Monoclonal Antibody to L-Selectin Attenuates Neutrophil Accumulation and Protects Ischemic Reperfused Cat Myocardium

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Background. Interaction of CD11/CD18 located on neutrophil membranes with its endothelial counter-receptor, intercellular adhesion molecule-1, plays a major role in polymorphonuclear leukocyte (PMN)-mediated endothelial dysfunction and myocardial injury associated with ischemia and reperfusion. However, PMN-derived L-selectin, which is thought to play an early role in PMN rolling along the vascular endothelium, has not been studied in a setting of myocardial ischemia and reperfusion.

Methods and Results. In this study, we evaluated the effects of a monoclonal antibody against L-selectin, DREG-200, in a feline model of myocardial ischemia (1.5 hours) and reperfusion (4.5 hours). DREG-200 (1 mg/kg) or an isotype-matched IgG1 antibody, MAb R3.1, which does not cross-react in cats, was administered as a bolus 10 minutes before reperfusion. In MAb R3.1-treated cats, myocardial ischemia followed by reperfusion resulted in significant coronary vascular endothelial dysfunction, elevated cardiac myeloperoxidase activity indicative of neutrophil accumulation in the ischemic myocardium, and severe myocardial injury. In contrast, administration of DREG-200 at 1 mg/kg significantly attenuated myocardial necrosis (14±4 versus 32±3 expressed as percentage of area at risk, P<.001) and attenuated coronary endothelial dysfunction (P<.01) associated with ischemia/reperfusion. Moreover, myeloperoxidase activity in the ischemic myocardium was significantly lower than MAb R3.1-treated cats (0.4±0.1 versus 0.9±0.2 U/100 mg tissue, P<.05).

Conclusions. These results demonstrate that blocking L-selectin with DREG-200 exerts a significant cardioprotective effect in a feline model of myocardial ischemia and reperfusion, indicating that L-selectin plays a significant role in mediating PMN accumulation and PMN-induced endothelial and myocardial injury after ischemia and reperfusion. (Circulation 1993;88:649-658)

KEY WORDS • L-selectin • blood cells • reperfusion • endothelium

Early reperfusion remains the most effective treatment to minimize myocardial injury and improve ventricular function in myocardial ischemia. However, accumulating evidence indicates that reperfusion itself may exert deleterious effects and result in enhanced myocardial injury. The process of reperfusion injury involves components of a typical inflammatory reaction in which polymorphonuclear leukocytes (PMNs) play an important role. Experimental strategies involving prevention of PMN activation or PMN depletion have been shown to reduce myocardial reperfusion injury. Moreover, monoclonal antibodies directed against either the common β-chain of the neutrophil adhesion glycoprotein complex (ie, CD-18) or an endothelial ligand for CD11/CD18 (ie, intercellular adhesion molecule-1 [ICAM-1]) has been shown to protect the myocardium from reperfusion injury, indicating that interaction of CD11/CD18 with its counterreceptor (eg, ICAM-1) plays an important role in PMN-mediated endothelial and myocardial injury associated with myocardial ischemia and reperfusion.

Another leukocyte adhesion molecule recently named L-selectin (originally LAM-1 or LECAM-1) has been reported to be a major mediator for the initial rolling of PMNs along the endothelium, a process that is essential for subsequent CD-18/ICAM-1-mediated PMN adherence and activation. Although several studies have shown that L-selectin–mediated initial PMN rolling is an important component in the inflammatory response, its role in ischemic/reperfusion tissue injury in which PMN activation and accumulation play a critical role has not been investigated. In this study, we evaluated the role of L-selectin in myocardial ischemia and reperfusion injury in cats by using DREG-200, a monoclonal antibody (MAb) to L-selectin. Our results indicate that DREG-200 significantly attenuates PMN accumulation in ischemic/reperfused myocardium and exerts marked endothelial and myocardial protection, indicating that L-selectin plays an important role in myocardial ischemia and reperfusion injury.
Methods

Isolation of Cat Neutrophils

Peripheral blood (20 mL) was collected from the femoral artery of adult male cats anesthetized with pentobarbital (30 mg/kg IV) and was anticoagulated with citrate-phosphate-dextrose solution (Sigma Chemical Co, St Louis, Mo) (1:4:10, vol:vol anticoagulant to whole blood) into round-bottom polycarbonate centrifuge tubes (Nalge, Rochester, NY). PMNs were isolated by a procedure modified from Lafrad and Olsen.\textsuperscript{17} Platelet-rich plasma (PRP) was obtained by centrifuging blood at 400g for 20 minutes at 4°C in a Sorvall RC2-B refrigerated centrifuge (Sorvall Instruments, Wilmington, Del). PRP was decanted and centrifuged at 2500g for 10 minutes to obtain platelet-poor plasma (PPP). PPP was then mixed with isotonic Percoll (Sigma Chemical Co) (9 vol Percoll:1 vol 1.5-mol/L NaCl) to produce Percoll-PPP density gradients of 80%, 62%, and 50%. Eight milliliters of 6% dextran (average molecular weight, 60 000 to 90 000; Sigma Chemical Co) was added to the erythrocyte-leukocyte pellet from the initial 400g centrifugation. After mixture by inversion, the erythrocytes were allowed to settle over a period of 50 minutes. The upper suspension containing leukocytes was recentrifuged at 1500g for 10 minutes. The pellets obtained were resuspended in 1 mL of 0.9% NaCl and layered onto the Percoll:PPP gradient. Centrifugation was then performed at 1500g for 40 minutes at 4°C in a Sorvall RC2-B refrigerated centrifuge. PMNs were collected from the 62%-80% interface and washed twice with phosphate-buffered saline (PBS) before being assayed for viability using trypan blue exclusion. PMN preparations obtained by this method were typically >95% pure and >95% viable, as previously reported.\textsuperscript{9,10}

