Role of leukocytes in uterine hypoperfusion and fetal growth retardation induced by ischemia-reperfusion

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Received 16 June 2000; accepted in final form 23 October 2000

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Role of leukocytes in uterine hypoperfusion and fetal growth retardation induced by ischemia-reperfusion. Am J Physiol Heart Circ Physiol 280: H1215–H1221, 2001.—We investigated leukocyte involvement in uterine hypoperfusion and intruterine fetal growth retardation (IUGR) induced by ischemia-reperfusion (I/R) in Sprague-Dawley rats. On day 17 of gestation, leukocyte accumulation in the uterus and placenta subjected to 30 min of ischemia, followed by reperfusion, was assessed by measuring myeloperoxidase (MPO) activity. Uterine MPO activity was significantly higher after 1 h of reperfusion than it was before ischemia (P < 0.05), without any increase in placental MPO activity. Immunohistochemical staining showed leukocyte accumulation in the uterus subjected to I/R. The effects of treatment with monoclonal antibodies against CD11a (WT1) and CD18 (WT3) at a dose of 0.8 mg/kg on uterine blood flow and IUGR were investigated. Laser-Doppler flowmetry demonstrated that uterine hypoperfusion at 2 h after ischemia (blood flow, −51.7 ± 1.2%; P < 0.01) was inhibited by WT1 and WT3 treatment. I/R-induced IUGR at full term (P < 0.05 vs. nonischemic horn) was prevented by WT1 and WT3 treatment on day 17. These results indicate that leukocyte accumulation may play an important role in the pathogenesis of uterine hypoperfusion and IUGR induced by I/R in pregnant rats.

UTEROPLACENTAL CIRCULATION plays an important role in the growth and health of the fetus, and decreased uteroplacental blood flow with a reduced oxygen supply and nutrients to the fetus is considered to be one of the major factors associated with idiopathic intruterine fetal growth retardation (IUGR). It has been demonstrated that the severity of IUGR is related to the extent of placental infarct. The infarcted placenta shows a lobular distribution of the area of ischemic necrosis, resulting from spiral artery occlusion (33). In addition, placental infarction seems to be associated with regional changes in blood flow within the placenta (29). These findings suggest that ischemia-reperfusion may occur in some placental lobules in IUGR and exacerbate this complication of pregnancy. Recently, Ishimoto et al. (15) and Tanaka et al. (32) developed a new model of IUGR produced by transient ischemia of the uteroplacental circulation, followed by reperfusion. In this model, delayed hypoperfusion in uterine blood flow was observed during the reperfusion period, which is considered to be a cause of the development of IUGR.

Leukocyte-endothelial cell interactions have been implicated in the pathogenesis of ischemia-reperfusion injury in various organs, including the heart, lung, and skeletal muscle (3, 14, 16, 18). In particular, leukocyte-endothelial cell adhesion appears to be a prerequisite for the development of vascular injury. Recent studies on ischemia-reperfusion injury have shown that prevention of leukocyte adhesion can attenuate postschismic tissue damage. Crinnion et al. (6) reported that administration of antibodies against CD11/CD18 glycoproteins, which play a major role in leukocyte adhesion to endothelial cells, improved postschismic muscle reperfusion. Clinical investigations (2, 5, 10, 19, 24) have provided evidence that activated leukocytes are associated with the development of IUGR and hypertensive disorders of pregnancy. Furthermore, several studies have shown that decreased uteroplacental blood flow is involved in the genesis of these pregnancy disorders. Thus we hypothesized that leukocytes may contribute to the development of reduced uterine blood flow.

