Abstract—The cardinal manifestations of the pregnancy-specific disorder preeclampsia, new-onset hypertension, and proteinuria that resolve with placental delivery have been linked to an extracellular protein made by the placenta, soluble fms-like tyrosine kinase 1 (sFlt1), that injures the maternal vasculature. However, the mechanisms by which sFlt1, which is heavily matrix bound, gain access to the systemic circulation remain unclear. Here we report that the preeclamptic placenta’s outermost layer, the syncytiotrophoblast, forms abundant “knots” that are enriched with sFlt1 protein. These syncytiotrophoblast knots easily detach from the syncytiotrophoblast, resulting in free, multinucleated aggregates (50–150 μm diameter) that are loaded with sFlt1 protein and mRNA, are metabolically active, and are capable of de novo gene transcription and translation. At least 25% of the measurable sFlt1 in the third-trimester maternal plasma is bound to circulating placental microparticles. We conclude that detachment of syncytiotrophoblast knots from the placenta results in free, transcriptionally active syncytial aggregates that represent an autonomous source of sFlt1 delivery into the maternal circulation. The process of syncytial knot formation, shedding of syncytial aggregates, and appearance of placental microparticles in the maternal circulation appears to be greatly accelerated in preeclampsia and may contribute to the maternal vascular injury that characterizes this disorder. (Hypertension. 2012;59:256-264.) · Online Data Supplement

Key Words: syncytiotrophoblast knots · syncytial aggregates · microparticles · sFlt1 · soluble VEGFR1 · preeclampsia

Preeclampsia (PE) affects 5% to 7% of all pregnancies and results in substantial morbidity both to the mother and the fetus. Although new-onset maternal hypertension, proteinuria, and edema are the hallmarks of this disorder, PE left unchecked can progress to seizures, acute liver injury, and death. The only known treatment for PE is delivery of the placenta. Although the pathogenesis of PE remains incompletely understood, the rapid and complete resolution of this disease after delivery implicates the placenta as a critical factor for the pathogenesis of the disease.

We and others have described a marked elevation of soluble fms-like tyrosine kinase 1 (sFlt1 or sVEGFR1) in the circulation of women with PE that is both proportional to the severity of the disease and is antecedent to the clinical manifestations. Heterologous expression of sFlt1 in pregnant rodents is sufficient to recapitulate the major features of PE. sFlt1 may mediate maternal injury by binding and sequestering trophic growth factors, such as vascular endothelial growth factor and placental growth factor, that are necessary for the maintenance of normal vascular endothelial function. Northern analysis of tissue arrays and measurement of its concentration in the uterine artery versus vein have implicated the placenta as the major source for circulating sFlt1 during pregnancy.

All of the known sFlt1 isoforms contain heparin-binding domains in the third and fourth immunoglobulin loops that fully account for its strong avidity to the extracellular matrix. This chemical property has raised questions about how sFlt1 made by placental cells can even gain access to the maternal circulation. Having observed that the outermost layer of the placenta, the syncytiotrophoblast, most strongly expresses sFlt1 and that placental material has been noted in the maternal circulation for decades, we hypothesized that syncytiotrophoblast knots shed into the maternal circulation are a significant source of circulating sFlt1 in PE. We performed experiments in preeclamptic placentas, third-trimester placenta...
tual organ cultures, and third-trimester maternal plasma to test this hypothesis. To circumvent the well-described confusion in terms, we have used “syncytial knots” to describe multinucleated structures that are loosely attached to the tips of placental villi in situ, “syncytial aggregates” to describe detached multinuclear structures of 50 to 150 μm recovered from placental washes and organ cultures, and “microparticles” to describe products isolated by high-speed centrifugation of placental washes, culture medium, or maternal plasma.19