Flow Cytometric Analysis of DREG-200 Binding to Cat Neutrophils

The production and characterization of the monoclonal antibody DREG-200 and MAb R3.1 have been described previously.\textsuperscript{18,19} MAb R3.1 is an isotype-matched monoclonal antibody that does not bind to cat PMNs.\textsuperscript{9} The binding of DREG-200 to L-selectin on cat neutrophils was determined by flow cytometric analysis of freshly isolated whole cat blood. Three additional cats were anesthetized with sodium pentobarbital (30 mg/kg IV), and 10 mL of peripheral blood was collected into sterile, 50-mL Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing 0.05 mL of 15% ethylene diamine tetracetic acid. The anticoagulated blood was transferred into Eppendorf microtubes (Brinkman Instruments Co, Westbury, NY) in 100-μL aliquots and maintained at room temperature. DREG-200 (40 μg/mL) or the control antibody, MAb R3.1 (40 μg/mL), was added directly to the whole blood aliquots. Leukotriene B\textsubscript{4} (Biomol Research Laboratories, Plymouth Meeting, Pa) was added to additional aliquots of whole blood at a final concentration of 100 nmol/L for 5 minutes to induce shedding of L-selectin from neutrophils, and then either DREG-200 or MAb R3.1 (40 μg/mL) was added to the whole blood. The aliquots of whole blood were placed on ice for 30 minutes and then centrifuged in an Eppendorf Micro centrifuge (Brinkman Instruments Co) for 30 seconds at 10 000g, and the supernatant was removed. The cell pellets were resuspended in 500 μL of Dulbecco’s PBS (Gibco, Grand Island, NY) containing 0.2% bovine serum albumin (Sigma Chemical Co). After centrifugation in the Micro centrifuge, the supernatant was discarded. F(ab’)\textsubscript{2} fragments of a goat anti-mouse IgG-phycoerythrin conjugate (Tago, Inc, Burlingame, Calif) was used as the secondary antibody at a 1:100 dilution, and the cell pellets were placed on ice for 30 minutes. The red cells were then lysed with a whole blood lysing reagent kit (Coulter Immunology, Hialeah, Fla) that consists of a lysing solution and fixative reagent. The remaining leukocytes were then washed twice in 100 μL of cold PBS, and the cells were resuspended in 300 μL of 1.0% paraformaldehyde (Sigma Chemical Co) and stored at 4°C in the dark until analysis. The binding of DREG-200 to neutrophils was then specifically determined by use of a Coulter EPICS flow cytometer (Coulter Immunology).

Immunohistochemical Evaluation of DREG-200 Interaction With Cat Coronary Endothelium

To evaluate whether DREG-200 also interacts with feline vascular endothelium, three cats were exposed to 90 minutes of ischemia followed by 20 minutes of reperfusion. The hearts were subsequently removed, and the aorta was immediately cannulated and perfused with Krebs-Henseleit (K-H) solution for 2 minutes at 50 mm Hg. After this 2-minute washout period, the K-H solution was switched to 4% paraformaldehyde in PBS, and the hearts were perfusion-fixed for 5 minutes. Full-thickness slices of the left ventricular wall (1 mm in thickness and 5 mm in width) were fixed for 2 hours at 4°C. Slices cut from the ischemic/reperfused region and the nonischemic region were dehydrated in a graded series of acetone at 4°C and embedded in Immunobed (Polysciences Inc, Warrington, Pa) at 4°C for 12 hours. Sections 5 μm thick were cut with glass knives and transferred to coated slides (Vectabond; Vector Laboratory, Burlingame, Calif).

Immunohistochemical procedures on plastic sections were performed in the following sequence according to a modification of the methods described previously by Beckstead et al\textsuperscript{20} using the avidin-biotin immunoperoxidase technique (Vectastain ABC Reagent; Vector Laboratory). Incubation of the primary antibody was carried out overnight at room temperature at dilutions of 1:10 and 1:100 in PBS. Control preparations for immunohistochemistry included omission of the primary antibody and also incubation with the primary antibody but with omission of the secondary antibody (biotinylated IgG). The sections were examined with a Zeiss microscope.

