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ACCRETA COMPLICATING COMPLETE PLACENTA PREVIA IS CHARACTERIZED BY REDUCED SYSTEMIC LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND EPITHELIAL-TO-MESENCHYMAL TRANSITION OF THE INVASIVE TROPHOBLAST

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Abstract

OBJECTIVE—To characterize serum angiogenic factor profile of women with complete placenta previa and determine if invasive trophoblast differentiation characteristic of *accreta*, *incretta* or *percreta* shares features of epithelial-mesenchymal-transition (EMT).

STUDY DESIGN—We analyzed gestational age matched serum samples from 90 pregnant women with either complete placenta previa (n=45) or uncomplicated pregnancies (n=45). Vascular-endothelial-growth-factor (VEGF), placental-growth-factor (PlGF) and soluble fms-like-tyrosine-kinase-1 (sFlt-1) were immunoassayed. VEGF and phosphotyrosine (P-Tyr) immunoreactivity was surveyed in histological specimens relative to expression of vimentin and cytokeratin-7.

RESULTS—Women with previa and invasive placentation [*accreta* (n=5); *incretta* (n=6); *percreta* (n=2)] had lower systemic VEGF (invasive previa: median [IQR]: 0.8[0.02–3.4] vs. control: 6.5[2.7–10.5] pg/mL, *P*=0.02). VEGF and P-Tyr immunostaining predominated in the invasive extravillous trophoblasts (EVT) which co-expressed vimentin and cytokeratin-7, a EMT feature and tumor-like cell phenotype.

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CONTRIBUTIONS TO AUTHORSHIP

MJW, EF, IAB and CSB formulated the hypothesis, designed the study, collected, analyzed, and interpreted the data and drafted the manuscript. MJW and CSB recruited patients and together with ST, MOB, EW, KC, AKS and CL collected biological specimens and followed the patients prospectively to the point of delivery. MJW and GZ conducted the ELISA assays and the immunohistochemistry experiments. IAB, MJW, CSB and CMS evaluated and performed the histological analysis of our specimens. All the co-authors participated with aspects of study design, critical interpretation of the data, contributed to writing of the paper and have reviewed and approved the final version.

CONCLUSIONS—Lower systemic free VEGF and a switch of the interstitial EVT to a metastable cell phenotype characterize placenta previa with excessive myometrial invasion.

Keywords

angiogenesis; invasion; myometrium; trophoblast; VEGF

INTRODUCTION

When the placenta is inserted into the lower uterine segment and covers partially or entirely the cervix, a diagnosis of placenta previa is established.¹ Placenta previa complicates approximately 0.3 to 0.8% of pregnancies and represents one of the most frequent causes of painless bleeding during the second half of gestation.^{1,2} Risk factors for placenta previa include a history of prior Cesarean delivery, uterine surgery, termination of pregnancy, smoking, advanced maternal age, multiparity, drug abuse and multiple gestations.¹ Although the risk factors for placenta previa are well defined, much less is known about its etiology. Furthermore, the underlying cause of the excessive myometrial penetration which characterizes placenta *accreta*, *increta* or *percreta* in the presence of attendant placenta previa remains largely unknown.¹ In normal pregnancy, development of the vascular system is essential for providing the embryo and fetus with an adequate supply of nutrients and oxygen. A variety of regulatory molecules play functional roles in controlling the process of trophoblast invasion and development during implantation and placentation.^{3,4,5} These include vasoactive and cell surface proteins, proteases, cytokines, chemokines and growth factors.⁵

Data derived from animal and human studies demonstrate that the signaling components of the vascular endothelial growth factor (VEGF) family including VEGF-A, Placental Growth Factor (PlGF) and their receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) are present in the decidua and play crucial roles in the normal development of the feto-placental vascular network.^{6,7,8,9,10} Kinase-insert Domain Receptor (KDR) is recognized as the central VEGF receptor in angiogenesis, while Flt-1 plays a supporting role.¹⁰ Engagement of Flt-1 and Flk-1/KDR receptors, by both VEGF and PlGF leads to various downstream activations which are responsible for endothelial cell proliferation, migration and survival.¹¹ Presence of phosphotyrosine (P-Tyr) was proposed as an immunohistochemical marker of VEGF-mediated receptor activation. Importantly, an alternatively spliced soluble form of Flt-1 (sFlt-1) is a modulator of the VEGF and PlGF activity.^{7,12} There is general consensus that the process of normal trophoblast invasion and placentation requires fine coordination among VEGF, PlGF and sFlt-1 and that oxygen tension has a key role in regulating their expression.^{11,13}