Materials and Methods

Study Population and Sample Collection Protocols

Biological samples (plasma and placenta) were collected from normal and preeclamptic patients. PE was defined as new onset of hypertension and proteinuria occurring after 20 weeks of gestation.20 Diagnosis of PE was confirmed by an obstetrician after review of the medical charts of the study participants. Approximately 5 mL of blood were collected from subjects via venipuncture, centrifuged at 3500g for 10 minutes, and the plasma was collected and stored at −80°C without thaw before analysis. For placental studies, several villous biopsies (2 cm) were excised from the maternal surface midway between the chorionic and basal plates, within 30 minutes of delivery, and the decidual layer was carefully removed. A portion was flash frozen in liquid nitrogen for RNA and protein analysis, and the remaining villous tissue collected was cut to 0.5 cm and rinsed in 50.0 mL of ice-cold PBS for 2 minutes. After rinsing, a portion of the villous tissue was flash frozen, and another portion of the villous tissue was used in explant cultures. The washes were combined (100 mL total) and filtered using a thin layer of gauze. The material in the filtrate was collected by centrifugation (800 g for 10 minutes), and then subjected to red blood cell lysis (red blood cell lysis solution, Roche Applied Sciences, Mannheim, Germany). After centrifugation and resuspension again in PBS, a portion of the pellet was used for microscopy and the remaining flash frozen in liquid nitrogen for mRNA and protein analyses. These human studies were approved by the institutional review boards at the Beth Israel Deaconess Medical Center and at the Magee-Womens Research Institute (Pittsburgh, PA), and subjects gave informed consent.

Immunohistochemistry and Electron Microscopy

Expression of sFlt1 protein in formalin-fixed and paraffin-embedded sections of placenta was evaluated using an anti-human Flt1 antibody that recognizes the N-terminal region of Flt1/sFlt1 (1:200 dilution, R&D Systems, Minneapolis, MN) and an ImmPRESS antigenic staining kit (Vector Laboratories, Burlingame, CA) according to published protocols.21 Evaluation of the hematoxylin-eosin and sFlt1 staining was performed by a single pathologist (I.E.S.) in a blinded fashion. Grading of Flt1/sFlt1 staining was done using the semiquantitative ordinal scale as follows: 1+ (focal trophoblast staining), 2+ (<50% of the villous trophoblast showing staining), 3+ (51% to 90% staining), and 4+ (>90% staining). Weak staining was considered negative (0).

Contents of the placental washes were fixed in 10% neutral-buffered formalin and then washed with PBS. Samples were centrifuged at 800 g for 10 minutes at 4°C and were placed into Histogel specimen medium and then embedded into paraffin. Four-micrometer sections were cut and stained for sFlt1 expression as described above. In addition, pellets of the placental washes were stained with Trypan blue and imaged at 10× and 40× magnifications using an Olympus digital camera.

For electron microscopy studies, placental wash effluents were fixed in 3% formaldehyde 3% glutaraldehyde in 0.1 mol/L of cacodylate buffer (pH 7.35, Toussimis Research Corporation Rockville, MD). The fixed cells/debris was processed in standard fashion (Epon embedded) for transmission electron microscopy, and images were acquired using a JEOL 1011 Transmission Electron Microscope, with a Hamamatsu Orca-HR Digital Camera (Advanced Microscopy Techniques, Woburn, MA).

Northern Blot Analysis

Total RNA was isolated from archived placental wash pellets using RNAwiz (Ambion, Austin, TX), and Northern blot analysis was performed using total RNA (20 μg) isolated from the washes as described previously.22 Two regions of the FLT1 mRNA (GenBank Accession No. X51602) spanning the region 250 to 881 (5’TATGGTCACTCTGGAACACCGGGGT and 5’ACTGGTGCTTCAGAGTGGAAGC, respectively) and 2300 to 3300 (5’CTAATGGTTGCCAGGCTT and 5’CCATTGTTACTCCTGGTATG) were amplified using PCR and were used as probes in the Northern blots using published protocols.

Villous Explant Cultures and Isolation of Syncytial Aggregates

Placental villous explant preparation and culture were carried out according to published protocols.23 Explants were incubated at 37°C for 24 hours on an orbital shaker (60 rpm, Becton, Stovall Life Science Inc, Greensboro, NC) under standard tissue culture conditions in a cell culture incubator (Napco Series 8000 WJ, Thermo Scientific, Marietta, OH). At the end of the incubation period, the explants were removed, blotted with sterile cotton gauze to remove any excess media, and flash frozen in liquid nitrogen and stored at −80°C. To isolate syncyntial aggregates, we cultured villous explants on Netwells (15 mm Netwell insert with 500 μm mesh size, Corning) as described.24 To evaluate for shed products, the placental material collected in the lower chamber was concentrated by centrifugation at 800 g for 5 minutes and used in further experiments.