PMN Adherence to Coronary Vascular Endothelium In Vitro

Cat PMNs were isolated as described above. Isolated autologous PMNs were then labeled with Zynaxis PKH2 fluorescent dye according to the method of Yuan and Fleming.\textsuperscript{21} Diluent (1 mL) was added to a loose cell pellet containing <10 million cells. One milliliter of PKH2-GL dye (4 μmol/L) was added to the cell suspension and then mixed for 5 minutes by inversion. PBS (2 mL) containing 10% PPP was added to stop the reaction, and another 5 mL of PBS underlay the suspension. Cells were then centrifuged at 400g for 10
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minutes at 4°C. The supernatant was removed, and the cells were resuspended in PBS and then recounted. This labeling procedure yields cells possessing normal morphology and function.21

The effect of DREG-200 on unactivated PMN adherence to endothelium was evaluated by an in vitro PMN adherence assay previously established in our laboratory.5,10,22 Normal unstimulated endothelium does not have surface expression of ligands for L-selectin. In this study, we stimulated endothelial cells by exposing coronary arteries to 90 minutes of ischemia and 20 minutes of reperfusion. To minimize the activation of PMNs, adherence was studied at 4°C, a temperature at which CD18-mediated PMN adherence to coronary vascular endothelium is minimal. The left anterior descending coronary artery (LAD), which was subjected to 90 minutes of ischemia and 20 minutes of reperfusion, and the left circumflex coronary artery (LCx), which was not subjected to ischemia, were both carefully removed so as not to disturb the endothelium. These artery segments were placed into warmed K-H solution. Isolated coronary vascular segments were carefully cleaned of fat and connective tissue and cut into rings 2 to 3 mm in length. These rings were then opened carefully and placed in 5-mL round cell culture dishes containing 3 mL of cold K-H solution. PMNs (4×10⁶ cells/mL) preincubated for 5 minutes with either DREG-200 (10 to 40 μg/mL) or MAb R3.1 (40 μg/mL) were added to the culture dishes. The preparations were then placed in a temperature-controlled (4°C) shaker and agitated at a rate of 120 agitations/min. After a 20-minute incubation period, the opened coronary artery rings were removed from culture dishes and dipped 3 or 4 times in fresh K-H solution to wash loose PMNs from the endothelium. These coronary rings were then placed endothelial side up on a glass slide. The number of PMNs adhering to the endothelial surface in five separate microscopic fields was counted by use of a Zeiss epifluorescent microscope (Carl Zeiss Inc, Thornwood, NJ) at a magnification of ×100, and PMN adherence was expressed as PMNs/mm² of endothelial surface.

**Myocardial Ischemia and Reperfusion in the Cat**

Adult male cats (weight, 2.5 to 3.9 kg) were anesthetized with sodium pentobarbital (30 mg/kg IV). An intratracheal cannula was inserted through a midline incision, and all cats were placed on intermittent positive-pressure ventilation (Harvard small animal respirator, Dover, Mass.). A polyethylene catheter was inserted into the right external jugular vein for supplementary pentobarbital infusion to maintain a surgical plane of anesthesia and for administration of drugs. An additional polyethylene catheter was inserted through the left femoral artery and positioned in the abdominal aorta for measurement of mean arterial blood pressure (MABP) via a Statham P23AC pressure transducer. After a midsternal thoracotomy, the pericardium was opened and a 2-0 silk ligature was placed around the LAD 8 to 10 mm from its origin. Standard lead II of the scalar ECG was used to determine heart rate and ST-segment elevation. The ECG and MABP were continuously recorded on a Grass model 7 oscillographic recorder (Grass Instrument Co, Quincy, Mass). The pressure-rate index, an approximation of myocardial oxygen demand, was calculated as the product of MABP and heart rate divided by 1000. ST-segment elevations were determined from an ECG recording at 50 mm/s every 20 minutes. Coronary collateral blood flow measurements were not made in this study. However, previous studies have shown that ischemic cat myocardium has only 7% collateral flow in this model of myocardial ischemia.9

After all surgical procedures were completed, the cats were allowed to stabilize for 30 minutes, at which time a baseline reading of ECG and MABP was recorded and an initial blood sample was drawn. Myocardial ischemia (MI) was produced by tightening the previously placed reversible ligature around the LAD to completely occlude the vessel. This was designated as time 0. After 1.5 hours of ischemia, the LAD ligature was untied, and the ischemic myocardium was reperfused (R) for 4.5 hours. Ten minutes before reperfusion, MAb DREG-200 (1 mg/kg) or a nonbinding control antibody, MAb R3.1 (1 mg/kg), was given as a bolus injection. The animals were randomly divided into three major groups consisting of six cats per group: sham MI+R cats receiving DREG-200, MI+R cats receiving MAb R3.1, and MI+R cats receiving DREG-200. A dose of 1 mg/kg DREG-200 was selected because this corresponds to 20 μg/mL, a blood concentration of DREG-200 at which a full inhibitory effect of PMN adherence was obtained in vitro. Moreover, pilot studies with four additional cats treated with 0.25 mg/kg of DREG-200 10 minutes before reperfusion exhibited a necrosis of 30±7% of the area at risk, a value not significantly different from previous findings with untreated ischemic reperfused cats.9 Sham MI+R cats were subjected to the same surgical procedures as MI+R cats, except that the LAD coronary artery was not occluded.