The current study was designed to investigate the involvement of leukocytes in uterine hypoperfusion and IUGR induced by ischemia-reperfusion in pregnant rats. We evaluated leukocyte accumulation in the uterus and placenta subjected to ischemia-reperfusion. In addition, we determined whether treatment with monoclonal antibodies (MAb) against CD11a and CD18 has any effect on postischemic uterine hypoperfusion and ischemia-reperfusion-induced IUGR.
LEUKOCYTES AND UTERINE BLOOD FLOW

MATERIALS AND METHODS

Surgical preparation of the animal model. Sprague-Dawley rats weighing 230–300 g were purchased from a local breeder (Sankyo Lab Service; Tokyo, Japan). The rats were housed in an environmentally controlled vivarium and had free access to a standard pellet diet and water. Animal care complied with the guidelines for the care and use of laboratory animals of Keio University School of Medicine. Ischemia-reperfusion of the uteroplacental circulation was performed as reported previously (15). Briefly, on day 17 of pregnancy, a low abdominal midline incision was performed on rats anesthetized with halothane (3% for induction, and then 0.5–1.0% for maintenance). Each rat was placed on a heating pad to maintain the deep rectal temperature between 36.5 and 37.0°C. After the uterine horns were inspected, the uterine vessels near the lower and upper ends of the right horn were occluded with two small artery clamps. The clamps were removed after 30 min of ischemia.

Evaluation of leukocyte accumulation in uterus and placental tissue during ischemia-reperfusion. Four uteri and placenta of the right horn were excised preischemia, after 30 min of ischemia, and after 60 and 120 min of reperfusion (n = 6–9 rats). Tissue-associated myeloperoxidase (MPO) activity was measured as a biochemical estimate of leukocyte accumulation by a modification of the method of Grisham et al. (11). Briefly, the excised uterus and placenta were quick-frozen separately in isopentane and then stored at −80°C until analysis. The tissue was then thawed, weighed, and homogenized in 10 vol of ice-cold 20 mM potassium phosphate buffer (pH 7.4). Two milliliters of tissue homogenate were sonicated for 10 s in 8 ml of ice-cold 50 mM potassium phosphate buffer (pH 7.4) and centrifuged at 20,000 g for 20 min. The supernatant was discarded, and the pellet was resuspended in 2 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma; St. Louis, MO) and recentrifuged. MPO activity was assessed by measuring the H₂O₂-dependent oxidation of 3,3′, 5,5′-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO present that produced an absorbance change of 1.0 at 655 nm at pH 7.4 and 37°C per minute. The concentration of protein in the uterine and placental homogenates was measured by using a protein assay reagent kit (Micro BCA, Pierce; Rockford, IL). The unit of enzyme activity was expressed as units per minute per milligram of tissue protein.

Immunohistochemical staining. The uterus and placenta in the ischemic horn were excised before ischemia and after 30 min of ischemia, followed by 120 min of reperfusion. The tissues were fixed in periodate-lysine paraformaldehyde for 8 h. Dissected samples were frozen in an optimum cold-temperature compound (Tissue-Tek, Miles; Elkhart, IN) in isopentane that had been precooled in liquid nitrogen. Then 6-mm cryostat sections were prepared, air-dried, and blocked in normal goat serum for 30 min at room temperature. The sections were incubated with an appropriate diluted mouse MAb WT1 directed against CD11a (Seikagakukogyo; Tokyo, Japan) overnight at 4°C. After the sections were washed in phosphate-buffered saline (PBS), the sections were then incubated with biotinylated goat anti-mouse IgG (Amersham; Buckinghamshire, UK) for 1 h at room temperature, washed in PBS, and incubated with Streptavidin-Fluorescein (Amersham) for 30 min at room temperature.

Contributions of CD11a and CD18 to leukocyte accumulation in uterus and placenta subjected to ischemia-reperfusion. In the treatment group (n = 5 rats), MAb WT1 or WT3 directed against CD18 (Seikagakukogyo) at a dose of 0.8 mg/kg iv in a volume of 1-ml PBS was administered 10 min before inducing ischemia. The control group was composed of rats receiving vehicle alone in the same manner (n = 5). On the basis of other studies (25) on ischemia-reperfusion injury using MAb WT1 and WT3 and in our preliminary experiments, we used the minimal effective dose of each MAb. To assess the contributions of CD11a or CD18 to leukocyte accumulation in the uterus and placenta, MPO activity in the uteruses and placentas subjected to 30 min of ischemia and 120 min of reperfusion in these groups was measured as described above. In this experiment, 0.5 ml of blood were collected from the femoral vein and the number of circulating total leukocytes and polymorphonuclear leukocytes (PMN) were counted immediately before ischemia and after 120 min of reperfusion.

