Placental Growth Factor

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Placental growth factor: a promising diagnostic biomarker for tubal ectopic pregnancy

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Abstract

Context—Tubal ectopic pregnancy is common but accurate diagnosis is difficult and costly. There is currently no serum test to differentiate tubal from intrauterine implantation and an effective biomarker of ectopic pregnancy would be a major clinical advance.

Objective—A key feature of successful intrauterine implantation is the establishment of a supportive vascular network and this has been associated with the activity of placental growth factor (PIGF). We hypothesized that the local decidual environment facilitates PIGF-dependent angiogenesis and that this pathway is not active in tubal implantation. We aimed to determine whether tubal implantation is manifest by an attenuation of the normal trophoblast PIGF-response and whether serum PIGF levels are different in ectopic compared to intrauterine pregnancy.

Design—Tissue and serum analysis.

Setting—A large UK teaching hospital.

Patients—Gestation-matched pregnant women undergoing surgical termination of pregnancy (viable intrauterine) (n=15), evacuation of uterus for embryonic missed miscarriage (non-viable intrauterine) (n=10) and surgery for tubal ectopic pregnancy (n=15).

Interventions—Trophoblast was examined by immunohistochemistry and quantitative RT-PCR, and serum was analyzed by ELISA.

Results—PIGF was localized to the cytotrophoblast cells. Expression of PIGF mRNA was reduced in trophoblast isolated from women with ectopic compared to intrauterine pregnancies ($P<0.05$). Serum PIGF was undetectable in women with tubal ectopic pregnancies and reduced, or undetectable, in miscarriage compared to viable intrauterine pregnancies ($P<0.01$).

Conclusions—Serum PIGF is a promising novel diagnostic biomarker for early pregnancy location and outcome, and large-scale studies are now required to determine its clinical utility.

Keywords

Angiogenesis; ectopic pregnancy; miscarriage

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Introduction

Implantation in the Fallopian tube occurs in 1-2% of pregnancies in the developed world and ectopic pregnancy remains a leading cause of pregnancy-related first trimester deaths (1). Tubal ectopic pregnancy, and its treatment, is also a considerable cause of maternal morbidity worldwide (1). It is associated with severe pelvic pain, acute hypovolemic shock and the need for blood transfusion as well as emergency surgical treatment with prolonged recovery. In the long-term, complications of treatment include ongoing pelvic pain, *de novo* adhesion formation, impairment of future fertility prospects and an increased chance of further ectopic pregnancy (2).

Current diagnosis of tubal ectopic pregnancy involves a combination of transvaginal ultrasound and measurement of serum human chorionic gonadotrophin (hCG) concentrations. However accurate and early ascertainment remains problematic and there are often delays in making the diagnosis and instigating treatment. Fewer than 50% of tubal ectopic pregnancies are diagnosed at the patient's initial presentation (3) and this rate has not improved in the last decade, despite advances in ultrasound imaging technology (4). The inevitable multiple visits and tests that are currently necessary are a sizeable expense for health services. As an example, we have calculated that, in the UK, the health services are spending an estimated $12.8 million each year, in direct costs alone, diagnosing and excluding ectopic pregnancy (4). There is an urgent unmet need for a biomarker for tubal ectopic pregnancy to allow quicker diagnosis and facilitate earlier and less invasive treatment (5,6).

During implantation the conceptus interacts with the local environment to facilitate its growth and development. One of the key features of successful implantation is the establishment of a supportive vascular network (7). Neo-vascularization depends on the induction of secreted pro-angiogenic growth factors. These are regulated by a combination of paracrine signaling molecules and hypoxia (8). Intrauterine implantation has been associated with the activity of placental growth factor (PIGF) (7-9). PIGF is a secreted pro-angiogenic protein with similarities to vascular endothelial growth factor (VEGF). It is has been identified at the implantation site and acts on neighboring cells, notably endothelial cells, through the receptors flt-1 (VEGFR1) and fkl-1/KDR (VEGFR2), to facilitate the development of a local blood supply (7,9).