Excessive trophoblast invasion is a frequent occurrence in pregnancies complicated by complete placenta previa (CPP).¹ While the pathogenesis of this phenomenon is still unknown, one possible explanation may be related to differences in oxygen tension in the lower uterine segment or in the uterine scar.¹⁴ Alternatively, the aforementioned risk factors for abnormal placentation may be responsible for the decreased proportion of the normally remodeled blood vessels in women with placenta *accreta*.¹⁵ Therefore, consistent with the concept of trophotropism, the unchecked trophoblast invasion of the myometrium may be the result of compensatory mechanisms aimed to meet the metabolic and oxygen requirements of the developing fetus.^{15,16,17} Alternatively, an abnormal trophoblast differentiation process with acquisition of an excessively invasive phenotype is possible. This paradigm is supported by the shared molecular pathways between normal trophoblasts and cancer cells.¹⁸ The observation that a transcription factor characteristic of metastatic epithelial cancers is up-regulated in placenta previa *accreta*, supports this view.¹⁹ Yet, the

specific signaling pathways lost or acquired during the process of abnormal trophoblast differentiation remain largely elusive.

Epithelial-to-Mesenchymal Transition (EMT) is a process characterizing invasive tumor cells which lose the epithelial phenotype to acquire mesenchymal features.²⁰ As a result of EMT, cells change their shape, lose their polarity and the cell-cell contact to manifest a motile phenotype. A characteristic feature of EMT is that cytoskeletal intermediate filaments of epithelial cells, initiate the expression of vimentin.²⁰ This is a significant observation given that under normal conditions the cytoskeletal intermediate filaments of epithelial cells express keratin only. Due to vimentin's direct role in mediating cell motility and thus tumors' invasive and metastatic potential, co-expression of vimentin with cytokeratin is considered a hallmark of EMT and of a metastable phenotype.^{20,21}

The defective development of the villous vascular tree as well as the abnormal secretion of placental VEGF, PlGF and sFlt-1 are now considered to be implicated in the development of several pathologic states of pregnancy such as preeclampsia and hypoxia induced intrauterine fetal growth restriction (IUGR).^{22,23,24,25} This body of knowledge and the evidence that hypoxia regulates the transcription of VEGF, PlGF and sFlt-1¹⁹ led us to test the hypothesis that women with CPP have an abnormal serum angiogenic profile which is further altered in the setting of the life-threatening placental invasive myometrial processes accompanying placenta *accreta*, *increta* or *percreta*. As a corollary to our hypothesis we proposed that the trophoblast in accreta may share pathological features of epithelial-to-mesenchymal (EMT) transition to explain its abnormal invasive phenotype.²⁶

MATERIALS AND METHODS

Study design and patient population

In a case-control study design we analyzed maternal blood serum samples from 90 women enrolled prospectively at Yale-New-Haven Hospital between May 2005 and January 2010. Our study group consisted of 45 consecutive singleton patients [gestational age (GA), median, interquartile range [IQR]: 31 [28–34] weeks], who were admitted with a diagnosis of vaginal bleeding due to CPP. Blood specimens from 45 healthy women (GA: 31 [28–33] weeks), pregnant with singletons were matched for GA and served as controls. All women provided signed informed consent under protocols approved by the Human Investigation Committee of Yale University. A detailed description of the exclusion criteria and clinical management of the patients following admission is available in the online only Data Supplement.

Ultrasonographic evaluation and diagnosis of the placenta previa

The prenatal diagnosis of CPP was based on gray-scale and Doppler sonography. A detailed Method of ultrasonographic diagnosis of CPP is available in the online Data Supplement.

Blood collection, storage and immunoassay procedures

All maternal blood samples were retrieved by venipuncture. For the study group women, blood collection was performed at the time of admission prior to steroid therapy or blood transfusion. Blood was allowed to clot. Serum samples were spun at 3000 g at 4°C for 20 min., the supernatant aliquoted and immediately stored at –80°C until VEGF, PlGF and sFlt-1 levels were measured using specific immunoassays.

ELISA assays for human unbound VEGF, PlGF and sFlt-1 were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN) by investigators unaware of

the diagnosis. A detailed description of the immunoassay procedures is available in Data Supplement.

