Abstract—Maternal spiral artery remodeling is the consequence of controlled trophoblast invasive interaction with the maternal cellular environment and is fundamentally important for successful placentation. In preeclampsia, trophoblast invasion is shallow, remodeling is incomplete, and vessels develop an inflammatory appearance, termed “acute atherosis.” We noted that, in our preeclampsia, human renin-human angiotensinogen transgenic rat model, complement component 3 (C3), and tumor necrosis factor-α were upregulated and heavily expressed in atherotic uteroplacental vessels. We next used coculture involving human trophoblasts, rat vascular smooth muscle cells (VSMCs), and human VSMCs to observe VSMC-trophoblast regulatory interactions. Tumor necrosis factor-α induced complement C3 and interleukin-6 expression in VSMCs. We found that trophoblasts were able to reduce VSMC C3 and interleukin-6 expression after the VSMCs were stimulated with tumor necrosis factor-α. However, a direct VSMC-trophoblast cell-cell contact was necessary for this anti-inflammatory response. We also studied double-transgenic VSMCs that express inflammatory components and exhibit accelerated proliferation (“synthetic” phenotype). Trophoblasts could not downregulate C3 in these cells. We then examined uteroplacental tissues from preeclamptic and control patients. In control deciduas, only traces of C3 staining were observed, and vessels were thin walled without thrombus formation. In preeclampsia, the decidual vessels showed atherosis, thrombus formation, and C3 expression. Our data suggest that fatally derived trophoblasts require direct cell-cell contact with maternally derived VSMCs to downregulate VSMC C3 and interleukin-6 expression and to avoid atherosis. The findings also implicate C3 in the placental vasculopathy observed in preeclampsia. (Hypertension. 2008;51[part 2]:554-559.)

Key Words: vascular remodeling ■ complement ■ preeclampsia ■ trophoblast ■ acute atherosis

P reecclampsia remains the most common cause of maternal and fetal morbidity worldwide and also represents a major long-term cardiovascular risk factor for mother and child.1 Trophoblast invasion and maternal uterine vessel adaptation are fundamental to normal pregnancy. Reduced trophoblast invasion may play an initial role in preeclampsia that is subsequently proselytized by other mechanisms.2 Trophoblasts originate from the external layer of the developing blastocyst, invade the maternal decidua, and subsequently reach the spiral arteries, where vascular remodeling is necessary for the tremendous product interchange occurring between mother and fetus. The vascular smooth muscle cells (VSMCs) are partially replaced or transdifferentiated so that the spiral arteries are converted to low-resistance, high-capacity vessels.3 In preeclampsia, this process seems to be impaired. The resultant vascular morphology has some resemblance to atherosclerosis and was termed “acute atherosis” by early investigators.4 Preeclampsia is associated with a local inflammatory response and so is normal pregnancy, albeit to a lesser extent.5 We showed earlier that complement component 3 (C3) participates in angiotensin (Ang) II–induced vasculopathy.6 VSMCs from double-transgenic rats (dTGRs) harboring the human renin and angiotensinogen genes showed increased proliferation and C3 expression compared with controls. Tumor necrosis factor (TNF)-α induced C3 mRNA slightly in control VSMCs but markedly in dTGR VSMCs. Girardi et al7 showed that complement activation is a required intermediary event in the pathogenesis of placental and fetal injury that leads to intrauterine growth retardation. In vitro studies on the trophoblast-VSMC interaction are sparse, particularly as related to preeclampsia. We used coculture techniques to focus on this interaction and

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speculated that VSMCs could produce inflammatory mediators, such as C3 and interleukin (IL)-6, that might be regulated by trophoblasts.

Methods

Patients, Trophoblast Preparation, and Placental Villous Explants Supernatant

We obtained placentas from normal pregnant and preeclamptic women who where delivered by cesarean section. Our committee on the protection of human subjects (institutional review board) approved the study, and written informed consent was obtained. Women were considered preeclamptic by the new onset of hypertension (blood pressure >140/90 mm Hg) and proteinuria (>300 mg/d or 2+ dipstick) in the third trimester of pregnancy.8

Primary trophoblasts were isolated from human term placental villous tissue according to Ugele et al8 and have been described earlier.9 Nontrophoblastic cells were removed by immunoadsorption with monoclonal mouse anti-human HLA-ABC antigen (Dako-Cytomation), bound to Dynabeads pan-mouse IgG antibody (Dynal Biotech). This procedure resulted in a population of trophoblasts of >95% viability, assessed by trypan blue, and >95% purity, as identified by the presence of rabbit anti-cytokeratin 7 (diluted 1:500, Dako). Isolated trophoblasts were immediately seeded for experiments.

