Coronary Endothelial and Cardiac Protective Effects of a Monoclonal Antibody to Intercellular Adhesion Molecule-1 in Myocardial Ischemia and Reperfusion

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Background. Intercellular adhesion molecule-1 (ICAM-1) is a major ligand on endothelial cells for adherence of activated polymorphonuclear leukocytes (PMNs). The major purpose of this study was to study the effects of RR1/1, a monoclonal antibody against ICAM-1 (i.e., MAb RR1/1), on myocardial injury and endothelial dysfunction associated with myocardial ischemia and reperfusion.

Methods and Results. Either MAb RR1/1 (2 mg/kg, n=7), an antibody that was found to bind selectively to endothelial cells in the cat, or MAb R3.1 (2 mg/kg, n=7), a nonbinding control antibody, was given as an intravenous bolus 10 minutes before reperfusion. Two hundred eighty minutes later, hearts were excised. The left ventricle area-at-risk (AAR) was similar in MAb RR1/1 (29±2%) and MAb R3.1 (30±3%) groups. In MAb R3.1-treated cats, 4.5 hours of reperfusion induced a significant myocardial injury (necrotic tissue/AAR, 28±2%), high myeloperoxidase activity (0.65±0.16 units/100 mg ischemic tissue), and a marked decrease in endothelium-dependent vasorelaxation in isolated left anterior descending coronary arteries (vasorelaxation to acetylcholine, 29±3%) with no change in endothelium-independent vasorelaxation (relaxation to NaNO₂, 91±3%). However, cats treated with MAB RR1/1 developed significantly less myocardial necrosis (10±2% of the AAR, p<0.01), lower myeloperoxidase activity in ischemic myocardial tissue (0.2±0.03 units/100 mg ischemic tissue, p<0.01), and enhanced vasorelaxant responses to endothelium-dependent relaxation to acetylcholine (53±5%) compared with ischemic/reperfused cats treated with MAb R3.1. Furthermore, addition of MAB RR1/1 in vitro significantly inhibited unstimulated PMN adherence to ischemic-reperfused coronary artery endothelium.

Conclusions. These results suggest that ICAM-1-dependent PMN adherence plays an important role in reperfusion injury, and that PMN adherence and infiltration contribute significantly to coronary endothelial dysfunction. (Circulation 1992;86:937-946)

KEY WORDS • polymorphonuclear leukocytes • endothelium • reperfusion • intercellular adhesion molecule

Ischemia/reperfusion--induced inflammatory tissue damage is mediated to a large extent by polymorphonuclear leukocytes (PMNs).1-3 Experimental strategies that have involved inhibition of PMN activation or depletion of PMNs have been shown to reduce myocardial injury after ischemia and reperfusion.4-6 Adherence of PMN to the vascular endothelial cells appears to represent one of the earliest steps in the pathogenesis of ischemia/reperfusion tissue injury. Moreover, recent experimental results indicate that PMN adherence to endothelial cells involves several adhesion molecules on the surface of both cell types. Among those adhesion receptors found on PMNs, the CD11/CD18 glycoprotein complex appears to play a critical role in PMN-endothelial interaction.7,8 In this connection, we have observed that a monoclonal antibody against the common β-chain of the CD11/CD18 family (i.e., MAB R15.7) significantly protected myocardial tissue from reperfusion injury and preserved endothelial function.9

Recently, an endothelial ligand for the CD18 complex has been identified and named intercellular adhesion molecule-1 (ICAM-1). This molecule appears to be constitutively expressed at low levels on the endothelial cell surface and can be markedly upregulated by cytokine stimulation. Administration of a monoclonal antibody against ICAM-1 inhibits activated PMN adherence to endothelial cells both in vivo and in vitro.10,11 However, the role of ICAM-1 interaction with PMN glycoproteins in ischemia/reperfusion--induced PMN adherence and accumulation in ischemic myocardial
tissue is unknown. Therefore, the purposes of this study were to determine the effect of a monoclonal antibody to ICAM-1 (i.e., MAb RR1/1) on cat PMN adherence to cat coronary vascular endothelium in vitro, evaluate the effect of MAb RR1/1 on ischemia/reperfusion–induced PMN accumulation in cardiac tissue, and investigate the effects of MAb RR1/1 given just before reperfusion on coronary endothelial dysfunction and myocardial injury associated with myocardial ischemia and reperfusion in cats.