Ultracentrifugation of Plasma and Explant Culture Medium

We subjected plasma samples obtained from normal pregnant (n = 12) and preeclamptic women (n = 16) to ultracentrifugation (100 000 rpm for 90 minutes, ~415 000g) after diluting them 10 times with ELISA assay calibration buffer (R&D Systems, Minneapolis, MN) or PBS. Similarly, the villous explant conditioned medium was centrifuged for 5 minutes at 800 g to remove larger particles and diluted 10 times with PBS and processed for ultracentrifugation.

Heparin-Agarose Enrichment of sFlt1 and Western Blot Analyses

sFlt1 in human plasma samples and conditioned medium from explant cultures was concentrated by heparin-agarose affinity chromatography using published protocols25 and Western blots performed as described previously.22 Briefly, the 100 000 rpm pellets were resuspended in 1 mL of PBS and were incubated with 25 μL of heparin-agarose beads (Sigma Chemical Company, St Louis, MO) at 4°C for 1 hour with continuous mixing. The heparin-agarose/sFlt1 conjugate was then centrifuged and the pellet washed 3 times with PBS buffer. After the final wash, the beads were resuspended in minimal volume of 1X Laemmli solution and Western blots performed using mouse monoclonal vascular endothelial growth factor R1 antibody (V4262; Sigma Chemical Company) that recognizes the amino acid terminus epitope present in both Flt1 and sFlt1.

ELISA and Placental Alkaline Phosphatase Colorimetric Assay

sFlt1 in culture medium and in maternal plasma, before and after 100 000 centrifugation, was measured by ELISA using the human vascular endothelial growth factor R1 Quantakine kit from R&D Systems (R&D Systems), following the manufacturer’s instructions. Sensitivity of the assay was 5.01 pg/mL, with an intra-assay coefficient of variation of 2.6% to 3.8% and an interassay coefficient of variation of 7.0% to 8.1%. Placental alkaline phosphatase activity in the 100 000-pellet fraction (same fraction as used in Western blot) was estimated using a kit from Abcam (ab83369-500, Abcam),
Cambridge, MA) according to the manufacturers instructions. The placental alkaline phosphatase activity was expressed as micromoles of p-nitrophenol release.

**Adenoviral Expression Studies**

Placental explants were incubated for 24 to 48 hours on a Netwell (Corning Life Sciences, Pittston, PA) that facilitates any cells and debris to move in to the lower chamber. The medium was collected and centrifuged at 800 g to harvest the debris. After red blood cell lysis, the concentrated debris was transduced with 2 μL of 1×10^10 plaque-forming units per milliliter adenovirus carrying green fluorescent protein (Vector BioLabs, Eagleville, PA) and assessed for green fluorescent protein expression after 12 to 24 hours following published protocols. Briefly, the debris was collected by centrifugation, resuspended in 30 to 50 μL of PBS, and spread on a microscopic slide and let air dry. The slides were dipped in ice-cold acetone for 30 minutes for fixation and PBS, and the nuclei were stained with 4’,6-diamidino-2-phenylindole blue (Molecular Probes Inc, Eugene, OR). The slides were mounted using Gelvetol, fluorescence microscopy was done using an Olympus digital camera, and the images were processed using the DP2-BSW program. In another experiment, the debris was transduced with adenovirus expressing truncated mouse sFlt1 protein for 48 hours. The medium was subjected to heparin-agarose enrichment and probed for sFlt1 expression using Western blots as described above.

**Results**

**sFlt1 Is Highly Expressed in Syncytial Knots**

Within Placentas of Women With PE

Compared with normal third-trimester placentas (N=9), F1t1&sFlt1 expression was significantly increased in the syncytial layer of preeclamptic placentas (N=12), with pronounced overexpression in syncytial knots (Figure 1A and 1B). Furthermore, the abundance of such knots was increased in preeclamptic placentas. Blinded scoring confirmed that F1t1&sFlt1 staining was markedly enhanced in the syncytial layer of preeclamptic placentas (Figure 1C; P<0.001).

