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Genetic Deletion of Placenta Growth Factor in Mice Alters Uterine NK Cells

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Placenta growth factor (PIGF; formerly PGF), a vascular endothelial growth factor gene family member, is expressed in human implantation sites by maternal uterine NK (uNK) and fetal trophoblast cells. Lower than normal concentrations of blood and urinary PIGF have been associated with impending onset of pre-eclampsia, a hypertensive disease of late human gestation characterized by limited intravascular trophoblast invasion. In pregnant rodents, delivery of the PIGF antagonist sFlt-1 or S-endoglin induces pre-eclampsia-like lesions. Mice genetically deleted in PIGF reproduce, but neither their implantation sites nor their uNK cell development are described. We combined real-time PCR of endometrium from nonpregnant and gestation day (gd)6–18 C57BL6/J (B6) mice with immunohistology to analyze PIGF expression in normal mouse pregnancy. To estimate the significance of uNK cell-derived PIGF, PIGF message was quantified in mesometrial decidua from pregnant alymphoid Rag2 null/common γ chain null mice and in laser capture-microdissected B6 uNK cells. Histopathologic consequences from PIGF deletion were also characterized in the implantation sites from PIGF null mice. In B6, decidual PIGF expression rose between gd8–16. uNK cells were among several types of cells transcribing PIGF in decidualized endometrium. Immature uNK cells, defined by their low numbers of cytoplasmic granules, were the uNK cells displaying the greatest number of transcripts. PIGF deletion promoted the early differentiation high numbers of binucleate uNK cells (gd8) but had no other significant, morphometrically detectable impact on implantation sites. Thus, in mice, PIGF plays an important role in successful uNK cell proliferation and/or differentiation. The Journal of Immunology, 2007, 178: 4267–4275.

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²Abbreviations used in this paper: PIGF; placenta growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; uNK, uterine NK; PL, placenta; gd, gestation day; MLAp, mesometrial lymphoid aggregate of pregnancy; γc, common γ-chain; LCM, laser capture microdissection; DB, decidua basalis; DBA, Dolichos biflorus agglutinin; AG, agranular; IM, immature; aRNA, antisense RNA; PAS, periodic acid-Schiff; WT, wild type.

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form a transient lymphoid structure known variously as the mesometrial lymphoid aggregate of pregnancy (MLAp), metrial gland, or decidualized mesometrial triangle. This mural structure surrounds the radial branches of the uterine artery as they arborize into the endometrial spiral arteries. Pregnancy-associated structural changes in murine spiral arteries are detected histologically at gd9–10, just before the gd11 onset of death in uNK cells. An analogous decline in human uNK cell numbers occurs about midgestation (22). Murine spiral arterial modification may differ from that seen in women because intralumenal trophoblast invasion occurs in arterial segments more restricted in proximity to the PL (terminal 150 μm; Ref. 20). By midgestation, most murine uNK cells (>65%) are intimately associated with endometrial vessels and 10% are intravascular (23), suggesting that uNK cells directly influence vascular smooth muscle, endothelium, and possibly conducted responses in these vessels.

PIGF null mice (2) are born at Mendelian frequency. They are healthy, fertile, and are reported to show only minor deficits in remodeling of retinal vessels and less angiogenesis in ovarian corpora lutea than normal controls. No information is available on uNK cells and their target spiral arterial and decidual tissues in PIGF null mice. This study was undertaken to characterize PIGF in mouse implantation sites.

Materials and Methods

Mice

Male and female C57BL6/J (B6) mice were purchased from The Jackson Laboratory, housed, and mated for timed pregnancies under standard conditions at the University of Guelph. A lymphoid (NK−, T−, B−) Rag2 null/ common γ chain (γc null) null mice on a B6 background were bred under barrier husbandry at the University of Guelph from foundation stock provided by Dr. J. P. Di Santo (Pasteur Institute, Paris, France). Some pregnant B6 and Rag2 null/γc null females were perfused with 4% paraformaldehyde, and their implantation sites were processed as paraffin-embedded blocks and shipped to Canada for histological study. No resorbing fetuses were detected in the PIGF null mice.