**Methods**

Adult male cats (2.3–3.8 kg) were anesthetized with sodium pentobarbital (30 mg/kg i.v.). An intratracheal cannula was inserted through a midline incision, and all cats were placed on intermittent positive-pressure ventilation (Harvard Apparatus small animal respirator, Dover, Mass.). A polyethylene catheter was inserted into the right external jugular vein for infusion of drugs or their vehicle. Another polyethylene catheter was positioned in the abdominal aorta through the left femoral artery 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 left anterior descending coronary artery (LAD) 8–10 mm from its origin. Standard lead II of the scalar ECG was used to determine heart rate (HR) and ST segment elevation. The ECG and MABP were continuously recorded on a Grass model 7 oscillographic recorder. ST segment elevations were determined manually from an ECG recording at 50 mm/sec every 20 minutes. The pressure-rate index (PRI), an approximation of myocardial oxygen demand, was calculated as the product of MABP and HR divided by 1,000.

**Experimental Protocol**

After completing all surgical procedures, the cats were allowed to stabilize for 30 minutes, at which time a baseline reading of ECG and MABP and an initial blood sample were 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 for 4.5 hours. Ten minutes before reperfusion, a monoclonal antibody directed against ICAM-1 of endothelial cells (MAb RR1/1) or a nonbinding control antibody, MAb R3.1, was given intravenously as a bolus. Animals were randomly divided into two major groups consisting of seven cats in each group: 1) MI plus MAb R3.1 (2 mg/kg) and 2) MI plus MAb RR1/1 (2 mg/kg). In a preliminary study, we found that 1 mg/kg MAb RR1/1 only partially protected myocardium from reperfusion injury, and we observed that a concentration of 40 μg/ml MABP RR1/1 was needed to exert its maximal inhibition effect on PMN adherence. Furthermore, a dose of 2 mg/kg MAb RR1/1 was found to be just in excess of that necessary to exert a 100% block of ICAM-1 at 5–6 hours in primates using flow cytometry (personal communication, Dr. R. Winquist). These data led to the final selection of 2 mg/kg as the dose of choice. An additional six cats were subjected to a sham MI procedure in which everything was identical to the MI cats except that the ligature around the LAD was not tightened. Three of these sham-MI cats received 2 mg/kg MAb RR1/1, and three received MAb R3.1.

**Plasma Creatine Kinase Analysis**

Arterial blood samples (2 ml) were drawn immediately before ligation and hourly thereafter. The blood was collected in polyethylene tubes containing 200 IU heparin sodium. Samples were centrifuged at 2,000g and 4°C for 20 minutes, and plasma was removed for biochemical analysis. Plasma protein concentration was assayed using the biuret method. Plasma creatine kinase (CK) activity was measured without knowledge of the group of origin of the cat using the method of Rosalki and expressed as international units per milligram of protein.

**Myocardial Tissue Analysis**

At the end of the 6-hour experimental period, the ligature around the LAD was retightened. Then, 30 ml of 0.5% Evans blue was injected into the left atrium to stain that 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 into warmed, oxygenated Krebs-Henseleit (K-H) solution. The left circumflex coronary artery (LCx) and LAD were isolated and removed for the subsequent study of coronary ring vasoactivity. Then, the right ventricle and great vessels were removed, and the left ventricle was sliced parallel to the atrioventricular groove in 3-mm-thick sections. The unstained portion of the myocardium (i.e., the total area-at-risk) was separated from the Evans blue-stained portion of the myocardium (i.e., the area-not-at-risk). The area-at-risk was again sectioned into 1-mm-thick slices and incubated in 0.1% nitroblue tetrazolium (NBT) 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 that did not stain was separated from the stained portion of the myocardium (i.e., the ischemic but nonnecrotic area) blindly by other investigators in our laboratory. All three portions of the left ventricular myocardium (i.e., nonischemic, ischemic nonnecrotic, and ischemic necrotic) were weighed, and the results are expressed as the area-at-risk indexed to the total left ventricular mass and the area of necrotic tissue indexed to the area-at-risk and as percent of the total left ventricular mass. The three portions of the myocardium were then stored at −70°C for later assay of myeloperoxidase activity, which was done in a blinded manner.

