Polymorphonuclear Leukocyte–Induced Vasocontraction and Endothelial Dysfunction

Role of Selectins

Toyoaki Murohara, Michael Buerke, Allan M. Lefer

Abstract The roles of selectin adhesion molecules (P- and L-selectin) and their counterreceptors sialyl Lewis a were investigated in polymorphonuclear leukocyte (PMN)–induced cat coronary vasocontraction and endothelial dysfunction. Unstimulated autologous PMNs (10⁶ cells/mL) were added to organ chambers containing cat coronary artery rings stimulated with either thrombin (2 U/mL) or hydrogen peroxide (100 μmol/L). PMNs elicited a significant vasoconstriction in thrombin- (119±14 μg) and hydrogen peroxide- (132±15 μg) stimulated coronary rings. This PMN-induced vasoconstriction was significantly attenuated by pretreatment with either an anti-P-selectin, an anti-L-selectin monoclonal antibody (ie, MAb PB 1.3 and MAb DREG-200), or a sialyl Lewis a–containing oligosaccharide (SLe a–OS). Endothelial function as assessed by endothelium-dependent vasorelaxation to acetylcholine was also significantly attenuated after PMN-induced vasoconstriction in stimulated coronary rings. This endothelial dysfunction was significantly prevented by either PB 1.3, DREG-200, or SLe a–OS. In contrast, endothelium-independent relaxation to acidified sodium nitrite was not altered by PMN incubation, indicating that vascular smooth muscle function was unaffected. Adherence of PMNs to coronary endothelium also significantly increased following stimulation of endothelium with either thrombin or hydrogen peroxide, but this was significantly attenuated by PB 1.3, DREG-200, or SLe a–OS. Thus, PMN-endothelial interaction mediated by either selectin adhesion molecules (ie, P-selectin and L-selectin) or sialyl Lewis a may play an important role in PMN-induced vasoconstriction and endothelial dysfunction. This mechanism may be important in the early endothelial dysfunction observed following reperfusion of an ischemic coronary vasculature.

Key Words • P-selectin • L-selectin • sialyl Lewis a oligosaccharide • nitric oxide

Acute myocardial ischemia often results from thrombotic occlusion of a large epicardial coronary artery. In these situations, angioplasty and thrombolysis are the most direct approaches to supply the myocardium with oxygen and nutrients. However, reperfusion itself leads to a significant degree of tissue damage called reperfusion injury.1-3 Polymorphonuclear leukocytes (PMNs) are believed to play a critical role in reperfusion injury4,5 because activated PMNs can release oxygen-derived free radicals, including superoxide anions, hydrogen peroxide, and proteolytic enzymes.5,6 On the other hand, vascular endothelial cells play an important regulatory role through the production of bioactive substances such as prostacyclin and endothelium-derived relaxing factor (EDRF).5,7 EDRF, identified as nitric oxide (NO), can be degraded by superoxide radicals.8 Activated PMNs elicit an endothelium-dependent vasoconstriction in rabbit aorta9 and cat coronary arteries10 that is mediated by superoxide anion–mediated inactivation of basal EDRF release. The interaction between PMNs and the vascular endothelium is required for the PMN-mediated reperfusion injury.3,11-13 Soon after reperfusion, PMNs start to roll along the endothelium. The rolling phenomenon may be mediated by P-selectin on the endothelial surface14,16 and constitutively expressed L-selectin on unstimulated neutrophils.16,17 The cooperation of P-selectin and platelet activating factor (PAF) expressed on the endothelium leads to the activation of the rolling PMNs (ie, shape change, shedding of L-selectin, and upregulation of CD11/CD18).18,19 This is further followed by the β2 integrin (CD11/CD18)–mediated firm attachment of PMNs to the endothelium.20 The counterreceptors for the selectins are considered to be carbohydrate-containing molecules such as sialyl Lewis a or sialyl Lewis x,16,21 while intercellular adhesion molecule–1 (ICAM-1) and ICAM-2 act as counterreceptors for CD11/CD18.20,22 Recently, a monoclonal antibody (MAb) directed against CD18 significantly attenuated PMN-induced vasoconstriction and endothelial injury in cat and dog coronary artery rings.23,24 Although P- and L-selectins mediate the initial phase of leukocyte rolling on the endothelium in reperfusion injury,1,2,23 the role of selectins in PMN-induced vasoconstriction and endothelial dysfunction has not yet been determined.

Therefore, the main purposes of the present study were to determine the role of selectins in PMN-induced vasoconstriction and endothelial dysfunction in thrombin- and hydrogen peroxide–stimulated cat coronary artery rings and to investigate the mechanisms of PMN adherence to coronary endothelium and relate this to PMN-induced vasoconstriction and endothelial dysfunction.

Methods

Preparation of Cat Coronary Artery Rings

Adult male cats (2.6 to 3.8 kg) were anesthetized with sodium pentobarbital (30 mg/kg IV). An intratracheal cannula was inserted.
immediately inserted through a midline incision, and cats were placed on intermittent positive-pressure ventilation (Harvard Small Animal Respirator, Harvard Apparatus Co). A percutaneous catheter was inserted through the right femoral artery and placed in the abdominal aorta. Immediately after obtaining 80 mL of blood for PMN isolation from the arterial catheter, a midsternal thoracotomy was performed, and the heart was rapidly excised and immersed in cold (4°C) oxygenated Krebs-Henseleit (K-H) solution. The coronary arteries were removed and placed in warmed K-H solution containing (in mmol/L) NaCl, 115.0; CaCl₂, 2.5; KH₂PO₄, 1.25; MgSO₄, 7H₂O, 1.19; NaHCO₃, 12.5, and glucose 10.0. Isolated coronary vessels were cleaned of fat and connective tissue and cut into rings 2 to 3 mm long for subsequent studies in organ chamber experiments and for determination of PMN-endothelial adhesion. In some rings used for organ chamber experiments, the endothelial layer was mechanically removed by inserting a narrow forceps into the lumen and gently rolling the ring back and forth several times on moistened filter paper. Removal of the endothelium was later functionally confirmed by the absence of relaxation to the endothelium-dependent vasodilator acetylcholine (ACh). The protocol for this experiment was accepted by the institution's Animal Care Committee and adhered to the National Institutes of Health (NIH) guidelines for use of experimental animals.

