Placental growth factor (PIGF) is linked to inflammation and metabolic disorders in mice with diet-induced obesity

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Abstract. Placental growth factor (PIGF), a member of the vascular endothelial growth factor (VEGF) sub-family, plays a major role in angiogenesis and vasculogenesis. Previous study demonstrated that PIGF-overexpressing transgenic (Tg) mice had gestational loss. In addition, PIGF secretion was up-regulated in isolated T lymphocytes (T-cell) upon CD3/CD28 stimulation, suggesting that PIGF could be a regulator of T-cell differentiation and development. T-cells are well known to play a critical role in obesity-induced inflammation. Therefore, to verify the possible link of diet-induced obesity (DIO) with inflammation and related metabolic disorders, such as insulin resistance, we fed high-fat diet (HFD) to Tg mice for 16 weeks. Adiposity and glucose intolerance significantly increase in Tg mice fed a HFD (Tg HFD) compared to wild-type (WT) mice fed HFD (WT HFD). In addition, macrophage infiltrations were significantly higher in the epididymal white adipose tissue (EWAT), liver, and pancreatic islets of Tg HFD mice compared to WT HFD mice. In the in vitro study, we showed that isolated CD4⁺ T-cells from Tg mice further differentiate into type 1 (Th1) and type 17 (Th17) helper T-cells via CD3/CD28 stimulation. Furthermore, we observed that the pro-inflammatory cytokines IL-6, IL-17, and TNFα, are remarkably increased in Tg mice compared to WT mice. These findings demonstrate that PIGF overexpression in T-cells might lead to inflammatory T-cell differentiation and accumulation in adipose tissue (AT) or metabolism-related tissues, contributing to the development of systemic metabolic disorders. Thus, PIGF may provide an effective therapeutic target in the management of obesity-induced inflammation and related metabolic disorders.

Key words: Placental growth factor, Inflammation, T-cell, Diet-induced obesity, Metabolic disorders
temic inflammation. Patients with these diseases have impaired insulin sensitivity and glucose homeostasis due to the up-regulation of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-17, and TNFα [10, 11]. Activated macrophages and T-cells are a major source of these inflammatory cytokines, in particular [10]. Infiltrated immune cells in AT releases abundant inflammatory factors such as IL-1 β, IL-6, IL-17, TNFα, and monocyte chemoattractant protein-1 (MCP-1) [12-14]. Particularly, increased AT macrophage levels result in the formation of crown-like structures (CL斯), which surround dead adipocytes, influencing AT remodeling, and inducing insulin resistance, as a result [15]. Numerous studies have suggested that diet-induced obesity (DIO) accelerates metabolic disorders through fatty liver and pancreatic inflammation, ultimately leading to type 2 diabetes (T2DM) [16, 17].

In this study, we elucidated the role of PlGF in inflammatory T-cell differentiation using our established Tg mice [18]. Type 1 (Th1) and type 17 (Th17) helper T-cell differentiation was more pronounced in Tg mice than WT mice (data not shown). However, research on the relationship between PlGF and inflammation in DIO still remains insufficient. Therefore, this study investigated the effect of elevated PlGF, and its role in inflammation and disease progression in mice with DIO.

Materials and Methods

**Transgenic mice and experimental protocol**

We generated Tg mice using the specific promoter for human T-cell CD2 [18]. Tg and WT littermate male mice were housed in laboratory cages at a temperature of 24°C and humidity of 50%, with a 12 h dark/light cycle (lights switched on at 7 a.m.). For the diet study, WT and Tg male mice were grouped separately, depending on their feed: a Normal diet [ND; FORMULA M07; 7 kcal % fat; FEEDLAB, Kyonggi-do, Korea (WT ND and Tg ND)] or a high-fat diet [HFD; 45 kcal % fat; Research Diets Inc., New Brunswick, NJ (WT HFD and Tg HFD)] for 16 weeks, starting with 8-week-old mice. Body weight and food intake were monitored weekly. In addition, the blood glucose levels were determined once a week with blood collected from the tail vein of mice, for 16 weeks, using a glucometer (ACCU-CHEK; Roche, New York, NY). The mice were ethically euthanized at the end of the experiment, and their blood and tissue samples collected. All animal experiments were performed according to the guidelines for animal experimentation and were approved by the Kyungpook National University Animal Care and Use Committee.

