Neutrophils Infiltrate Resistance-Sized Vessels of Subcutaneous Fat in Women With Preeclampsia

Courtney E. Leik, Scott W. Walsh

Abstract—We examined if there is systemic vascular inflammation and neutrophil infiltration in women with preeclampsia. Resistance-sized vessels (10 to 200 μm) of subcutaneous fat were evaluated from normal nonpregnant women, normal pregnant women, and preeclamptic women. Immunohistochemical staining was performed for: (1) interleukin-8 (IL-8), a potent neutrophil chemokine; (2) intercellular adhesion molecule-1 (ICAM-1; CD54), an endothelial cell adhesion molecule; and (3) CD66b, a neutrophil antigen. Vessels of preeclamptic patients had intense IL-8 staining in the endothelium and vascular smooth muscle, as compared with little or no staining for normal pregnant and normal nonpregnant patients. ICAM-1 was expressed on the endothelium of all patient groups. In preeclamptic patients, ICAM-1 was also expressed on vascular smooth muscle. Vessels of preeclamptic patients had significantly more CD66b staining of neutrophils than did normal pregnant or normal nonpregnant patients. There were significantly more vessels stained, more vessels with neutrophils flattened and adhered to endothelium, more vessels with neutrophils infiltrated into the intima, and more neutrophils per vessel. In conclusion, in women with preeclampsia, there was significant infiltration of neutrophils into maternal systemic vasculature associated with inflammation of the vascular smooth muscle indicated by increased expression of IL-8 and ICAM-1. Neutrophil infiltration provides a reasonable explanation for endothelial and vascular smooth muscle dysfunction in preeclampsia because neutrophils produce toxic substances, which may explain clinical symptoms. (Hypertension. 2004;44:1-6.)

Key Words: neutrophils • preeclampsia • interleukins • cell adhesion molecules • endothelium • adipose tissue

Neutrophils mediate innate immunity to protect an individual from infection by generation of reactive oxygen species (ROS) and release of granules containing proteolytic enzymes.1 These toxic products kill bacteria, but they can also damage host tissue. There is accumulating evidence of neutrophil involvement in inflammatory diseases not associated with infection.

Preeclampsia is a hypertensive disorder of human pregnancy and a leading cause of maternal and fetal morbidity and mortality.3 Clinically, it is diagnosed as maternal hypertension with proteinuria. Pathological edema is usually present. The pathophysiology of preeclampsia is associated with neutrophil activation,4 oxidative stress,5,6 and endothelial cell dysfunction.7 Greer et al first reported that neutrophils were activated by measuring a significant increase in neutrophil elastase in the maternal circulation.8 Subsequently, Tsukimori et al and others reported a significant increase in superoxide production by neutrophils obtained from preeclamptic women.9-11 Barden et al reported upregulation of neutrophil β-integrins.12 Neutrophil activation likely occurs as they circulate through the intervillous space and are exposed to oxidized lipids secreted by the placenta.5,13,14 Although neutrophils are activated, their role in the pathogenesis of preeclampsia is not known. One role could be to act as cellular carriers of oxidative stress from the placenta to the maternal circulation by adhering and infiltrating maternal systemic vasculature.

Neutrophil infiltration into vascular tissue requires vascular expression of a chemotactic agent to attract neutrophils, endothelial expression of adhesion molecules to bind neutrophils to the endothelium, as well as neutrophil activation. To study if neutrophils adhere to endothelium and infiltrate systemic resistance-sized vessels in women with preeclampsia, we used immunohistochemistry to evaluate: (1) vascular smooth muscle expression of interleukin-8 (IL-8), a potent neutrophil chemotactic agent and activator of neutrophils; (2) endothelial expression of intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule that binds β-integrins on the neutrophil surface, causing them to adhere and flatten onto the endothelium before infiltration; and (3) neutrophil activation and infiltration into systemic vascular tissue. We evaluated neutrophils in preference of other leukocytes because: (1) they are the most abundant of the leukocytes; (2) their numbers increase in pregnancy;3 (3) their numbers...
further increase in preeclampsia; and (4) they produce toxic substances (thromboxane, ROS, myeloperoxidase, and tumor necrosis factor-α), which could be responsible for vasoconstriction and vascular cell dysfunction. We hypothesized that in women with preeclampsia, there would be increased expression of vascular smooth muscle IL-8 and endothelial ICAM-1 coincident with infiltration of neutrophils into maternal systemic vascular tissue.

Methods

Subcutaneous fat biopsy samples were collected from patients at MCV Hospitals of Virginia Commonwealth University Medical Center. Fat biopsy samples were collected at the time of cesarean section from normal pregnant patients (n=6) and patients with preeclampsia (n=5), or at the time of abdominal or minimally invasive surgery from normal nonpregnant patients (n=4). Preeclampsia was defined according to criteria of the American College of Obstetricians and Gynecologists. Women in active labor were excluded from the study. All patients were nonsmokers. Informed consent was obtained before surgery. This study was approved by the Virginia Commonwealth University Office of Research Subjects Protection. Procedures were in accordance with institutional guidelines.