The normal response to implantation is an augmented secretion of PIGF and this increase is reflected systemically, such that it can be measured in serum (10,11). The role of PIGF in the development of an intrauterine vascular network is highlighted by its relationship to pre-eclampsia, which is associated with reduced placental vascularization (12). Lower maternal serum levels of PIGF in early pregnancy correlate with a greater risk of developing pre-eclampsia in the third trimester (13).

In order to grow, and cause harm, an ectopic pregnancy needs to develop a supportive blood supply and angiogenesis also occurs at tubal implantation sites. It is not known whether PIGF is involved in increasing the vascularization of the Fallopian tube in ectopic implantation. However it is known that another pro-angiogenic growth factor, vascular endothelial growth factor (VEGF) is involved. VEGF and its receptors are up regulated at the tubal implantation site in ectopic pregnancy compared to elsewhere in the Fallopian tube (14). In addition, serum VEGF is increased in women with ectopic compared to intrauterine pregnancies (15).

We suspected that the angiogenic signature of a pregnancy is dependent on the trophoblast local environment and is different in intrauterine and tubal implantation. We therefore hypothesized that tubal implantation is manifest by an attenuation of the normal trophoblast

PIGF-response and that serum PIGF levels would be reduced in ectopic compared to intrauterine pregnancy. We aimed to assess the expression of PIGF in trophoblast from intrauterine and ectopic pregnancies and determine whether serum PIGF assessment could be useful as a diagnostic biomarker for ectopic pregnancy.

Material and Methods

Tissue collection

Ethical approval for this study was obtained from Lothian Research Ethics Committee (LREC 04/S1103/20) and informed written consent was obtained from all patients before sample collection. Serum samples were obtained from women (age 18-45 years) undergoing surgical termination of pregnancy (TOP, USS-confirmed viable intrauterine, n=15, group 1, mean gestation 57.8 days, mean hCG 96289.2u/L [range 31766-185815]), surgical management of embryonic missed miscarriage (USS-confirmed non-viable intrauterine, n=10, group 2, mean gestation 56.9 days, mean hCG 12929.3u/L [range 271-32526]) and surgical management of tubal pregnancy (n=15, group 3, mean gestation 57.6 days, mean hCG 3651.7u/L [range 203-12161]).

Pure trophoblast samples, uncontaminated by decidualized endometrium or Fallopian tube, were successfully obtained from group 1 (n=7), group 2 (n=4) and group 3 (n=4). None of the women undergoing surgical management of tubal ectopic pregnancy presented acutely with hemodynamic shock, and all required serial serum beta-HCG and ultrasound monitoring prior to diagnosis. Pregnancy tissue was obtained by suction curettage from groups 1 and 2 and by salpingectomy from group 3. The trophoblast was isolated from the decidualized endometrium and Fallopian tube macroscopically and (a) immersed in RNAlater™ (Ambion, Austin, TX) at 4°C overnight then flash frozen and stored at −70°C; and (b) fixed in 10% neutral buffered formalin overnight at 4°C, transferred to 70% ethanol, and wax embedded for subsequent hematoxylin and eosin staining and immunohistochemistry. An expert gynecological histopathologist, using standard morphological assessment, excluded the presence of contaminating decidualized endometrium and Fallopian tube in the samples of trophoblast.

Immunohistochemistry

Immunohistochemistry was performed using standard techniques. The goat polyclonal PIGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 50-fold in normal horse serum. Antibody binding was visualized using diamino benzidine (DAB) staining. Goat IgG, diluted to the same concentration as the PIGF antibody, was used as a negative control.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from the trophoblast as detailed in the manufacturers' protocol (Qiagen, Crawley, West Sussex, UK) that included a DNAse treatment step. The concentration and quality of the extracted RNA was assessed using a Bioanalyzer (Agilent Technologies Ltd., Wokingham, Berkshire, UK) and 200ng was reverse transcribed into cDNA using random hexamers (Applied Biosystems, Foster City, CA). Taqman quantitative RT-PCR was then used to measure gene expression levels. Primers and probes for PIGF were designed using the Universal Probe Library software (Roche, Welwyn Garden City, Hertfordshire, UK) and validated prior to experiments. Taqman RT-PCR reactions were performed under standard conditions and all analyses were performed in triplicate. Relative gene expression comparisons were made to an appropriate human control cDNA that was included in all reactions. Using the 2-ΔΔCt method, mRNA expression results were normalized against 18S and expressed as relative PIGF expression compared to the internal calibration sample.