**Determination of Tissue Myeloperoxidase**

The myocardial activity of myeloperoxidase, an enzyme occurring virtually exclusively in neutrophils, was determined using the method of Bradley et al as modified by Mullane et al. The myocardium was homogenized in 0.5% HTAB (Sigma Chemical Co., St. Louis, Mo.) and dissolved in 50 mM potassium phosphate buffer at pH 6 using a Polytron (PCU-2) homogenizer. Homogenates were centrifuged at 12,500g, 4°C for 30 minutes. The supernatants were then collected and reacted with 0.167 mg/ml of o-dianisidine dihydrochloride (Sigma Chemical) and 0.0005% H2O2 in 50 mM
phosphate buffer at pH 6.0. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of myeloperoxidase is defined as that quantity of enzyme hydrolyzing 1 mmol peroxide/min at 25°C. The assays were performed without knowledge of the group to which each cat belonged. To determine whether exposure of the ischemic-reperfused myocardium to NBT has any effect on myeloperoxidase assay, we made the following additional observations in three cats. These cats were subjected to 90 minutes of ischemia and 4.5 hours of reperfusion and received only 0.9% NaCl. Myocardial area-at-risk was determined by the same method as mentioned above, and each heart was randomly separated into two subgroups. Half of the myocardium was incubated with NBT for 15 minutes, and another half was incubated with 0.9% NaCl. Subsequently, myeloperoxidase activity was assayed separately. The results indicate that exposure of the ischemic/reperfused myocardium to NBT had no effect on myeloperoxidase assay (i.e., 0.71±0.09 units/100 mg tissue with NBT incubation versus 0.69±0.08 units/100 mg tissue with 0.9% NaCl incubation, p>0.5). Furthermore, we also observed that Evans blue staining of the normal non–ischemic/reperfused myocardium had no significant effect on myeloperoxidase assay (0.003±0.001 units/100 mg tissue with Evans blue stain versus 0.004±0.002 units/100 mg tissue without Evans blue stain).

Isolated Coronary Ring Studies

Both LAD and LCx coronary segments (i.e., i.d., 300–500 mm) were removed and placed into warmed K-H buffer consisting of (in mM): NaCl 118, KCl 4.75, CaCl2 · 2H2O 2.54, KH2PO4 1.19, MgSO4 · 7H2O 1.19, NaHCO3 12.5, and glucose 10.0. Isolated coronary vessels were cleaned and cut into rings 2–3 mm in length. 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., Quincy, Mass.) to record changes in force in a Grass model 7 oscillographic recorder. The baths were filled with 10 ml of K-H buffer and aerated at 37°C with a gaseous mixture of 95% O2–5% CO2. Coronary rings were initially stretched to give a preload of 0.5 g of force and equilibrated for 60–90 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 nM U-46619 (9,11-epoxymethanoy-PGH2, Upjohn Co., Kalamazoo, Mich.), 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 nM acetylcholine (ACH) was added to the bath. After the response stabilized, the rings were washed and allowed to equilibrate to baseline again. The procedure was repeated with A-23187 (1, 10, 100, and 1,000 nM) and then with NaNO2 (0.1, 1, 10, and 100 μM). NaNO2 was prepared by dissolving the compound in 0.1N HCl and titrating it to pH 2.0. Titrating distilled water to pH 2.0 and adding aliquots to buffer in the bath did not produce any vasorelaxation.