Placental villous explants from control and preeclamptic placentas were cultured in cell dishes, as described earlier.10 After 24 hours, the supernatant was collected and immediately snap frozen.

The Preeclampsia Rat Model and VSMC Preparation

We studied a preeclamptic rat model (female TGR, human angiotensinogen [hAOGEN]×male TGR, human renin [hREN]) and age-matched control rats (Sprague-Dawley [SD]×SD) at day 21 of pregnancy. Dams harboring the hAOGEN gene develop hypertension and proteinuria in the second half of pregnancy when mated with sires harboring the hREN gene as described earlier.12 The converse cross was not affected. We also used VSMCs from male transgenic rats harboring the human renin and angiotensinogen genes (dTGRs) and male control rats (SD). American Physiology Society guidelines for animal care were obtained (permit G.0268/06). Aortic VSMCs were isolated from male dTGRs and male SD rats, as described previously.6 VSMCs from passages 2 to 8 were used for experiments.

Male human aortic VSMCs were purchased from Lonza and used according to the manufacturer’s protocol. We used human VSMCs only in the experiment for Supplemental Figure S2 (available online at http://hyper.ahajournals.org).

Coculture Experiments

Each coculture experiment used trophoblasts and VSMCs obtained from individual specimens. VSMCs were cultured together with trophoblasts. This coculture was performed over 72 hours without changing the media. VSMCs were isolated from individual specimens. VSMCs were cultured together with trophoblasts and treated identically to the coculture situation. All of the groups of each experiment were performed as 6 replicates. Each experiment was performed ≥6 times. Transwell membranes (Corning) with 0.4-μm pore sizes were used to prevent cell-cell contact; only the conditioned medium was able to overcome the membrane pores. VSMCs were seeded in the lower and trophoblasts at the upper one.

We isolated total mRNA with the TRIzol (Invitrogen) method according to the manufacturer’s protocol and as described earlier.13 For quantification, the target sequences were normalized in relation to the glycerinaldehydephosphate dehydrogenase product. By using Primer Express 2.0 (Applied Biosystem), primer and probes (Biotek) were designed to recognize exclusively cDNA from Rattus norvegicus and are given in Table S1.

The target gene expression was related to the expression in the TNF-α treated VSMC monoculture, which was set to 100%. The mRNA expression results are expressed as means±SEM. All of the data were tested with SPSS 12.0 for normal distribution with the Kolmogorov-Smirnov test. Normally distributed data were analyzed with the t test. Otherwise, the Mann-Whitney test was applied. P<0.05 was considered statistically significant.

We seeded human trophoblasts and/or rat VSMCs on LabTek chamber slides (Nalgene Nunc), fixed the cells with methanol, and immediately stored them at −20°C. Staining was performed with 1:500 diluted fluorescein-conjugated goat IgG fraction to rat complement C3 (Cappel). Preparations were examined under a Zeiss Axioscope-2 microscope, and digital images were acquired with AxioVision 3.0. The same experiment was performed with human trophoblasts and/or human VSMCs. The rat complement C3 2-site ELISA (Kamiya Biomedical) and the rat IL-6 sandwich ELISA (R&D Systems) were used according the manufacturer’s protocol to measure C3 or IL-6 protein in the rat VSMC culture supernatant.

Immunohistochemistry

We sampled human decidua tissue underlying the placenta from control and preeclamptic patients (5 patients per group) with a vacuum suction technique and embedded in paraffin, as described previously.14 A 5-μm serial section was stained with hematoxylin/eosin to reveal spiral artery athertic lesions or was stained with C3 (1:100, clone B-9, Santa Cruz Biotechnology), which was detected with a 1:500 diluted Cy3-labeled secondary antibody (Jackson Immunoresearch).

Rat placentas with the attached mesometrial triangle were collected from pregnant SD and pregnant preeclamptic rats. After snap freezing, the tissue was serial sectioned and stained with fluorescein-conjugated IgG fraction to complement C3 (1:1000, Cappel); smooth muscle-α actin (1:50, clone CP 47, Calbiochem) was detected with a 1:500 diluted Cy3-conjugated secondary antibody (Jackson Immunoresearch) or TNF-α (1:50, clone M-18, Santa Cruz Biotechnology) was detected with a 1:500 diluted fluorescein isothiocyanate–conjugated secondary antibody (Jackson Immunoresearch).

Please see the data supplement for extensive immunohistochemistry protocols.