Effects of MAb against CD11a and CD18 on uterine blood flow during ischemia-reperfusion. Uterine blood flow was measured by laser-Doppler flowmetry as described previously (15). Briefly, pregnant rats (day 17) were anesthetized with halothane (3% for induction, then 0.5–1.0% for maintenance) and placed on a heating pad to maintain a body temperature close to 37°C. The femoral artery was cannulated for monitoring mean arterial pressure (MAP). The femoral vein was also cannulated for drug administration. After a midline incision was made, right uterine blood flow was measured with a laser-Doppler flowmeter (model LBF-3F, Biomedical Science; Kanazawa, Japan), avoiding areas with large vessels. After the rats were stabilized, MAb WT1 or WT3 at a dose of 0.8 mg/kg in a volume of 1-ml PBS was administered intravenously 10 min before onset of ischemia in the treatment group (n = 5 rats). The right uterine horn was then subjected to 30 min of ischemia, followed by 120 min of reperfusion. In the control group (n = 5), rats received vehicle alone in the same manner. MAP and uterine blood flow were continuously monitored and recorded with a computer-based data acquisition system (model MP100WS, Biopac System; Galeta, CA). The blood flow data were averaged every 5 min and expressed as the percent change from preischemic blood flow (10-min period before occlusion of uterine vessels).

Effects of MAb against CD11a and CD18 on fetal growth. On day 17 of pregnancy, rats were given MAb WT1 or WT3 at a dose of 0.8 mg/kg in a volume of 1-ml PBS intravenously 10 min before ischemia in the treatment group (n = 5 rats). Control rats received the same amount of vehicle (n = 5). Ischemia was induced in the right uterine horn for 30 min as described above. After reperfusion, each animal received an intraperitoneal injection of 50 mg of cefazolin sodium (Cefamezine, Fujisawa Pharmaceuticals; Osaka, Japan), and the incision was closed. On day 21 of gestation (4 days after the start of reperfusion), the live fetuses and placentas were delivered by cesarean section with the dam anesthetized with halothane (3% for induction, and then 0.5–1.0% for maintenance) and were weighed to the nearest 0.001 g. The ratio of the mean fetal and placental weights in the ischemic horn to that of the nonischemic horn was calculated.

Statistical analysis. All values are expressed as means ± SE. Comparisons of the mean fetal and placental weights in the ischemic and nonischemic horns were made with the unpaired Student’s t-test. Data on MPO activity and leukocyte counts were initially analyzed by using a one-way ANOVA. Dunnett’s post hoc test was used to determine which groups were statistically different from the preischemic value. ANOVA for repeated measures was used to compare uterine blood flow and MAP in the treatment group versus the control group. When a significant difference was detected, an unpaired Student’s t-test was used to compare
values obtained from the two groups at the same time interval. Differences in uterine blood flow between preischemia and each reperfusion period for the vehicle-treated control group were analyzed by using a one-way ANOVA, followed by Dunnett’s post hoc test. A $P$ value of $<0.05$ was considered statistically significant.

RESULTS

Evaluation of leukocyte accumulation in uterine and placental tissue during ischemia-reperfusion. The preischemic MPO activity of uterus and placenta was $56 \pm 9.0$ and $32 \pm 6.0$ U·mg$^{-1}$·min tissue protein$^{-1}$, respectively. The MPO activity in the uterus significantly increased at 60 and 120 min of reperfusion, compared with preischemic values ($P < 0.05$). In contrast, placental MPO activity did not change substantially throughout the experimental period (Fig. 1). We observed no infiltration of CD11a-positive leukocytes into the uterine tissue before ischemia (Fig. 2A). In contrast, samples obtained from rats after 30 min of ischemia and 120 min of reperfusion exhibited the infiltration of CD11a-positive leukocytes in the uterine tissue (Fig. 2B).