Subcutaneous fat biopsy samples were used because subcutaneous fat is a highly vascularized tissue representative of the systemic vasculature and it is easily obtained at the time of surgery. Vessels of subcutaneous fat were stained for IL-8, ICAM-1, and CD66b, a granulocyte-specific antigen. CD66b is upregulated and secreted on granulocyte activation. CD66b staining primarily represents neutrophils because they comprise 96% of the granulocyte population.

Details on patients, fat collection, immunohistochemistry, data analysis, and statistical analysis are available in an online supplement at http://www.hypertensionaha.org.

Results

Clinical data for normal nonpregnant, normal pregnant, and patients with preeclampsia are summarized in the Table.

<table>
<thead>
<tr>
<th>Clinical Data for Patient Groups</th>
<th>NNP (n=4)</th>
<th>NP (n=6)</th>
<th>PE (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, y</td>
<td>40.2±6.1*</td>
<td>27.5±4.5</td>
<td>24.6±5.5</td>
</tr>
<tr>
<td>Prepregnancy BMI</td>
<td>31.0±4.2</td>
<td>26.6±6.5</td>
<td>29.2±7.9</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>128.2±9.4</td>
<td>119.5±12.6</td>
<td>178.0±12.8†</td>
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<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>81.4±9.3</td>
<td>76.7±8.2</td>
<td>111.0±12.3†</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>ND</td>
<td>ND</td>
<td>3.7±0.6 (n=3)</td>
</tr>
<tr>
<td>Parity</td>
<td>NA</td>
<td>2.8±1.2</td>
<td>1.2±1.1*</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>NA</td>
<td>39.1±0.8</td>
<td>33.7±3.8†</td>
</tr>
<tr>
<td>Infant birth weight, g</td>
<td>NA</td>
<td>3291±533.8</td>
<td>2004±1027*</td>
</tr>
</tbody>
</table>

Values are mean±SD.

NNP indicates normal nonpregnant; NP, normal pregnant; PE, preeclamptic; ND, not determined; NA, not applicable.

*P<0.05.

†P<0.01.

Results for IL-8, ICAM-1, and CD66b are shown in Figure 1. Normal nonpregnant patients had little or no vascular staining (Figure 1A). Staining results for IL-8 are shown in Figure 2. Normal nonpregnant patients had little or no vascular staining (Figure 2A). Staining intensity for ICAM-1 was significantly lower for NP patients than for PE or NNP patients. Numbers in parentheses represent OD measurements (background OD = 50). ODs were highly correlated with staining scores (IL-8, r=0.92; ICAM-1, r=0.56; CD66b, r=0.84). Data are expressed as mean±SE of total vessels scored for each patient group. ***P<0.001.
2a), whereas normal pregnant patients had light staining, including light staining in vascular smooth muscle (Figure 2b). Serial sections of a patient with preeclampsia are shown in Figure 2c to 2f, with staining for IgG as negative control, factor VIII to identify endothelial cells, IL-8, and α-smooth muscle actin to identify vascular smooth muscle cells. In contrast to normal nonpregnant and normal pregnant patients, patients with preeclampsia had intense IL-8 staining in endothelium, as well as intense IL-8 staining in vascular smooth muscle. Figure 2g shows another example of intense IL-8 staining in vascular smooth muscle verified by staining for α-smooth muscle actin in a serial section (Figure 2h).

Staining results for ICAM-1 are shown in Figure 3. In normal nonpregnant women, ICAM-1 was primarily localized to endothelial cells (Figure 3a). In normal pregnant women, ICAM-1 was primarily in the endothelium, with some on the vascular smooth muscle (Figure 3b). In women with preeclampsia, ICAM-1 was present not only on the endothelium but also on the vascular smooth muscle. Serial sections of a patient with preeclampsia are shown in Figure 3c to 3f with staining for IgG as negative control (Figure 3c), factor VIII (Figure 3d), ICAM-1 (Figure 3e), and α-smooth muscle actin (Figure 3f) demonstrating intense diffuse staining of ICAM-1 in the vascular smooth muscle. Figure 3g shows another example of ICAM-1 staining of the vascular smooth muscle verified by staining for α-smooth muscle actin in Figure 3h.

To verify specificity of the CD66b antigen for neutrophils, leukocytes were isolated from blood by histopaque density centrifugation. Neutrophils showed intense staining for CD66b, whereas monocytes and lymphocytes did not stain.