Cat PMN Isolation and Labeling

Peripheral blood (20–40 ml) was collected from the femoral artery of pentobarbital (30 mg/kg i.v.) anesthetized adult male cats and anticoagulated with citrate-phosphate-dextrose solution (Sigma Chemical) (1:4:10, vol/vol anticoagulant to whole blood) into round-bottom polycarbonate centrifuge tubes (Nalge, Rochester, N.Y.). PMNs were isolated by a procedure modified from Lafrado and Olsen.16 Platelet-rich plasma (PRP) was obtained by centrifuging blood at 400g for 20 minutes in an Econospin Tabletop Swinging Bucket Centrifuge (Sorvall Instruments, Wilmington, Del.). PRP was decanted and centrifuged at 2,500g for 10 minutes to obtain platelet-poor plasma (PPP). PPP was then mixed with isotonic Percoll (Sigma Chemical) (9 vol Percoll:1 vol 1.5 M NaCl) to produce Percoll-PPP density gradients of 80%, 62%, and 50%. Five milliliters of 4% dextran (average molecular weight, 60,000–90,000; Sigma Chemical) was added to the erythrocyte-leukocyte pellet from the initial 400g centrifugation. After mixture by inversion, the erythrocytes were allowed to settle for 50 minutes. The pellets 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 62–82% interface and washed twice with 0.9% NaCl before being assayed for viability using Trypan blue exclusion. PMN preparations obtained by this method were typically >95% pure and >95% viable.

Isolated autologous PMNs were then labeled with Zynaxis PKH2 fluorescent dye according to the method of Yuan and Fleming.17 One milliliter of diluent was added to a loose cell pellet containing <10 million cells. One milliliter of PKH2-GL dye (4 μM) was added to the cell suspension and then mixed for 5 minutes by inversion. Two milliliters of phosphate-buffered saline (PBS) (containing 10% PPP in PBS) was added to stop the reaction, and another 5 ml of PBS was underlayered in 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 then recounted. This labeling procedure yields cells possessing normal morphology and function.17

Immunohistochemical Localization of ICAM-1

To assess the cross-reactivity of MAb RR1/1 with feline endothelium, streptavidin-peroxidase immunohistochemical localization of ICAM-1 was conducted according to the method of Lutty et al.18 Three additional cats were subjected to 90 minutes of LAD coronary occlusion followed by 270 minutes of reperfusion, and the hearts were isolated. Tissue samples were taken from the nons ischemic and ischemic zones and frozen in O.C.T tissue embedding compound (Tissue TEC, Miles, Elkhart, Ind.). Then, 10-μm sections were cut at −20°C and fixed in 2% paraformaldehyde in PBS (140 mM NaCl, 10 mM NaPO4, pH 7.4) for 5 minutes at 4°C. After fixation, the sections were washed in PBS, permeabilized in absolute methanol for 5 minutes at −20°C, and then air dried. Endogenous peroxidases were inhibited by a 5-minute incubation in 3% hydrogen peroxide. After washing in PBS, the tissue was blocked with 2% goat serum and then washed again in PBS. Nonspecific binding of streptavidin and biotin was prevented by using the ABC Blocking Kit (Vector, Burlingame, Calif.) as recommended by the manufacturer. MAb RR1/1 was diluted with 1% bovine serum albumin (BSA; Pentax Fraction V, Miles) in PBS and used at titers of 1:200.
1:2,000, 1:1,000, and 1:500 for 24 hours at 4°C. After incubation with primary antibody, the sections were washed in PBS for 30 minutes and then incubated with biotinylated goat anti-mouse immunoglobulin (IgG) (1:500; Kirkegaard and Perry, Gaithersburg, Md.) for 30 minutes. This secondary antibody was preincubated for 30 minutes at 37°C with cat serum (1 part antibody to 10 parts serum) before final dilution with PBS containing 1% BSA. The tissue sections were then incubated in streptavidin labeled with peroxidase (1:500) and then washed in PBS for 15 minutes. After washing with PBS, 3 amino-9-ethylcarbazole (AEC; Sigma Chemical) was used as the peroxidase indicator: 12 ml of stock 8 mM AEC in absolute dimethyl sulfoxide was added to 100 ml of 0.1 M sodium acetate (pH 5.1) and 0.8 ml of 3% H2O2. After 15 minutes of developing, the slides were washed twice in distilled water, one of the two sections on each slide was counterstained with Harris’ hematoxylin, and the coverslips were applied with Kaiser’s glycerogel. Localization of MAb RR1/1 was visualized by a red reaction product.