Histology and immunohistochemistry

Following delivery, placental tissues of the placenta previa cohort were examined by a perinatal pathologist. In those patients requiring hysterectomy (n=14), 13 pathology reports documented the presence and degree of placental invasion (*accrete* n=5; *increta* n=6; *percreta* n=2). An additional woman with CPP but no accreta developed post-partum uterine atony that required hysterectomy for haemostatic purpose. For all CPP cases complicated by abnormal invasion of the myometrium we evaluated a full thickness biopsy of the placenta with the underlying uterine wall as well as a biopsy of the uterine wall, opposite to the placental insertion site. For comparison, placental bed biopsies (decidua basalis) were retrieved from 5 women who had an uncomplicated gestation and an elective Cesarean delivery (CD) at GA: 37 [37–38] weeks. These tissues were used as the best possible tissue controls given that performance of a placental insertion site biopsy in women with healthy pregnancies is neither possible nor ethically acceptable at 35 [34–36] weeks which is the median GA at delivery for the CPP group. Additional descriptions of clinical characteristics, details on antibodies and immunostaining techniques are presented in Data Supplement.

Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnov test. Comparisons among groups were performed using Student's paired t-tests, Wilcoxon's Signed Rank Test, McNemar's and Mann-Whitney tests. Data are reported as median and IQR or average $\pm$ standard error mean (SEM), as appropriate. For immunoassay results, logarithmic transformations were applied before statistical comparisons were performed. Relationships between variables (correlations) were explored using Spearman's Rank order correlations. We used SigmaStat version 2.03 (SPSS, Inc., Chicago, IL) and MedCalc (Broekstraat, Belgium) software. A $P < 0.05$ was considered to indicate statistical significance. Sample size calculations were based on our prior data on serum levels of angiogenic factor concentration in women with preeclampsia. 24 It was estimated that 10 patients in each group would be necessary to detect differences equal to the standard deviation in serum concentrations of angiogenic factors in women with placenta previa compared to controls (80% power, $\alpha=0.05$, paired t-test). Additional subjects were enrolled to facilitate a finer comparison between groups required to account for confounders and possible effect modifiers such as maternal age, parity and/or race.

RESULTS

Clinical characteristics of women

We present the demographic, clinical and pregnancy outcome characteristics of our cohort in Table 1. Women with CPP were significantly older, were of significantly higher gravidity and parity and had a significantly higher number of prior CDs when compared to controls. The vast majority of the women with CPP experienced at least one episode of vaginal bleeding during pregnancy [episodes of bleeding: 1 [1–2]]. The fetuses of both CPP and control women were of appropriate growth for GA. Women with CPP more often received antenatal steroids, blood transfusions and were delivered at an earlier GA compared to controls. As expected, the frequency of CD, hysterectomy and histologically confirmed placenta *accreta*, *increta* or *percreta* was higher in the group of women with CPP. In multivariate analysis, the only variables significantly associated with the occurrence of CPP were increased parity ($P=0.027$) and advanced maternal age ($P=0.002$), both known as risk factors for placenta previa.

Circulating levels of sFlt-1, PlGF and VEGF in women with CPP

In Fig. 1 we present the maternal serum concentrations of sFlt-1, PlGF and VEGF in women with CPP. The sFlt-1 and PlGF were detected in all maternal serum samples. Conversely, VEGF levels were below the limit of detection in 16 cases of CPP and in 10 controls. Overall, women with placenta previa had similar maternal serum levels of sFlt-1 (CPP: 1,363 [953–2,047] vs. control: 1,414 [1,196–1,865] pg/mL, $P=0.550$, Fig. 1A) and PlGF (CPP: 421 [285–824] vs. control: 492 [300–815] pg/mL, $P=0.710$, Fig. 1B). When all CPP cases were analyzed together, there were no statistical differences in free VEGF levels compared to controls (CPP: 1.4 [0–6.1] vs. control: 3.0 [0.2–9.5] pg/mL, $P=0.08$, Fig. 1C). These results maintained for all 3 analytes even after the cases of placenta *accreta*, *incretta* or *percreta* were excluded from the analysis (sFlt-1: $P=0.324$; PlGF: $P=0.138$; VEGF: $P=0.489$).

Given that the ratio between pro- and anti-angiogenic factors may be more relevant than their absolute levels we also analyzed the relative ratios among the 3 angiogenic factors in our cohort. There were no significant differences in either sFlt-1/PlGF ($P=0.802$), PlGF/sFlt-1 ($P=0.809$) or VEGF/sFlt-1 ($P=0.221$) ratios between women with CPP and controls. Correcting for GA did not change the results.

