Hypoxia-Induced Upregulation of CD11b Expression in Granulocytes


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Abstract. The aim of this study is to evaluate leukocyte-endothelial adhesion during ischemia/reperfusion injury. Polymorphonuclear neutrophils (PMN) were isolated from healthy volunteers and incubated in the presence of 2% O₂ (hypoxia) or 21% O₂ (normoxia) at 37°C for two hours. In some experiments, whole blood was subjected to hypoxia or normoxia prior to PMN isolation. Flowcytometric analysis and adhesion assays were carried out using these PMN. Moreover, adhesion assay was carried out using PMN, prepared from the patients who underwent infrarenal aortic aneurysmectomy with aortic clamping at various time points as an in vivo model of ischemial reperfusion injury. Flowcytometric analysis revealed an increased expression of CD11b in PMN subjected to hypoxia compared with those subjected to normoxia prior to isolation. Interestingly, these phenomena were not observed with PMN isolated prior to being subjected with hypoxia. Enzyme-linked immunosorbent assay (ELISA) using serum prepared from whole blood subjected to hypoxia revealed elevated levels of tumor necrosis factor (TNF)-α compared with those subjected to normoxia. PMN obtained during aneurysmectomy exhibited an increased adhesion to activated human umbilical vein endothelial cells (HUVEC), compared with those taken from the same patients prior to the operation. In contrast, PMN prepared after aortic clamp, exhibited a significant reduced adhesion to activated HUVEC. Hypoxic condition, induced CD11b expression on PMN in vitro. PMN subjected to hypoxic condition in vivo exhibited an increased adhesion to activated endothelium. Elevated level of serum TNF-α may be involved in this phenomenon.

Introduction

Ischemia/reperfusion (I/R) injury has been one of the most serious problems after vascular reconstruction in chronic critical limb ischemia or acute arterial ischemia. Numerous studies suggested that leukocyte-endothelial interactions played an important role in pathogenesis of these conditions. The release of mediators due to hypoxia may result in the expression of surface adhesion molecules on endothelial cells [1,2] as well as polymorphonuclear neutrophils (PMN) [1,3,4]. It has recently been shown that hypoxia increases the adherence of granulocytes to endothelial cells through an increased production of platelet-activating factor (PAF) by hypoxic endothelial cells [1]. Korthuis et al. [5] have achieved leukocyte depletion using leukocyte filter, as the result, prevented the increases in vascular permeability and vascular resistance. Crinnion et al. [6] have reported that neutrophil recruitment and muscle infarction are reduced using anti-MAC 1 monoclonal antibody. Monoclonal antibodies (mAb) to CD11b appear to have a beneficial effect in canine myocardial injury [7]. So far, the importance of PMN-endothelial interaction has been emphasized in I/R injury [8,9], but no direct evidence has been provided whether hypoxia directly affects PMN function or not.

The aim of this study is to assess the possible effects of hypoxia on I/R and we also extended our research utilizing a sample obtained from patients who underwent an elective aneurysmectomy to investigate in vivo effect of PMN adhesion to vascular endothelial cells after I/R.

Materials and Methods

Reagents

RPMI-1640, Dulbecco’s phosphate buffered saline (DPBS) and Hank’s balanced salt solution (HBSS) were obtained from Sigma. Fetal bovine...
serum (FBS) was purchased from GIBCO BRL (Grand Island, NY). Paraformaldehyde was purchased from Fisher Scientific (Springfield, NJ). Recombinant human interleukin (IL)-1β was obtained from Biogen (Cambridge, MA). Biscarboxyethyl-carboxyfluorescein acetoxymethyl ester (BCECF) was purchased from Molecular Probes (Eugene, OR). mAb to CD11a, CD11b, CD18, and sialyl Lewis x (sLex) were obtained from Pharmingen (San Diego, CA), mAb to L-selectin was obtained from Serotec (Oxford, England). Human umbilical vein endothelial cells (HUVEC) were isolated and established in culture, as previously described by Yoshida et al. [10]. Primary cultures were serially passaged (≤ 1:3 split ratio) and maintained in Medium 199 buffered with 25 mmol/L Hepes and supplemented with 10% FBS, endothelial cell growth factor (25 μg/ml), and porcine intestinal heparin (50 μg/ml). For experimental use, subcultured (passage 2 or 3) endothelial cells were plated on gelatin-coated 35-mm tissue culture dishes.