Controls included sections incubated with no primary antibody (MAb RR1/1) and sections incubated with goat or mouse serum (1:500) instead of MAb RR1/1. No reaction product was visualized in any of the control sections taken from either the nonischemic or ischemic zone of ischemic/reperfused hearts. However, reaction product was observed in endothelium of tissue sections incubated with primary antibody (i.e., MAb RR1/1). Furthermore, more reaction product was observed in the endothelium of sections isolated from the ischemic zone than those isolated from nonischemic zone. Some weak staining was observed in the interstitium of a few ischemic/reperfused tissue sections. This staining appeared to be on macrophages localized in the myocardium. These results clearly demonstrate that MAb RR1/1 cross-reacts with ICAM-1 on the endothelial surface in cat coronary vessels.

Effect of MAb RR1/1 on Activated PMN Adherence to Stimulated Coronary Artery Endothelium

In three control cats that were not subjected to ischemia/reperfusion, normal LAD and LCx coronary segments were carefully removed so as not to disturb the endothelium and placed into warmed K-H solution. Isolated coronary vessels were cleaned of fat and connective tissue and cut into rings 2–3 mm in length. These rings then were opened carefully and placed into 5-ml round cell culture dishes containing 3 ml of K-H solution and MAb RR1/1 (40 μg/ml), or MAb R3.1 (40 μg/ml) was added. After 10 minutes of preincubation with MAb RR1/1 or MAb R3.1, PMNs (4 × 10^5 cells/ml) were added in the absence of any activator and incubated for 20 minutes. Coronary artery strips were then removed from the culture dishes, and PMN adherence to endothelium was assessed as described above.

Statistical Analysis

All values in the text, table, 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 test. Values of p≤0.05 were considered statistically significant.

Results

Inhibitory Effect of MAb RR1/1 on PMN Adherence to Coronary Vascular Endothelium In Vitro

To determine whether MAb RR1/1 inhibits cat PMN adherence to cat endothelial cells, we observed the effect of MAb RR1/1 on PMN adherence to endothelium in vitro. To upregulate ICAM-1 on endothelial surface and upregulate CD11/CD18 on PMN surface, we stimulated endothelium with tumor necrosis factor-α (TNFα) for 2 hours and stimulated PMNs with fMLP for 20 minutes. Stimulating the coronary vascular endothelium with TNFα and activating PMNs with fMLP induced a significant increase in PMN adherence to endothelial cells. Addition of MAb RR1/1 inhibited stimulated PMN adherence to endothelium in a concentration-dependent manner (Figure 1). At a concentration of 40 μg/ml, a maximal inhibitory effect was obtained (about 75%). Figure 2 summarizes the results obtained from eight to 10 coronary strips. Very few
nonactivated neutrophils adhered to unstimulated coronary endothelium, whereas large numbers of fMLP (100 nM) activated neutrophils adhered to the TNFα-stimulated endothelium. Addition of MAb RR1/1 (40 μg/ml) before addition of PMNs and fMLP significantly inhibited neutrophils from adhering to the coronary endothelium, whereas addition of a nonbinding control antibody had no effect. These results clearly show that the anti-ICAM-1 antibody (i.e., RR1/1) is effective in preventing cat neutrophils from adhering to cat coronary endothelium.

Inhibitory Effect of MAb RR1/1 on Unstimulated PMN Adherence to Ischemic/Reperfused Coronary Artery Endothelium

Upregulation of ICAM-1 expression on endothelial cell surface in response to cytokine stimulation takes at least 1 hour. To determine whether ischemia/reperfused cat coronary artery endothelium exhibited any changes in their adhesive properties and whether basally expressed ICAM-1 on endothelial surface plays any important role in neutrophil adherence to endothelium, we studied the adherence of unstimulated PMNs to the ischemic/reperfused coronary artery endothelium and the effect of MAb RR1/1 on this response. Figure 3 summarizes these results. After 90 minutes of ischemia and 20 minutes of reperfusion, unstimulated PMN adherence to coronary artery endothelium was significantly increased, indicating that the adhesiveness of ischemic/reperfused coronary artery endothelium was markedly enhanced. Furthermore, when MAb RR1/1 was incubated with ischemic/reperfused coronary artery segments for 10 minutes before addition of PMNs, PMN adherence to cat coronary endothelium was significantly decreased by about 48% ($p<0.01$). Addition of a nonbinding control isotype antibody had no effect on PMN adherence. These results indicate that ischemic/reperfused cat coronary arteries exhibited a significant enhancement of adhesiveness and that basally expressed ICAM-1 present on cat vascular endothelium plays an important role in PMN adherence after ischemia/reperfusion.