**Washing of Preeclamptic Placentas Releases Syncytial Aggregates**

Based on the anatomic orientation of the syncytial knots at the tips of placental villi, we hypothesized that they may easily detach from this outermost layer of the placenta. To test this, we gently flushed placentas from preeclamptic pregnancies with PBS and collected the effluent. The effluent contained large structures of which the size of 50 to 150 μm was consistent with detached syncytial knots (Figure 2). Closer examination demonstrated that these structures were always membrane bound and always multinucleated, again consistent with a syncytial origin (Figure 2B through 2F). We repeated this experiment in placentas from normal term pregnancies and observed very few multinucleated structures in the effluent (Figure 2A). These data showed that the abundant syncytial knots in preeclamptic placentas might easily detach from the syncytial layer to become free aggregates of syncytial origin.

**sFlt1 mRNA and Protein Are Elevated in Preeclamptic Placental Effluents**

Having previously observed that gentle washing of preeclamptic placentas reduced the abundance of sFlt1 mRNA, we now asked whether the released syncytial aggregates in the effluent contained sFlt1. To test this, we first performed immunohistochemistry on syncytial aggregates isolated by low speed spin (800 g) of placental washes from normal and preeclamptic third-trimester placentas and observed that the latter stained much stronger for sFlt1 (Figure 3A through 3D) and the related angiogenic protein endoglin (see Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org).

Next, we performed Northern analysis to quantify the relative amount of sFlt1 mRNA associated with this liberated placental material. Both normal and preeclamptic placental effluents demonstrated the 7.4-kb band that corresponds with membrane-bound Flt1 and the 2 smaller bands (3.0 and 2.4 kb) corresponding with alternatively spliced products encoding soluble forms of Flt1 (Figure 3E), but the relative amounts of sFlt1-encoding bands were markedly higher in preeclamptic placental effluents (Figure 3F). Finally, sFlt1-encoding mRNA was not significantly altered in peripheral blood mononuclear cells obtained from preeclamptic subjects, suggesting that loss of peripheral blood cells was unlikely to account for the reduction in sFlt1 mRNA following placental washes (see Figure S2).

**sFlt1 Is Associated With Microparticles in Villous Explant Culture Medium**

To rule out the possibility that the observed syncytial aggregates were artifacts of physical manipulation, we next placed

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Immunohistochemistry for soluble fms-like tyrosine kinase 1 (Flt1&sFlt1) expression in normal and preeclamptic placentas. Immunohistochemical staining and analysis of placental tissues from normal pregnancy (n=9) and preeclampsia (n=12) for Flt1&sFlt1 expression were performed. A and B show a representative staining of normal and preeclamptic placenta, at term, respectively. The red arrowheads represent syncytial knots. Magnification: ×400. C shows a graphical representation of the quantitation of the Flt1&sFlt1 staining.
villous explants from preeclamptic and normal term placentas in organ culture and collected the medium at 48 hours for further analysis. Although sFlt1 protein has been demonstrated previously in the culture medium using this technique, it has generally been assumed that this solely represents free secreted protein in solution. We asked whether sFlt1 was also bound to microparticles. Centrifugation at 100 000 rpm (≈415 000g) for 90 minutes reduced the amount of sFlt1 by 30% in the conditioned media for both normal pregnant and preeclamptic villous explants (Figure 4A). To demonstrate that the reduction of sFlt1 after centrifugation was related to placental microparticle-bound sFlt1, we solu-

Figure 2. Analyses of placental washes from normal and preeclamptic placentas. Representative photomicrograph of Trypan blue staining of the contents of the placental washes obtained from normal (A) and preeclamptic women (B). C through F show different sized syncytial aggregates in preeclamptic placental effluents. Red blood cells and leukocytes can be seen in the background.

Figure 3. Expression of soluble fms-like tyrosine kinase 1 (sFlt1) mRNA and protein in syncytial debris obtained from placental washes. A to D show sFlt1 staining by Immunohistochemistry (IHC) of the syncytial knots obtained from placental washes from 2 normal pregnant women (A and B) and from 2 preeclamptic women (C and D). Scale bar: 20 μm in all of the panels. RNA obtained from placental washes of normal pregnant (NP; n=5) and preeclamptic women (PE; n=6) was analyzed by Northern blot. A representative blot from 2 samples of each category is shown in E and the quantitation in a graph in F. *P<0.05 by ANOVA.
bilized the pellet and assayed for sFlt1 and placental alkaline phosphatase. Pellets obtained from culture medium from preeclamptic women showed significantly higher amounts of sFlt1 (Figure 4B) and higher placental alkaline phosphatase than their normal counterparts (Figure 4C). These results show that shedding of the placental microparticle material can occur spontaneously rather than being a simple artifact of aggressive flushing and that this release is exaggerated in preeclamptic placentas. Moreover, these data suggest that approximately one third of the secreted sFlt1 is associated with released microparticles.