RNA isolation from C57BL6/J and Rag2 null/γc null mesometrial endometrium

Total RNA was extracted, using RNeasy mini kit (Qiagen) per the manufacturer’s instructions, from mesometrial endometrium of nonpregnant uteri or from dissected decidua basalis (DB) or MLAp of implantation sites. Similar tissues from a single pregnant uterus were pooled. Briefly, 30 mg of tissue were disrupted by homogenization and the tissue lysate was centrifuged (15,000 × g, 3 min). The cleared lysate was mixed with 700 μl of 70% ethanol. The reaction mixture was loaded on a RNeasy mini column and centrifuged (8,000 × g, 30 s). Columns were washed (500 μl each of wash buffer), centrifuged (8,000 × g, 2 min), and RNA was eluted in 50 μl of nuclease-free water. cDNA was prepared from these RNA samples using First-Strand cDNA synthesis kit (Amersham Biosciences).

LCM of uNK cells, RNA isolation, and cDNA synthesis

Implantation sites collected for LCM from gd6–12 B6 mice were embedded using Cryomats (ThermoShandon) in a plastic mold over dry ice. Cryostat sections were cut at 7 μm, placed on glass slides (Fisher Scientific), and either stored at −80°C for subsequent use or stained immediately using a rapid Dolichos biflorus agglutinin (DBA) lectin-staining protocol to identify uNK cells as described previously (24). DBA lectin staining produces a distinct brown coloration over the surface of all uNK cells and over the membranes encapsulating uNK cell cytoplasmic granules once granules begin to develop. LCM was performed using the PixCell IIe system (Arcturus Engineering). Stained uNK cells were evaluated at ×400 microscopic magnification to estimate the number of cytoplasmic granules and the diameter of each cell. Both of these parameters normally increase as uNK cells mature (21). Approximately 200–300 morphologically homogeneous uNK cells of each of four distinct subtypes were collected by focal laser melting of the thermoplastic film from the cap onto the individual desired sites using multiple implantation sites for cell harvesting (24). The four uNK cell subtypes used were as follows: 1) agranular (AG; DBAα, <9-μm diameter); 2) immature (IM; DBAα, ~13-μm diameter, <5 cytoplasmic granules); 3) granular (DBAβ, 20- to 30-μm diameter, heavily granulated <10 cytoplasmic granules); or 4) senescent (DBAβ, >30-μm diameter, apoptotic nuclei, vacuolated cytoplasm, irregular rather than rounded shape >10 cytoplasmic granules). Each subtype was harvested on the gd when it was the most abundant uNK cell type present. RNA extraction from captured uNK cells was done using the Picopure RNA isolation kit (Arcturus), and two rounds of antisense RNA (aRNA)
amplification were conducted using MessageAmp II aRNA kit (Ambion) per manufacturer’s instructions. The aRNA obtained after RNA amplification from the LCM-isolated uNK cells and total RNA extracted from implantation sites were reverse transcribed using the First-Strand cDNA synthesis kit (Amersham Biosciences). The resultant cDNA was used as a template for real-time PCR.

Quantitative real-time PCR

Real-time PCR (LightCycler; Roche Diagnostics) was used to quantify PlGF expression relative to β-actin in mesometrial endometrium and in uNK cells. Primers were designed using the Primer 3 software program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.ebi). PlGF primers are forward primer (TCTGGGTCTAGAAGCTGTC) reverse primer (GGACACAGGGACTGGAAT), product size 222 bp and B-actin forward primer (TCTGGGCACCACCTTCTA) reverse primer (AGGCATGGACACAGGACTGGAAT), product size 190 bp. For quantification of PlGF expression, the SYBR Green PCR kit (Qiagen) was used. LightCycler reactions were performed in 20 μl of total reaction volume, using the manufacturer’s instructions. PCR products were gel purified using the Wizard DNA purification system (Promega), and/or plasmid DNA with specific inserts were quantified and diluted serially to generate standard curves for the gene of interest. The resulting amplified products were sequenced to reconfirm amplification efficiency. Each reaction mixture contained 2 μl of cDNAs, 10 μl of SYBR Green I master mix, 1 μl of sense and antisense primers (0.5 μM each), and 6 μl of PCR-grade water supplied with the kit. The LightCycler program for each gene was denaturation (94°C, 15 min); PCR amplification and quantification (95°C, 10 s, 58°C, 5 s, 72°C, 20 s) with the fluorescence measurement at specific acquisition temperatures for 5 s, repeated for 45 cycles. The melting program was 70–95°C at the rate of 0.1°C/s with continuous fluorescence measurement, with the final cooling step at 40°C. Data were quantified using RelQuant LightCycler analysis software.