Of the three analytes, maternal sFlt-1 levels correlated directly with GA in both previa ($R=0.438$, $P<0.001$) and control cases ($R=0.404$, $P=0.007$). When correcting for GA at sample collection there was a significant but inverse correlation between free serum VEGF and sFlt-1 ($R=-0.507$, $P<0.001$), consistent with the functionally opposing roles of these proteins.

Circulating levels of sFlt-1, PlGF and VEGF in women with placenta previa and aberrant invasion of the myometrium

We further restricted our analysis to patients with histologically confirmed *accreta*, *incretta* or *percreta*. We found no difference in the maternal serum levels of sFlt-1 (CPP with *accreta*, *incretta* or *percreta*: 1,343 [792–2,325] vs. control: 1,301 [821–1,661] pg/mL, $P=0.758$, Fig. 2A) or PlGF (CPP with *accreta*, *incretta* or *percreta*: 823 [292–1,029] vs. control: 370 [249–572] pg/mL, $P=0.137$, Fig. 2B). However, we found that women with aberrant invasion of the myometrium had significantly lower serum VEGF levels compared to their matched control group (CPP with *accreta*, *incretta* or *percreta*: 0.8 [0.02–3.4] vs. control: 6.5 [2.7–10.5] pg/mL, $P=0.02$, Fig. 2C). The ratios of the 3 angiogenic factors were not affected in the above clinical scenario ($P>0.05$ for all).

Histological and immunohistochemical examination of placental insertion sites in women with previa and excessive invasion

Given the observed decrease in free VEGF in the peripheral blood of women with placenta *accreta*, *incretta* or *percreta*, we sought to investigate the pattern of VEGF expression in the hysterectomy specimens of these cases. For comparison we used a full thickness uterine wall biopsy retrieved from a location opposite to the placental insertion site (CPP) and placental bed biopsies of women with adequate trophoblast invasion ($n=5$). For all immunohistochemical studies presented below, specificity of staining was confirmed by slides where the first antibody was omitted (negative controls). In Fig. 3 we illustrate our histological and immunohistochemical findings in a representative case of placenta *percreta*. Serial sections through the placenta previa implantation site (Fig. 3A–D) showed abundant extracellular matrix and collagen stained blue with Masson trichrome (Fig. 3A). These findings were consistent with an insertion site located in close proximity to the uterine cervix. Characteristic of all *accreta* biopsies was the direct apposition of placental villi to the myometrium as exemplified in Fig. 3A.

Numerous EVT's were seen scattered deep below the basal plate as judged by their bright red cytoplasm, vesicular nucleus (Fig. 3A) and intense staining for cytokeratin-7 (Fig. 3B). In contrast, vimentin positive decidual cells were virtually absent at the implantation site of women with placenta previa complicated by accreta (Fig. 3C). Expression of vimentin was confined predominantly to fibroblasts and interestingly to the interstitial EVT's (iEVT), Fig. 3C, left insert). In contrast to villous syncytiotrophoblast cells (Fig. 3C, right insert) which were vimentin negative, iEVT's in placenta *accreta* cases appeared to co-express vimentin along with cytokeratin-7. Relative to these cellular landmarks, the most conspicuous VEGF immunoreactivity was found in the iEVT's and endothelial cells lining the blood vessels (Fig. 3D). In addition, we observed an abundant extracellular VEGF staining consistent with the increased affinity of VEGF for components of the extracellular matrix (i.e. proteoglycans).²⁷ This finding was also apparent in the pattern of VEGF staining within the uterine wall opposite from the placental insertion site (Fig. 3F–I) and of the amniochorionic membrane that remained attached to decidua parietals in Cesarean hysterectomy specimens (Fig. 3I, lower insert). At this site, extracellular VEGF signal appears localized among the muscle fibers and within the lax connective tissue of the decidua. Intense VEGF staining was found in amnion epithelial cells, decidual cells, EVT's of the choriodecidua and fibroblasts resident cells within the myometrium or the decidua.