**Ex Vivo Organ Culture Recapitulates Syncytial Knot Formation and Spontaneous Syncytial Aggregate Release**

To evaluate further the nature of syncytial material being released by the preeclamptic placenta, we performed third-trimester placental organ cultures on Netwell inserts and collected the culture medium below the mesh for further characterization (Figure 5A). This technique has previously only been used for the isolation of microparticles from first-trimester placental organ culture.23 Cytological analysis of the culture medium below the mesh revealed membrane-bound knot-like structures containing multiple nuclei that appear to be “budding” off a main branch (Figure 5B). We also observed free membrane-bound particles 50 to 150 μm in diameter containing multiple nuclei (Figure 5C and 5D). Both the size and composition of these aggregates exactly mirrored the contents of effluents from placentas (Figure 2) and the spontaneously released material in villous explant cultures of preeclamptic placenta (Figure 3). These results, therefore, suggest that ex vivo culture of third-trimester placentas on Netwell results provides an efficient method for studying the spontaneous release of syncytial aggregates.

**Released Syncytial Aggregates Are Viable and Metabolically Active**

We applied the Netwell method on third-trimester placental organ culture to collect syncytial aggregates for further study. Several previous reports have termed these structures “debris,” implying that they are dead or nonviable material. Transmission electron microscopy of these structures showed that the plasma membrane was organized into a microvillous structure and that the cytoplasm contained several nuclei,
numerous mitochondria, and other organelles (Figure 6A and 6B). The internal composition of these aggregates and the fact that we had detected both sFlt1 mRNA and protein in them (Figures 3 and 4) suggested to us that these structures could have gene expression capacity. To test this, we infected aggregates with green fluorescent protein–encoding adenovirus (Figure 6C). Aggregates were fluorescent for 48 hours after infection, demonstrating that they possess both transcriptional and translational activity and the energetic capacity required for these processes. We repeated this experiment with sFlt1 adenovirus and obtained a comparable result (Figure 6D). Finally, to confirm the ability of these microparticles to synthesize sFlt1 protein from endogenous sFlt1 mRNA, we performed a pulse-chase experiment with $^{35}$S, immunoprecipitated the conditioned medium with anti-Flt1, and confirmed the presence of new protein by autoradiography (see Figure S3). These results show that spontaneously released syncytial aggregates from late-pregnancy placentas possess a spectrum of biological capacities, including the ability to synthesize sFlt1 protein from endogenous stores of its mRNA.

**Released Microparticles Show Antiangiogenic Properties**

Pellets obtained by ultracentrifugation of the culture medium from the placental explant cultures were resuspended in growth medium and were used in endothelial tube formation assays, a standard tool for assessing angiogenic activity. PE suspensions showed significant inhibition of endothelial tube formation (see Figure S4A and S4B) that was reversed by the addition of exogenous vascular endothelial growth factor (see Figure S4C). Quantitation of the tube lengths is presented in Figure S4D.

**Circulating Microparticles of Syncytial Origin Contribute $\geq 25\%$ of the sFlt1 in the Plasma of Normal Pregnancy and Preeclamptic Subjects**

