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Angiogenic dysfunction in molar pregnancy

David KANTER, MD¹, Marshall D. LINDHEIMER, MD², Eileen WANG, MD¹, Romana G. BORROMEO, MD³, Elizabeth BOUSFIELD, MD⁴, S. Ananth KARUMANCHI, MD⁵, and Isaac E. STILLMAN, MD⁶

¹ Department of Obstetrics & Gynecology, The University of Chicago

² Department of Obstetrics & Gynecology and Medicine, The University of Chicago

³ Department of Obstetrics & Gynecology, Makati Medical Center

⁴ Department of Pathology, Makati Medical Center

⁵ Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School

⁶ Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School

Abstract

Objective—Molar pregnancy is associated with very early-onset preeclampsia. Since excessive circulating anti-angiogenic factors may play a pathogenic role in preeclampsia, we hypothesized that molar placentas produce more anti-angiogenic proteins than normal placentas.

Study design—This retrospective case-control study utilized a semiquantitative immunohistochemical technique to compare histological sections of molar placentas to normal controls. Tissue slides were treated with two antisera: one recognized the anti-angiogenic markers fms-like tyrosine kinase receptor 1 (Flt1) and its soluble form (sFlt1), while the other recognized vascular endothelial marker CD31. Stain intensity was graded from 1+ (strong focal staining) to 4+ (91%–100% staining).

Results—Molar placentas (n=19) showed significantly more staining than controls (n=16) for Flt/sFlt1 ($P < 0.0001$).

Conclusion—There was a significant difference in Flt1/sFlt1 immunostaining intensity when molar placentas were compared to controls. This supports a hypothesis that the phenotype of preeclampsia in molar pregnancy may result from trophoblasts overproducing at least one anti-angiogenic protein.

Correspondence: David Kanter, MD, The University of Chicago, Department of Obstetrics and Gynecology, 5841 S. Maryland Ave. MC2050, Chicago, Illinois 60637. Telephone: 773-834-2229 (office) Fax: 773-702-5159 (office).

Study cities: Chicago, Illinois; Boston, Massachusetts; and Makati City, Republic of the Philippines

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Keywords

Anti-angiogenic factors; fms-like tyrosine kinase receptor; hydatidiform mole; molar pregnancy; sFlt1

Introduction

Hydatidiform mole and preeclampsia are two disorders unique to pregnancy. Both have dysfunctional placentas that are integral to each disease process. Hydatidiform mole, or molar pregnancy, is a group of disorders of genomic imprinting characterized by varying degrees of trophoblastic proliferation and hydropic change of the chorionic villi.

The forms of molar pregnancy are termed complete and partial (1). Complete moles are characterized by a normal 46XX karyotype, with both sets of chromosomes typically of paternal origin. Villi are voluminous and show diffuse hydropic (edematous) changes, with cytologically atypical hyperplastic trophoblasts. No fetal tissue is present. In contrast, partial moles are usually triploid. Villi show focal hydropic changes, with minimal cytological atypia. Fetal tissue may be present. Compared to complete moles, partial moles are less likely to evolve into choriocarcinoma. Edematous or hydropic villi, regardless of molar form, typically display cistern formation, namely a central acellular space. Such villi are often avascular or display markedly reduced vessel density.

The incidence of molar pregnancy varies greatly geographically, and appears related to ethnicity (2), being more frequent in Southeast Asia (1/1000–2/1000 in Japan and China) than North America (0.5/1000–1/1000) (2). Molar pregnancy is a risk factor for very early-onset preeclampsia (3), a disorder in which increased sFlt1 has been implicated in its pathogenesis (4–7).

In the past, molar pregnancies were suspected when vaginal bleeding, increased uterine size for gestational age, and elevated β -hCG were observed in early pregnancy. Hyperemesis and preeclampsia before midgestation raised suspicion further (3). This clinical picture has changed, as widespread use of β -hCG measurement and ultrasound have led to the earlier diagnosis of molar pregnancy. Treatment by evacuation of the uterus often occurs prior to the presentation of many of the previous hallmark signs and symptoms. Consequently, while vaginal bleeding remains the most common presenting symptom, others, such as increased uterine size, hyperemesis, and very early-onset preeclampsia are significantly less common (3). However, in developing nations, where access to healthcare is limited, the classic symptoms described above may be more prevalent.

Preeclampsia is customarily diagnosed when new-onset hypertension and proteinuria occur after midgestation. While the etiology of preeclampsia remains unclear, two of its phenotypes, hypertension and proteinuria, and its characteristic renal lesion, glomerular endotheliosis, are believed to be caused by an excess of circulating soluble fms-like tyrosine kinase 1 (sFlt1), an endogenous anti-angiogenic protein that enters the maternal circulation after being overproduced in the placenta. Specifically, the soluble factor sFlt1 antagonizes, or decreases, free maternal circulating levels of angiogenic proteins such as free vascular endothelial growth factor (VEGF) and free placental growth factor (PlGF) (4,5,8). The free circulating angiogenic factors VEGF and PlGF are critical for endothelial growth, differentiation, and vascular integrity (9). They also decrease vascular resistance and blood pressure.

