Abstract—Preeclampsia is associated with increased levels of the circulating antiangiogenic factor sFlt-1 and with an excessive shedding of placenta-derived multinucleated syncytial aggregates into the maternal circulation. However, it remains unclear whether these aggregates are transcriptionally active in the maternal organs and can, therefore, contribute to the systemic manifestations of preeclampsia. In this study, we measured placental soluble fms-like tyrosine kinase-1 (sFlt-1) mRNA levels in preeclamptic- and control placental and performed RNA in situ hybridization to localize the main placental expression site of sFlt-1 mRNA. Because the maternal lung is the first capillary bed that circulating syncytial aggregates traverse, we studied the presence and persistence of placental material in lungs of preeclamptic and control subjects. To confirm the placental origin of these aggregates in maternal lungs, immunohistochemistry for the placenta-specific marker hCG (human chorionic gonadotropin) and Y chromosome in situ hybridization were performed. Using human placental tissue, we found that syncytial knots are the principal site of expression of the antiangiogenic factor sFlt-1. In addition, autopsy material obtained from women with preeclampsia (n=9) showed significantly more placenta-derived syncytial aggregates in the lungs than in control subjects (n=26). Importantly, these aggregates still contained the antiangiogenic factor sFlt-1 after their entrapment in the maternal lungs. The current study confirms the important role of syncytial knots in placental sFlt-1 mRNA production. In addition, it shows a significant association between preeclampsia and larger quantities of sFlt-1 containing syncytial aggregates in maternal lungs, suggesting that the transfer of syncytial aggregates to the maternal compartment may contribute to the systemic endothelial dysfunction that characterizes preeclampsia. (Hypertension. 2013;62:608-613.) ● Online Data Supplement

Key Words: endothelial cells ■ hypertension, pregnancy-induced ■ placental circulation ■ preeclampsia ■ sFlt-1 protein, human

Preeclampsia is a severe, pregnancy-specific syndrome that is characterized by endothelial dysfunction and presents with hypertension and proteinuria after the 20th week of gestation. Therapeutic options are limited beyond delivery of the fetus and placenta and, therefore, preeclampsia remains one of the major causes of fetal and maternal morbidity and mortality worldwide, and particularly in developing countries.1

The widespread endothelial dysfunction that characterizes preeclampsia is believed to be because of an imbalance between pro- and antiangiogenic factors.2,3 The placenta is a major source of circulating antiangiogenic factors during both normal and preeclamptic pregnancies.3–6 In preeclampsia, in particular, the outermost layer of the placenta, the syncytiotrophoblast, forms knots that contain high amounts of the antiangiogenic protein soluble fms-like tyrosine kinase-1 (sFlt-1).7 These syncytial knots are released into the maternal circulation, thereby becoming syncytial aggregates that can become lodged in maternal organs.8–10 Importantly, a recent study showed that on their release, circulating syncytial aggregates remain transcriptionally active and likely serve as an autonomous source of sFlt-1 protein within the maternal circulation.7

We hypothesized that in preeclampsia, syncytial knots are the primary placental site of sFlt-1 production and that increased numbers of sFlt-1-containing syncytial aggregates are retained in the maternal lungs. To test this hypothesis, we first studied the expression of sFlt-1 in both normal and preeclamptic placentas. Next, we used placentar- and fetus-specific markers to investigate the presence of sFlt-1–containing...
syncytial aggregates in the lungs of women with preeclampsia and control subjects.