**Glucose and insulin tolerance tests**

Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed as described previously [19]. Briefly, mice were put on overnight fast, prior to the GTT and ITT, after 16 weeks of feeding. Basal glucose levels of the mice, during fasting, were measured with a glucometer using blood collected from the tail vein. Next, the mice were intraperitoneally injected with glucose (1.5 g/kg body weight) and insulin (2 U/kg body weight), and blood glucose levels were determined after 15, 30, 60, and 120 min using a glucometer. Glucose levels were also assessed by calculating area under the curve (AUC) from GTT and ITT data.

**CD4+ T-cell purification**

Single cell suspensions were prepared from the spleens of WT and Tg mice, and placed in red blood cell lysis buffer (0.15 M NH₄Cl and 0.1 mM Na₂EDTA) for 30 min at room temperature to eliminate erythrocytes. CD4+ T-cells were isolated using anti-CD4 antibody-coupled magnetic cell sorting micro-beads (Miltenyi Biotec, Auburn, CA). CD4+ T-cells were purified to >95% purity, as analyzed by flow cytometric staining with anti-CD4 and anti-CD8 antibodies.

**Maintenance in culture and activation of CD4+ T-cells**

The purified CD4+ T-cells were plated at a density of 5 x 10⁶ cells/well into six-well culture plates (Nunc, Roskilde, Denmark). One group was stimulated by pre-coating with 1 µg/mL anti-CD3 antibody (BD Biosciences, San Jose, CA) and treating with 4 µg/mL soluble anti-CD28 monoclonal antibody (BD Biosciences, CA), whereas the other group of cells was left untreated. CD4+ T-cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco, NY) and 0.1% β-mercaptoethanol (Gibco, NY), in a humidified cell culture incubator containing 5% CO₂ at 37°C.

**Enzyme-linked immunosorbent assay (ELISA)**

The CD4+ T-cells, isolated from the spleen of 8-week-old WT and Tg mice, were stimulated by activated CD3/CD28 in vitro. After stimulation, the supernatant was collected, and cytokine (PlGF, TNFα, IL-1β, IL-17, and IL-6) levels were measured in the supernatant by using
commercially available enzyme-linked immunosorbent assays (R&D system, MA). In addition, using the same methods as above, we confirmed the cytokine levels in the serum of WT and Tg mice fed a ND or HFD for 16 weeks.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from the liver of WT and Tg mice using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Quantitative RT-PCR was performed using a StepOnePlus™ PCR system (Applied Biosystems, Foster City, CA), which can detect SYBR green fluorescence (Takara, Shiga, Japan) after cDNA hybridization (Takara, Shiga, Japan). The set of primers used for RT-PCR are shown in Table 1, and β-actin was used as an internal quantitative control.

Histological analysis

Mice were sacrificed at 16 weeks, following the start of DIO. Mouse EWAT, liver, and pancreas were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) (Sigma Aldrich, St. Louis, MO) for routine histological analysis. For immunohistochemistry (IHC), 5 μm sections of paraffin-embedded EWAT were treated with Proteinase K for 30 min, and blocked with 5% BSA and 0.5% Tween 20 for another 30 min at room temperature. To examine the degree of macrophage infiltration, EWAT, liver, and pancreas sections were incubated with macrophage marker antibody (RM0029-11H3; Santa Cruz Biotechnology, CA) at room temperature for 1 h. Isotype control antibodies (Sigma Aldrich, St. Louis, MO) were used as a negative control. Each slide was washed three times in phosphate buffered saline with Tween 20 (PBST). After the final wash step, the staining was visualized using the EnVision™ Detection System (Dako, Glostrup, Denmark) in a humid chamber for 30 min. Positive cells were detected using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, San Francisco, CA).