Cardiac Electrophysiological and Hemodynamic Changes

In three sham-MI cats, we observed that intravenous administration of MAb RR1/1 at a dose of 2 mg/kg had no effect on any of the measured hemodynamic, ECG, or biochemical variables. Furthermore, there were no significant differences in any of the variables observed initially between the two MI groups of cats. However, within a few minutes of LAD occlusion, the ST segment became elevated and peaked at 20–40 minutes after coronary occlusion. After reperfusion, the ST segment decreased to nearly control values. There was no significant difference in peak ST segment elevation between the two MI groups (0.18±0.05 versus 0.19±0.05 mV), indicating that the ischemic insult was similar in these two groups. At reperfusion, there was a noticeable increase in the incidence of premature ventricular contractions (PVCs). One cat in each group developed ventricular fibrillation that was resistant to cardioversion and were thus excluded from further study. There was no overall difference between the MI groups in the number of PVCs occurring after reperfusion, indicating that MAb RR1/1 does not appear to exert any overt arrhythmic effect. Immediately after coronary occlusion, the PRI decreased in all of the cats. There were no significant differences between the two MI groups at any of the hourly PRI readings, suggesting that administration of MAb RR1/1 had no significant effect on systemic hemodynamics and thus no effect on myocardial oxygen demand during MI and reperfusion.

Effect of MAb RR1/1 on Reperfusion Myocardial Injury

In control sham-MI cats, the plasma CK activity increased only slightly throughout the 6-hour observation period, reaching a final value of $12±2.9$ IU/μg protein. Administration of MAb RR1/1 in the sham-MI cats did not change the CK activity (final plasma CK activity, $13±3.2$ IU/μg protein), indicating that administration of MAb RR1/1 had no effect on CK clearance. In addition, we added MAb RR1/1 to aliquots of cat plasma obtained from cats subjected to MI plus 0.9% NaCl, and the samples were within 6% of each other in
the presence and absence of the antibody. Thus, MAb RR1/1 does not affect the CK assay. In the two MI groups, plasma CK activity increased slightly during MI. In MI cats treated with nonbinding control antibody, MAb R3.1, plasma CK activity increased significantly during the 4.5 hours of reperfusion, reaching a final value of 65±6.1 IU/µg protein. In contrast, MI cats treated with MAb RR1/1 developed significantly lower CK activities compared with MI cats treated with the MAb R3.1. Although there was no significant difference in CK activity at the first two readings after reperfusion, the later increases in plasma CK activity were significantly attenuated (final value, 40.7±4.8 IU/µg protein, p<0.01 compared with MAb R3.1–treated group), suggesting that administration of MAb RR1/1 protected the myocardium from reperfusion injury.

To verify plasma CK activity as an index of preservation of ischemic tissue and to determine the effects of MAb RR1/1 on the degree of myocardial salvage of ischemic or necrotic tissue after reperfusion, we also 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 of the total left ventricular mass. There was no significant difference in the area-at-risk expressed as percentage of total left ventricle between groups, indicating that a comparable initial ischemic insult existed. However, the necrotic area either expressed as percentage of area-at-risk or of total left ventricular mass was significantly lower (p<0.01) in cats treated with MAb RR1/1, indicating that MAb RR1/1 significantly prevented myocardial damage induced by ischemia/reperfusion (Figure 4).

Prevention of PMN Accumulation in Ischemic Myocardial Tissue by MAb RR1/1

Infiltration of the ischemic region by neutrophils during reperfusion has been thought to be one of the major mechanisms responsible for reperfusion injury. We measured myeloperoxidase activity of the three different portions of the myocardium as a marker for neutrophil accumulation in ischemic tissue. It is evident that in the nonischemic myocardium (i.e., area-not-at-risk), myeloperoxidase activity was very low in both MI groups, and there was no significant difference between them, indicating that few neutrophils infiltrated in the nonischemic myocardium. However, MI cats receiving MAb R3.1 exhibited a marked increase in myeloperoxidase activity in the ischemic region, with a 10–12-fold increase in myeloperoxidase activity in the necrotic myocardium. In contrast, MAb RR1/1–treated ischemic cats exhibited a significantly lower myeloperoxidase activity in ischemic nonnecrotic myocardial tissue but not in the necrotic myocardial tissue (Figure 5). These results indicate that adherence and infiltration of neutrophils to ischemic nonnecrotic myocardium was markedly inhibited by the monoclonal antibody against ICAM-1 (i.e., MAb RR1/1).