Methods

Patient Selection and Tissue Collection
Placentas were obtained from preeclamptic (n=32) and control (n=37) subjects who delivered at the Leiden University Medical Center (LUMC), The Netherlands from 2007 through 2010. All women gave written informed consent. Parallel to the collection of placenta material, autopsy samples from women who died during pregnancy were obtained via a nationwide search using the Dutch PALGA system, a histopathology and cytopathology network and archive that includes all pathology laboratories within The Netherlands. The paraffin-embedded lung samples obtained from 9 preeclampsia patients and 26 pregnant control subjects were provided by collaborating laboratories. The control subjects were women who died because of a cause other than a hypertensive disorder of pregnancy. The cause of death in each case was confirmed using the records of the National Maternal Mortality Committee of the Dutch Society of Obstetrics and Gynecology. To investigate the effect of pregnancy on maternal autopsies, an additional control group (n=11) comprising nonpregnant, nonhypertensive women was included. All tissues were coded and handled anonymously in accordance with the Dutch National Ethics Guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This study was approved by the ethics committee of the LUMC.

Placental sFlt-1 mRNA Expression
SYBR Green quantitative polymerase chain reaction was performed to quantify the placental sFlt-1 mRNA levels. The expression of sFlt-1 was normalized to the expression of hypoxanthine phosphoribosyltransferase and glyceraldehyde-3-phosphate dehydrogenase. All cDNA samples were measured in duplicate. In addition, in situ hybridization was performed to identify the cells in the placenta that synthesized sFlt-1 mRNA. Accordingly, an RNA probe was prepared to specifically recognize sFlt-1 but not Flt-1 mRNA. Four placentas per group were examined.

Immunohistochemistry
To test for the presence of placentalis material in the maternal lungs, lung tissues from women with preeclampsia were stained immunohistochemically for the trophoblast-specific marker human chorionic gonadotropin (hCG). If hCG-positive syncytial aggregates were observed, sequential sections were stained for Flt-1 protein to determine whether these syncytial knots still contained this antiangiogenic protein. The control group was also screened using hCG staining to determine the specificity of these syncytial aggregates to preeclampsia. Sections were incubated with an antihuman β-hCG antibody (1:1600; DakoCytomation) or an antihuman Flt-1 antibody (1:100; R&D Systems). Binding of the primary antibody was visualized using the appropriate secondary antibodies with diaminobenzidine as the chromogen. Placental tissue served as a positive control.

Y Chromosome In Situ Hybridization
A digoxigenin (DIG)-labeled DNA probe that specifically recognizes the Y chromosome was used to determine whether the putative syncytiotrophoblast aggregates in the maternal lungs were of fetal origin. Sections of lungs from women who had carried a male fetus were incubated with the DIG-labeled probe. To visualize the probe, the sections were incubated first with a mouse anti-DIG monoclonal antibody (Sigma-Aldrich) followed by goat anti–mouse IgG Alexa-647 (Invitrogen).

Quantification of Staining
The number of sFlt-1 mRNA-positive syncytial knots was counted by 2 independent observers who were blind with respect to the groups. Two observers also scored the lung sections for the absence or presence of hCG. When hCG-positive multinucleate aggregates were present, the sequential sections were tested for the colocalization of hCG with Flt-1 protein and the Y chromosome.

Results

Clinical Data
Placentas were investigated from women with preeclampsia (n=32) and pregnant controls (n=37). Gestational age was significantly lower in the women with preeclampsia (mean, 30.6 weeks; SD, 1.3 weeks) than in the control subjects (mean, 39.6 weeks; SD, 1.7 weeks; P<0.05). Clinical data of the women whose lungs were investigated are provided in the Table. Furthermore, in these women, the presence of pulmonary edema was investigated at the clinical, gross, and microscopic levels. Neither the presence of clinical symptoms of pulmonary edema nor the evidence of pulmonary edema on either gross or microscopic examination differed significantly between the groups.

Increased Placental sFlt-1 mRNA Expression in Preeclampsia
To compare the levels of sFlt-1 mRNA in the preeclamptic and control placentas, quantitative polymerase chain reaction was used to measure sFlt-1 mRNA. On average, the placental sFlt-1 mRNA levels were 6-fold higher in the preeclamptic placentas than in the placentas obtained from control subjects (P<0.001, Mann–Whitney test). The preeclamptic placentas had more intense sFlt-1 staining (measured using in situ hybridization) than control placentas, particularly in the syncytial knots (Figure 1). In addition, the number of syncytial knots was significantly higher in the women with preeclampsia than in the control subjects (P<0.05; Figure 1). As expected, the sense control probe was negative in all samples (Figure 1).