**Autologous Cat Neutrophil Isolation**

Autologous cat neutrophils were isolated by a modification of the method of Laffredo and Olsen.²⁴ Peripheral blood (80 mL) was collected from the femoral artery of pentobarbital-anaesthetized (30 mg/kg IV) adult male cats and was anticoagulated with citrate-phosphate-dextrose solution (Sigma Chemical Co). A polyethylene catheter was inserted through the right femoral artery and placed in the abdominal aorta. Immediately after obtaining 80 mL of blood for PMN isolation from the arterial catheter, a midsternal thoracotomy was performed, and the heart was rapidly excised and immersed in cold (4°C) oxygenated Krebs-Henseleit (K-H) solution. The coronary arteries were removed and placed in warmed K-H solution containing (in mmol/L) NaCl, 115.0; CaCl₂, 2.5; KH₂PO₄, 2.5; MgSO₄, 7H₂O, 1.19; NaHCO₃, 12.5, and glucose 10.0. Isolated coronary vessels were cleaned of fat and connective tissue and cut into rings 2 to 3 mm long for subsequent studies in organ chamber experiments and for determination of PMN-endothelial adhesion. In some rings used for organ chamber experiments, the endothelial layer was mechanically removed by inserting a narrow forceps into the lumen and gently rolling the ring back and forth several times on moistened filter paper. Removal of the endothelium was later functionally confirmed by the absence of relaxation to the endothelium-dependent vasodilator acetylcholine (ACh). The protocol for this experiment was accepted by the institution's Animal Care Committee and adhered to the National Institutes of Health (NIH) guidelines for use of experimental animals.

**Isolated Cat Coronary Artery Ring Study**

Isolated cat coronary artery rings were mounted between two stainless steel hooks and suspended in organ chambers connected to force-displacement transducers (FT-03; Grass Instruments Co) in order to record changes in isometric force. Two stainless steel hooks and suspended in organ chambers containing nonstimulated cat coronary artery rings were incubated with thrombin (2 U/mL; Sigma) for 10 minutes or with hydrogen peroxide (100 μmol/L; Sigma) for 60 minutes. Following incubation with these stimulants, the rings were washed with K-H solution, and unstimulated autologous PMNs (10⁶ cells/mL) were then added to the organ chambers. Vascular responsiveness to unstimulated PMNs was observed for 20 minutes, after which the rings were washed and allowed to restabilize for 15 minutes. After the developed force returned to baseline, PMN and thrombin or hydrogen peroxide responsiveness to cumulative concentrations of ACh and NaNO₂ was again obtained in the presence of U-46619–induced tone. After the response stabilized, the rings were washed again and allowed to equilibrate for 15 minutes to reach baseline force.

After obtaining pre-PMN vasodilator responses to ACh and NaNO₂, coronary artery rings were incubated with thrombin (2 U/mL; Sigma) for 10 minutes or with hydrogen peroxide (100 μmol/L; Sigma) for 60 minutes. Following incubation with these stimulants, the rings were washed with K-H solution, and unstimulated autologous PMNs (10⁶ cells/mL) were then added to the organ chambers. Vascular responsiveness to unstimulated PMNs was observed for 20 minutes, after which the rings were washed and allowed to restabilize for 15 minutes. After the developed force returned to baseline, PMN and thrombin or hydrogen peroxide responsiveness to cumulative concentrations of ACh and NaNO₂ was again obtained in the presence of U-46619–induced tone. Maximum values of vasorelaxation to both ACh and NaNO₂ were compared with those obtained before PMN incubation.

The concentration of stimulator (ie, thrombin and hydrogen peroxide) and amount of PMNs used in the present study were determined according to initial concentration-response relations and previous studies.¹²,²⁶,²⁷ In preliminary experiments we studied the vasoconstriction of coronary rings to different PMN concentrations (1×10⁵ to 2×10⁶ cells/mL) with a fixed concentration of thrombin (2 U/mL) or hydrogen peroxide (100 μmol/L), and we also examined the vasoconstriction to different concentrations of thrombin (0.5 to 5 U/mL) or hydrogen peroxide (1 to 100 μmol/L). The vasoconstrictor response to unstimulated PMNs was dependent on both PMN and thrombin or hydrogen peroxide concentration (Fig 1). From these pilot experiments we determined the amounts of PMNs (10⁵ cells/mL) and optimal concentrations of thrombin (2 U/mL) and hydrogen peroxide (100 μmol/L). These concentrations were used in all subsequent studies reported in this article.

To investigate the role of P-selectin, L-selectin, sialyl Lewis' carbohydrate, or PAF in these phenomena, we examined the effects of PB 1.3, an anti−P-selectin MAb (20 μg/mL), along with its control nonblocking isotype, MAb NBP 1.6 (20 μg/mL); DREG-200, an anti-L-selectin MAb (20 μg/mL), along with a murine immunoglobulin G (IgG) isotype control, MAb R 3.1 (20 μg/mL); sialyl Lewis'−containing oligosaccharide (SLex−OS; 500 μmol/L); sialyl Lewis'−containing oligosaccharide (Le⁺−OS; 500 μmol/L); and a PAF receptor antagonist, WEB-2170 (20 μg/mL) on PMN−induced vasoconstriction and endothelial function.

