOVA with Bonferroni correction. $n = 5$. * $P < 0.05$. NS, non-significant.

analysed both beta cell proliferation and beta cell neogenesis. We found a substantial increase in beta cell proliferation in pregnant mice, which also appeared to be the cause of increase in beta cell mass during pregnancy, and its reduction by L-NAME injection. We then analysed expression of a specific pancreatic endocrine determinant transcription factor, Ngn3, in islet-deprived pancreatic cells, as a marker of beta cell neogenesis. This approach was taken as mature beta cells have been shown to express sustainedly low levels of Ngn3 (32,48–51), which would have affected our results if islets had been included in the analyses. Our method allows examination of beta cell neogenesis from duct cells or other exocrine cells, outside islets. Our result, however, did not support such a possibility, which argued against the involvement of beta cell neogenesis in pregnancy, and was consistent with most previous reports (32,34–40).

To define a causal link between reduced growth in beta cell mass and attenuated PLGF levels, we used a gain-of-function experiment by providing L-NAME-treated mice with exogenous PLGF in subcutaneous implant pumps, along with L-NAME injections. We adjusted doses of PLGF so that serum PLGF was

exactly rescued to levels in mice without L-NAME treatment. This dose is perhaps species-dependent (rats, mice or other) and strain-dependent.

We found that exogenous PLGF partially rescued BP and proteinuria, suggesting an essential but not exclusive role of PLGF in control of PE-related vessel damage. However, such results have been published by others and are not a focus of the current study. We also checked islet vessel density by CD31 staining, and did not detect significant difference in experimental settings. The effect of PLGF on beta cell growth may not require development of islet capillaries (60,61). Thus, PLGF may activate specific signal transduction in islet endothelial cells after binding to VEGFR1 in these cells, which triggers production and secretion of trophic factors to promote proliferation of adjacent beta cells in a paracrine manner.

Interestingly, we found that exogenous PLGF significantly rescued beta cell proliferation, and the subsequent increase in beta cell mass, in L-NAME-treated pregnant mice, which provides compelling evidence that shortage in PLGF is responsible for impaired beta cell growth and higher occurrence of GDM in patients with PE. Thus, in the current study, we have illustrated a meta-

bolic pathway in which PLGF regulates not only maternal and foetal vessel dynamics but also beta cell mass, in response to metabolic need. Our study has thus highlighted the importance of PLGF in prevention and treatment of GDM in patients with PE.

Acknowledgement

This work is financially supported by National Natural Science Foundation of China (NSFC) no.: 81372486.

Conflict of interest

None disclosed.

Author contributions

Conceived and designed the experiments: JL. Performed the experiments: JL, HY, GC, QG and LC. Analysed the data: JL. Wrote the paper: JL.

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