Presence of hCG-Positive Aggregates in Maternal Lungs Is Significantly Associated With Preeclampsia
Because hCG was highly expressed within the syncytial knots, we considered hCG to be a suitable specific marker to study the presence of syncytiotrophoblast aggregates in maternal lungs. hCG-positive multinucleate aggregates were observed in the lungs of 6 of the 9 women with preeclampsia. After

Table. Clinical Data

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PE (n=9)</th>
<th>Controls (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age at death, y</td>
<td>33.3 (4.6)</td>
<td>32.0 (5.2)</td>
</tr>
<tr>
<td>Death postpartum, %†</td>
<td>100</td>
<td>42.3</td>
</tr>
<tr>
<td>GA at birth, wk*</td>
<td>35.2 (3.0)</td>
<td>38.5 (3.2)</td>
</tr>
<tr>
<td>Death postpartum, h</td>
<td>107.4 (157.2)</td>
<td>96.8 (180.0)</td>
</tr>
<tr>
<td>Death during pregnancy, %†</td>
<td>0</td>
<td>57.5</td>
</tr>
<tr>
<td>GA at death, wk</td>
<td></td>
<td>22.3 (10.6)</td>
</tr>
<tr>
<td>Death-autopsy time, h</td>
<td>19.7 (13.8)</td>
<td>25.7 (14.7)</td>
</tr>
<tr>
<td>Sex offspring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, %</td>
<td>57.1</td>
<td>47.8</td>
</tr>
<tr>
<td>Female, %</td>
<td>42.9</td>
<td>52.2</td>
</tr>
<tr>
<td>Parity</td>
<td>0.7 (1.1)</td>
<td>1.0 (1.2)</td>
</tr>
</tbody>
</table>

Data are given as mean±SD. GA indicates gestational age; and PE, preeclampsia. *P<0.05; †P<0.01.
the observation that syncytial aggregates were present in the lungs of women with preeclampsia, we also stained control lung sections for hCG. Syncytial aggregates were observed in the lung samples of 6 of the 26 pregnant control subjects. Importantly, the women with preeclampsia had a significantly higher number of syncytial aggregates per 100 mm² lung tissue (P<0.05, Mann–Whitney test; Figure 2). Syncytial aggregates were found in the pregnant control subjects whose gestational age was 10 to 40 weeks and in women with preeclampsia with a gestational age of 32 to 39 weeks. We performed a separate analysis to exclude any potential effect of gestational age. Because the shortest gestational age in the preeclampsia group was 30 weeks, we performed an analysis in which we excluded the control subjects with a gestational age shorter than 30 weeks. Importantly, although the gestational age of the resulting subset of controls was now similar to the women with preeclampsia, the number of syncytial aggregates remained significantly higher in the preeclampsia group (P<0.05; Figure S1 in the online-only Data Supplement). Aggregates were observed in subjects who died ≤13 days after delivery. The lung samples obtained from the additional control group of nonpregnant, nonhypertensive women contained no syncytial aggregates. The number of aggregates was not associated with gestational age, maternal age, or the severity of preeclampsia.

Syncytiotrophoblast Aggregates in the Maternal Lung Retain the sFlt-1 Protein

To test our hypothesis that syncytial aggregates retain sFlt-1 protein after transferring to the maternal compartment and becoming entrapped in the maternal lung, we stained the hCG-positive aggregates in the maternal lung samples for Flt-1 protein. Staining sequential sections for Flt-1 and hCG revealed that these proteins were colocalized within the aggregates (Figure 2). In the preeclampsia group, 56% of all hCG-positive aggregates were also positive for Flt-1; in contrast, in the control group, 26% of the syncytial aggregates were positive for Flt-1 protein (P<0.05; Figure 3).