At the end of the 6-hour experimental period, the ligature around the LAD was tightened. Thirty milliliters of 0.5% Evans blue dye was injected into the left atrium to stain the area of myocardium that was perfused by the patent coronary arteries. The area at risk was thus determined by negative staining. The heart was rapidly excised and placed in warmed, oxygenated K-H buffer consisting of (in mmol/L): NaCl 118; KCl 4.75; CaCl₂·2H₂O 2.54; KH₂PO₄ 1.19; MgSO₄·7H₂O 1.19; NaHCO₃ 12.5; and glucose 10.0. The LCx and the LAD were isolated and removed for subsequent study of coronary ring vasoactivity. The right ventricle and great vessels were then removed, and the left ventricle was sliced parallel to the atroventricular groove in 3-mm-thick sections. The unstained portion of the myocardium (ie, the total area at risk) was separated from the Evans blue-stained portion of the myocardium (ie, the area not at risk). The area at risk was again sectioned into 1-mm-thick slices and incubated in 0.1% nitroblue tetrazolium in phosphate buffer at pH 7.4 and 37°C for 15 minutes. The tetrazolium dye forms a blue formazan complex in the presence of coenzymes and dehydrogenases. The irreversibly injured or necrotic portion of the myocardium at risk, which did not stain, was separated from the stained portion of the myocardium (ie, the ischemic but nonnecrotic area). The nonischemic, nonnecrotic, and ischemic necrotic portions were subsequently weighed, and the results were expressed as the area at risk indexed to the total left ventricular mass,
the area of necrotic tissue indexed to the area at risk, and the area of necrotic tissue indexed to the total left ventricular mass. All three portions of myocardial tissue were stored at −70°C for subsequent determination of myeloperoxidase (MPO) activity, which was done in a blinded manner.

Determination of Myocardial MPO Activity

The myocardial activity of MPO, an enzyme occurring virtually exclusively in neutrophils, was determined by the method of Bradley et al as modified by Mullane et al.24 The myocardium was homogenized in 0.5% HTAB (Sigma Chemical Co) and dissolved in 50-mmol/L potassium phosphate buffer at pH 6.0 with a Polytron (PCU-2) homogenizer. Homogenates were centrifuged at 12,500g at 4°C for 30 minutes. The supernatants were then collected and reacted with 0.167 mg/mL of O-dianisidine dihydrochloride (Sigma Chemical Co) and 0.0005% H₂O₂ in 50 mmol/L phosphate buffer at pH 6.0. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide per minute at 25°C.

Isolated Coronary Ring Studies

Both LAD and LCx coronary segments (i.d. 300 to 500 μm) were removed and placed into warmed K-H buffer. Isolated coronary vessels were cleaned and cut into rings 2 to 3 mm long. The rings were then mounted on stainless steel hooks, suspended in 10-mL tissue baths, and subsequently connected to FT-03 force displacement transducers (Grass Instrument Co) to record changes in force on a Grass model 7 oscillographic recorder, as previously described. The baths were filled with 10 mL of K-H buffer and aerated at 37°C with a gas mixture of 95% O₂ and 5% CO₂. Coronary rings were initially stretched to give a preload of 0.5 g force and equilibrated for 60 to 90 minutes. During this period, the K-H buffer in the tissue baths was replaced every 20 minutes. After equilibration, the rings were then exposed to 100 nmol/L U-46619 (9,11-epoxymethano-PGH₂, Biomol Research Laboratories), a thromboxane A₂ mimetic, to generate about 0.5 g of developed force. Once a stable contraction was obtained, 0.1, 1, 10, and 100 nmol/L acetylcholine (ACh), a receptor-mediated endothelium-dependent vasodilator, was added to the bath. After the response stabilized, the rings were washed and allowed to equilibrate to baseline once again. The procedure was repeated with A-23187 (1, 10, 100, and 1000 nmol/L), a receptor-independent endothelium-dependent vasodilator, and then with acidified NaNO₃, an endothelium-independent vasodilator (0.1, 1, 10, and 10 μmol/L). NaNO₃ was prepared by dissolving the compound in 0.1N HCl and titrating it to pH 2.0.

Statistical Analysis

All values in the text and figures are presented as mean±SEM of n independent experiments. All data were subjected to ANOVA followed by the Bonferroni correction for post hoc t tests. Probabilities of P<.05 were considered to be statistically significant.