Effects of MAb against CD11a and CD18 on leukocyte accumulation in uterus and placenta subjected to ischemia-reperfusion. MPO activity of uterine tissues in the vehicle-treated control and the MAb WT1- and WT3-treated groups are shown in Fig. 3. Intravenous administration of MAb WT1 or WT3 at a dose of 0.8 mg/kg significantly suppressed the increase in uterine MPO activity at 120 min of reperfusion ($P < 0.01$). The levels of uterine MPO activity in the MAb WT1- and WT3-treated groups did not differ significantly from the preischemic level. There were no significant differences in placental MPO activity between the control and the treated groups (data not shown). The number of circulating total leukocytes and PMNs remained unchanged.
Effects of MAb against CD11a and CD18 on uterine blood flow during ischemia-reperfusion. Changes in uterine blood flow relative to the preischemic level measured by laser-Doppler flowmetry are shown in Fig. 4. In the vehicle-treated control group, uterine blood flow decreased gradually and reached 78.2 ± 6.0% of the preischemic level at 60 min of reperfusion \((P < 0.01 \text{ vs. the preischemic level, respectively})\). Additionally, uterine blood flow at 90 and 120 min of reperfusion were 71.1 ± 4.7 and 48.2 ± 1.2% of the preischemic level, respectively \((P < 0.01 \text{ vs. the preischemic level, respectively})\). In contrast, treatment with MAb WT1 or WT3 prevented the development of uterine hypoperfusion. In our study, the relative uterine blood flow during the ischemic period in the control and MAb WT1- and WT3-treated groups was 25.5 ± 0.32, 36.76 ± 7.62, and 30.88 ± 2.91% of the preischemic levels, respectively, with no significant difference observed. There were no significant differences in the change of MAP between the control and the treatment groups during the experimental periods (Fig. 5).

Effects of MAb against CD11a and CD18 on fetal and placental weights. In the vehicle-treated control group, the mean fetal weight was significantly increased in the ischemic horn compared with the nonischemic horn \((3.191 ± 0.072 \text{ and } 3.684 ± 0.161 \text{ g, respectively, } P < 0.05)\). Similarly, the mean placental weight in the ischemic horn was significantly lower than that of the nonischemic horn \((0.349 ± 0.0018 \text{ and } 0.421 ± 0.0018 \text{ g, respectively, } P < 0.05)\). The mean fetal-to-placental weight ratio in the ischemic horn to nonischemic horn was 86.6 ± 3.9 and 82.8 ± 1.9%, respectively. In contrast, administration of MAb WT1 and WT3 significantly inhibited the reductions in fetal body and placental weights induced by ischemia-reperfusion. In the MAb WT1- and WT3-treated groups, the mean fetal and placental weights in the ischemic horn did not differ significantly from those in the nonischemic horn (Figs. 6 and 7).

### Table 1. Effects of treatment with monoclonal antibodies against CD11a and CD18 on total leukocyte and PMN counts

<table>
<thead>
<tr>
<th></th>
<th>Total Leukocyte Counts</th>
<th>PMN Count</th>
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<tr>
<td></td>
<td>Before</td>
<td>120 min after reperfusion</td>
</tr>
<tr>
<td>I/R</td>
<td>5,925 ± 543</td>
<td>5,062 ± 469</td>
</tr>
<tr>
<td>I/R + WT1</td>
<td>5,124 ± 878</td>
<td>5,920 ± 998</td>
</tr>
<tr>
<td>I/R + WT3</td>
<td>6,193 ± 938</td>
<td>7,183 ± 911</td>
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Values are means ± SE of 5 dams in each group. PMN, polymorphonuclear neutrophil; I/R, ischemia-reperfusion plus vehicle; I/R + WT1, ischemia-reperfusion plus WT1 treatment; I/R + WT3, ischemia-reperfusion plus WT3 treatment. Monoclonal antibodies (MAb) against CD11a (WT1) or CD18 (WT3) at a dose of 0.8 mg/kg or vehicle were administered intravenously 10 min before inducing ischemia. Number of circulating total leukocytes and polymorphonuclear leukocytes was counted immediately before ischemia and after 120 min of reperfusion. Treatment with MAb WT1 or WT3 did not affect total leukocyte and PMN counts.