ELISA

PIGF levels were measured in patient serum using a commercially available ELISA (R&D Systems, Abingdon, Oxfordshire, UK) following the manufacturers' recommended method. According to the manufacturers, the minimal detectable dose is typically <7pg/mL. Our standardization confirmed that we could reliably detect PIGF concentrations >1pg/mL.

Statistical analysis

After confirmation of normality of distribution the samples were analyzed using ANOVA with a value of $P<0.05$ being statistically significant. Where significant differences existed pair-wise comparison was conducted using the Bonferroni method. All statistical analyses were conducted using commercially available Prism software (GraphPad Software Inc., San Diego, CA).

Results

PIGF protein is localized to cytotrophoblast cells

Using immunohistochemistry, PIGF protein was localized to cytotrophoblast cells (Figure 1A). No staining was observed in the negative control sections. While PIGF expression was obvious in cytotrophoblast cells from intrauterine pregnancies, attempts to localize PIGF in tubal ectopic pregnancies was unsuccessful.

PIGF mRNA expression is lower in trophoblast from women with tubal ectopic compared to intrauterine pregnancies

We used quantitative RT-PCR to assess whether there was any difference in PIGF mRNA expression in cytoptrophoblast cells from tubal ectopic compared to intrauterine pregnancies. PIGF mRNA expression was significantly lower in trophoblast cells isolated from women with tubal ectopic pregnancies (n=4) compared to women with non-viable (miscarriage, n=4) and viable (STOP, n=7) intrauterine pregnancies ($P<0.05$) (Figure 1B). These numbers were smaller than anticipated due to the fact that it was not possible to obtain trophoblast cells from all of the patients recruited into the study. This resulted from the technical difficulties involved in identifying trophoblast cells and the fact that some samples had to be excluded due to decidual contamination. However, serum was obtained from all patients (see below).

Serum PIGF levels are lower in women with tubal ectopic and non-viable intrauterine pregnancies

Using ELISA, serum PIGF concentrations were shown to be significantly lower in women with tubal ectopic pregnancies (n=15) (too low to be determined) and non-viable intrauterine pregnancies (n=10) compared with viable intrauterine pregnancies (n=15) ($P<0.01$) (Figure 1C). Despite similar hCG concentrations, the PIGF assay was sufficiently sensitive to differentiate a tubal ectopic pregnancy from a non-viable intrauterine pregnancy in three of the cases (Figure 1D).

Discussion

This study reports on novel data supporting the concept that there are differences in the angiogenic signature of the invading trophoblast in tubal ectopic and intrauterine pregnancies of similar gestations. We show that the angiogenic factor PIGF is reduced in trophoblast cells from tubal ectopic pregnancies when compared to that of intrauterine pregnancies. In addition, we show that this difference can be assessed systemically and that serum PIGF levels are reduced in tubal ectopic pregnancy and miscarriage. This highlights

that the differential secretion of angiogenic molecules associated with embryo implantation could be used to define a diagnostic biomarker strategy.

Normally at the maternal-fetal interface, expression of PIGF mRNA is high in trophoblast cells but quantitative studies have confirmed that preeclamptic trophoblast cells also expresses less PIGF mRNA and protein than a normal pregnancy (7.16). The reduction in transcription of PIGF seen in preeclampsia has been attributed to low oxygen tensions that are known to decrease PIGF expression (16). Thus, a potential explanation for our finding is that implantation in the unfavorable Fallopian tube is associated with increased tissue hypoxia. However, the precise intrinsic molecular and cellular mechanisms that function to regulate PIGF gene expression in human trophoblast cells are not known.

It is perhaps not surprising that serum PIGF levels are attenuated with tubal implantation. Levels of PIGF are also abnormal in women with preeclampsia and the link between abnormalities in trophoblast invasion and the generalized maternal endothelial dysfunction seen in preeclampsia is thought to be due to systemic release of placental angiogenic factors (13,17). However, the current data relating to PIGF in preeclampsia are conflicting: some authors showing a decrease and others no significant differences (17-20). This can be explained in part by differences in methodology, experimental design and population heterogeneity among the studies.