The percentage of vessels stained for CD66b was significantly greater for patients with preeclampsia than normal pregnant or normal nonpregnant patients (Figure 4a). There was also greater adherence and flattening of neutrophils along
Figure 3. Representative sections of vessels in subcutaneous fat immuno-stained for ICAM-1. a, Normal nonpregnant. b, Normal pregnant. c to f, Sequential sections of a PE patient (c, IgG-negative control; d, factor VIII; e, ICAM-1; f, α-smooth muscle actin). g and h, Sequential sections of a PE patient (g, ICAM-1; h, α-smooth muscle actin). Vessels of normal nonpregnant showed brown staining of ICAM-1 in the endothelium, but not vascular smooth muscle. Leukocytes also express ICAM-1; the bold arrow indicates one stained in the vessel lumen. Vessels of normal pregnant showed staining for ICAM-1 primarily in endothelial cells. Vessels of PE showed intense and diffuse brown staining for ICAM-1 of endothelium and vascular smooth muscle. Staining for α-smooth muscle actin in sequential sections confirmed ICAM-1 staining of vascular smooth muscle in PE. (VL indicates vessel lumen; VSM, vascular smooth muscle; EC, endothelial cell. All images are 400 x magnification. Scale bars indicate 50 μm.

Figure 4. Staining of resistance-sized vessels for CD66b, a neutrophil marker. Normal nonpregnant and normal pregnant vessels had little neutrophil involvement as compared with PE vessels. In PE, there were significantly more vessels with staining for neutrophils (a), more vessels with neutrophils flattened and adhered to endothelial cells (b), more vessels with neutrophils infiltrated into intimal space (c), and higher number of neutrophils per vessel (8-μm-thick section) (d). Data are expressed as mean±SE. **P<0.01, ***P<0.001.
the endothelium (Figure 4b) and greater infiltration into the intima (Figure 4c). The total number of neutrophils adhered to endothelium and infiltrated into intima per stained vessel was greater for patients with preeclampsia (Figure 4d).

Representative sections of vessels for CD66b staining of neutrophils are shown in Figure 5. There was no staining for IgM-negative controls (data not shown). Few vessels for normal nonpregnant (Figure 5a) and normal pregnant patients (Figure 5b) had staining for CD66b except for occasional staining of neutrophils in the lumen or lightly attached to the endothelium. Stained neutrophils in normal nonpregnant and normal pregnant patients were generally rounded and it was rare to find neutrophils flattened to endothelium or infiltrated into the intima. In contrast, there was massive neutrophil involvement of vessels of women with preeclampsia with neutrophil adherence and flattening onto endothelium and infiltration into the intima. In some cases, vessels were almost occluded with neutrophils (Figure 5c through 5h).

Discussion

Staining for IL-8, a potent neutrophil chemotactic agent, was significantly greater in women with preeclampsia than normal pregnant or normal nonpregnant women. IL-8 staining was observed on vascular smooth muscle, in addition to endothelium, in women with preeclampsia. This finding is significant because it demonstrates inflammation of vascular smooth muscle in preeclampsia, and increased expression of vascular smooth muscle IL-8 establishes a concentration gradient for IL-8 from the circulation to the vascular smooth muscle. This provides a mechanism for transendothelial migration of neutrophils to the vascular smooth muscle because neutrophils migrate along a concentration gradient to IL-8.17

ICAM-1 was expressed on the endothelium in all groups. Endothelial ICAM-1 expression appeared to be downregulated in normal pregnancy. The most notable observation for ICAM-1 was that in patients with preeclampsia, ICAM-1 was expressed intensely on vascular...
smooth muscle. This corroborates the IL-8 data that there is inflammation of vascular smooth muscle in preeclampsia, because ICAM-1 is expressed by a variety of tissues under conditions of inflammation. Other investigators have reported markers of systemic inflammation in women with preeclampsia. The percentage of vessels stained for CD66b was significantly greater in women with preeclampsia than in normal pregnant or normal nonpregnant women. There were significantly more vessels with neutrophils adhered and flattened along the endothelium and within the intima in women with preeclampsia as compared with controls. The percentage of vessels with neutrophils present within different portions of the vessel correlated with an overall greater number of neutrophils per vessel. Diffuse vessel staining also was present in women with preeclampsia and, most likely, reflected secretion of CD66b from activated neutrophils or neutrophils undergoing apoptosis.

These new data may explain previous findings in preeclampsia. A study by Roggensack et al showed increased expression of nitrotyrosine, suggestive of peroxy nitrite formation, a strong prooxidant, in the vasculature of women with preeclampsia. A simultaneous increase in the expression of endothelial nitric oxide synthase and a decrease in the expression of superoxide dismutase also were observed. Our findings may explain the source of superoxide for the formation of peroxy nitrite, because the rapid interaction of neutrophil superoxide with endothelial nitric oxide in the presence of deficient superoxide dismutase would produce peroxy nitrite. This would result in an endothelial deficiency of nitric oxide, which could result in increased vaso motor tone and hypertension. Our data, in conjunction with Roggensack’s data, strongly suggest that there is localized systemic oxidative stress throughout the maternal vasculature, which would result in lipid peroxidation leading to vascular inflammation and dysfunction.

Perspectives
The present study is the first to our knowledge to show that there is vascular smooth muscle inflammation and neutrophil infiltration into maternal systemic vascular tissue in preeclampsia. Neutrophil infiltration could explain clinical symptoms of hypertension, proteinuria, and edema.

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References
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