Having performed a series of ex vivo experiments with third-trimester human placentas to establish structural and functional features of shed trophoblast material, we returned to the maternal circulation to ask how much shed microparticles of syncytial origin contribute to circulating levels of sFlt1 in normal and preeclamptic pregnancies. After a low-speed spin to remove cellular components, we centrifuged plasma at 100,000 rpm ($=415,000g$) for 90 minutes to collect microparticles into a pellet. We measured the sFlt1 concentration in plasma before and after centrifugation. In plasma from third-trimester normal pregnancies ($N=12$ subjects), these values were $6779\pm 1458$ and $5116\pm 1228$ pg/mL, respectively. In plasma from preeclamptic subjects ($N=15$), centrifugation reduced the sFlt1 concentration from $30,486\pm 6108$ to $25,381\pm 5710$ pg/mL. Therefore, in both clinical settings, free microparticle–associated sFlt1 accounted for $\approx 25\%$ of the total sFlt1 concentration in plasma samples from both groups (Figure 7A).
Our results show that third-trimester placentas from preeclamptic women have more syncytial knots that are more heavily loaded with sFlt1 protein compared with those from normal pregnancies. Gentle flushing of these placentas selectively releases more trophoblast mass in the form of syncytiotrophoblast origin, we performed communoprecipitation by pulling down the syncytiotrophoblast marker syncytin 1 and blotting for sFlt1 (Figure 7C).

**Discussion**

Next, we performed Western analysis on the centrifuged pellets and observed that preeclamptic plasma was enriched for sFlt1 bound to these microparticles (Figure 7B). Finally, to confirm that the sFlt1 in these pellets was of syncytiotrophoblast origin, we performed communoprecipitation by pulling down the syncytiotrophoblast marker syncytin 1 and blotting for sFlt1 (Figure 7C).

Our results show that third-trimester placentas from preeclamptic women have more syncytial knots that are more heavily loaded with sFlt1 protein compared with those from normal pregnancies. Gentle flushing of these placentas selectively releases more trophoblast mass in the form of syncytiotrophoblast aggregates from the preeclamptic placentas than their normal counterparts. Liberation of syncytiotrophoblast aggregates could not be attributed to aggressive handling, because preeclamptic placentas in organ culture spontaneously released aggregates of identical size and multinuclear composition into the medium. This shed material contained both sFlt1 protein and mRNA. Placement of third-trimester placental explants over a 500-μm mesh in organ culture not only enabled efficient isolation of syncytial aggregates but also suggested that this shed material may arise by syncytiotrophoblast fission from the underlying syncytiotrophoblast. Isolated aggregates were multinuclear, rich in cytoplasmic organelles, and capable of de novo gene expression, demonstrating that these structures were not only viable but also biologically active. Finally, we returned to the third-trimester maternal circulation, where we could demonstrate that ≥25% of plasma sFlt1 was associated with microparticles. Although placental heparinase upregulation has been implicated recently as one factor that may contribute to the release of sFlt1 into systemic circulation, our data suggest that release of syncytial microparticles may be an important additional factor that contributes to the elevated sFlt1 in human PE.

Based on these results, we speculate that third-trimester placentas spontaneously form living syncytial sprouts/knots (Figure 1) that detach from placental villi through fission (Figure 5), liberating membrane-bound multinuclear structures (Figures 2 and 3) that we called syncytial aggregates that possess critical biological capacities (Figure 6), including the ability to synthesize sFlt1 protein from endogenous stores of mRNA. Because each phase of this sequence is exaggerated in PE—in situ knots (Figure 1) followed by liberated sFlt1-expressing syncytial aggregates (Figure 3) that then perhaps further dissociate to sFlt1-associated microparticles (Figure 7)—we also speculate that accelerated knot/sprout formation within the placenta may be an early event in PE that enhances the delivery of sFlt1 into the maternal circulation.

Normal pregnancy is characterized by trophoblast turnover and shedding, as evidenced by the detection of trophoblastic microparticles in the maternal circulation throughout pregnancy and by the appearance of detached syncytial aggregates with euchromatic nuclei, labeled “syncytial sprouts,” in term human placentas. Based on the current results, we speculate that third-trimester placentas spontaneously form syncytial sprouts/knots (Figure 1) that detach from placental villi through fission (Figure 5), liberating membrane-bound multinuclear structures (Figures 2 and 3), termed syncytial aggregates, that possess critical biological capacities (Figure 6), including the ability to synthesize sFlt1 protein from endogenous stores of mRNA. Therefore, although apoptotic, necrotic, or “aponecrotic” placental material loaded with sFlt1 protein may well be released into the maternal circulation, our data suggest that term placentas secrete or shed biologically active syncytial aggregates that, in turn, may release smaller microparticles with similar biological capacities into the maternal circulation, akin to the release of platelets from megakaryocytes residing in the bone marrow.