Statistical analyses

Data are expressed as the mean ± SD from at least three independent experiments. The significance of differences between groups was evaluated using the Student’s t-test. p < 0.05 was considered significant.

Results

DIO in Tg mice displays increased adiposity and insulin resistance

Our previous study had indicated that PlGF secretion is up-regulated in isolated T-cells, stimulated by activated CD3/CD28, suggesting that PlGF can regulate T-cell differentiation and development [18]. Therefore, in this study, we determined whether PlGF overexpression in T-cells is involved in adiposity and obesity-induced inflammation. First, we performed a basic examination of the 8-week-old mice prior to the diet study (Fig. 1). Body weight, blood glucose, GTT, and ITT results did not differ between WT and Tg mice (Fig. 1A–D). WT and Tg mice were then grouped further depending on their feed, either a ND or HFD, over 16 weeks. We confirmed that body weight was significantly increased in Tg HFD compared to WT HFD. However, the body weights of Tg mice fed a ND (Tg ND) were similar to those of WT mice on ND (WT ND) (Fig. 2A). Food intake was monitored weekly; there was no difference in food intake between ND and HFD mice (Fig. 2B). At 16 weeks, following completion of the diet experiment, GTT and ITT were performed. Glucose levels obtained from GTT and ITT results did not differ between WT and Tg mice (Fig. 1A–D). Food intake was monitored weekly; there was no difference in food intake between ND and HFD mice (Fig. 2B). At 16 weeks, following completion of the diet experiment, GTT and ITT were performed. Glucose levels obtained from GTT and ITT were higher in Tg HFD mice than in WT HFD mice. A significant difference was observed 60 min after injection (Fig. 2C, D). Glucose tolerance was
also assessed by calculating AUC for glucose levels from GTT and ITT data. We confirmed that AUC glucose levels were higher in Tg HFD mice than in WT HFD mice (Fig. 2E, F). Furthermore, Tg HFD mice also developed significant hyperinsulinemia than WT HFD mice (Fig. 2G). The increased blood glucose and hyperinsulinemia of DIO model is an indicator for insulin resistance [11]. Therefore, these results indicated that adiposity and insulin resistance are increased in Tg HFD mice.

**DIO in Tg mice leads to EWAT hypertrophy and dysregulates gene expression in metabolism-related tissues**

We confirmed histological changes in a representative white adipose tissue, EWAT. H&E staining showed that adipocytes were larger in Tg HFD than in WT HFD mice (Fig. 3A). Furthermore, Tg HFD mice also developed significant hyperinsulinemia than WT HFD mice (Fig. 2G). The increased blood glucose and hyperinsulinemia of DIO model is an indicator for insulin resistance [11]. Therefore, these results indicated that adiposity and insulin resistance are increased in Tg HFD mice.

**PlGF overexpression affects macrophage infiltration in metabolism-related tissues**

To investigate this issue further, we performed qPCR analysis using several primers related to adipogenic and insulin sensitivity markers from EWAT and liver. Tg ND mice had no significant change in adipogenic, lipolytic, and insulin sensitivity markers compared to WT ND mice (data not shown). Nevertheless, Tg HFD mice expressed higher levels of adiposity markers, PPARγ-2 and FABP-4 mRNA. In addition, insulin sensitivity markers, such as IRS-1 and GLUT-4, were lower in Tg HFD mice (Fig. 3D). Similarly, hepatic lipogenic markers were significantly increased in Tg HFD mice compared to WT HFD mice (Fig. 3E). These results are tangible proofs that PlGF increases adiposity and metabolic disorders in Tg HFD mice.
Fig. 2 DIO in Tg mice displays increased adiposity and insulin resistance.