Y Chromosome In Situ Hybridization Strongly Supports the Idea That Multinucleate Aggregates Are of Fetal Origin

To confirm our hypothesis that the multinucleated syncytial aggregates in the maternal lung were of placental, and therefore fetal, origin, we performed Y chromosome in situ hybridization in lung samples obtained from women who were carrying a male fetus. A sequential section was used to investigate colocalization with hCG and Flt-1. We observed Y chromosome positive aggregates in the maternal lung samples, and sequential sections showed colocalization between the Y chromosome and both hCG and Flt-1 (Figure 2).

Discussion

Here, we report that multinucleate aggregates in the maternal lungs originate from the syncytiotrophoblast, and that these aggregates retain the antiangiogenic protein sFlt-1. Syncytial knots, which become syncytial aggregates on release from the placenta, are rich in sFlt-1 mRNA and protein, suggesting that these structures are the primary placental site of sFlt-1 production. The systemic spread of these syncytial aggregates was confirmed by the presence of hCG-positive multinucleate aggregates in the lungs of pregnant women, and the number of syncytial aggregates in the maternal lungs was significantly higher in the women with preeclampsia. Colocalization of hCG with both the Y chromosome and the sFlt-1 levels strongly supports the idea that these aggregates are of fetal origin and shows that these aggregates contain sFlt-1 even after their release from the placenta.

Our finding that syncytial knots are the primary placental site of sFlt-1 mRNA synthesis is in agreement with the observations that syncytial knots have the highest placental levels.
of sFlt-1 protein and that these knots are more numerous in the setting of preeclampsia. Syncytial knots detach readily from the placenta, becoming syncytial aggregates that circulate in the maternal blood. It has long been known that circulating placental material, most likely trophoblast cells, can reach maternal organs, particularly the lungs. Using colocalization of hCG with the Y chromosome, we show that the placental multinucleate aggregates in the maternal lung were derived from the syncytiotrophoblast. Interestingly, these placenta-derived aggregates in the maternal lung still contained sFlt-1 protein. This observation supports the idea of circulating syncytial aggregates as a mechanism of sFlt-1 release into the maternal circulation. Importantly, we also found that preeclampsia was associated with a significantly higher number of syncytial aggregates within the maternal lung tissue. During both preeclamptic and normal pregnancies, the placenta is the principal source of sFlt-1. However, previous research has shown that ≈25% of all sFlt-1 is derived from shed syncytial aggregates. It has been estimated that near the end of pregnancy, ≈3 g of this placental material is shed into the maternal circulation (ie, into the maternal lungs) daily. Therefore, the cumulative quantity of these circulating aggregates, and their relative contribution to total sFlt-1 production, should not be underestimated.

The lungs of the women with preeclampsia contained a significantly higher percentage of sFlt-1–positive syncytial aggregates than the control samples. This observation further supports the idea that preeclampsia is associated both with an increased number of circulating syncytial aggregates and with increased sFlt-1 expression within these aggregates. By releasing sFlt-1, these aggregates may contribute to the systemic endothelial dysfunction that is characteristic of preeclampsia.