Results

We searched for complement C3 expression in uteroplacental vessels from preeclamptic rat dams harboring the hAOGEN gene that had been mated with hREN-bearing sires. We stained for complement C3 expression (green) and smooth muscle-α actin (red) in rat uteroplacental vessels (Figure 1A). Complement C3 expression was increased in vessels of preeclamptic transgenic rats compared with controls. Fluorescence microscopy showed that C3 and smooth muscle-α actin were colocalized in VSMCs in the vessel media. In SD controls, α-actin was clearly visible; however, no C3 staining was observed. Furthermore, rat uteroplacental TNF-α expression (red) was increased in preeclamptic vessels (transgenic rat [hAOGEN]×transgenic rat [hREN]) compared with controls (Figure 1B). This expression was most prominent in the media, but also in the adventitia. SD controls showed weak TNF-α staining. These findings raise the possibility that the complement system and its inflammatory pathways are involved in the preeclampsia model.

We next established a coculture model (Figure S1). Small round trophoblasts (star) and larger VSMCs with pseudopodia (arrow) are shown with phase contrast microscopy in Figure S1A. Representative quantitative RT-PCR amplification by TaqMan RT-PCR showed that C3 mRNA expression was confined to rat VSMCs, as shown in Figure S1B. No C3 expression was detected in human trophoblasts using the rat
primer/probe set. This finding shows that our rat primer/probe set detected only rat C3 expression. The human and rat C3 primer sequences used in establishing the coculture model were different, as shown in Figure S1C, so that cross-reactions between the human and rodent mRNA can be excluded. These findings outline the coculture model and document that the C3 source that we investigated in this study is from rat VSMCs and not from human trophoblasts.

We next searched for C3 expression in our coculture model and showed quantitative C3 mRNA expression by TaqMan RT-PCR (Figure 2). Under basal conditions, coculture with trophoblasts had no effect on VSMC C3 expression, as shown in Figure 2A. C3 expression was induced by TNF-α in VSMCs. Coculture with trophoblasts reduced C3 expression by ~50% (P < 0.05). The same coculture conditions that included introducing a transwell membrane that precluded direct cell-cell contact blocked any decrease in C3 expression in VSMCs. These findings suggest that trophoblasts influence VSMCs to inhibit their production of C3. A transwell filter blocks this effect, suggesting that cell-cell contact and not secreted molecules are responsible. The protective effect is confined to primary trophoblasts; immortalized trophoblasts or cell lines (eg, the choriocarcinoma BeWo cell line) were ineffective (data not shown). Figure 2B corroborates the notion that conditioned medium from control villous explants was not responsible, because its presence did not abrogate C3 mRNA expression in the face of TNF-α.

As shown in Figure 2C, with an expanded ordinate, conditioned medium of preeclamptic villous explants increased C3 expression of VSMCs. TNF-α evoked a response double in magnitude. However, in the presence of conditioned “preeclamptic” medium and TNF-α, the C3 expression of these cells was raised to double the magnitude observed in any of our experiments.

We next investigated rat VSMCs obtained from hypertensive dTGRs (Figure 2D). These VSMCs have been termed “synthetic” by others, because of their propensity to produce inflammatory mediators, compared with VSMCs from control SD rats. Again, these data indicate that TNF-α induced C3 expression in these cells. Contact with trophoblasts could not ameliorate C3 expression in the hypertensive VSMCs. These studies suggest that human trophoblasts can reduce the inflammatory activity (in terms of C3 expression) of VSMCs by direct contact and not via the medium. The medium from preeclamptic placental explants, in contrast,
provides a stimulatory effect. The C3 production of primed
VSMCs exhibiting a “synthetic” phenotype is not ameliorated
by contact with human trophoblasts.

We next investigated C3 protein expression by immuno-
cytochemistry. We found (Figure S2) that C3 protein expres-
sion in rat VSMCs was not detectable in nonstimulated rat
VSMC monoculture, as shown in Figure S2A. TNF-α in-
duced a green cytoplasmatic staining, which is clearly visible
above the autofluorescence background (Figure S2B). A
coculture has been conducted in the presence of TNF-α. The
photomicrograph shows that the VSMCs (as opposed to
Figure S2B) show no cytoplasmatic C3 staining. To corrob-
orate these findings from rat VSMCs, we performed similar
experiments from human VSMCs. Trophoblasts reduced
TNF-α–induced C3 protein expression in human VSMCs.
These immunocytochemical experiments corroborate the
above findings shown on the mRNA level, namely that
human trophoblasts can modulate VSMC inflammatory re-
sponses in terms of generating C3 mRNA and the respective
C3 protein.