In addition to demonstrating the biological capacity of third-trimester shed trophoblast material, the current findings also add to the existing literature in other ways. First, deportation of living placental material, followed by de novo translation of preexisting mRNA, may be a new mechanism by which sFlt1 is delivered into the maternal circulation. Second, although sFlt1 protein has been identified on circulating placental particles and an increase in circulating
particles has been associated with PE,18 no explanation has been proposed for how these particles are formed. This is perhaps because late-pregnancy particles in the maternal circulation have been assumed to be dead material. To our knowledge, ours are the first data that physically connect circulating microparticles to syncytial knots by suggesting that shed syncytial aggregates are the intermediary form. Third, if syncytial knots give rise to circulating sFlt1-expressing microparticles through the shed aggregates, and if these phenomena are quantitatively stronger in PE, our data suggest that accelerated syncytial knot formation is a proximal event in the pathogenesis of PE, in agreement with previous reports.16 Regardless, it is unlikely that syncytial knots within the intact placenta simply represent an artifact of tangential sectioning, as has also been proposed.16

Important questions remain for future investigation. Metabolically active microparticles appear to begin forming in the first trimester.24 Does PE, therefore, represent the same process, but accelerated? If so, what fraction of peripheral sFlt1 protein is contributed by viable sFlt1-expressing microparticles versus dead/inactive microparticles that are already preloaded with sFlt1 protein? Also, what PE-specific mechanisms drive the induction of knot formation, deportation of syncytial aggregates, and microparticle generation? Conversely, could enhanced sFlt1 production somehow trigger syncytial knot formation? Second, we present novel evidence that living syncytial aggregates arise from syncytial sprouting and fission, but the molecular apparatus of nuclear aggregation and cytokinesis remains to be described. Third, different mechanisms for sFlt1 export from the placenta have been described, including the shedding of dead syncytial material17 and the release of matrix-bound sFlt1 by matrix-dissolving enzymes, such as heparinase.16 Although upregulation or accumulation of heparinase has not been shown to occur in the preeclamptic human placenta,16 it will be of interest to determine the relative contributions of these processes to maternal sFlt1 exposure. Fourth, we have established some of the biological capacity of syncytial aggregates by demonstrating de novo gene expression, but other functions, including regulation of inflammation and immunity, may also be important.38 It would also be important to determine whether proinflammatory stimuli and other factors, such as angioten- sin autoantibodies, that have been linked with PE pathogenesis may induce syncytial knot and microparticle formation. Finally, it has been suggested that shed syncytial aggregates get trapped in the capillary beds of lung tissue, where they further undergo disaggregation or apoptosis/necrosis to release the smaller microparticles into the systemic circulation.39–41 The relative contribution of these processes to the formation of trophoblast microparticles within the maternal circulation remains to be determined.

Perspectives

The present studies show that syncytial knots in the third-trimester placentas may give rise to biologically active microparticles that can translate packaged sFlt1 mRNA into protein. In turn, this process may be a novel means by which sFlt1 and other toxic proteins, such as soluble endoglin, may be delivered into the maternal circulation, where they mediate the major manifestations of PE. Our findings not only have direct implications for the care of women and unborn children with this disease but may also advance our understanding of fundamental cell biological processes. Future work on the basic biology of syncytialization may shed clues on the molecular defect in PE.42,43

Acknowledgments

We thank Dawn McCullough and Saira Sallaludhin for help in sample procurement and Saumya Hegde and Poorna Natarajan for technical assistance.

Sources of Funding

This work was funded by National Institutes of Health grants RO3 HD055219-03 (to A.R.) and P01 HD030367 (to C.A.H. and A.J.). S.R. is supported by a Harvard Diversity and Community Partnership Faculty Fellowship Award. The Gulbenkian Programme for Advanced Medical Education is sponsored by Fundação Calouste Gulbenkian, Fundação Champalimaud, and Ministério da Saúde e Fundação para a Ciência e Tecnologia (Portugal). S.A.K. is an investigator of the Howard Hughes Medical Institute.

Disclosures

S.A.K. 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.K. reports having served as a consultant to Roche and Beckman Coulter and has financial interest in Aggamin LLC. The remaining authors report no conflicts.