In control studies, unstimulated PMNs were added to the organ chambers containing nonstimulated cat coronary artery rings (ie, without thrombin or hydrogen peroxide stimulation). In these unstimulated coronary artery rings, unstimulated PMNs induced only a negligible vasoconstriction (ie, less than 20%) and endothelial dysfunction. Maximum vasorelaxation to ACh after PMN incubation was 90±4.5% (n=7), which was not significantly different from the pre-PMN responses (92±2.2%). We also examined endothelium-dependent vasorelaxation to ACh before and after incubation of coronary artery rings with thrombin (2 U/mL) for 10 minutes, hydrogen peroxide (100 μmol/L) for 60 minutes, or K-H solution for 60 minutes without
Autologous Cat PMN Adherence to Stimulated Coronary Endothelium

PMNs isolated by the procedure described above were labeled with a fluorescent dye (PKH2-GL; Sigma Immuno-chemical Co) according to the method of Yuan and Fleming. In brief, 1 mL dye diluent was added to a loose cell pellet containing about 10 million PMNs. One milliliter PKH2-GL (4 μmol/L) was added to the cell suspension, mixed, and then incubated for 5 minutes. Two milliliters of PBS containing 10% PPP was added to stop the labeling reaction, and another 5 mL PBS was added to the suspension. Cells were then centrifuged at 400g for 10 minutes at room temperature. The supernatant was removed, and the cells were resuspended in PBS and recounted. This labeling procedure does not affect the normal morphology or function of cat PMNs.

Cat coronary rings were prepared as described above. Rings were then carefully opened with microsurgery scissors and placed with the endothelial surface up in culture dishes filled with 3 mL oxygenated K-H buffer at 37°C. To stimulate endothelial cells, coronary rings were incubated with 2 μmol/L thrombin for 10 minutes or with 100 μmol/L hydrogen peroxide for 60 minutes. After this incubation, coronary segments were removed and placed in another cell-culture dish filled with fresh oxygenated K-H solution. These ring segments were incubated with K-H solution (vehicle), PB 1.3 (20 μg/mL), NBP 1.6 (20 μg/mL), SLe-OS (500 μmol/L), Le-OS (500 μmol/L), or WEB-2170 (20 μg/mL) for 10 minutes. Labeled PMNs (400,000 PMN/mL) were then added gently to the bottom of cell-culture dishes and incubated for 20 minutes. During this period, culture dishes were agitated in a shaker bath at 37°C. After the incubation, coronary ring segments were removed, placed on glass slides, and covered with a coverslip. Labeled PMNs that adhered to the endothelial surface were counted by using epifluorescence microscopy (Nikon Diaphot, Nikon Inc). Adherent neutrophils on five regions of each vessel segment were randomly counted and expressed as mean number of PMNs per square millimeter of endothelial surface.

Cat Aortic Endothelial Cell Culture

Endothelial cells were isolated from the thoracic aorta of cats by collagenase (1.0 mg/mL) digestion and grown to confluence on 1.0% gelatin-coated 35-mm culture dishes. Endothelial cells in culture were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, endothelial cell growth supplement, and antibiotics at 37°C under a humidified atmosphere of 5% CO₂ and 95% air. Confluent cultures of the cells exhibited the typical cobblestone pattern and were positive for factor VIII-related antigen as detected by indirect immunofluorescence.

Flow Cytometric Analysis of Cultured Endothelial Cells and Isolated Leukocytes

The binding of MAAb PB 1.3 to P-selectin on cultured cat aortic endothelial cells with or without stimulation by thrombin (2 μmol/L) was determined by employing a fluorescence-activated cell sorter (FACS) flow cytometer (Becton-Dickinson). Endothelial cells in confluent cultures in two to three passages were detached from their culture dishes by incubating the cells with Dulbecco's PBS containing 5 mmol/L EGTA and 1 mmol/L EDTA (pH 7.4) at 37°C for 20 minutes. Cells were collected by centrifugation at 500g for 5 minutes and were resuspended in Dulbecco's modified Eagle's medium. Some aliquots of cell suspension were incubated with thrombin (2 μmol/L), and others were incubated with PBS (control) at 37°C for 10 minutes. After incubation, cell suspensions were immediately fixed with 1.0% paraformaldehyde (Sigma) and then centrifuged at 500g for 5 minutes. Each endothelial cell pellet was incubated with 20 μg/mL anti-P-selectin MAAb PB 1.3 at 4°C for 30 minutes. Cells were washed with PBS with 0.2% bovine serum albumin (BSA) and incubated with secondary antibody (ie, a 1:100 diluted goat anti-mouse IgG phycoerythrin conjugate; Tago Inc) at 4°C for 30 minutes. Cells were washed with PBS with 0.2% BSA, and the final cell pellet was resuspended in 400 μL 10% paraformaldehyde and
was immediately analyzed by an FACS flow cytometer (Becton-Dickinson).

The binding of MAb DREG-200 to L-selectin on isolated cat neutrophils was also determined by FACS flow cytometry. Cat PMNs were isolated by the method described above. Immediately after isolation, PMNs (5 × 10⁶ cells/200 μL PBS) were fixed with 1 mL 1.0% paraformaldehyde. PMNs were collected by centrifugation at 500×g for 5 minutes, and the cell pellet was incubated with 20 μg/mL DREG-200 at 4°C for 30 minutes. PMNs were washed with PBS with 0.2% BSA, and the cell pellet was incubated with secondary antibody (ie, a 1:100 diluted goat anti-mouse IgG phycoerythrin conjugate; Tago) at 4°C for 30 minutes. Cells were washed again with PBS with 0.2% BSA and finally resuspended with 400 μL 1.0% paraformaldehyde and immediately analyzed by flow cytometry.