Histological and morphometric procedures

Histological serial sections (7 μm) of implantation sites from PlGF null mice and their congenic controls were prepared. Some sections were stained with H&E for general morphology. Other sections were stained with periodic acid-Schiff’s (PAS) reagent to reveal uNK cell granules. uNK cells were enumerated in two regions, the DB and the MLAp, using an eyepiece micrometer. For each implantation site studied, a 1-mm² area of tissue was scored on 11 medial sections, spaced to provide non-overlapping uNK cells (42 μm apart). Three implantation sites were used for each mother studied. The same 11 sections were used for morphometry (Optimas 6.2) of the spiral arteries. Three regions were outlined for each vessel: the lumen surface of the endothelium, the more densely stained matrix in the vessel wall that, in many vessels, was obscured or interrupted by uNK cells and therefore estimated as uniform over the gap area, and an unusual peripheral circumference of mixed cell composition that appeared to be a structural component of the spiral arterial walls. Areas of these regions were compared as ratios. The total surface areas of the PL, DB, and MLAp were also estimated by making three circumscribing measurements. These dimensions were the entire mesometrial side of the implantation site, the PL, including the giant cells, and the MLAp as the area from the uterine circular smooth muscle to the serosa. The area of the DB was estimated by subtraction of the sum of the latter two areas from the first area.

Immunohistochemistry and lectin histochemistry

To detect PlGF and to identify trophoblast cells, immunohistochemistry was performed using implantation sites from B6 and alymphoid mice. The primary Abs (either anti-mouse PlGF Ab (Abcam) or wide-spectrum
screening cytokeratin Ab (DakoCytomation)) were applied to the sections at 1/1000 concentration overnight at 4°C. Negative controls used isotype-matched Ab to estimate nonspecific binding of the secondary Ab. PlGF staining was conducted after removal of paraffin blocking of endogenous peroxidase using 0.2% hydrogen peroxide in methanol for 20 min, and blocking of nonspecific binding using 2% BSA at room temperature. Cytokeratin staining was conducted after proteinase-K Ag retrieval for 6 min followed by blocking in 1% BSA for 30 min. Sections were then incubated (30 min, room temperature) with HRP-labeled goat anti-mouse IgG (Southern Biotechnology Associates) at 1:500 and revealed using NovoRED for 10 min (Vector Laboratories). Between each step, tissue sections were washed with PBS. Sections were counterstained with hematoxylin before viewing and photography using a Leica photomicroscope.

To identify IM, nongranulated as well as granulated uNK cells in implantation sites from PlGF null mice and their congenic controls, the standard DBA lectin-staining protocol of Paffaro et al. was used (21). ExtrAvidin peroxidase conjugate (Sigma-Aldrich) was used to detect bound biotinylated lectin and was revealed with 3,3'-diaminobenzidine in TBS with 0.1% hydrogen peroxide. These sections were counterstained with hematoxylin.

Statistical analyses

Statistical analyses used ANOVA (SAS software 8.2; SAS Institute). A value of \( p < 0.05 \) was considered significant.

Results

Quantification of PlGF message in endometrium of B6 mice

To address endometrial expression of PlGF message during pregnancy, real-time PCR was used to analyze mesometrial samples from virgin or gd6–18 B6 mice (Fig. 1). PlGF expression was detected in nonpregnant (virgin) B6 endometrium, and this level of expression was not altered at gd6 or -8. By gd10, PlGF expression increased to gd16, then declined at gd18.