**DISCUSSION**

Reduced uterine blood flow is considered to be involved in the development of IUGR and hypertensive disorders of pregnancy, such as preeclampsia and pregnancy-induced hypertension (PIH). For example, clinical investigations (27, 28) that used Doppler ultrasound showed that abnormal uterine artery Doppler velocimetry reflecting reduced uterine blood flow is observed in these pregnancy disorders. In addition, it has been reported (21, 31) that trophoblastic invasion of the uterine spiral arteries in the uteroplacental bed is commonly inadequate, providing anatomic evidence...
for decreased maternal blood supply to the placenta. In the present study, we confirmed that a delayed hypo-perfusion of uterine tissue was induced by transient ischemia of the uteroplacental circulation, as reported previously (15). The Wigglesworth system, in which ligation of a single uterine artery produces a progressive decrease in fetal weight, has been widely employed for the creation of IUGR in animals (7, 33). However, changes in uterine blood flow cannot be examined by using the Wigglesworth system. To our knowledge, there is no useful in vivo system for investigating factors associated with uterine hypoperfusion that cause IUGR. Thus this newly developed ischemia-reperfusion model of IUGR will be useful to investigate leukocyte involvement in the genesis of decreased uterine blood flow. Several authors (8, 20) reported a relationship between fetal weight and placental blood flow in the animal model of IUGR, showing that fetal growth is regulated by the rate of placental blood flow. In our model, postischemic uterine hypoperfusion may cause a decrease in placental blood flow. Thus reperfusion-induced uterine hypoperfusion appears to be one of the major factors associated with the genesis of IUGR in our model.

Several studies (2, 5, 10, 19, 24) have demonstrated that activated leukocytes are associated with development of IUGR, preeclampsia, and PIH. Johnston et al. (19) reported that the concentration of neutrophil elastase in maternal plasma is elevated in pregnancies with IUGR, suggesting that leukocyte activation and degranulation occur in this disorder. Similar results have been demonstrated in pregnancies with preeclampsia and PIH. In other organs, recent studies (3, 14, 16, 18) on ischemia-reperfusion injury have demonstrated that reperfusion-induced leukocyte adhesion to endothelium is an essential step in postischemic perfusion impairment. Thus we hypothesized that leukocytes may contribute to the development of decreased uterine blood flow.

The present study demonstrates that MPO activity is significantly increased in uterine tissue subjected to 30 min of ischemia and 60 min of reperfusion. Because tissue-associated MPO activity is considered to be a standard indirect measure of leukocyte infiltration (11), our results indicate that leukocytes infiltrate uterine tissue after 60 min of reperfusion. In contrast, placental MPO activity did not increase during the reperfusion period, indicating that leukocyte accumulation did not occur in placentas subjected to ischemia-reperfusion. The histological observation that there are more CD11a-positive leukocytes in this uterine tissue supports our data regarding MPO activity. Similarly, it has been demonstrated (4, 9) that leukocyte activation is confined to the maternal circulation, and significantly increased numbers of elastase-positive neutrophils may be found in the basal decidual region of pregnancies with PIH. Although the causes for the lack of leukocyte accumulation in the placenta remain to be clarified, an antiadhesive mechanism that prevents leukocyte accumulation may exist in placental tissue.