MABs Directed Against Adhesion Molecules and Soluble SLe-OS

MAb PB 1.3 binds to P-selectin and blocks the interaction between P-selectin and the sialyl Lewis*-carbohydrate ligand. MAB NBP 1.6 also recognizes but does not block the action of P-selectin. Both are murine IgG, MABs raised against human P-selectin; they were generous gifts from Dr J.C. Paulson of the Cytel Corp, San Diego, Calif. The complete characterization of these antibodies has been described. The concentrations of MABs PB 1.3 and MAB NBP 1.6 used in the present study (ie, 20 μg/mL) were based on previous studies.

MAb DREG-200 was raised against human L-selectin and was generously supplied by Dr T.K. Kishimoto (Boehringer-Ingelheim Pharmaceuticals, Inc). MAB R 3.1 is an isotype-matched MAb that does not bind to cat L-selectin. The binding of MAB DREG-200 to L-selectin on cat neutrophils was confirmed by flow cytometric analysis. The production and characterization of the MAB DREG-200 and MAB R 3.1 have been described. The concentrations of MAB DREG-200 and MAB R 3.1 used in the present study (ie, 20 μg/mL) were based on previous works.

The soluble SLe*-OS NeuAcα2,3Galβ1,4-(Fucα1,3) GlcNAcβ1,3Galβ1-0-CH₂CH₃ and its nonsialylated form (Le⁺-OS) were generous gifts from Dr J.C. Paulson of Boehringer-Ingelheim Pharmaceuticals.

PAF Receptor Antagonist WEB-2170

WEB-2170 (8[R,S]-6-[2-chlorophenyl]-8,9-dihydro-1-methyl-8-[4-morpholinylcarbonyl]-4H,7H-cyclopenta[4,5][thieno][3,2-ff][1,2,4]triazolo[4,3-a]1,4 diazepine), a specific PAF receptor antagonist, was supplied by Boehringer-Ingelheim AG. The concentration of WEB-2170 used (20 μg/mL) was based on previous work. WEB-2170 was readily soluble in K-H solution (pH 7.4, 37°C). The concentrations of SLe*-OS and Le⁺-OS used in the present study (300 μmol/L) were based on previous works.

MAb R 15.7, an IgG MAb directed against the common β-chain (CD18) of the neutrophil adherence glycoprotein (ie, β2 integrin), was generously supplied by Dr Robert Rothlein of Boehringer-Ingelheim Pharmaceuticals.

Statistical Analysis

All values are presented as means ±SEM based on n independent experiments. All data were subjected to ANOVA followed by Fisher’s t test for evaluation of the difference between groups. Probabilities of .05 or less were considered statistically significant.

Results

PMN-Induced Vasoconstriction in Thrombin- or Hydrogen Peroxide-Stimulated Cat Coronary Artery Rings

Representative recordings of unstimulated PMN-induced vasoconstriction and vasodilator responses to both ACh and acidified NaNO₂ before and after PMN-induced vasorelaxation in a thrombin-stimulated coronary artery ring are shown in Fig 2. PMNs induced a gradual significant coronary artery vasconstriction (center). PMNs elicited a vasorelaxation in thrombin- or hydrogen peroxide-stimulated coronary artery rings (119±14 and 132±15 mg, respectively); however, in control unstimulated coronary artery rings, PMNs did not induce a significant vasorelaxation (Figs 3 and 4) (P<.01 versus stimulated rings). Moreover, neither thrombin (2 U/mL) nor hydrogen peroxide (100 μmol/L) alone (ie, without PMNs) altered isometric coronary artery tone (data not shown).

Effects of MABs Directed Against Adhesion Molecules and SLe*-OS on the PMN-Induced Vasoconstriction

Dentative recordings of vasorelaxation to both ACh and NaNO₂ before and after PMN-induced vasoconstriction in thrombin-stimulated coronary artery rings in the presence of PB 1.3 or NBP 1.6 are shown in Fig 2 (center). PB 1.3 but not control MAB NBP 1.6 markedly inhibited PMN-induced vasorelaxation. The contraction to unstimulated PMNs was significantly attenuated by pretreatment with PB 1.3 (20 μg/mL) in thrombin- and hydrogen peroxide-stimulated coronary artery rings (P<.01 versus vehicle of MAb; Figs 3 and 4). However, the vasorelaxation to PMNs was not significantly attenuated by NBP 1.6 (20 μg/mL) in either thrombin- or hydrogen peroxide-stimulated coronary artery rings (Figs 3 and 4).

The vasoconstriction to PMNs (10⁶ cells/mL) was also significantly attenuated by pretreatment of the anti-L-selectin MAB DREG-200 (20 μg/mL) to 73±12 mg in thrombin-stimulated and 70±22 mg in hydrogen peroxide-stimulated coronary artery rings (P<.05 versus vehicle of MAb; Figs 3 and 4). However, the PMN-induced vasoconstriction was not significantly inhibited by the isotype IgG control MAB R 3.1 (20 μg/mL) in either thrombin- or hydrogen peroxide-stimulated coronary artery rings. Because the inhibitory effect of the anti-L-selectin MAB was less than that of the anti-P-selectin MAB, we determined concentration-response relations of MAB DREG-200 on PMN-induced vasoconstriction in thrombin-stimulated coronary artery rings. DREG-200 (10 to 20 μg/mL) elicited a concentration-related inhibition of PMN-induced vasoconstriction. However, 40 μg/mL DREG-200 did not elicit any further inhibitory effect compared with 20 μg/mL of the MAB. DREG-200 (5 μg/mL) did not significantly inhibit PMN-induced vasoconstriction.