Localization of immunoreactive PlGF in B6 and alymphoid mouse implantation sites

To establish whether the endometrial cells expressing PlGF included or were restricted to only uNK cells, immunohistochemistry was conducted on gd10 implantation sites from B6 (Fig. 2A) and from alymphoid mice (Fig. 2B). In B6 sites, strong staining was detected in multiple endometrial cell types, including uNK cells. Significant stromal staining was noted perivascular to the spiral arteries and in smooth muscle cells. Spiral arterial endothelium appeared to be nonreactive. In alymphoid mouse implantation sites that are characterized by the absence of uNK cells and MLAp development and by decidual hypocellularity (19), PlGF was abundantly expressed in the PL. Weakly positive stromal cells were present in DB. Myometrial cells and the smooth muscle cells and endothelia of the spiral arteries appeared to be negative.

Quantification of PlGF message in endometrium of alymphoid mice

PlGF message was essentially undetectable in the mesometrial endometrium of nonpregnant alymphoid mice (Fig. 1). Pregnancy induced stable expression of PlGF to gd12. At gd6 and -8, endometrial expression of PlGF in alymphoid mice was equivalent to that in B6 mice. This result suggests that lymphocytes are not the cells in murine DB responsible for the early, postimplantation gain in PlGF expression. Between gd10–16, there was significantly less PlGF transcription in DB from alymphoid mice than from B6 mice (Fig. 1). This difference suggests that, at midgestation, most
decidual PlGF is lymphocyte-derived. *PlGF* transcription from nonlymphoid cell types showed further waves of elevation at gd14 and -18. Transcript numbers at gd14 were still significantly lower in alymphoid than in B6 mice but at gd18, there was no significant difference. These data indicate that lymphocytes have the potential to contribute to angiogenesis in virgin and pregnant uteri.

*PlGF* expression in B6 uNK cells

No DBA lectin-reactive lymphocytes are present in virgin B6 uteri or in virgin or pregnant uteri from alymphoid mice. To establish when *PlGF* transcription occurs during the maturation of uNK cells from proliferating AG to heavily granulated, postmitotic cells, pools of 200–300 morphologically homogeneous DBA lectin-reactive uNK cells (brown) were localized around vessels and scattered in MLAp and DB. The gd10 images show overall morphology, and gd12 images focus on cross-sectional cuts of the coiled spiral arteries (*) at higher power showing their dilation. g. Represents the negative control in which the specific sugar ligand N-acetyl galactosamine was used to block DBA lectin binding. (Magnification a, b, d, e, ×200; magnification c, f, and g, ×400.) B. Comparison of the midsagittal cross-sectional areas (mean ± SD) of MLAp, DB, and PL from *PlGF* null mice to congenic controls at gd10 and -12. The total surface areas for these regions were estimated by making three circumscribing measurements in the entire mesometrial side of the implantation site, the PL, including the giant cells, and the MLAp. C. Cytokeratin-reactive trophoblasts (brown, indicated by arrows) are seen predominantly in the PL layer of *PlGF* null (a and b) and WT (c and d) mice at gd10 (a and c) and gd12 (b and d), respectively. Images b and d represent cytokeratin positive trophoblasts associated with spiral arteries in the DB layer in *PlGF* knockout (KO) and WT mice, respectively. M, Mesometrial side (maternal arteries entry side). Magnifications, ×400.
in endometrium at gd16 and -18 must be attributed to cell types other than uNK cells.

General histology and morphometric measurements of implantation sites in PlGF null mice

To determine whether PlGF deficiency modifies implantation sites, sites from PlGF null mice and their congenic controls were studied. Sites from PlGF null mice were different from the controls grossly and histologically (Fig. 4A). PlGF null implantation sites were grossly larger. Histopathology attributed this observation to more abundant endometrial (maternal) subregions (Fig. 4B). Of note was the significant enlargement of the MLAp at gd12. This endometrial subregion is where more IM, proliferative uNK cells are found in normal mice (20). PlGF null PLe were significantly smaller (p < 0.05) than the control PL at gd10 and -12 (Fig. 4B) and had not assumed a fully mature shape. Placentae from the null mice were pointed and appeared to retain the shape of the preceding ectoplacental cone stage of development. Cytokeratin reactivity was demonstrated in the PL (Fig. 4C) of both PlGF knockout and wild-type (WT) mice. At gd12, some cytokeratin-reactive migrating trophoblasts were present in DB in both PlGF null and their WT control mice.

uNK cells were numerous in PlGF null mice, found in appropriate microdomains, and declined after midgestation (Fig. 5, A and B). uNK cells were significantly more abundant within the MLAp of PlGF null mice than in controls. Many of these cells had scant cytoplasm (an unusual finding) and were smaller, less granular (i.e., appeared more IM) than in the congenic controls. The remaining uNK cells found in the MLAp were extremely large. uNK cell numbers in DB resembled controls although there was more heterogeneity in cell size.