Our histological examination of the placental bed biopsies of pregnancies with physiologic invasion (Fig. 4A–D) demonstrated that vimentin positive decidual cells (Fig. 4C) displayed the most abundant VEGF immunoreactivity (Fig. 4D). VEGF staining was also found in the few scattered iEVT's as well as in endovascular EVT's (eEVT) within the wall of transformed spiral arteries (cytokeratin: Fig. 4B and VEGF: Fig. 4D, high magnification inserts). These findings were in contrast to the site of aberrant placentation and invasion of the myometrium where the chief cellular localization of VEGF was within the cytoplasm of iEVT's (Fig. 3D).

In Fig. 5 we illustrate additional representative patterns of VEGF immunoreactivity in *accreta*, *increta* or *percreta* cases. Consistent findings in *accreta* regions were the abundant presence of VEGF signal in multinucleated giant cells (MNGC) (Fig. 5A, insert 1), stagnant blood trapped in intra-placental lakes (Fig. 5A, insert 2), fibrinoid and areas of aberrant vascularization (Fig. 5A, insert 3). A known distinctive feature of invasive placentation is the relative paucity of MNGCs seen in late normal placentation.^{16,28} Our semiquantitative analysis confirmed this observation (% MNGC average $\pm$ SEM, *accreta*: $3\pm1\%$ vs. normal placental bed biopsies: $36\pm13\%$, $P=0.001$). In Fig. 5B we show a representative micrograph of VEGF immunostaining in endothelium and blood trapped in vascular lacunae (Fig. 5B) in a pattern evoking a functional state of VEGF stimulation. These findings were consistently present in cases with aberrant invasion but not placental biopsies retrieved from women with normal placentation (data not shown). The macroscopic aspect of placental lakes is shown in Fig. 5D. In Fig 5E presents micrographs of the same case stained for smooth muscle α -actin illustrating the immediate confrontation of the placenta and iEVT's with myometrial tissue.

Phosphorylation of specific tyrosine residues on the cytoplasmic tail of VEGF receptors and of other downstream targets is associated with activation of signaling pathways.¹¹ As it can be determined from Fig. 6, in placental bed biopsies from patients with normal invasion, the scant P-Tyr signal was mostly localized to blood vessel endothelium while iEVT's and MNGC were virtually P-Tyr negative (Fig. 6A). In contrast, as it could be determined from serial sections of a representative *accreta* case stained with P-Tyr (Fig. 6B), VEGF (Fig. 6C), Masson trichrome (Fig. 6D), cytokeratin (Fig. 6F) or vimentin (Fig. 6G), iEVT's were P-Tyr positive (Fig. 6B) and localized to areas with ample cellular and extracellular VEGF staining (Fig. 6C). This observation would be consistent with activation of VEGF downstream signaling in patients with excessive placental invasion.

COMMENT

Herein, we found that women with CPP have circulatory levels of sFlt-1, PlGF and VEGF similar to those expected for GA. That the process of excessive myometrial invasion in the setting of CPP is characterized by significantly lower maternal serum levels of VEGF was an unexpected finding.

Following apposition and attachment of the blastocyst to the uterine wall, the process of normal placentation requires a symbiotic interaction between fetal and maternal tissues. This process includes among others cytotrophoblast invasion of the inner third of the myometrium.²⁹ The invading trophoblasts breach and remodel the spiral arteries enmeshed in the matrix of decidualized cells and myometrial junctional zone. The regulation of their sophisticated behavior remains unknown, but trophoblast oxygen sensing seems to play a key role.^{13,14}

Local oxygen concentrations are thought to be critically involved in both EVT proliferation and VEGF expression.^{11,13,14} Hypoxia stimulates EVT proliferation and VEGF mRNA expression, while normoxia has an inhibitory activity. A common view is that the placenta implants and grows better in the highly vascularized fundus, anterior and posterior uterine wall.¹ We first reasoned that implantation in areas with less blood supply such as the lower uterine segment, cervix or uterine scar may lead to a suboptimal blood supply to the placenta. We thus proposed that in the setting of a CPP, the hypoxic placenta will release into the maternal circulation an array of putative anti-angiogenic factors such as sFlt-1. If true, this process should have mimicked, at least at the biochemical level one of the phenomena accompanying preeclampsia.^{22,24,30} Given that prior to our research no other studies evaluated the circulatory levels of angiogenic factors in women with CPP, we predicted that binding of VEGF and PlGF by the over-expressed sFlt-1 could lead to decreased circulatory levels of the former two factors. Another possible scenario could have been that the relatively low oxygen tension in the poorly vascularized lower uterine segment and/or uterine scar would result in altered levels of VEGF and PlGF. Evidence in support of this premise comes from previous reports which suggest that, *in vitro*: 1) hypoxia promotes angiogenesis and up-regulates VEGF;³¹ 2) hypoxia decreases PlGF expression in trophoblast³¹ but increases PlGF transcription in cultured fibroblasts.³² Our experimental findings, however, refuted all these hypotheses. Evaluation of the myometrial oxygenation level in women with CPP is not possible due to the emergency character of this obstetrical condition and ethical issues. However, the present data provides indirect evidence that the oxygen demands of the placenta and developing fetus of women with placenta previa are most frequently reached when trophoblast penetration of the lower uterine segment myometrium is appropriate. The observation that women with placenta previa and physiologic myometrial invasion have a normal serological profile of VEGF, PlGF and sFlt-1, is consistent with none of the fetuses in our cohort being growth restricted and with the literature refuting placenta previa as an independent risk factor for IUGR or preeclampsia.^{33,34}