![Binding of DREG-200 to Cat Neutrophils](image)

FIG 1. Flow cytometry histogram of log fluorescence intensity of MAb DREG-200 and control MAb R3.1 at 40 μg/mL. The control antibody (MAb R3.1) peak is 2.1 and the DREG-200 peak is 47.2 mean channel fluorescence. Thus, a significant increase in mean channel fluorescence was observed, indicating a high degree of cross-reactivity of DREG-200 with feline neutrophils.

Results

Binding of DREG-200 to Cat Cells and Tissue

Flow cytometric analysis of freshly isolated whole blood from three cats clearly indicates that DREG-200 does bind to feline neutrophils, as shown in Fig 1. Unstimulated cat neutrophils stained 75.0±9.3% positive for DREG-200, compared with 5.1±0.3% positive staining for the control IgG antibody, MAb R3.1 (P<.01). In addition, stimulation with leukotriene B₄ for 5 minutes resulted in the shedding of L-selectin, and the percentage of neutrophils staining positive for L-selectin under these conditions was 10.1±4.6% (P<.01 from DREG-200 unactivated).

Binding of DREG-200 to cat neutrophils resulted in a mean channel fluorescence of 25.5±7.0 compared with 2.3±0.1 for MAb R3.1 (P<.01). Furthermore, after stimulation of the neutrophils with leukotriene B₄, mean channel fluorescence decreased to 3.2±0.4 (P<.01). The addition of leukotriene B₄ had no effect on the surface binding of the control antibody R3.1.

Immunohistochemical studies failed to demonstrate any binding of DREG-200 to either normal control or ischemic/reperfused cat coronary endothelial cells. These results clearly show that DREG-200 does not cross-react with cat vascular endothelium and is specific for neutrophils.

Effect of DREG-200 on Unactivated PMN Adherence to Coronary Vascular Endothelium In Vitro

To determine whether binding of DREG-200 to neutrophils actually blocks cat PMN-endothelium interaction, we directly observed the effect of DREG-200 on neutrophil adherence to cat coronary vascular endothelium in vitro. When unactivated PMNs were added to the normal nonischemic reperfused LCx coronary segments and incubated at 4°C for 30 minutes, only about 20 to 30 PMNs/mm² adhered to the endothelial surface. This basal level of adherence of unactivated PMNs to normal unactivated coronary endothelium was not significantly influenced by incubation of PMNs with DREG-200, indicating that the counterreceptor for L-selectin is not present and active on unstimulated cat
coronary endothelial cells. However, when unactivated PMNs were added to the coronary segments isolated from the cats subjected to 90 minutes of ischemia and 20 minutes of reperfusion, a threefold to fourfold increase in PMN adherence was observed. Preincubation of PMNs with DREG-200 significantly attenuated this response, but preincubation of PMNs with MAb R3.1 had no effect (Fig 2). These results indicate that the endothelial ligands for L-selectin are significantly upregulated after 90 minutes of ischemia and 20 minutes of reperfusion, and preincubation of PMNs with an L-selectin antibody blocked the interaction of constitutively expressed L-selectin on PMN surface with the newly expressed ligands on the endothelial surface. Fig 3 illustrates the concentration-response relation of DREG-200 on unstimulated PMN adherence to ischemic/reperfused coronary vascular endothelium. Addition of DREG-200 inhibited PMN adherence to the endothelium in a concentration-dependent manner with a maximal inhibition of about 70% occurring at 20 μg/mL of DREG-200.

Effect of In Vivo Administration of DREG-200 on Myocardial Ischemia and Reperfusion

The effects of administration of DREG-200 10 minutes before reperfusion on PMN accumulation and myocardial reperfusion injury were studied in a well-established cat myocardial ischemia and reperfusion model. In four sham myocardial ischemia and reperfusion cats, we observed that intravenous administration of DREG-200 (1 mg/kg) had no detectable effect on any of the measured hemodynamic, ECG, or biochemical variables. Thus, DREG-200 at the dose regimen used appeared to be devoid of any significant side effects that could interfere with this study. There were also no significant differences in any of the variables observed initially between the two MI+R groups of cats. Additionally, there were no significant differences between the sham MI+R and MI+R groups at any of the hourly pressure-rate index readings, suggesting that administration of DREG-200 exerted no significant effect on systemic hemodynamics and thus did not alter myocardial oxygen demand during myocardial ischemia and reperfusion (Fig 4). A few minutes after LAD occlusion, the ST segment of the ECG became significantly elevated and peaked at 20 to 40 minutes after coronary occlusion. After reperfusion, the ST segment decreased to nearly control values, indicating an effective degree of reperfusion. There was no significant difference in peak ST-segment elevation between the two MI+R groups (0.18±0.02 versus 0.19±0.02), indicating that the ischemic insult was comparable in these two MI+R groups. At reperfusion, there was a noticeable increase in the incidence of premature ventricular contractions in all cats. One MI cat treated with DREG-200 and two MI cats treated with MAb R3.1 developed ventricular fibrillation, which was successfully converted to a normal sinus rhythm. There was no obvious overall difference between the MI groups in the number of premature ventricular contractions occurring after reperfusion, indicating that DREG-200 did not appear to exert any overt antiarrhythmic effect. Myocardial ischemia and reperfusion did not significantly change the number of circulating PMNs in vehicle-treated cats. The PMN counts 10 minutes before coronary occlusion, 10 minutes before reperfusion, and 10 minutes and 4.5 hours after reperfusion were 6731±241/μL, 7125±317/μL, 7394±314/μL, and 6859±253/μL, respectively. Thus, the number of circulating PMNs did not change over the course of the experiment. Moreover, there was no significant difference in numbers of circulating PMNs between the two MI+R groups before myocardial ischemia (7611±206/μL in DREG-200 treated group, NS), and immediately before DREG-200 administration (ie, 10 minutes before reperfusion) (7418±302/μL). Furthermore, administration of DREG-200 at 1 mg/kg had no significant effect on circulating PMNs. In this regard, the PMN counts at 10 minutes and 4.5 hours after reperfusion were 7793±294/μL and 7258±217/μL, respectively. These results clearly indicate that administration of DREG-200 at 1 mg/kg in cats does not result in leukopenia. Therefore, any protective effects of