(A) Body weight and (B) food intake were monitored weekly in WT and Tg mice fed a ND (WT ND and Tg ND) or HFD (WT HFD and Tg HFD) for 16 weeks (n = 5–6, each group). (C) GTT and (D) ITT were performed in WT and Tg mice fed either a ND or HFD (n = 8–9, each group). (E, F) Glucose tolerance was assessed by calculating AUC using GTT and ITT data. (G) Serum insulin levels in WT and Tg mice fed a ND or HFD (n = 5–6, each group). Values represent the mean ± SD. * p < 0.05; ** p < 0.01, vs. WT HFD.
mice (Fig. 3A). According to IHC, macrophages positive for CLSs were more strongly detected in Tg HFD than in WT HFD mice, whereas there were no positive signals in ND mice (Fig. 4A).

DIO is associated with chronic systemic inflammation, which leads to inflammation of tissues including liver, muscles, and pancreatic islets, ultimately leading to diabetes mellitus [10]. To further elucidate how the increase of immune cell infiltration in Tg HFD mice contributes to systemic inflammation, we examined other metabolism-related tissues such as the liver and pancreas. In IHC analysis, macrophage infiltrations were more strongly detected in the liver and pancreas of Tg HFD mice than in those of WT HFD mice (Fig. 4B, C).

PIGF overexpression regulates T-cell differentiation

In a previous examination of the effect of PIGF overexpression in T-cells [18], Th1 and Th17 helper T-cell differentiation was found to be more in Tg mice than in WT mice (data not shown). According to our data, Tg HFD mice showed more inflamed phenotypes, along with insulin resistance, compared to WT HFD mice, suggesting alteration of T-cell differentiation in Tg mice as a possible cause.

To verify this possibility of T-cell differentiation in CD4+ T-cells, we isolated CD4+ T-cells from the spleen of 8-week-old WT and Tg mice, and measured their cytokine production after stimulation by activated CD3/CD28 in vitro for 48 h. Both TNFα (Th1 type) and IL-17 (Th17 type) cytokines were highly secreted by the T-cells in Tg mice, in response to CD3/CD28 stimulation (Fig. 5A). We confirmed that Th1 and Th17 helper T-cells differentiate to a greater extent in Tg mice.

We observed stronger insulin resistance, adequate
presence of CLSs, and elevated tissue inflammation in Tg mice, indicating a higher risk of metabolic disorders in them, relative to WT mice. We attributed these results to the activation of inflammatory T-cell differentiation in Tg mice in response to DIO. To demonstrate this, we confirmed the cytokines in the serum of WT and Tg mice fed either a ND or HFD for 16 weeks. We observed that TNFα and IL-17, which are representative inflammatory Th1 and Th17 cytokines, were remarkably increased with IL-6 in the serum of Tg mice (Fig. 5B, C). These results are consistent with the aforementioned findings of greater Th1 and Th17 helper T-cell differentiation in Tg mice (Fig. 5A). Taken together, we observed that pronounced development of obesity-induced inflammatory
conditions in Tg HFD mice compared to WT HFD mice.

**Discussion**

PlGF is a member of the VEGF sub-family, a key regulator in angiogenesis and vasculogenesis. It is expressed in almost all cell types, including adipocytes [1]. In pathological conditions, such as inflammation and ischemia, PlGF is known to induce angiogenesis [1, 3-5, 20]. In a previous study, we had generated Tg mice that overexpress PlGF in T-cells, under the control of the human CD2 promoter. In this study, we indicated that PlGF secretion was up-regulated in isolated T-cells, stimulated by activated CD3/CD28, hence suggesting PlGF as a regulator of T-cell differentiation in an autocrine or paracrine manner [18]. As a result, T-cells in Tg mice differentiate into inflammatory Th1 and Th17 subtypes more frequently or easily (data not shown).

In the current in vitro study, the CD4+ T-cells isolated from the spleen of Tg mice indicated greater inflammatory Th1 and Th17 helper T-cell differentiation (Fig. 5A), thereby emphasizing the role of PlGF in T-cell differentiation and development.