Figure 2. A, Sections of placenta and sequential sections of maternal lungs that were stained for hCG or Flt-1 (using immunohistochemistry) or the Y chromosome (using in situ hybridization). To simplify the terminology, we use syncytial knots to describe multinucleated structures that are loosely attached to the tips of placental villi in situ and syncytial aggregates to describe detached multinuclear structures within the maternal lungs. The left column shows a placenta obtained from a preeclamptic patient, and the middle and right columns represent the lungs obtained from 2 women who were pregnant with a boy and died because of preeclampsia. The various stains are shown horizontally. Within the preeclamptic placenta, the hCG (a) and Flt-1 (b) proteins are most abundantly present within the syncytial knots (arrowheads). In addition, Y chromosome in situ hybridization (c) shows the presence of the Y chromosome in the nucleus (visible as red puncta). d and g, The presence of hCG-positive aggregates (arrowheads) in the lungs of 2 women who died because of preeclampsia. e and h, Flt-1 staining patterns in sections that were sequential to the sections shown in d and g, respectively. These images demonstrate that within the maternal lungs, hCG-positive aggregates also contain Flt-1 protein. The next sequential sections were used to perform Y chromosome in situ hybridization (f and i). These images show that the multinucleate aggregates contain Y chromosomes (arrowheads), indicating that it is very unlikely that these aggregates are not of fetal origin. Altogether, the figure demonstrates colocalization of hCG, Flt-1, and the Y chromosome in the multinucleate aggregates (arrowheads). B, Additional examples of multinucleate aggregates in maternal lungs, with colocalization of hCG (a–d) and Flt-1 (e–h). Each row shows matched sequential sections. C, The number of hCG-positive syncytial aggregates within the maternal lungs of women with preeclampsia (n=9) and control subjects (n=26; *P<0.05).
This finding is also consistent with the observation that pre-eclamptic placentas contain more syncytial aggregates that are heavily loaded with sFlt-1 than placentas obtained after uneventful pregnancies.

In addition, the presence of syncytial aggregates in maternal organs, particularly in the early stages of pregnancy, may play a key role in the development of immune tolerance. As early as gestational week 10, we observed syncytiotrophoblastic aggregates in maternal lungs. Because preeclampsia rarely presents before 20 weeks of gestation, we could not investigate the presence of syncytial aggregates in the lungs of women with preeclampsia early in pregnancy. We did, however, observe syncytiotrophoblastic aggregates in the lungs of women with preeclampsia at gestational week 32 and later, and other groups have reported the presence of trophoblast fragments in maternal blood in earlier stages of pregnancy. Altogether, circulating syncytial aggregates are present early in pregnancy, and we and others have found a strong association between increased shedding of syncytial aggregates and preeclampsia. Thus, one may speculate that the release and transfer of syncytial aggregates to the maternal compartment is an early event in the pathogenesis of preeclampsia. However, the relative contribution of sFlt-1 expression in these aggregates in early pregnancy is unclear.

The presence and persistence of fetal cells in maternal organs may also have both short-term and long-term implications for postpartum maternal health. Syncytial aggregates that remain in the maternal lungs may undergo further disaggregation, forming smaller microparticles. These sFlt-1–loaded microparticles may, via their release into the systemic maternal circulation, contribute to endothelial dysfunction in maternal organs other than the lungs. We found that even 13 days after delivery, hCG-positive syncytial aggregates can be detected within the maternal lungs. This finding supports the idea that placenta-derived syncytial aggregates may be involved in the postpartum complications that are associated with preeclampsia. Preeclampsia usually resolves rapidly after delivery, and its resolution is reflected by a parallel decrease in sFlt-1 levels. However, in a subset of women, the symptoms and complications of preeclampsia can persist or present several days after delivery. Syncytial aggregates remain transcriptionally active ≤48 hours after delivery, and estimates suggest that during pregnancy, 25% of all circulating sFlt-1 is derived from circulating syncytial aggregates. Therefore, we propose that these aggregates may play an important role in postpartum (pre)eclampsia.

It must be acknowledged that the placentas in our study were not obtained from the same women from whom we obtained the lung tissues. Therefore, the preeclampsia phenotype of the women whose lung tissues were investigated might have been more severe than the phenotype of the women who provided the placentas. As a consequence of this potential mismatch between phenotypes, we were unable to correlate placental sFlt-1 production to the portion of sFlt-1–loaded syncytial aggregates in the maternal lungs. To overcome this complication, an animal model could be used to study the association between placental sFlt-1 production and lung pathology.

Trophoblast cells are likely not the only fetal cell population that is present in the maternal lung. A previous study using mice suggested that fetal cells in the maternal lung comprised a mixture of cell types that includes trophoblasts, mesenchymal stem cells, and cells from the immune system. We have now confirmed the presence of trophoblast cells in the human maternal lung. In the long run, the release of vital cells from the placenta may result in chimerism, as fetal cells can be retained in the maternal blood and organs for decades after delivery. Because retained fetal cells have stem cell–like properties, it can be speculated that these cells provide a mechanism through which maternal health can be affected for decades after pregnancy.