![Graph showing concentration-response relation of DREG-200 on inhibition of unstimulated neutrophil adherence to ischemic (90 minutes) and reperfused (20 minutes) coronary vascular endothelial cells. Data are expressed as percent inhibition of adhered polymorphonuclear leukocytes (PMNs) compared with maximal adherence without antibody. Values are mean±SEM for eight coronary vascular rings.](http://circ.ahajournals.org/)

![Graph showing effects of in vitro addition of DREG-200 and MAb R3.1 20 μg/mL on unstimulated neutrophil adherence to nonischemic/reperfused left circumflex (LCX) coronary endothelium and ischemic/reperfused left anterior descending (LAD) coronary endothelium. Data are expressed as numbers of polymorphonuclear leukocytes (PMNs) per square millimeter. Bar heights are means, brackets indicate ±SEM, and numbers at the bottom of the bars are numbers of coronary rings studied.](http://circ.ahajournals.org/)
DREG-200 in cat myocardial ischemia and reperfusion could not be attributed to changes in the number of circulating PMNs.

To observe the effects of DREG-200 on the degree of actual myocardial salvage of ischemic or necrotic tissue after reperfusion, we directly measured the area at risk of the ischemic heart and the amount of necrotic cardiac tissue expressed as a percentage of either the area at risk or total left ventricular mass. These findings are summarized in Fig 5. There was no significant difference in the area at risk expressed as percentage of total left ventricle between the groups, indicating that a comparable degree of ischemic jeopardy existed between the two MI+R groups. However, the necrotic area expressed as percentage of either area at risk or total left ventricular mass was significantly lower (P<.001) in cats treated with DREG-200, indicating that blocking L-selectin with DREG-200 significantly attenuated myocardial damage induced by ischemia and reperfusion.

Accumulation of neutrophils in the ischemic region during reperfusion has been thought to be one of the major mechanisms responsible for reperfusion injury. We measured MPO activity of the three different portions of the myocardium as a marker for neutrophil accumulation in ischemic tissue. These data are summarized in Fig 6. It is evident that in the nonischemic myocardium (ie, area not at risk), MPO activity was very low in both MI groups, and there was no significant difference between them, indicating that few neutrophils were present in the nonischemic myocardium. However, MI cats receiving only the control antibody, MAb R3.1, exhibited a marked increase in MPO activity in the ischemic region, developing a sevenfold to ninefold increase in MPO activity in the necrotic myocardium. In contrast, DREG-200–treated ischemic cats exhibited a significantly lower MPO activity in both ischemic nonnecrotic myocardial tissue and necrotic myocardial tissue. These results indicate that adherence and accumulation of neutrophils in ischemic/reperfused myocardium were markedly inhibited by the antibody against L-selectin.

Effect of DREG-200 on Endothelial Dysfunction

Since endothelial dysfunction is an early and critical event in reperfusion injury, we also tested endothelial function by comparing vasoactivity of isolated coronary artery rings with two endothelium-dependent vasodilators, ACh and A-23187, and with an endothelium-independent vasodilator, acidified NaNO₂. Fig 7 illustrates typical recordings of LAD coronary rings obtained from sham MI+R cats or MI+R cats receiving DREG-200 or MAb R3.1. Coronary rings isolated from sham MI+R cats exhibited full relaxation to the endothelium-dependent vasodilator ACh as well as to the

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**FIG 4.** Graph showing pressure-rate index (PRI) expressed as mm Hg×(beats per minute [bpm])/1000 sampled hourly during the 6-hour observation period. All values are mean±SEM for six cats in each group. No significant differences occurred among the three groups at any time or within any group after addition of antibody. MI, myocardial ischemia.