The most unusual finding in PlGF null implantation sites was a striking elevation in the frequency of binucleated uNK cells (Fig. 6A). Significantly higher numbers of nonmature (i.e., low numbers of cytoplasmic granules), nonsenescent (intact nuclear morphology and absence of cytoplasmic vacuolation) binucleated uNK cells were quantified in MLAp and DB of PlGF null mice between gd8 to -12 than in controls (Fig. 6B). The percentage of uNK cells that were binucleated at gd8 in MLAp and DB of PlGF null mice was 30 and 40% (controls 5 and 8%), respectively. At gd10 the percentages were 15 and 10% (controls 3 and 6%), and at gd12 binucleated cells were 30 and 15% (controls 8 and 5%) of the uNK cells in MLAp and DB, respectively.

Normal murine uNK cells promote spiral artery remodeling (Fig. 5A). The effect of PlGF deletion on this uNK cell function was assessed by measurement of spiral artery to lumen area ratios (Fig. 6C). At gd10, the mean vessel area to lumen area ratios in PlGF null mice was significantly higher (less modification) than in the congenic controls (p < 0.05). No significant differences were found at gd12, suggesting that delayed rather than impaired uNK cell function is associated with the anomalies observed in IM uNK cells of this strain. Vessel structure in the DB of PlGF null mice differed from...
controls at all times studied. The smooth muscle coat around the spiral arteries was thicker than in control implantation sites.

**Discussion**

The goal of these studies was to characterize PlGF in murine implantation sites and to evaluate whether the PlGF null mouse displayed features that would make it valuable for studies of the pathogenesis of pre-eclampsia. In humans, low levels of PlGF have been associated with pre-eclampsia (25–30), and endometrial PlGF is reported to be an exclusive product of uNK cells in the late menstrual cycle. PlGF is also found in uNK cells from early, elective pregnancy terminations (5).

PlGF has three spliced forms, PlGF-1, PlGF-2, and PlGF-3 (31, 32). In the rhesus monkey, where three early stages of pregnancy (lacunar, early villous, and villous PL stages) were studied for PlGF expression by immunohistology (33), weak, transient stromal expression was found on vascular smooth muscle cells and glandular epithelium. Stronger staining appeared in decidual cells as pregnancy progressed, but these cells were not further characterized by lineage or morphology. Because mice have only a single

**FIGURE 6.** A, Binucleated uNK cells in PlGF null mice at gd10. Unusually high numbers of PAS-positive binucleated uNK cells (a) was a striking feature in early to midgestation implantation sites of PlGF null mice. Image b (binucleated uNK cell) represents a DBA lectin-stained uNK cell at high magnification (×1,000) with two distinct nuclei. Images c and d represent uNK cells stained with PAS and DBA, respectively, in PlGF WT controls. DBA-reactive uNK cell from PlGF WT control is shown in image e at high magnification. (Magnification, a and e, ×400; magnification, b and d, ×1,000.) B, Enumeration of bi- or multinucleate uNK cells in MLAp and DB in PlGF WT and PlGF null mice at gd8, -10, and -12. Significantly more binucleated uNK cells were found in PlGF null mice compared with their congenic controls (*, p < 0.05; **, p < 0.001). C, Comparison of ratios for spiral arterial mean vessel area to mean lumen area ± SD for PlGF WT and PlGF null mice at gd10 and -12. At gd10, the ratio was significantly higher for PlGF null mice than WT. No significant difference was present at gd12.
PIGF (gene 3), full analysis of PIGF expression in a detailed gestational time course is possible.