Carbohydrate-containing molecules such as sialyl Lewis* or sialyl Lewis* are thought to be ligands for the selectins. We investigated the effects of soluble SLe*-OS on the unstimulated PMN-induced vasorelaxation. The vasorelaxation to PMNs (10⁶ cells/mL) was significantly attenuated by pretreatment with SLe*-OS (300 μmol/L) to 12±5 mg in thrombin-stimulated and 22±6 mg in hydrogen peroxide-stimulated coronary artery rings (P<.01 versus oligosaccharide vehicle, respectively; Figs 3 and 4). However, the vasorelaxation to PMNs was not significantly attenuated by Le⁺-OS (500 μmol/L).
To determine the effects of the β2 integrins on the PMN-induced vasoconstriction in thrombin-stimulated coronary arteries, we tested a MAb directed against CD18 (MAb R 15.7). At a concentration of 20 μg/mL, R 15.7 attenuated the PMN-induced contraction to 18±5 mg (P<.01 versus vehicle) in five coronary rings.

Endothelial Function Before and After Unstimulated PMN–Induced Vasocontraction

To evaluate whether endothelial function is altered after PMN-induced vasocontraction, we tested the ability of coronary artery rings to relax to ACh and NaNO₂ both before and after PMN-induced vasocontraction (Fig 2). Before incubation with PMNs, coronary artery rings elicited a full relaxation to both ACh and NaNO₂, whereas after PMN-induced vasocontraction, endothelium-dependent relaxation to ACh was markedly attenuated, but endothelium-independent relaxation to NaNO₂ was not altered. Thus, endothelium-dependent relaxation to ACh was significantly reduced after PMN-induced vasocontraction in both thrombin- and hydro-
TABLE 1. Vasorelaxation to ACh and NaNO₂ Before and After PMN-Induced Vasocontraction in Thrombin-Stimulated Cat Coronary Artery Rings

<table>
<thead>
<tr>
<th>Group</th>
<th>Vehicle (14)</th>
<th>PB 1.3 (7)</th>
<th>NBP 1.6 (7)</th>
<th>DREG-200 (7)</th>
<th>R 3.1 (6)</th>
<th>SLex-OS (6)</th>
<th>Lex-OS (6)</th>
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<tr>
<td>Pre-PMN ACh</td>
<td>89±2.9</td>
<td>90±1.8</td>
<td>90±3.7</td>
<td>86±1.4</td>
<td>85±1.0</td>
<td>86±2.0</td>
<td>87±1.3</td>
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<tr>
<td>Pre-PMN NaNO₂</td>
<td>91±1.9</td>
<td>92±2.7</td>
<td>90±3.3</td>
<td>85±1.7</td>
<td>84±2.4</td>
<td>87±1.3</td>
<td>85±0.7</td>
</tr>
<tr>
<td>Post-PMN ACh</td>
<td>48±5.7†</td>
<td>82±4.1‡</td>
<td>56±5.7†</td>
<td>63±4.7‡</td>
<td>54±3.4*</td>
<td>85±1.2§</td>
<td>57±3.4†</td>
</tr>
<tr>
<td>Post-PMN NaNO₂</td>
<td>91±2.3</td>
<td>93±3.1</td>
<td>86±3.7</td>
<td>85±1.7</td>
<td>86±2.6</td>
<td>88±1.4</td>
<td>85±1.5</td>
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</tbody>
</table>

ACh indicates acetylcholine; NaNO₂, acidified sodium nitrite; PMN, polymorphonuclear leukocyte; SLex-OS, sialyl Lewis-x-containing oligosaccharide; and Le⁰-OS, nonsialylated Lewis⁰-containing oligosaccharide. Numbers in parentheses are numbers of independent experiments; data are expressed as means±SEM. Values are percent relaxation of precontraction.

*P<.05, †P<.01 vs pre-PMNs; ‡P<.05, §P<.01 vs vehicle.

Effects of SLex-OS and MAbs Directed Against P-Selectin and L-Selectin on Endothelial Dysfunction After PMN-Induced Vasoconstriction

The effects of anti-selectin agents (ie, either SLex-OS or MAbs directed against P- or L-selectin) were tested on the endothelial dysfunction developing after PMN-induced vasoconstriction. Fig 2 (center) shows representative recordings of vasodilator responsiveness to ACh and NaNO₂ before and after PMN-induced vasoconstriction in thrombin-stimulated coronary artery rings in the presence of anti-P-selectin MAb PB 1.3 or control MAb NBP 1.6. Before PMN incubation, coronary artery rings elicited a full relaxation to both ACh and NaNO₂. After PMN-induced vasoconstriction, the endothelial-dependent relaxation to ACh was preserved in MAb PB 1.3-treated rings and markedly reduced in NBP 1.6-treated rings, whereas the direct relaxation to NaNO₂ was unaltered. Tables 1 and 2 summarize the values for the maximum vasorelaxations to both ACh and NaNO₂ obtained in rings stimulated with either thrombin (2 U/mL) or hydrogen peroxide (100 μmol/L) before and after PMN-induced vasoconstriction. Before PMN incubation, coronary artery rings elicited full relaxation to both ACh and NaNO₂ in all groups; these values did not differ significantly. Treatment with either MAb PB 1.3 (20 μg/mL), MAb DREG-200 (20 μg/mL), or SLex-OS (500 μmol/L) significantly attenuated the endothelial dysfunction to ACh in both thrombin- and hydrogen peroxide-stimulated coronary artery rings compared with vehicle-treated rings. In contrast, neither of the control antibodies (ie, NBP 1.6 or R 3.1) nor Le⁰-OS preserved the endothelium-dependent relaxation to ACh in thrombin- and hydrogen peroxide-stimulated coronary artery rings compared with vehicle-treated rings. As expected, the endothelium-independent relaxation to NaNO₂ was not affected after PMN-induced vasoconstriction in any group.