We next examined quantitative IL-6 mRNA expression to
supplement our complement findings (Figure 3). Coculture
with trophoblasts had no effect on IL-6 expression in VSMCs
under basal expression. IL-6 expression was induced by
TNF-α in VSMCs and could be significantly reduced by
coculture with trophoblasts. Thus, the inflammatory response
was not confined to C3 but could also be demonstrated for
IL-6. We show in Figure 3B that IL-6 and C3 expression
correlate significantly in our coculture model.

We then studied other components of the complement
cascade. We first looked for C5 and C7 expression in VSMCs
to determine whether the C5 to C9 membrane attack complex
was activated in our system. We could not find any expres-
sion of C5 or C7 after TNF-α stimulation in our VSMCs,
whether they were accompanied by human trophoblasts (data
not shown). To corroborate C3 protein expression above and
beyond protein immunohistochemistry, we used an ELISA
for C3 (Figure 4A). C3 was released at very low levels in the
supernatant of VSMC cells. The expression was markedly
increased after TNF-α stimulation. IL-6 was also secreted
from VSMCs (Figure 4B). The release in the supernatant was
induced by TNF-α. We next examined decidual tissues from
preeclamptic and control patients. The top part of Figure 4C
shows control decidua with only traces of C3 staining. The
vessel cross-section appears healthy without thrombus forma-
in. In contrast, the bottom portion of the panel shows a
thrombus-occluded decidual spiral artery vessel of a pre-
eclamptic pregnancy with features of acute atherosis. Immu-
nohistochemistry shows ample C3 expression in this same
evessel wall.

**Discussion**

We observed that C3 and TNF-α were expressed in the
placental artery walls of female preeclamptic rats. We found
that invading trophoblasts modulate the VSMC inflammatory
response in terms of producing C3 and IL-6. This protective
effect requires direct cell-cell contact. Moreover, the super-
natant of preeclamptic placental villous explants potentiates

Figure 3. A, Quantitative IL-6 mRNA
expression is shown. Coculture with tro-
phoblasts had no effect on IL-6 expres-
sion in VSMCs under basal expression.
IL-6 expression was induced by TNF-α in
VSMCs and could be reduced by cocul-
ture with trophoblasts (P < 0.05). B, IL-6
and C3 expression correlate significantly
in our coculture model.

Figure 4. A, ELISA for C3 was performed.
C3 was released in the supernatant of
VSMCs. The expression was increased after
TNF-α stimulation. B, ELISA for IL-6 was
performed. IL-6 is also secreted from
VSMCs. TNF-α increased the release of
IL-6. C, Decidual tissue stained with hema-
toxylin/eosin (left) and for C3 (right). Deci-
dual tissues from a control pregnancy show
normal vessels and only trace C3 staining
(top). On the other hand, decidual tissues
from a preeclamptic patient showed acute
atherosis with vascular thrombus occlusion.
A parallel section showed prominent C3
staining in the vessel wall (bottom; scale
bar: 100 μm).
C3 expression. Finally, we observed that the C3 complement fraction is present in the vascular wall of uteroplacental vessels in human preeclampsia. Normal pregnancy is a unique situation of immunologic tolerance. We suggest that invading trophoblasts of fetal origin modulate innate immunity in VSMCs of maternal origin and downregulate production of inflammatory mediators and propose that cell-cell contact is necessary for this purpose. Perturbation of this process plays a permissive role in atherosclerosis and preeclampsia development.

Successful placentation is the consequence of controlled trophoblast interactions with the maternal cellular environment. Extravillous trophoblasts migrate into the maternal spiral arteries, partially replacing endothelial cells and the musculoelastic tissue that normally maintains vessel integrity. This process converts the uterine spiral arteries into low-resistance, high-capacity vessels allowing sufficient blood flow across the maternal-fetal interface to meet the demands of the growing fetus. Interstitial invasion of the trophoblasts precedes endovascular invasion and brings the trophoblast into direct cell-cell contact with VSMCs. The mechanism whereby trophoblasts interact with VSMCs and replace the maternal endothelial lining of uterine arteries is unknown. A limited trophoblast invasion and incomplete maternal vessel remodeling has been associated with both preeclampsia and fetal growth restriction, whereas an excessive trophoblast invasion is associated with invasive mole, placenta acrera, and choriocarcinoma. Few reports have specifically examined interactions between trophoblasts and spiral artery VSMCs. Most studies have been limited to immunohistochemical analysis of placental and uterine tissues or placental explant models. We developed our coculture cell model to examine interactions between pure populations of VSMCs and trophoblasts.