### PMN Preparation

PMN were isolated from citrated blood drawn from normal volunteers using dextran sedimentation followed by Ficoll-hypaque centrifugation. Hypotonic lysis was used to remove erythrocytes. Then, PMN (1 x 10^6/ml) were subjected to a hypoxic condition (2% O_2) (hypoxia) or a normoxic condition (21% O_2) (normoxia) for 2 hours at 37°C. In some experiments, whole blood was directly subjected to a hypoxic condition or a normoxic condition for two hours at 37°C, then PMN were isolated as described above.

### Flowcytometric Analysis

PMN were prepared for flowcytometry using previously described methods [11]. Briefly, PMN (0.5 x 10^6 per condition) were washed with RPMI-1640 contained +1% FBS, and incubated in the presence of indicated mAb for 45 minutes. After three washings with RPMI + 1% FBS, fluorescein isothiocyanate (FITC)-goat anti-mouse polyclonal immunoglobulin (Ig) G was added at 1:200 dilution ratio and incubated for 45 minutes. After an additional three washings, the PMN were fixed with 3% paraformaldehyde and surface expression of adhesion molecules in PMN was measured by a fluorescence activated cell sorter (FACS) (Becton-Dickinson). A total of 10,000 cells were analyzed for each measurement.

### Adhesion Assay

A human leukocyte cell line, HL-60 cells, prelabeled with the fluorescent dye BCECF were added (1.6 x 10^6 cells/well in RPMI + 1% FBS) to monolayers of HUVEC in 35-mm dishes. After incubation under static adhesion assay conditions, nonadherent HL-60 cells were removed by washing three times with RPMI + 1% FBS, and the monolayer-associated HL-60 cells were collected into HBSS + 5 mM ethylenediaminetetraacetic acid (EDTA) and their fluorescence measured in a fluorescent plate reader (Perceptive Bysystems), as previously described (Yoshida M, 1996) [10].

### Tumor Necrosis Factor (TNF)-α Measurement

Whole blood drawn from healthy normal volunteers in the presence of anti-coagulant were subjected to a hypoxic condition (2% O_2) or a normoxic condition (21% O_2) for two hours at 37°C. Plasma fraction was collected after centrifugation at 2,500 r.p.m. for 15 minutes. TNF-α in plasma was measured using the human TNF-α ultrasensitive Enzyme-Linked Immunosorbent Assay (ELISA) (COSMO-BIO, Tokyo, Japan).

### PMN Preparation During Vascular Surgery Operation

Blood samples were collected from the patients who underwent an elective aneurysmectomy and a bifurcated graft replacement for infrarenal abdominal aortic aneurysm (n = 5) as described in Table 1 after obtaining informed consent. The abdominal aorta and bilateral external iliac arteries (EIA) were clamped while the proximal and distal anastomosis were accomplished. Blood samples were taken from the left femoral vein at four different time points, 1. prior to the aortic clamping (control), 2. just before the aortic declamp, 3. 5 minutes after the aortic declamp, 4. 20 minutes after the aortic declamp. PMN were isolated from each blood sample as described above and adhesion assay was carried out under static condition.

### Results

First, we examined whether hypoxia modulated surface expression of adhesion molecules on PMN. PMN were isolated from whole blood, drawn from healthy volunteers in the presence of anti-coagulant, then were subjected to a hypoxic condition or a normoxic condition for two hours at 37°C (PMN hypoxia). Flowcytometric analysis revealed that no significant changes were observed for CD11a, CD11b, CD18 expression between those subjected to hypoxia (black line) and normoxia (gray area) (Figure 1, PMN hypoxia). In contrast, when PMN were subjected to hypoxia prior to isolation from whole blood, expression of CD11b was significantly increased (Figure 1, whole blood hypoxia). The expression levels of other adhesion molecules (CD11a and CD18) were not changed under these assay conditions.

To evaluate adhesion capabilities of these PMN subjected to hypoxia prior to isolation from whole blood, adhesion assay was conducted under static condition. As shown in Figure 2, the number of adherent PMN to HUVEC activated with IL-1β for four hours were not significantly changed between these subjected to pre-isolation hypoxia and normoxia. Adhesion assay utilizing PMN subjected to post-isolation hypoxia also

### Table 1.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (y.o.)</th>
<th>Diagnosis</th>
<th>Comorbidity</th>
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<td>1.R. AAA, rt. IIAA</td>
<td>post gastrectomy</td>
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<tr>
<td>5.</td>
<td>Male</td>
<td>75</td>
<td>1.R. AAA</td>
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