TABLE 2. Vasorelaxation to ACh and NaNO₂ Before and After PMN-Induced Vasocontraction in Hydrogen Peroxide-Stimulated Cat Coronary Artery Rings

<table>
<thead>
<tr>
<th>Group</th>
<th>Vehicle (7)</th>
<th>PB 1.3 (7)</th>
<th>NBP 1.6 (6)</th>
<th>DREG-200 (6)</th>
<th>R 3.1 (6)</th>
<th>SLex-OS (9)</th>
<th>Lex-OS (7)</th>
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<tbody>
<tr>
<td>Pre-PMN ACh</td>
<td>90±1.5</td>
<td>90±2.9</td>
<td>90±3.5</td>
<td>91±1.5</td>
<td>91±2.7</td>
<td>93±3.3</td>
<td>93±3.3</td>
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<tr>
<td>Pre-PMN NaNO₂</td>
<td>89±2.6</td>
<td>90±2.0</td>
<td>88±4.4</td>
<td>92±3.1</td>
<td>89±2.1</td>
<td>90±1.6</td>
<td>90±3.1</td>
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<tr>
<td>Post-PMN ACh</td>
<td>50±6.4†</td>
<td>85±3.1‡</td>
<td>61±5.8*</td>
<td>68±5.0‡</td>
<td>65±7.5*</td>
<td>90±2.6§</td>
<td>62±3.0†</td>
</tr>
<tr>
<td>Post-PMN NaNO₂</td>
<td>85±3.3</td>
<td>90±2.4</td>
<td>89±3.1</td>
<td>86±2.4</td>
<td>85±1.0</td>
<td>90±2.3</td>
<td>84±1.9</td>
</tr>
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</table>

ACh indicates acetylcholine; NaNO₂, acidified sodium nitrite; PMN, polymorphonuclear leukocyte; SLex-OS, sialyl Lewis-x-containing oligosaccharide; and Le⁰-OS, nonsialylated Lewis⁰-containing oligosaccharide. Numbers in parentheses are numbers of independent experiments; data are expressed as means±SEM. Values are percent relaxation of precontraction.

*P<.05, †P<.01 vs pre-PMNs; ‡P<.05, §P<.01 vs vehicle.
Adherence of Unstimulated PMNs to Thrombin- and Hydrogen Peroxide–Stimulated Cat Coronary Endothelium

Because P-selectin might be upregulated on the endothelial surface after stimulation with thrombin or hydrogen peroxide, we further quantified PMN adherence to stimulated cat coronary endothelium. Figs 5 and 6 summarize the results of unstimulated PMN adherence to thrombin- or hydrogen peroxide–stimulated coronary endothelium. PMN adherence was markedly enhanced by endothelial stimulation with either thrombin (2 U/mL) or hydrogen peroxide (100 μmol/L) (P < .01 versus unstimulated control coronary endothelium). This increased adhesiveness of unstimulated PMNs to stimulated coronary endothelium was significantly attenuated by treatment with either MAb PB 1.3 (P < .01 versus MAb vehicle), MAb DREG-200 (P < .05 versus MAb vehicle), or SLe\(^x\)-OS (P < .01 versus vehicle). In contrast, treatment with MAb NBP 1.6 (20 μg/mL), MAb R 3.1 (20 μg/mL), or Le\(^x\)-OS (500 μmol/L) did not significantly attenuate the PMN adherence to stimulated coronary endothelium. Thus, selectin-blocking agents markedly attenuate PMN adherence to either thrombin- or hydrogen peroxide–stimulated coronary vascular endothelium.

Binding of Anti-L-Selectin MAb DREG-200 to Isolated Cat Neutrophils

Flow cytometric analysis of isolated PMNs from three cats clearly indicated that anti-L-selectin MAb DREG-200 binds to isolated cat neutrophils. Unstimulated cat neutrophils stained 89±8% positive for DREG-200 compared with 5±1% positive for the control PMNs (ie, only secondary antibody–treated cells). Binding of DREG-200 to cat neutrophils resulted in a mean channel fluorescence of 52.4±7.2 compared with 2.1±0.4 for the secondary antibody alone (P < .01).

Binding of Anti-P-Selectin MAb PB 1.3 to Cultured Cat Aortic Endothelial Cells

Flow cytometric analysis of cultured cat aortic endothelial cells demonstrated that the anti-P-selectin MAb PB 1.3 does bind to cat endothelial cells and that P-selectin expression is markedly enhanced after stimulation with thrombin (2 U/mL; Fig 7). Cat endothelial cells incubated with only the secondary antibody revealed 6% positive staining for cells and a mean channel fluorescence of 1.5 (ie, nonspecific background fluorescence). However, after stimulation with thrombin (2 U/mL), the binding of PB 1.3 to cat endothelial cells markedly increased to a mean channel fluorescence of 310 compared with 42.5 for the nonstimulated control endothelial cells. As a result, 97% of the stimulated endothelial cells were positive for P-selectin.
Mechanism of Unstimulated PMN-Induced Vasoconstriction in Stimulated Coronary Artery Rings

We further examined the mechanism of the unstimulated PMN-induced vasoconstriction. Fig 8A shows a representative recording of vasoconstriction induced by PMNs (10⁶ cells/mL) in a thrombin-stimulated cat coronary artery ring. In contrast, in an endothelium-denuded ring (Fig 8B), the vasoconstriction to PMNs was virtually absent. The addition of human superoxide dismutase markedly attenuated the PMN-induced vasoconstriction in a ring with an intact endothelium. D, A nitric oxide synthase inhibitor, N⁴-monomethyl-L-arginine (L-NMMA; 100 μmol/L), also markedly inhibited PMN-induced vasoconstriction in rings with an intact endothelium. W indicates wash; ACh, acetylcholine; and ISO, isoproterenol.