Effect of MAb RR1/1 on Endothelial Dysfunction

Because endothelial dysfunction is an early event in reperfusion injury, we also tested endothelial function by comparing vasoactivity of coronary artery rings to the endothelium-dependent vasodilators ACh and A-23187 and to the endothelium-independent vasodilator, NaNO₂. Figure 6 illustrates a typical recording of LAD coronary rings obtained from sham-MI cat or MI cats receiving MAb R3.1 or MAb RR1/1. The coronary rings isolated from sham-MI cat exhibited a full relaxation to either endothelium-dependent vasodilator, ACh, or endothelium-independent vasodilator, NaNO₂. However, response of coronary rings obtained from an MI cat receiving MAb R3.1 to the endothelium-dependent vasodilator ACh was almost totally abolished at 4.5 hours after reperfusion, but these rings relaxed fully when the endothelium-independent vasodilator NaNO₂ was added. In contrast, endothelium-dependent vasodilation of the rings obtained from cats treated with MAb RR1/1 was significantly preserved. Figure 7 summarizes the vasorelaxant responses to ACh, A-23187, and NaNO₂ in isolated cat LAD and LCx coronary artery rings. Clearly, the response of ischemic LAD coronary rings to the endothelium-dependent vasodilators was significantly preserved in the MAb RR1/1–treated ischemic cats. The vasorelaxant response of paired nonischemic LCx coronary rings were studied as a control. The nonischemic LCx rings, whether obtained from MAb R3.1–treated group or MAb RR1/1–treated ischemic cats, showed complete relaxation to ACh, A-23187, and
the impairment of endothelial function and myocardial injury associated with ischemia/reperfusion.21–23

Current evidence indicates that PMNs contribute to endothelial dysfunction associated with ischemia/reperfusion by at least three mechanisms. First, adhered and activated PMNs may release reactive oxygen metabolites near the endothelium. These oxygen-derived free radicals, particularly superoxide free radicals, have been shown to inactivate endothelium-derived relaxing factor (EDRF) and thus induce significant endothelial dysfunction.24,25 Second, activated PMNs may release a variety of cytokines, including interleukin-1 (IL-1) and TNF. We have previously demonstrated that TNF inhibits EDRF release from the vascular endothelium.26 Third, PMNs are capable of damaging endothelial cells and increasing endothelial permeability through the release of other cytotoxic substances, including proteases, cationic proteins, and collagenases.27

PMN-induced endothelial injury over time may significantly contribute to reperfusion-induced myocardial injury. The endothelial dysfunction and injury may not only result in impaired vasodilation but may also lead to platelet and neutrophil adhesion, aggregation, and activation, thus increasing the risk of further ischemic injury.28 Furthermore, PMN may induce direct injury of the myocardium by the neutrophil-derived cytotoxic substances, such as proteases and reactive oxygen species. In this connection, activated PMNs were shown to adhere to cardiac myocytes by a CD18-ICAM-1-dependent mechanism29 and release their cytotoxic substances near these cardiac muscle cells, thus inducing myocyte injury. Furthermore, aggregated PMNs can mechanically obstruct capillaries and thereby contribute to exacerbation of the ischemic myocardial injury.30,31

An initial step in neutrophil-mediated injury in a variety of models of inflammation is the increased adhesion of neutrophils to vascular endothelium before pseudopod formation and diapedesis into the perivascular space. Recently, it has been demonstrated that PMN adherence to endothelial cells involves several adhesion molecules on the surface of both cell types.8 Among those adhesion receptors found on PMNs, the CD11/CD18 glycoprotein complex appears to play a critical role in PMN–endothelial cell interaction. It has been demonstrated that a number of factors, including fMLP, leukotriene B4, C5a, TNFα, calcium ionophore, and phorbol esters, may upregulate the surface expression of CD11/CD18 complex within minutes, thereby increasing PMN adherence to endothelial cells.32–34 Alternatively, it was recently reported that these factors may increase PMN adhesiveness by altering the conformation of CD11b/CD18.35,36