Given the association between molar pregnancy and very early-onset preeclampsia, we hypothesized that placentas from molar pregnancies would produce more anti-angiogenic proteins than normal controls.

Materials and methods

Subject selection

This was a retrospective case-control study examining archived tissue and patient records. Cases (partial, complete, and invasive moles) were from Makati Medical Center, Makati, Republic of the Philippines, while controls (first-trimester normal placentas) were from The University of Chicago Hospitals. The Institutional Review Board (IRB) at The University of Chicago approved this study. IRB approval at Makati Medical Center was not necessary because no therapeutic treatment was rendered. The following clinical data were obtained from each subject's medical record: subject age, estimated gestational age, medical history, previous history of gestational trophoblastic disease, presenting signs and symptoms (including signs and symptoms of preeclampsia), entry β -hCG, and qualitative measurement of proteinuria. The pathologic diagnosis was available for all samples. Additional data included baseline pre-pregnancy blood pressure, entry blood pressure, and blood pressure 6–12 weeks after uterine evacuation. Preeclampsia was defined as new-onset hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg) plus de novo proteinuria (qualitative, 1+; or, quantitative, ≥ 300 mg/day) (8).

Placentas from subjects undergoing elective pregnancy termination during the first trimester were used as controls. Control subjects with known risk factors for preeclampsia were excluded. This included diagnoses of end-stage renal disease, vasculitis, poorly controlled hypertension, or poorly controlled diabetes mellitus.

Morphologic evaluation

Formalin-fixed, paraffin-embedded tissue was processed and stained with hematoxylin and eosin using routine methods. Two sets of immunoperoxidase studies were then performed. Villous vascular density was documented using the endothelial marker CD31 (antibody against Clone 1A10, together with the Bond Polymer Detection system currently supplied by Leica Microsystems Inc., Bannockburn, IL). Fms-like tyrosine kinase receptor 1 (Flt1) and its soluble form (sFlt1) were identified using a goat anti-human VEGF R1/Flt1 antibody (Catalog Number: AF321, R & D Systems, Minneapolis, MN) and an anti-goat Cell and Tissue Staining Kit (Catalog Number: CTS 008, R & D Systems, Minneapolis, MN). Antigen retrieval was performed using citrate buffer and microwave heating. Two preparations were made for all samples, one with a primary antibody dilution of 1:80, and the other 1:200. Initial evaluation of the Flt1 preparations was done using the 1:80 antibody dilution. In an attempt to increase the discriminating power of the technique, Flt1 immunoperoxidase preparations were repeated using a primary antibody concentration of 1:200. The remainder of the protocol was identical.

Evaluation of the histologic and immunoperoxidase sections was performed by a single pathologist (IES) in a blinded fashion. Grading of villous trophoblast Flt1 staining (1:200 dilution) was done using a semiquantitative ordinal scale as follows: 1+ (strong focal trophoblast staining), 2+ (less than 50% of the villous trophoblast showing staining), 3+ (51%–90% staining), and 4+ (91%–100% staining). Weak staining was considered to be negative.

A two-sided t-test was used to determine statistical significance. Statistical analysis was performed with the use of Stata 10 SE (StataCorp LP, College Station, Texas).

Results

There were 20 cases and 16 controls selected for analysis. One case was excluded because pathologic review showed invasive disease. The remainder of the diagnoses were confirmed by hematoxylin and eosin review. One control had well-managed hypertension and was included in the analysis. Final analysis was performed on 19 cases and 16 controls.

As expected, hydropic villi showed markedly reduced vessel density by CD31 staining (Figure 1A–B). Semiquantitative analysis revealed that the molar tissue showed significantly more staining for Flt1/sFlt1 than controls (Figure 1C–D). The spikeplot (Figure 2) shows the distribution of stain intensity as a function of disease state.

Data on gestational age was unavailable for 1 case and 2 controls. With the available data, the mean gestational age of subjects comprising the cases was 14.1 weeks (95% CI, 11.7–16.5 weeks) while that of controls was 8.4 weeks (95% CI, 7.3–9.4 weeks). The median staining intensity for cases was 4+ (10th centile, 3; 90th centile, 4), while that for controls was 2+ (10th centile, 1; 90th centile, 3) (Table 1). The mode for cases was 4+ (11/19 observations, or 58%), while that for controls was 2+ (7/16 observations, or 44%).

There was a statistically significant difference in stain intensity between cases and controls. A Mann-Whitney U-test yielded $P=0.0001$. If we assume that for our sample size parametric testing is valid, a t-test yielded $P<0.0001$.