TABLE 3. Effects of WEB-2170 on the Unstimulated PMN-Induced Vasoconstriction, Endothelial Dysfunction, and Adherence to Thrombin-Stimulated Coronary Endothelium

<table>
<thead>
<tr>
<th>Condition</th>
<th>PMN-Induced Vasoconstriction, mg</th>
<th>Relaxation to ACh Pre-PMN, %</th>
<th>Relaxation to ACh Post-PMN, %</th>
<th>Adherence to Endothelium, cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control unstimulated</td>
<td>19.8±8.6</td>
<td>94±3.4</td>
<td>92±2.5</td>
<td>21±2.5</td>
</tr>
<tr>
<td>Thrombin stimulated</td>
<td>115±12</td>
<td>90±2.6</td>
<td>48±5.7†</td>
<td>110±6.0</td>
</tr>
<tr>
<td>Thrombin+WEB-2170</td>
<td>27±4.7*</td>
<td>88±3.5</td>
<td>75±3.1*</td>
<td>94±13</td>
</tr>
</tbody>
</table>

PMN indicates polymorphonuclear leukocyte; ACh, acetylcholine. Data are means±SEM for 5 to 12 coronary rings. *P<.01 vs thrombin-stimulated; †P<.01 vs pre-PMN.
implicates them in the further aggravation of leukocyte capillary plugging, thus contributing to the no-reflow phenomenon, and could lead to reduced myocardial oxygen supply after reperfusion. PMN-induced vasoconstriction is attenuated by a specific MAb directed against the β2 integrin adhesion molecule (CD18). Furthermore, several investigators recently reported that MAbs directed against adhesion molecules such as P-selectin, L-selectin, and CD18 significantly inhibited inflammatory tissue injury to the heart and lungs in vivo. Thus, adhesion molecules have become important mediators playing a pathophysiological role in reperfusion injury.

In the present study, nonactivated autologous cat PMNs elicited a vasoconstriction and subsequent endothelial dysfunction (ie, reduced endothelium-dependent relaxation to ACh) in isolated coronary arteries that were stimulated with either thrombin or hydrogen peroxide. This contraction was accompanied by a significant increase in adherence of PMNs to the stimulated endothelium. The PMN-induced vasoconstriction, endothelial dysfunction, and increased adherence of PMNs to the endothelial surface were all significantly attenuated by anti-selectin therapy, either by blocking P-selectin or L-selectin alone with a specific MAb or by blocking the action of selectins with a soluble ligand SLex-OS. To our knowledge, this is the first study demonstrating a significant role of selectins or a selectin ligand, sialyl Lewis x, in PMN-induced vasoconstriction and endothelial dysfunction.

PMN recruitment to inflammatory tissue is mediated by the sequential action of multiple adhesion molecules. Initial interaction between PMNs and the endothelium (ie, PMN rolling) is mediated by the selectin family. When endothelial cells are stimulated with inflammatory agents such as thrombin, histamine, or hydrogen peroxide, P-selectin is translocated to the endothelial surface from Weibel-Palade bodies within 5 to 10 minutes. P-selectin also translocates to the endothelial surface 10 minutes after reperfusion of ischemic coronary arteries in the cat. Furthermore, P-selectin upregulation occurs on the endothelium of large epicardial coronary arteries as well as postcapillary venules. In the present study, coronary arteries stimulated with thrombin or hydrogen peroxide exhibited a PMN-induced vasoconstriction, endothelial dysfunction, and enhanced PMN adherence to the endothelium. These responses were significantly attenuated by anti-P-selectin MAb PB 1.3 but not by a control antibody, NBp 1.6, which recognizes P-selectin but does not block P-selectin-mediated PMN-endothelial interaction. Furthermore, P-selectin was significantly upregulated after thrombin stimulation in cultured cat aortic endothelial cells as assessed by flow cytometric analysis. These results provide strong evidence that P-selectin-mediated PMN-endothelial interaction plays an important role in the phenomena described in this study.

L-selectin is constitutively expressed on the surface of leukocytes and is also believed to play an important role in the initial PMN rolling step during inflammation. In the present study, PMN-induced vasoconstriction, endothelial dysfunction, and increased adherence of PMNs to stimulated endothelium were significantly but more mildly attenuated by an anti-L-selectin MAb (DREG-200) compared with either PB 1.3 or SLex-OS. However, these values were not inhibited by a control isotype, MAb R 3.1. These results indicate that L-selectin-mediated PMN-endothelial interaction also plays a role in the present phenomena. Constitutively expressed L-selectin is shed from the leukocyte surface after activation. However, L-selectin expression on isolated cat PMNs was positive in 90% of cells by flow cytometric analysis. Thus, at least according to present methods of analysis, L-selectin was not markedly shed during PMN isolation. However, the efficacy of this anti-L-selectin MAb appears to be less than that of the anti-P-selectin MAb or SLex-OS in the present study. This finding persisted even after using a higher concentration of DREG-200 (ie, 40 μg/mL). The reason for this is unknown, but there are some possibilities. First, L-selectin might be shed to some extent after addition to the organ chamber, which was bubbled with 95% O2 and 5% CO2 and maintained at 37°C. Second, we observed an interaction between unstimulated PMNs and thrombin- or hydrogen peroxide-stimulated coronary endothelium in the present study. As shown by flow cytometry, 97% of the endothelial cells were positive for P-selectin after stimulation with thrombin. Thus, it is highly likely that an interaction between PMNs and endothelium via P-selectin and SLex-OS exists after treatment with anti-L-selectin MAb, resulting in an incomplete inhibition of PMN-induced endothelial dysfunction.

In contrast to P- and L-selectin, E-selectin is expressed via de novo synthesis of the protein over 4 to 6 hours after activation of endothelium by cytokines (eg, tumor necrosis factor-α, interleukin-1β), endotoxin, or ischemia/reperfusion. In this regard, E-selectin may not be as critical as the other selectins in the initial rolling process occurring in early inflammatory conditions such as ischemia/reperfusion. Winquist et al have shown that a MAb directed against E-selectin failed to protect against myocardial ischemia/reperfusion injury in monkeys.