In this study we did not have the ability to evaluate the maternal levels of VEGF in the first trimester which is an important time during the process of normal implantation and placentation. Nor did we have the ability to examine serially the maternal concentrations of angiogenic factors in a correlative fashion with the depth of trophoblast invasion. It is still possible for hypoxia to exercise a stimulatory effect on invasion at the previa site in the initial phase of implantation or until the oxygen demands of the placenta and fetus are reached. Once the critical oxygen threshold is attained, a negative regulation of VEGF gene expression should confer a protective mechanism against excessive myometrial invasion. However, in some placenta previa cases, the oxygen requirements and a down-regulation of

VEGF gene expression are achieved only after excessive penetration of the myometrium (*accreta*, *incretta*) or just after invasion of major pelvic vessels or visceral organs (*percreta*). Further studies remain to provide evidence in support of this model.

The observation that women with CPP and invasive placentation have lower or undetectable level of circulating free VEGF was provocative. Several explanations are possible. A plethora of regulatory molecules have been demonstrated to play functional roles in the process of normal decidualization, control of trophoblast adhesion, invasion and directionality of penetration.³⁵ During the process of implantation, cytotrophoblast cells detach from the anchoring sites and invade the maternal decidual stroma. These cells, collectively called EVT, differentiate primarily into interstitial and endovascular EVT. Owing to their plethora of proteases, iEVT degrade the extracellular matrix and promote cell migration. Decidua opposes the expansive nature of iEVTs through the activity of tissue inhibitors of the matrix metalloproteinases, and a variety of coagulation proteases.³⁶ One of the proposed mechanisms through which the iEVTs lose their invasive phenotype is through syncytial-type fusion into MNGCs.^{16,37} The secretion of VEGF by MNGCs is likely one of the signals initiating and coordinating vascularization in the decidua and placenta during implantation.³⁸ That the number of MNGCs is reduced in the basal decidua of women with placenta previa accreta, has been repeatedly confirmed by a previous study as well as the current study.¹⁶ Thus, a lower number of MNGCs may account for the reduced levels of VEGF in women with placenta previa complicated by *accreta*.

Our immunohistochemical findings that vimentin and cytokeratin are co-expressed *in-vivo* in the iEVTs penetrating beneath the basal plate in *accreta*, *incretta* and *percreta* cases are novel. These findings are suggestive of a striking similarity among accreta EVT and cancer cells. Previous *in vitro* data generated in a cultured trophoblast cell system are in support of our findings.³⁹ Vimentin is a filament protein, widely used as a marker for mesenchymal cells, such as fibroblasts and decidual cells.²⁶ Conversely, cytokeratin is a marker for epithelial cells including the cytotrophoblast. It is well recognized that acquisition of vimentin immunoreactivity by epithelial cells represents evidence for EMT, which is a process characteristic for cancer progression and metastasis.²⁶ The normal invasive migratory EVT do not express markers of EMT such as vimentin and retain epithelial characteristics such as cytokeratin.³⁵ One possibility is that maintenance of epithelial markers ensures that differentiation occurs in order to limit invasion of the underlying tissues. Our results suggest that the invasive EVT phenotype of placenta accreta may mimic a mesenchymal tumor-like, phenotype. Arguments in favor of the above interpretation are: **1)** during tumorigenesis epithelial cells switch to a metastable cell phenotype characterized by expression of dual mesenchymal and epithelial markers before they fully transform into mesenchymal cells; **2)** immortalized EVT cell lines engineered to maintain the invasive phenotype (HTR8/SVneo and Swan 71) gain expression of vimentin;⁴⁰ **3)** the trophoblasts of the invasive complete hydatiform mole express typical features of EMT.⁴¹