As anticipated from the reports of others (34), PIGF (Fig. 2) was abundantly expressed in the murine PL including trophoblast giant cells. Endometrial transcription of PIGF also occurred throughout gestation (Fig. 1).

Gestationally induced endometrial (decidual) expression occurred at times inconsistent with a conclusion that uNK cells were the exclusive source for endometrial PIGF (i.e., in both early (gd6) and late gestation (gd16–18)). The study of aliphoid mice also implicated nonlymphoid maternal sources during the first half of gestation. The elevation in PIGF transcription in aliphoid mice during the second half of gestation could be due to migrating trophoblast cells of fetal origin. There is considerable variation in the degree of fetal cell contamination of dissected mouse decidua (35), and study of a larger number of gd16–18 animals would be needed to confirm the apparent drop in PIGF transcription in gd16 aliphoid mice. In normal immunocompetent B6 mice, immunoreactive PIGF was found throughout the implantation site. Immunoreactive PIGF is associated with uNK cells and with smooth muscle cells of large endometrial arteries and of the myometrial-decidual junction. These data support a previously documented role for PIGF in smooth muscle cells (36) and may explain the unusual vascular morphology observed in PIGF null mouse implantation sites.

The major histological feature found in implantation sites from PIGF null mice was the unusual elevation of binucleate uNK cells in both the MLAp and DB early in pregnancy (gd8). In normal mice, binucleate uNK cells usually represent <4% of the uNK cell population in midgestation DB and they are senescent cells (20, 37). By term, binucleate cells are ~10% of normal uNK cells. As shown in Fig. 6A, binucleate uNK cells in PIGF null mice are not morphologically senescent, and they appear to retain their functional capacity to migrate to spiral arteries and into DB (Fig. 6B). Whether these binucleate cells retain functions other than migration and whether any of their functions are at levels equivalent to mononucleate uNK cells cannot be evaluated from this histological study. The uNK cell function of spiral arterial modification was observed in PIGF null implantation sites but with slightly delayed kinetics. This process could result from exclusive actions of mononucleate uNK cells, which are reduced from normal numbers in these implantation sites by the skewed ratio toward binucleate cells or to the combined actions of mononucleate and binucleate uNK cells with modest functional impairment of the binucleate cells.

Functions attributed to PIGF are the support of growth, differentiation, and migration of endothelial and trophoblast cells (38). Our data suggest that terminal stages in the proliferation and cytokinesis of uNK cells during their lineage maturation also require PIGF, whereas nuclear division does not. PIGF does not appear to be required for commitment to uNK cell maturation or for uNK cell migration. The mechanism for PIGF-mediated effects during uNK cell maturation is likely cell intrinsic because studies of others addressing VEGFR1 did not demonstrate the receptor on murine uNK cells (39). Because only 30% of the gd8 uNK cells of PIGF null mice were binucleate, it is also possible that study of these mice has identified two subsets of uNK cells, perhaps those with and without angiogenic potential or possibly uNK cells of thymic and nonthymic origins (40, 41).

uNK cell division occurs predominantly in the MLAp (20), and the great enlargement of this tissue may reflect its content of very large cells because the absolute numbers of uNK cells, whereas elevated above the controls, would not be adequate to account for the gain in absolute area and in proportion to the MLAp within PIGF null implantation sites. A mild aberration in placental shape and reduced placential growth were also seen in implantation sites from PIGF mice. These outcomes may reflect a separate role for PIGF in trophoblast growth and differentiation. Alternatively, they could arise from a mechanism similar to that more readily recognized in uNK cells in which differentiation of trophoblast cells is being skewed away from proliferative cells (spontiotrophoblast) toward endo-replicating trophoblast giant cells. We expected that the complete absence of PIGF would significantly impact mouse implantation sites and block spiral arterial modification. This effect was not observed. Small PL were found but spiral arterial modification was only slightly delayed in PIGF null mice, not prevented as in uNK cell-deficient mice (42). Whether the pathology seen in implantation sites of PIGF null mice could be reversed by VEGF rather than PIGF treatment is a question that, when answered, may shed light on whether VEGF therapy of women at risk for pre-eclampsia will be successful.

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**Disclosures**

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