Online Supplement

Autoantibody-mediated complement C3a receptor activation contributes to the pathogenesis of preeclampsia

By

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Expanded Methods

Blood pressure measurement
The systolic blood pressure was non-invasively measured by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT) \(^1-3\). Blood pressure was measured at the same time daily (±1 hour) while the mice were kept warm using a warming pad.

Quantification of Proteinuria
We quantified urinary albumin by ELISA (Exocell, Philadelphia, PA) and measured urinary creatinine by a picric acid colorimetric assay kit (Exocell, Philadelphia, PA). We used the ratio of urinary albumin to urinary creatinine as an index of urinary protein.\(^1-5\)

Histological analysis
We harvested placentas and kidneys of mice and fixed them in 4% formaldehyde overnight at 23 °C. Tissues were infiltrated and embedded in paraffin. We cut 4-μm serial sections and stained them with H&E by standard techniques.\(^6-7\) Placental histological quantification was carried by quantifying the number of calcifications/field under ×10 magnification. Placental sections were examined under the microscope and the number of calcifications was counted in each field and then plotted as number of calcifications recorded per field.

C3 and C3aR immunostaining in mouse placentas and kidneys and human placentas
Mouse placentas and kidneys were harvested on GD18 and human placenta explants were collected after 24 hours incubation. Human placentas were isolated immediately after delivery. Tissues were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Sections of 4μm were cut and stained with anti-mouse C3 (cat. no. LS-C22283, LifeSpan BioSciences, WA), anti-human C3 (cat. no. sc-20137, Santa Cruz, CA), and anti-human & mouse C3aR (cat. no. sc-20138, Santa Cruz, CA) at a dilution of 1:100 in a humidified chamber at 4 °C overnight. Following the primary antibody incubation, anti-mouse IgG HRP detection kit (cat. no.551011, BD Pharmingen, San Diego, CA) or anti-rabbit ABC Staining System (cat. no. sc-2018, Santa Cruz) were used to detect the C3 staining. The immunohistochemical staining for C3 (brown) was quantified by Image-Pro Plus software (Media Cybernetics, Bethesda, MD). The intensity of the brown staining (positive for C3) was measured, and the average densities of 6–10 areas per placenta was determined and averaged to get a mean value.

CD34 dual immunostaining in placenta and quantification
Mouse Placentas were harvested on GD18 and human placenta explants were collected after 24 hours incubation. Tissues were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Sections of 4μm were cut and stained with anti-mouse CD34 (cat. no. 553731, BD Pharmingen) or anti-human CD34 (cat. no. 555820, BD Pharmingen) at a dilution of 1:100 in a humidified chamber at 4 °C overnight. Following the primary antibody incubation, rat IgG ABC Kit (cat. no. AK-5004, Vector Laboratories, Burlingame, CA) or mouse IgG ABC Kit (cat. no.551011, BD Pharmingen) were used to incubate the slides at room temperate for 30
minutes. Then Alkaline Phosphatase Substrate Kit (cat. no. SK-5100, Vector Laboratories, Burlingame) was used to detect the C3 staining. The fluorescence red staining for CD34 was quantified by Image-Pro Plus software (Media Cybernetics) as before. The density of the red staining (positive for C3) was measured, and the average densities of 6–10 areas per placenta was determined to get a mean value.

**Human placental villous explant collection and culture**

Human placentas were obtained from normotensive patients who underwent an elective term cesarean section at Memorial Hermann Hospital in Houston. The explant culture system was conducted as before. On delivery, the placentas were placed on ice and submerged in phenol red-free DMEM containing 0.2% BSA and 1.0% antibiotics. 5 to 7 chorionic villous explant fragments were carefully dissected from the placenta and transferred to 24-well plates for an overnight equilibration period at 37°C and 5% CO2. All of the initial processing occurred within 30 minutes of delivery. The next day, the explants were incubated with IgG from normotensive pregnant women (NT-IgG; 1:10 dilution), IgG from PE patients (PE-IgG; 1:10 dilution) ± SB290157 (C3aR antagonist, 1μM, cat. no. #559410, Calbiochem) or SB290157 (1μM) alone. The dosage of SB290157 was adapted from experiments described previously. After 24 hours, the collection medium was siphoned and stored at -80°C, and the villous explants were lysed or fixed overnight in 10% formalin for embedding in paraffin wax for further analysis.

**Western blot analysis**

C3 protein in human and mice placentas was detected by western blotting analysis. Frozen tissues were used for protein extraction. We ran proteins on 8% SDS-PAGE gels and transferred the gels to a nitrocellulose membrane. We then incubated membranes with commercially available antibody to the C3 (1:200, cat. no. sc-20137, Santa Cruz) as the primary antibody.

**Enzyme-linked immunosorbent assays for sFlt-1**

The concentrations of sFlt1 in mouse plasma and human placenta explants supernatant were determined quantitatively using commercial kits (R&D Systems).
References:


Supplementary Data and Figure Legends

Figure S1. **C3aR protein in mouse placenta.** C3aR was examined by immunohistochemistry (scale bar=200 μm). Placenta tissue from NT-IgG injected pregnant mice was incubated with C3aR antibody (right) or blocking serum (left, negative control). The brown staining indicates C3aR signal. C3aR protein was evident in trophoblast cells of mouse placenta.

Figure S2. **C3aR protein in human placenta.** C3aR was examined by immunohistochemistry (scale bar=100 μm). Placenta tissue from normotensive pregnant women was incubated with C3aR antibody (right) or blocking serum (left, negative control). The brown staining indicates C3aR signal. C3aR protein was evident in trophoblast cells of human placenta.