VEGF is a well-known orchestrator of the events leading to development of cancer. In addition, it has been shown that VEGF induces acquisition of EMT features, including expression of vimentin.⁴² Lash *et al.* observed that *in-vitro* VEGF, but not PlGF enhanced EVT in a dose dependent manner.⁴³ Interestingly, none of the tested angiogenic growth factors affected EVT proliferation.⁴³ These results are in agreement with our findings of increased vimentin immunostaining in accreta specimens. Vimentin expression is known to enhance cell motility. We saw abundant extracellular VEGF staining. In addition, the observed elevated P-Tyr in women with accreta hint that VEGF may play an active functional role. In point, our findings of EMT in the iEVT of *accreta* may well be a functional consequence of VEGF's stimulation of EVT motility at the local level.

A study by Tseng et al. has previously noted increased VEGF expression in a cohort of patients with placenta accreta.⁴⁴ However, it is significant to note that the study did not evaluate the maternal circulatory levels of VEGF. Furthermore, Tseng et al., included patients delivered in the second trimester as well as those with co-morbid conditions. In our study, all our study group patients were diagnosed with CPP, had no associated co-morbidities and delivered in the third trimester. Confounding factors such as preeclampsia were also excluded in our study.

The sonographic appearance of intraplacental sonolucent spaces (lakes or lacunae) is a frequent finding in cases of suspected placenta accreta.⁴⁵ The pathophysiology behind the occurrence of placental lacunae is unknown, but their presence suggests an aberrant vascular remodeling pattern.⁴⁶ We observed prominent VEGF staining in the vascular lacunae of the patients with previa accreta. A similar finding was not encountered in the placental biopsies of normally invaded basal plates.

In summary, we propose that the molecular mechanisms responsible for the excessive invasion of the myometrium may facilitate local accrual of VEGF and a significant decrease in the maternal circulatory levels of this angiogenic factor. VEGF may play a central role in the process of pathological programming of EVTs toward increased motility and invasiveness, two features that intersect with the metastatic phenotype of cancerous cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Angiogenic factors in women with complete placenta previa
Circulating levels of free sFlt-1 (A), PlGF (B) and VEGF (C) in women with CPP (n=45) versus gestational age matched controls (n=45). Red lines mark group medians.

Figure 2. Angiogenic factors in women with complete placenta previa and abnormal invasion of the myometrium

Circulating levels of free sFlt-1 (A), PlGF (B) and VEGF (C) in women with placenta previa and invasive placentation (*accreta*, *increta*, *percreta*, $n=13$) versus gestational age matched controls ($n=13$). Red lines mark group medians.

Figure 3. Representative histological sections demonstrating immunolocalization of cytokeratin, vimentin and vascular endothelial growth factor (VEGF) in a representative case of placenta previa with excessive invasion (percreta, 36^{5/7} weeks at delivery)

Serial sections through the placental insertion site (A–D) or through the opposite site myometrium (F–I) were stained with either Masson trichrome (A&F) or monoclonal antibodies against cytokeratin (B&G), vimentin (C&H) or VEGF (D&I) (Bar: 200µm). For each panel, the areas delimited by the squares noted as 1 (illustrating characteristics of interstitial extravillous trophoblasts) or 2 (illustrating characteristics of villous syncytiotrophoblasts) are further shown as higher magnification captions (Bar: 50µm) below each panel. For the opposite site, below each panel a caption of the amniochorion and choriodecidua is presented (Bar: 200µm). Panels E&J had primary antibody omitted and are marked as negative (Neg). Abbreviations: am, amnion; bv, blood vessel; ch-de, choriodecidua; de, decidua; myo, myometrium.

Figure 4. Cellular and extracellular VEGF immunoreactivity in a representative placental bed biopsy of a pregnancy with normal invasion (37^{0/7} weeks at delivery)

Serial sections through the placental insertion site (A–D) were stained with either Masson trichrome (A) or monoclonal antibodies against cytokeratin (B), vimentin (C) or VEGF (D) (Bar: 200µm). For each panel, the area delimited by the square is further shown as higher magnification caption below each panel (Bar: 50µm). Panel E: had primary antibody omitted and is marked as negative (Neg, Bar: 50µm). Abbreviations: *bv*, blood vessel; *dc*, decidual cells; *eEVT*, endovascular extravillous trophoblasts; *iEVT*, interstitial extravillous trophoblasts.