Selectin adhesion molecules have common carbohydrate ligands (eg, sialyl Lewis x and sialyl Lewis y). SLex-OS but not its nonsialylated analogue Le x-OS significantly protects against endothelial dysfunction, myocardial necrosis, and PMN adherence to coronary endothelium in cats subjected to ischemia/reperfusion. In the present study, SLex-OS significantly inhibited the unstimulated PMN-induced vasoconstriction, endothelial dysfunction, and adherence to thrombin- or hydrogen peroxide-stimulated coronary endothelium. These results provide clear evidence that the initial PMN-endothelial interaction between the selectins and sialyl Lewis x may play an important role in the early events leading to PMN-induced tissue injury such as vasoconstriction and endothelial dysfunction.

The second step in the adherence cascade involves firm attachment of PMNs to the endothelium. Integrin-immunoglobulin adhesion mechanisms (eg, CD11/CD18–ICAM-1 or –ICAM-2) are primarily involved in this second step, along with upregulation of β2 integrins on the PMN surface. The initial rolling step of PMNs on the endothelium is considered to be necessary for the subsequent PMN activation and firm attachment to the endothelium. In this regard, the present results clearly indicated that inhibition of initial PMN-endothelial interaction (ie, rolling) by SLex-OS or antibodies...
directed against P-selectin or L-selectin can effectively attenuate subsequent PMN activation, thus preventing firm attachment. Although PMNs may predominantly adhere to capillaries and postcapillary venules in vivo, several investigators have shown that neutrophils do adhere to the walls of large epicardial coronary arteries or large mesenteric arteries after ischemia/reperfusion. Our present findings of enhanced PMN adherence to the coronary artery endothelium stimulated with thrombin or hydrogen peroxide extend these findings.

In the present study, PMN-induced vasocontraction was dependent on the presence of an intact endothelium. PMN-induced contractions were markedly attenuated by the addition of either human superoxide dismutase, a superoxide radical scavenger, or by a competitive inhibitor of NO synthase, L-NMMA. In addition, the PMN-induced vasoconstriction was not inhibited by indomethacin or by scavengers of either hydrogen peroxide or hydroxy radical. These results suggest that PMN-induced contraction is mediated by inactivation of basally released NO by superoxide radicals released from PMNs. These findings are generally consistent with studies in isolated vessels. One difference is that we employed unstimulated PMNs in the current study, which failed to elicit vasoconstriction, endothelial dysfunction, or increased adhesiveness in coronary arteries without stimulation of the endothelium by thrombin or hydrogen peroxide. These findings suggest the following series of events. First, unstimulated PMNs start to roll on the stimulated endothelial surface mediated by a selectin–SLIC carbohydrate interaction. PMNs are subsequently activated and release oxygen free radicals, resulting in vasocostriction and endothelial dysfunction of the coronary vessel. Finally, reduced NO release favors adherence of the PMNs to the endothelium. These events further support the theory that there is an endothelial trigger (ie, endothelial dysfunction) followed by a neutrophil amplification mechanism. In this regard, Shappel et al recently reported that inhibiting PMN adherence to endothelial cells reduced neutrophil production of oxygen free radicals by at least 90%.

Another interesting aspect of this study is the inhibitory role of the PAF receptor antagonist WEB-2170 in PMN-induced vasocontraction and endothelial dysfunction. PAF is coexpressed with P-selectin on the endothelial surface after stimulation with thrombin, histamine, or oxygen-derived free radicals. Recently, Lorant et al demonstrated that PAF coexpressed with P-selectin on the endothelial surface facilitates the subsequent activation of PMNs after P-selectin–mediated PMN rolling. They further suggested that the increased expression of P-selectin alone could not prime the PMN activation, but P-selectin in concert with PAF is able to activate the PMNs. In the present study, the PAF receptor antagonist WEB-2170 significantly attenuated the PMN-induced vasoconstriction and endothelial dysfunction in thrombin-stimulated coronary artery rings. However, WEB-2170 did not significantly attenuate the PMN adherence to thrombin-stimulated coronary endothelium. These results suggest that although PAF is coexpressed with P-selectin on the endothelial surface, PAF might play a critical role in the PMN adherence but could contribute to subsequent endothelial dysfunction. This line of evidence is supported by the finding that WEB-2170 significantly protected against endothelial dysfunction and myocardial necrosis after ischemia-reperfusion in cats. In conclusion, we have demonstrated that unstimulated autologous cat PMNs elicit an endothelium-dependent vasocontraction, an endothelial dysfunction, and an increased adherence of PMNs to the endothelial surface in isolated coronary arteries stimulated with thrombin or hydrogen peroxide. This endothelial dysfunction was significantly attenuated by MAbs directed against either P-selectin or L-selectin or by SLIC, indicating that selectin-mediated PMN-endothelial interaction plays an important early role in the PMN-induced endothelial dysfunction. Thus, selectin adhesion molecules may be important early mediators of endothelial dysfunction in settings in which an inflammatory response occurs. This endothelial dysfunction may lead to other PMN-endothelial interactions that act to propagate vascular dysfunction and contribute to reperfusion injury.

Acknowledgments

This work was supported by research grant No. GM-45434 from the National Institutes of Health. Dr Murohara was supported by a postdoctoral fellowship from the Japan Heart Foundation. Dr Buerke was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. We thank Robert Craig and Dr Kirk A. Milhaou for the expert technical assistance during the course of these investigations.

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Polymorphonuclear leukocyte-induced vasocontraction and endothelial dysfunction. Role of selectins.
T Murohara, M Buerke and A M Lefer

Arterioscler Thromb Vasc Biol. 1994;14:1509-1519
doi: 10.1161/01.ATV.14.9.1509

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