The association of DIO with T-cell differentiation and accumulation in metabolism-related tissues such as AT, liver, and pancreas, is well known to contribute to the development of systemic inflammation and related metabolic disorders [16, 21]. We confirmed from the diet study that TNFα and IL-17, which are inflammatory Th1 and Th17 cytokines respectively, were remarkably increased in the serum of Tg mice compared to that of WT mice. Furthermore, Tg mice showed enhanced cytokine production with dietary stimulation such as HFD, compared to that seen for Tg ND mice (data not shown).
These results indicated that the increased T-cell differentiation and infiltration in Tg HFD mice might contribute to their severe inflammation.

We found that immune cell infiltrations, particularly macrophages, occurred more in the EWAT, liver, and pancreas of Tg HFD mice compared to those of WT HFD mice. In DIO, the inflammatory response is initiated by macrophage recruitment. The macrophages infiltrate tissues to secrete TNFα and MCP-1, which are chemotactic factors [22], and further accelerate the inflammation and infiltration of various immune cells. According to a recent study, T-cells are the first to migrate to AT, and contribute to inflammatory cell activation prior to macrophage infiltration [23]. Considering this, along with the results from the present study, we propose that the increased inflammatory T-cell differentiation and infiltration in Tg HFD mice might contribute to the recruitment of macrophage, whose synergistic effect in metabolism-related tissues leads to the severe inflammation.

Pro-inflammatory cytokines and chemokines also affect major metabolism-related tissues, including liver, muscle, and pancreatic islets, contributing to metabolic syndromes [15, 17, 21]. The imbalance between pro-inflammatory and anti-inflammatory cytokines could lead to chronic inflammation [24-27], associated with metabolic disorders such as insulin resistance or glucose intolerance. Our results show an increase in the features of CLSs, pancreatitis, and fatty liver disease accompanied by impaired glucose homeostasis. Overall, these findings indicate that Tg HFD mice manifest the key pathological features attributable to increased inflammation.

Despite the increased cytokine production in Tg ND mice, our results confirmed that Tg ND mice have little influence on the insulin sensitivity or glucose homeostasis compared to the WT ND mice. In addition, no apparent phenotypic changes were observed in Tg ND mice. We speculated that the effect of moderately enhanced cytokine production in Tg mice, after the relatively short exposure of 16 weeks or less to a ND, may not be sufficient to cause metabolic disorders and pathologic condition. Further studies would be needed to confirm this with long-term diet exposure.

Interestingly, our results indicated that Tg HFD mice gained weight more readily than WT HFD mice, despite the same food consumption between WT and Tg mice. PlGF is widely known as an angiogenesis factor; the relationship between angiogenesis and PlGF in adiposity was confirmed in a recent study [8, 9]. It demonstrates that PlGF is important role during first stages of AT formation. Particularly, de novo formation of blood vessels is impaired in the PlGF-deficiency mice fed HFD. PlGF-deficiency mice also showed lower blood vessel size in EWAT, with decreased adiposity [8]. In line with these observations, a possible explanation could be that an increase of AT vasculature with metabolic stimulation (HFD) induces more AT expansion with increasing adiposity in our Tg mice model.

Another possible mechanism could be via PlGF-induced increase in infiltration of mesenchymal stem cells (MSCs), which then differentiate into endothelial cells and adipocytes [28]. Particularly, it suggests that PlGF, in addition to other factors such as bone morphogenetic proteins (BMPs), may contribute to the recruitment of bone marrow derived MSCs. PlGF is also expressed in the stromal vascular cell fractions (SVFs), which contain MSCs from the AT [8]. Therefore, PlGF might contribute to the extrinsic adipogenic differentiation potential by MSCs recruitment, and remodeling but their association requires further examination. In conclusion, we have confirmed that overexpression of PlGF in vivo leads to adiposity and impaired glucose homeostasis accompanied by elevated inflammation in DIO model. These results suggest that PlGF overexpression in T-cells may lead to T-cell differentiation and accumulation in AT or metabolism-related tissues, which contribute to the development of systemic inflammation and related metabolic disorders. Therefore, PlGF could be a potential target for therapeutic intervention aimed at regulation of inflammation and related metabolic disorders in DIO.

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**Disclosure**

None of the authors have any potential conflicts of interest associated with this research.
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