Figure 5. Focal patterns of VEGF and smooth muscle actin immunoreactivity in a case of placenta previa with percreta, (29^{5/7} weeks at delivery)
 Magnification for **A&B**: Bar: 200 μ m. The boxed areas in Panel **A** noted as 1, 2, or 3 are shown as higher magnification captions (Bar: 50 μ m) illustrative of VEGF staining in interstitial extravillous trophoblasts (iEVT) including few terminally differentiated multinucleated giant cells (MNGC), placental lakes, aberrant blood vessels (abv) and intra-placental fibrin, respectively. Panel **B** shows dilated vascular spaces with entrapped VEGF immunoreactivity. Panel **C** had primary antibody omitted and is marked as negative (Neg, Bar: 200 μ m.). Panel **D** shows the gross morphologic aspect of the sectioned placental biopsy illustrative of intra-placental lacunae which likely represent focal VEGF sinks and sites of functional VEGF hyperstimulation. Staining for smooth muscle actin (panel **E**, bar 200 μ m)

illustrates the histological intermingling of placental villi, iEVTs and myometrial fibers. The boxed areas in Panel **E** noted as 4 and 5 are shown to the right as higher magnification captions (Bar: 50µm). Abbreviations: *MNGC*, multinucleated giant cells; *iEVT*, interstitial extravillous trophoblasts; *SCT*, syncytiotrophoblast; *fib*, fibrinoid; myo, myometrium; plac, placenta; abv, aberrant vascularization.

Figure 6. Representative histological sections demonstrating immunolocalization of Phosphotyrosine (P-Tyr) Vascular Endothelial Growth Factor (VEGF), Cytokeratin and Vimentin in a normal placental bed biopsy and a case of placenta previa with excessive invasion Representative patterns of P-Tyr immunoreactivity in a normal placental bed biopsy (36^{2/7} weeks at delivery, marked as Control: **A&E**) versus a case of previa with excessive invasion (36^{5/7} weeks at delivery, marked as previa accreta: **B–D&F–H**). Serial sections through the placental insertion sites were stained with either monoclonal antibody against P-Tyr (**A&B**), VEGF (**C**), cytokeratin (**E&F**), vimentin (**G**), or stained histochemically with Masson trichrome (**D**) (Bar: 50µm). Panel **H** had primary antibody omitted and is marked as negative (Neg, Bar: 50µm). **Abbreviations:** *bv*, blood vessel; *dc*, decidual cells; *iEVT*, interstitial extravillous trophoblasts; *MNGC*, multinucleated giant cell.

Table 1

Demographic, clinical and outcome characteristics of enrolled women (n=90).

Variable	Placenta Previa n=45	Controls n=45	P value
<i>Characteristics at enrollment</i>			
Maternal age, years *	34 [31–38]	29 [23–33]	<0.001
Non-Caucasian race, n [%] †	16 [36]	14 [31]	0.823
Gravidity *	3 [2–4]	2 [1–3]	<0.001
Parity *	1 [1–2]	1 [0–1]	0.004
Gestational age, weeks *	31 [28–34]	31 [28–33]	0.917
Ultrasound diagnosis of previa, n [%] †	45 [100]	0 [0]	<0.001
Vaginal bleeding (≥1 episode), n [%] †	38 [84]	0 [0]	<0.001
Number of prior Cesarean sections †			0.003
0 (no prior Cesareans)	21	35	
1 prior Cesarean	14	9	
≥ 2 prior Cesareans	10	1	
<i>Outcome characteristics</i>			
IUGR, n [%] †	0 [0]	0 [0]	1.000
Antenatal steroids, n [%] †	33 [73]	0 [0]	<0.001
Blood transfusion, n [%] †	11 [24]	0 [0]	<0.001
Gestational age at delivery, weeks *	35 [34–36]	39 [39–40]	<0.001
Cesarean delivery, n [%] †	45 [100]	18 [40]	<0.001
Cesarean hysterectomy, n [%] †	14 [31]	0 [0]	<0.001
Histologically confirmed			
accreta, increta, or percreta, n [%] †	13 [29]	0 [0]	<0.001

* Data presented as median [interquartile range] and analyzed by Wilcoxon's Signed Rank test

† Data presented as n [%] and analyzed by McNemar's test.