Campbell et al used a bilayer coculture model between endothelial cells and trophoblasts. They showed that endothelial cells downregulated trophoblast migration in normal pregnancy, whereas trophoblasts from preeclamptic pregnancies altered endothelial matrix metalloproteinases. Cartwright et al developed a coculture model, where fluorescent-labeled trophoblasts were seeded over artery segments embedded in fibrin gels or perfused into arteries mounted on a pressure myograph. They found that trophoblast prepared the decidual spiral artery for endovascular trophoblast migration. Red-Horse et al studied the vascular effects of invasive human trophoblasts in vivo by transplanting placent al villi to the fifth mammary fat pads or beneath the kidney capsules of severe combined immune deficiency mice. They observed robust trophoblast interactions with resident murine blood vessels with remodeling. Inflammation and reactive oxidative species production are a part of normal pregnancy. According to the “free radical theory of development,” the fetoplacental unit depends on differential oxygen supplies, gradients in the intracellular redox state and a “physiological inflammatory status.” We observed TNF-α expression in the control rat placenta; however, the expression was clearly increased in the preeclamptic cross. Redman and Sargant introduced the concept that the inflammatory response is exacerbated in preeclampsia and may account for its clinical features. The disorder could develop when the maternal system fails to respond to fetal anti-inflammatory commands. A similar low-grade systemic inflammatory response characterizes adults with hypertension, arterial disease, obesity, or diabetes, which are conditions that also strongly predispose to preeclampsia. Such constitutions could possibly lead to an abnormal maternal response to pregnancy. In our coculture model, VSMCs derived from the dTGR hypertensive model behaved differently than control VSMCs. Their proatherogenic response to TNF-α could not be reduced by trophoblast coculture. The molecular mechanisms leading to the failure of trophoblasts to downregulate C3 in hypertensive VSMCs are unknown. Disturbed cell-cell communication between trophoblasts and hypertensive VSMCs may be responsible. Chronic exposure to Ang II or genetic alterations induces increased expression of genes involved in proliferation and inflammation, such as reduced nicotinamide-adenine dinucleotide phosphate oxidase and adhesion molecules in hypertensive VSMCs. Furthermore, these VSMCs are more susceptible to TNF-α-induced nuclear factor κB activation.

Trophoblasts reduced C3 and IL-6 expression to TNF-α stimulation in control VSMCs by means of cell-cell interaction. Cell-cell interaction is probably reduced in conditions with shallow trophoblast invasion that lead to intrauterine growth retardation. As a consequence, acute atherosclerosis involves acute inflammatory-like fibrinoid necrosis and lipid-laden foam cells could develop. In preeclampsia, these lesions develop over a shorter time period than in chronic hypertension-related vasculopathies occurring in nonpregnant women. Acute atherosclerosis in a placental biopsy specimen that contains ≥1 spiral artery occurs on average in 30% of placental bed biopsies from preeclamptic women. We found complement upregulated C3 both in acute spiral artery atherosclerosis from preeclamptic patients and in our preeclamptic rat model. C3 is important in immune defenses but may also have several other biological functions in cell differentiation and proliferation. C3 is increased in atherosclerotic lesions and contributes to the development of atherosclerosis in vivo. We have shown previously that complement activation is a major participant in mediating Ang II-induced end-organ damage in vivo and identified TNF-α as an important inducer of C3. Lin et al reported that young spontaneous hypertensive rats showed increased C3 expression and increased VSMC proliferation. Both were blocked by C3 downregulation. Lin et al speculated that the C3 gene could be responsible for the synthetic phenotype and exaggerated growth of VSMCs from spontaneous hypertensive rats. The C3 could influence surrounding cells in the vessel, such as endothelial cells or leukocytes.

Sinha et al were the first to show that Clq, C3, and C9 are increased in preeclamptic placentas and suggested that a complement pathway-mediated immune process contributes to the condition. Recently, Girardi et al showed that complement activation is required as an intermediary event in the pathogenesis of placental and fetal injury leading to intrauterine growth retardation and that complement activation causes dysregulation of the angiogenic factors required for normal placental development. We were not able to demonstrate
activation of the membrane attack complex in our coculture model and did not test this hypothesis in the course of these studies.

**Perspectives**

Vascular remodeling is a key feature in the normal maintenance of any cardiovascular system. We demonstrated that disturbed cell-cell communication between trophoblasts and VSMCs can lead to functional abnormalities in VSMCs, including a proatherogenic response. Cell contact most likely involves surface adhesion molecules, gap junction proteins, integrins, and integrin receptors. We are now in a position to investigate the nature of this cell-cell interaction.

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None.

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