**FIG 5.** Bar graph showing tissue wet weight of area at risk as a percentage of the total left ventricular wet weight and of the necrotic tissue either as a percentage of area at risk and of the total left ventricle for the two MI+R groups. Heights of bars are means; brackets represent ±SEM for six cats. Statistical significance is indicated. MI, myocardial ischemia.
endothelium-independent vasodilator, NaNO₂. In contrast, ACh-induced relaxation of coronary rings obtained from MI+R cats receiving the nonbinding control antibody MAb R3.1 was significantly depressed, whereas ACh-induced relaxation of the rings obtained from cats treated with DREG-200 was significantly preserved. Coronary rings isolated from all groups relaxed fully to the endothelium-independent vasodilator NaNO₂. Fig 8 summarizes the full vasorelaxant responses to ACh, A-23187, and NaNO₂ in isolated cat LAD coronary artery rings. Endothelium-dependent relaxation of ischemic LAD coronary rings to the highest concentration of ACh and A23187 was significantly greater in DREG-200–treated MI+R cats than in MAb R3.1–treated cats. However, the peak responses to NaNO₂ were comparable in all groups. Thus, DREG-200 significantly protected against the loss of endothelium-dependent relaxation observed in coronary artery rings isolated from cats subjected to myocardial ischemia and reperfusion.

Discussion

Neutrophil recruitment to sites of tissue injury is mediated by multiple adhesion molecules, which are sequentially required for the attachment of the cells to the blood vascular endothelium and their subsequent extravasation into the surrounding tissue.25,26 These adherence receptors are now grouped into three main families, which include the integrin family (eg, CD11/CD18), the immunoglobulin superfamily (eg, ICAM-1), and the selectin family (eg, L-selectin, P-selectin).27,28

Present evidence indicates that neutrophil extravasation at sites of acute inflammation is at least a three-step process.14 First, PMNs within the flowing blood must recognize and bind to vascular endothelial cells activated by inflammatory stimuli. The adhesion strength of this initial interaction may be insufficient to overcome intravascular shear force completely, resulting in the characteristic PMN rolling phenomenon.14 The second step of this process involves adhesion strengthening, ensuring a cessation of rolling by PMNs and firm attachment to the endothelium. This is followed by the third step, characterized by transmigration of PMNs through the vascular wall. Numerous studies have shown that the integrin-immunoglobulin adhesion pathway (eg, CD11a/CD18–ICAM-1) is primarily involved in the second step of this process.19,27 Monoclonal antibodies directed against either CD11/CD18 on the PMN surface or ICAM-1 on the endothelial surface significantly inhibit PMN adherence to endothelial cells27 and their accumulation in ischemic/reperfused myocardial tissue.9,10 However, recent studies indicate that integrins interacting with their counterreceptors on endothelial cells are not responsible for PMN rolling along the endothelial surface under shear stress.12 Administration of a monoclonal antibody against CD11/CD18 does not inhibit PMN rolling along the endothe-
lium, although it significantly inhibits PMN firm adherence and subsequent transendothelial migration.14

PMN rolling along the endothelium has recently been demonstrated to be mediated by adhesion molecules belonging to the selectin family,27,29 which consists of E-selectin (endothelial-leukocyte adhesion molecule-1, ELAM-1), P-selectin (GMP-140), and L-selectin (LAM-1).27 E-selectin is expressed only on endothelial cells after stimulation by cytokines such as interleukin-1β and tumor necrosis factor-α.30 Its expression requires protein synthesis, and 4 to 6 hours is necessary for its peak surface expression.30 In contrast, P-selectin is stored in platelets and Weibel-Palade bodies of endothelial cells,31,32 and within minutes of activation of endothelial cells by thrombin, histamine, or free radicals, P-selectin is rapidly redistributed to the surface of the cell, where it can promote adherence of neutrophils, monocytes, and platelets.31,33

L-selectin was first described in the mouse as the MEL-14 antigen, the "homing" receptor for lymphocyte binding to high endothelial venules of peripheral lymph nodes.34 Recent experiments demonstrate that the expression of L-selectin also occurs on other cells, including hematopoietic progenitor cells, immature thymocytes, monocytes, eosinophils, and neutrophils.11,15 The functional role of L-selectin on the surface of neutrophils and monocytes as well as that of L-selectin found on the surface of lymphocytes appears to facilitate cell adhesion to vascular endothelium in the process of extravasation.27 L-selectin is believed to modulate the first step of neutrophil migration out of the vasculature (ie, neutrophil rolling in the microcirculation).32 Monoclonal antibodies to L-selectin block up to 80% of intravascular neutrophil rolling and thus inhibit adherence in mesenteric venules.32,35 Thus, it is thought that interaction of L-selectin on PMNs with its counterreceptor(s) on endothelial cells plays a major role in mediating PMN rolling along the endothelium, the first step in a sequence of PMN interactions with the endothelium.