A growing body of evidence indicates that leukocyte adherence to endothelium may contribute to the development of postischemic perfusion impairment. Recent studies have demonstrated that leukocyte integrin CD11/CD18 has an essential role in establishing this adhesive interaction. The CD11/CD18 glycoproteins are classified into a family of three heterodimers, each consisting of variable \(\alpha\)-subunit (CD11a, CD11b, and CD11c) and a common \(\beta_2\)-subunit (CD18). For exam-
ple, pretreatment with MAb to CD11a, CD11b, or CD18 has been shown to prevent or attenuate reperfusion-induced tissue damage and postischemic perfusion impairment in other organs (6, 16, 18, 26, 30, 35). However, the role of leukocyte adhesion in uterine perfusion impairment has not been investigated. Thus we evaluated the effects of MAb to CD11a (WT1) and CD18 (WT3) on uterine blood flow during ischemia-reperfusion. In the present study, treatment with WT1 and WT3 prevented the postischemic hypoperfusion in uterine blood flow. Because administration of these antibodies before ischemia suppressed leukocyte accumulation in uterine tissues at 120 min of reperfusion, CD11/CD18-dependent adherence appears to be involved in the development of uterine hypoperfusion induced by ischemia-reperfusion. Furthermore, our results show that administration of WT1 and WT3 before ischemia inhibits the reduction in the mean fetal weight in the ischemic horn. Recently, Barden et al. (2) reported that neutrophils derived from women with preeclampsia showed increased expression of CD18 and CD11b, compared with those from normal pregnant women. Maemura et al. (24) demonstrated that expression of CD18, CD11a, and CD11b was elevated in pregnancies with IUGR or preeclampsia compared with normal pregnancies. Thus our results provide additional evidence that leukocytes may contribute to the genesis of reduced uterine blood flow.

Previous experiments have shown that immunoneutralization of P selectin and intercellular adhesion molecule-1 (ICAM-1) reduces leukocyte accumulation and postischemic perfusion impairment in other organs, suggesting that these adhesion molecules may contribute to leukocyte-dependent perfusion impairment (17, 23). Of the mediators that have been implicated in reperfusion-induced leukocyte accumulation, platelet-activating factor (PAF) is also shown to have an important role in ischemia-reperfusion injury in other organs (22). In the clinical setting of pregnancy complications, several authors (1, 13) reported that the serum from patients with preeclampsia shows increased ICAM-1 expression, supporting the hypothesis that the endothelial cells are activated in hypertensive disorders of pregnancy. Recent preliminary studies in our laboratory have demonstrated that PAF antagonists are effective in preventing postischemic uterine hypoperfusion and IUGR (unpublished data). Further studies in our model are needed to elucidate the contributions of ICAM-1 and P selectin to the development of uterine perfusion impairment and IUGR.

The present study shows that uterine blood flow returns to the preischemic level early after reperfusion, and administration of MAb against CD11a and CD18 prevents the reperfusion-induced uterine hypoperfusion. In addition, our previous experiments (15) have demonstrated that oxygen-derived free radicals may contribute to the genesis of postischemic uterine hypoperfusion. Although the precise mechanism responsible for reperfusion-induced uterine hypoperfusion in our model remains to be clarified, some pathological events during reperfusion, including leukocyte adhesion to endothelium and production of reactive oxygen metabolites, may contribute to uterine perfusion impairment. In other organs, the compressive effect of edema secondary to leukocyte-dependent increases in microvascular permeability has been shown to be the major cause of this problem in tissues that cannot expand as fluid accumulates in the interstitial spaces (18, 26). On the other hand, reduced deformability and swelling of endothelial cells and leukocytes during ischemia-reperfusion appear to promote luminal occlusion of venules by leukocyte plugging (12). The formation of interstitial edema may have little effect on uterine perfusion impairment, because uterine tissue can be expanded in our model.

In conclusion, the present study shows that leukocyte accumulation significantly increases in uterine tissue during the reperfusion period. Furthermore, we observed that treatment with a MAb to CD11a or CD18 prevents postischemic uterine hypoperfusion and ischemia-reperfusion-induced IUGR. Our data suggest that leukocytes may play an important role in the genesis of uterine hypoperfusion and IUGR induced by ischemia-reperfusion in pregnant rats.

This work was supported in part by a grant from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

12. Gute D and Korthuis RJ. Role of leukocyte adherence in reperfusion-induced microvascular dysfunction and tissue injury. In: Physiology and Pathophysiology of Leukocyte Adhesion,