Perspectives

In conclusion, we have demonstrated that multinucleate aggregates in the maternal lungs originate from the syncytiotrophoblast, that their presence is significantly associated with preeclampsia, and that these aggregates retain the antiangiogenic protein sFlt-1. Further studies are needed to determine the relevance and relative contribution of trophoblast cells, and other cell types, to maternal health. Likewise, understanding what drives the formation, detachment, and transfer of syncytial knots to the maternal compartment, and why these knots produce sFlt-1, are important questions to be investigated. Nevertheless, this report highlights the importance of investigating further the role that syncytial aggregates play in preeclampsia and its complications.

Acknowledgments

We thank Kimberley Veraar and Malu Zandbergen for their excellent technical support.

Disclosures

S.A. Karumanchi is a coinventor of multiple patents related to angiogenic proteins for the diagnosis and therapy of preeclampsia. These patents have been licensed to multiple companies. S.A. Karumanchi reports having served as a consultant to Roche, Siemens, and Beckman Coulter and has financial interest in Aggamin LLC. The other authors report no conflicts.

References


**Novelty and Significance**

**What Is New?**

- Within the placenta, syncytial knots are the principal site of expression of the antiangiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1).
- Placenta-derived syncytial aggregates that become lodged in the maternal lungs retain the antiangiogenic factor sFlt-1.
- Preeclampsia is associated with significantly higher quantities of sFlt-1–loaded syncytial aggregates within the maternal lung.

**What Is Relevant?**

- Although the precise cause of preeclampsia and its complications remains unknown, the condition is associated with excessive shedding of placental material into the maternal circulation.
- Placenta-derived syncytial aggregates within the maternal lungs contain sFlt-1 and may contribute to the systemic endothelial dysfunction that characterizes preeclampsia.

- Furthermore, retained fetal cells within the mother may have stem cell–like properties, thereby providing a mechanism through which maternal health can be affected for decades after pregnancy.

**Summary**

The current study confirms the important role of syncytial knots in placental sFlt-1 mRNA production. In addition, it demonstrates a significant association between preeclampsia and the presence of increased quantities of sFlt-1 containing syncytial aggregates in maternal lungs. These observations suggest that the transfer of syncytial aggregates into the maternal compartment likely contributes to the systemic endothelial dysfunction that characterizes preeclampsia.
Preeclampsia Is Associated With the Presence of Transcriptionally Active Placental Fragments in the Maternal Lung

Aletta J. Buurma, Marlies E. Penning, Frans Prins, Joke M. Schutte, Jan Anthonie Bruijn, Suzanne Wilhelmus, Augustine Rajakumar, Kitty W.M. Bloemenkamp, S. Ananth Karumanchi and Hans J. Baelde

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PREECLAMPSIA IS ASSOCIATED WITH THE PRESENCE OF TRANSCRIPTIONALLY ACTIVE PLACENTAL FRAGMENTS IN THE MATERNAL LUNG

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Figure S1. Number of syncytial aggregates in preeclamptic women and control subjects whose gestational age was over 30 weeks.

Figure S2. Number of syncytial aggregates in preeclamptic women and control subjects who died postpartum.
Figure S1: Number of syncytial aggregates in preeclamptic women and control subjects whose gestational age was over 30 weeks.

When comparing this subset of patients, the mean gestational age of the preeclamptic women (n=9) was 35.1 weeks, whereas the gestational age of the control subjects (n=14) was 38.0 weeks. The number of aggregates was still higher in the women with preeclampsia ($p<0.05$).
Figure S2: Number of syncytial aggregates in preeclamptic women and control subjects who died postpartum.

In this subset of patients, we compared the number of aggregates of the women with preeclampsia (n=9) and control subjects (n=11). The number of aggregates was still higher in the women with preeclampsia ($p<0.05$).