Comments

Our results document significantly increased Flt1/sFlt1 expression in molar placentas as compared to controls. This was accompanied by decreased villous vessels, as confirmed by CD31 staining, within the molar tissue suggesting reduced angiogenesis. These findings have implications regarding the association of molar gestations with very early-onset preeclampsia.

Koga et al. noted that circulating levels of sFlt1 in molar pregnancies ($n=7$) were 2-fold to 3-fold higher than those in gestationally aged-matched controls ($n=21$) (10). This observation and our data, when combined with numerous reports that demonstrate a pathogenic role for sFlt1 in early-onset preeclampsia (11), are consistent with the following hypothesis: The very early-onset preeclampsia associated with molar gestation may be due to excess production of anti-angiogenic factors, in particular sFlt1, by trophoblastic tissue.

This hypothesis is based on the assumption that the more intense staining noted is the result of greater production of sFlt1 and not due to impaired processing or release of the protein. This assumption is supported by studies documenting higher circulating levels of sFlt1 in preeclamptic patients whose placentas have been shown to express higher levels of sFlt1 (4). However, the techniques used in this study cannot differentiate between a “mass effect,” in which the increased trophoblastic mass of a molar placenta produces more total anti-angiogenic proteins, and a “cell effect,” in which each individual trophoblast of a molar placenta produces more anti-angiogenic proteins. Placental mRNA studies might clarify this distinction.

There are several strengths to this study. First we are unaware of other published series that attempt to establish up-regulation of placental anti-angiogenic factors as the potential link between molar pregnancy and preeclampsia. Our exclusion criteria ensured that confounders were not included in the control group. The final pathology was known for all studied specimens, and sample size was such that a more robust statistical test, the t-test, could be used to reject the null hypothesis.

However, this pilot study has limitations. The archived tissue was from two separate institutions, creating two potential issues. First, there was a discrepancy in ethnicity between cases and controls, the former primarily Asian in origin while the controls were not. Second, specimen handling and processing for paraffin-embedded blocking may have differed between subject groups, and this might result in differences in the antigen expression of each group of tissues. Of course, both sets of samples were processed simultaneously for antigen retrieval in this study. Another limitation was that interpretation of stain intensity could have been subject to bias. While the pathologist grading the immunostaining was unaware of the reported pathologic diagnosis, total blinding is not possible given that molar histopathology is distinctly different from that of normal placenta. However, we believe the magnitude of the differences precluded such bias.

Still another issue is that the primary antibody generated against Flt1/sFlt1 recognized both membrane-bound and soluble forms of fms-like tyrosine kinase 1. sFlt1 is a splice variant of Flt1 (i.e., VEGFR-1) and only contains the extracellular ligand-binding portion of the receptor. It does not have the transcellular or cytoplasmic portions (5). Because our antibody cannot differentiate Flt1 from sFlt1, our current technique cannot determine which variant accounts for the statistically significant difference in immunostaining.

Finally, there is potential selection bias due to our use of miscarried placentas as controls. Up to 60% of miscarriages are due to aneuploidy (12). Therefore, these controls may be inherently abnormal. Moreover, a number of aneuploid miscarriages result from trisomy (12), and angiogenic dysfunction is a known occurrence in trisomy 13 (13). Although there was a significant difference in mean gestational age between cases and controls, this should not affect our results. This is because Levine et al. have previously demonstrated that, for the gestational age range utilized in our study, there are no significant differences in serum sFlt1 levels between subjects who developed preeclampsia after midgestation and those who did not (14).

In summary, our data suggest that placentas from molar gestations produce more anti-angiogenic proteins than early pregnancy placentas from women without hydatidiform disease. This finding may account for the paucity of vessels seen within hydropic villi, and may establish a link between molar pregnancy and very early-onset preeclampsia. Future research will be directed to more quantitative measures of sFlt1, in addition to other angiogenic proteins, such as soluble endoglin, VEGF, and PlGF.

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Figure 1. Control vs. molar placenta histology

Representative photomicrographs comparing a control (A, C) and molar case (B, D). Normal villous vascular density as assessed by CD31 immunostaining (A). In contrast the hydropic villi of a complete mole show no vessels (B). (C), from the same control, shows only focal trophoblastic staining for Flt1/sFlt1 (1+), while the same mole (D) shows diffuse positivity (4+). All images taken at the same magnification (10x, original) and on identical preparations.

Figure 2. Distribution of stain intensity

This spikeplot shows the distribution of Flt1/sFlt1 stain intensity for cases (left) and controls (right). Statistical analysis showed a significant difference in stain intensity when cases are compared to controls.

Table 1

Stain intensity

Diagnosis	n	Mean (stain)	Median (stain)
Case	19	3.52632	4
Control	16	2.125	2

This table shows the median stain intensity (fourth column) for cases and controls. The mode (data not shown) for cases was 4+, while for controls it was 2+. The number of observations for each diagnosis (n) is in the second column. Cases showed greater stain intensity than controls, $P < 0.0001$.