It is interesting to note that there is a discrepancy between local expression of PIGF mRNA in trophoblast from women with miscarriage and that detected systemically in the serum from this group of women. It is possible that mRNA expression does not associate with levels of secreted protein. Even though we detected PIGF protein localization in trophoblast from women with miscarriage, we did not have enough pure trophoblast tissue available to determine if there was a relationship between tissue protein concentrations by Western blot analysis related to levels measured in the serum.

Although the pattern of serum PIGF concentrations in the different groups was reminiscent of hCG concentrations, it is noteworthy that the PIGF assay was sufficiently sensitive to differentiate an ectopic pregnancy from a miscarriage with a similar hCG concentration in three of the cases included in our study (see Figure 1D). Thus, the role of serum PIGF either in isolation, or in combination with other biomarkers, in the diagnosis of tubal ectopic pregnancy should be investigated in large-scale prospective studies in a carefully selected pregnancy population. In addition, the role and regulation of PIGF in embryo implantation should be addressed further to dissect the role of the trophoblast cell and its environment in this process. We believe that further diagnostic biomarkers of ectopic pregnancy could be identified by understanding the pathological angiogenesis seen in response to tubal implantation.

Précis

There is differential expression of placental growth factor in trophoblast from intrauterine or tubal sites and its measurement is a novel biomarker of pregnancy outcome.

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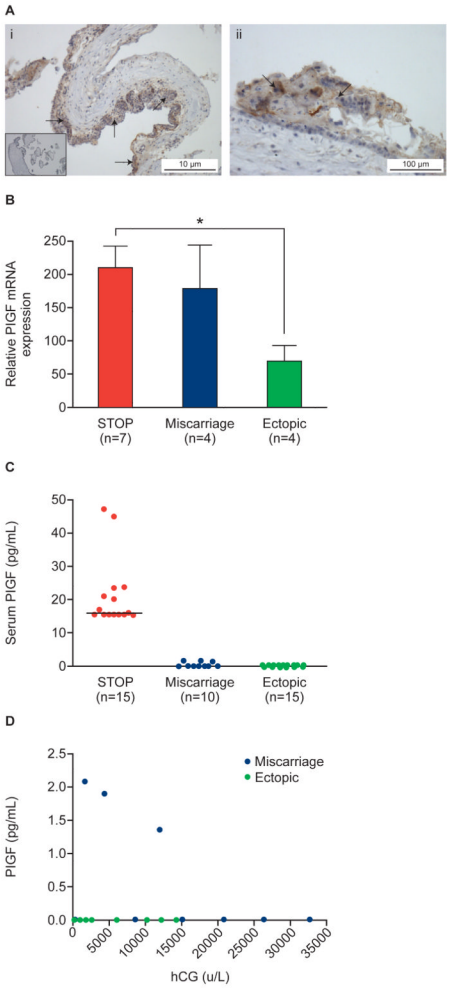

Figure 1.

(A) (i) and (ii) Immunohistochemistry demonstrated localization of PIGF protein to cytotrophoblast cells (see arrows) isolated from a woman with a viable intrauterine pregnancy (surgical termination of pregnancy, STOP). Negative control (insert). A similar pattern of expression was seen in cytotrophoblast cells isolated from non-viable pregnancies (miscarriage) but attempts to localize PIGF expression in tubal ectopic pregnancies was unsuccessful (data not shown). (B) Quantitative RT-PCR showed that PIGF mRNA expression was significantly lower in trophoblast isolated from women with tubal ectopic (n=4) compared to women with non-viable (miscarriage, n=4) and viable (STOP, n=7) intrauterine pregnancies ($P<0.05$). (C) ELISA showed that serum PIGF concentrations were significantly lower in women with tubal ectopic (n=15) (too low to be determined) and non-viable intrauterine pregnancies (miscarriage, n=10) compared with viable intrauterine pregnancies (STOP, n=15) ($P<0.01$). (D) Scatter plot of PIGF against hCG concentrations from women with tubal ectopic (n=15) and non-viable intrauterine pregnancies (miscarriage, n=10). The PIGF assay was sufficiently sensitive to differentiate a tubal ectopic pregnancy from a non-viable intrauterine pregnancy in three of the cases.