In vitro studies have shown that activation of PMNs with various chemotactic agents results in a rapid increase in L-selectin binding affinity followed by a rapid shedding of the L-selectin molecule from the cell surface.36,37 Conversely, the CD11b/CD18 complex, a major integrin adhesion molecule, exhibits a rapid and sustained upregulation on activation of the leukocyte.38 These results suggest that CD11/CD18 and L-selectin on the PMN surface mediate fundamentally distinct adhesion events. Based on the present data concerning the pattern of PMN-endothelium interaction, many investigators12,14,15 now believe that selectin- and integrin-mediated PMN-endothelium interaction may occur in a cooperative and sequential fashion. L-selectin-mediated rolling of the neutrophils along the endothelium may prolong interaction time between PMNs and the surface of the endothelium to promote PMN activation. As the neutrophil becomes activated, L-selectin is shed and the integrins are upregulated on the surface, allowing for the neutrophil to adhere more strongly to the endothelium and begin its migration.12,14 Therefore, blockade of either the selectin pathway mediating PMN rolling or of the integrin pathway mediating adherence should markedly reduce PMN accumulation in inflammatory tissue.

Previous studies indicate that monoclonal antibodies directed against either the common β-chain of neutrophil adhesion glycoproteins (ie, CD18) or a major endothelial ligand for the CD18 complex (ie, ICAM-1) significantly attenuate endothelial dysfunction and myocardial injury after myocardial ischemia and reperfusion.9,10,39-41 However, the role of selectin-mediated initial PMN-endothelium interaction in reperfusion injury has not been studied. In the present study, we evaluated the role of L-selectin in myocardial reperfusion injury by observing the effects of administration of a monoclonal antibody to L-selectin (ie, DREG-200) on endothelial dysfunction and myocardial necrotic injury. Our results clearly show that blocking L-selectin with DREG-200 significantly attenuates unstimulated PMN adherence to ischemic/reperfused coronary endothelium, diminishes PMN accumulation in ischemic/reperfused myocardial tissue, significantly preserves endothelial function, and dramatically attenuates reperfusion-induced myocardial necrotic injury. To the best of our knowledge, this is the first observation that L-selectin-mediated PMN-endothelium interaction plays a significant role in myocardial reperfusion injury. These results implicate the early rolling phenomenon of PMNs in the microcirculation as a key step in triggering the adherence cascade of events leading to endothelial
dysfunction and to neutrophil amplification of the injury to the myocardium. Our data indicate that the DREG-200 cross-reacts significantly with L-selectin on the surface of neutrophils, as shown by flow cytometry. Activation of the neutrophil by leukotriene B4 caused shedding of the L-selectin from the neutrophil surface. Moreover, we could not detect any binding of DREG-200 to either control or ischemic/reperfused cat coronary endothelium by immunohistochemistry. Thus, DREG-200 does not appear to cross-react with either P-selectin or E-selectin in the cat, confirming the findings of von Andrian et al14 in the rabbit.

In our previous studies, we have reported that a monoclonal antibody against either CD18 on neutrophils (ie, MAB R15.7)9 or ICAM-1 on the endothelial cells (ie, MAB RR1/1)10 exerts significant endothelial and myocardial protective effects in the same model of ischemia/reperfusion as that used in the present study. The protective effects of DREG-200 in this model of reperfusion injury were comparable to those obtained with the MAB against ICAM-1 (ie, MAB RR1/1). In both cases, the blocking antibodies were compared against an isotype control nonbinding monoclonal antibody. The results obtained with the control antibodies are equivalent to those obtained with 0.9% NaCl as a vehicle control in previous studies.42 However, neither RR1/1 nor DREG-200 exerted as marked a protection against PMN accumulation and myocardial injury as MAB R15.7 in this model of ischemia/reperfusion. These differences may arise because PMNs adhere to the endothelium and migrate to the myocardium via CD18-dependent but L-selectin- and ICAM-1–independent mechanisms. Indeed, in this study, even 40 μg/mL of DREG-200 resulted in a 72% inhibition of PMN adherence, a value comparable to that obtained with 40 μg/mL of MAB RR1/1, whereas 20 μg/mL of MAB R15.7 almost totally inhibited PMN adherence to cat coronary endothelium.9 Alternatively, the affinity of the DREG-200 receptors on PMNs may be lower than that of MAB R15.7 receptors on PMNs.

In conclusion, we have demonstrated that a monoclonal antibody to L-selectin significantly inhibited PMN adherence to ischemic/reperfused coronary endothelium in vitro, and in vivo administration of DREG-200 just before reperfusion significantly attenuated myocardial injury and endothelial dysfunction. To our knowledge, this is the first in vivo study examining the role of L-selectin in myocardial ischemia/reperfusion injury. These results confirm that the initial L-selectin–mediated interaction of PMNs with the endothelium plays an important role in myocardial ischemia/reperfusion injury.

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