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Transcriptionally Active Syncytial Aggregates in the Maternal Circulation May Contribute to Circulating Soluble Fms-Like Tyrosine Kinase 1 in Preeclampsia
Augustine Rajakumar, Ana Sofia Cerdeira, Sarosh Rana, Zsuzsanna Zsengeller, Lia Edmunds, Arun Jeyabalan, Carl A. Hubel, Isaac E. Stillman, Samir M. Parikh and S. Ananth Karumanchi

Hypertension. 2012;59:256-264; originally published online January 3, 2012;
doi: 10.1161/HYPERTENSIONAHA.111.182170
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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http://hyper.ahajournals.org/content/59/2/256

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**Manuscript Title:** Transcriptionally active syncytial aggregates in the maternal circulation may contribute to circulating sFlt1 in preeclampsia

Figure S1: Expression of endoglin in synytial aggregates

Figure S2: Expression of sFlt1 mRNA in peripheral blood mononuclear cells

Figure S3: *De novo* protein synthesis in cultures of syncytial aggregates

Figure S4: Endothelial tube formation assay using syncytial debris
**Figure S1.** Expression of endoglin in syncytial aggregates
Placental washes obtained from normal (Panels A-B) and preeclamptic placentas (C-F) were analyzed for endoglin expression by immunohistochemistry.
Figure S2. *sFlt1* expression in peripheral mononuclear cells
Northern blot analysis of total RNA isolated from peripheral blood mononuclear cells (PBMCs) women with normal pregnancy and preeclampsia. Five microgram of total RNA from 10 patients in each group were pooled and 50 µg of total RNA per lane was analyzed. As a control 15 µg of total placental RNA from a single preeclamptic patient was used.
Supplementary Figure S3

Figure S3. De novo protein synthesis in cultures of syncytial aggregates

Placental syncytial aggregates obtained from 12 Netwells were pooled and incubated with methionine and cysteine free DMEM with 200 µCi of $^{35}$S (Easy Tag™ Express $^{35}$S Protein Labeling Mix, Perkin Elmer, Waltham, MA) for 72 hrs. Total protein was prepared from the debris pellet in 1X Laemmli buffer (10 mM Tris, pH 6.8, 1% SDS, 10% glycerol) with protease inhibitors. The cell extract and the culture supernatant were diluted 1:1 with RIPA buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium Deoxycholate, 0.1% SDS and protease inhibitors) and precleared using protein A/G agarose (Santa Cruz Biotech) for 30 min. The clarified supernatants were incubated with 1.0 µg of human VEGFR1 antibody (mouse monoclonal, V4262, Sigma Chemicals co., St Louis, MO). The immune complexes were captured with protein A/G agarose and after 3 washes with RIPA buffer and the bound proteins were eluted in 1X Laemmli buffer. Proteins were separated on a SDS containing 8% polyacrylamide gel and transferred on to a PVDF nitrocellulose membrane. After coating the membrane three times with with ENHANCE™ (Perkin Elmer, Waltham, MA) the membrane was exposed for autoradiography for 5-7 days.

Immunoprecipitated $^{35}$S-labeled sFlt1 from the syncytial aggregates (lane 1) and supernatant (lane 2) is shown in the figure.
Supplementary Figure S4. *Endothelial tube formation assay using syncytial debris*

Twenty thousand human umbilical vein endothelial cells (HUVEC) were plated onto Matrigel-pre-coated wells of a 48 well plate. These were then incubated at 37 °C for 12-16 hours with 200µl of post ultracentrifugation pellets (after brief sonication) from preeclamptic placental explants (n=4) that were resuspended in 200µl of 2.5% serum containing media with or without addition of 200 ng of exogenous VEGF. Tube formation was then quantified using an inverted phase-contrast microscope at X40 (Nikon TE 300, Nikon Corporation). Images were acquired using a Leica DFC350FX camera controlled with LeicaFirecam version 1.5 (Leica Microsystems Imaging Solutions). Total length of tube-like structures per microscopic field was enumerated using Image J freeware (Image J, National Institutes of Health).

A representative micrograph of control and debris treated is shown in Panels A-B. The anti-angiogenic effect of the debris could be reverted by pre-incubating with recombinant VEGF, panel C. Quantitation of the tube formation is shown in Panel D. *p<0.05 by ANOVA.