When stimulated with different factors, the endothelial surface can express several distinct ligands for PMN adherence, including GMP-140, ELAM-1, and ICAM-1.37–39 Among these adhesive proteins, ICAM-1 appears to be a major ligand for CD18-dependent PMN adherence to endothelial cells.40 Smith et al40,41 suggested that the unstimulated neutrophils adhere to surfaces via CD11a/CD18–ICAM-1 receptor ligand interaction, but chemotactically stimulated neutrophils adhere to purified ICAM-1 or endothelial cells by both CD11a/CD18–ICAM-1 and CD11b/CD18–ICAM-1 binding. Furthermore, upon stimulation by cytokines, the surface of myocyte may express ICAM-1 and thus induce CD18-
ICAM-1–dependent neutrophil–myocyte interaction. An antibody against either neutrophil CD18 (i.e., MAb R15.7) or ICAM-1 of the myocyte (MAb CL18/6) is highly effective in blocking PMN adherence to myocytes and their subsequent release of oxygen-derived free radicals.

Unlike GMP-140 and ELAM-1, ICAM-1 has been found to be constitutively expressed on the endothelial cell surface, although it can be markedly upregulated upon stimulation by cytokines, including TNF, and IL-1β. Early studies indicated that upregulation of ICAM-1 by cytokines requires protein synthesis and takes 2–3 hours. However, recent evidence clearly indicates that basally expressed ICAM-1 on the endothelial surface may play a significant role in PMN–endothelial cell interaction. Argenbright et al. observed that administration of a monoclonal antibody against ICAM-1 significantly inhibited C5a–activated PMN adherence to rabbit unstimulated endothelial cells in vivo. In addition, Gasic et al. demonstrated that perfusion of canine carotid arteries with H2O2 for only 20 minutes induced a significant increase in PMN adherence and that this response could be almost completely inhibited by CL18/6, a MAb against ICAM-1. Furthermore, Goldman et al. recently reported that thromboxane A2 may mediate PMN diapedesis after ischemia/reperfusion by activation of neutrophil adhesion receptors, which in turn interacts with basally expressed ICAM-1. Administration of a MAb against ICAM-1, MAb RR1/1, the monoclonal antibody that was used in this study, significantly reduced PMN diapedesis induced by ischemic plasma. Thus, basally expressed ICAM-1 may play an important role in PMN adherence to endothelial cells during early reperfusion. Furthermore, circulating TNFα concentrations were shown to increase significantly after ischemia/reperfusion. Therefore, the expression of ICAM-1 may be additionally upregulated by cytokines or other substances in ischemic/reperfused vessels during the later stages of reperfusion.

The exact mechanisms of PMN–endothelial cell interaction during early reperfusion is not clear. Recent experimental results suggested that link between GMP-140 (P-selectin) and platelet-activating factor (PAF) may play an important role in PMN adherence to endothelial cells. When endothelial cells are stimulated with thrombin, histamine, or free radicals, endothelial surface coexpression of PAF and GMP-140 occurred. GMP-140, which is constitutively present in Weibel-Palade bodies of endothelial cells and is rapidly expressed on the endothelial surface, may bind to a receptor on the neutrophil, tethering it to endothelial cells. PAF, which can be rapidly synthesized, may also bind to its receptor on the PMNs, serving as a signal for upregulation of CD11/CD18 glycoproteins on the PMN plasma membrane and subsequent activation of the PMNs. The tethering component mediated by GMP-140 facilitates PAF interaction with its receptor on the PMN and enhances the PAF-stimulated adhesiveness. Once activated, PMNs may adhere to unactivated endothelial cells, indicating that constitutively expressed ligands on the endothelial surface play an important role. One of the most likely candidates for this kind of endothelial ligand may be ICAM-1. Recent evidence indicates that PAF synthesis is significantly increased after ischemia and reperfusion and that the oxidative burst occurring just after reperfusion may upregulate GMP-140. Thus, it is quite possible that this link between GMP-140 and PAF occurs after ischemia/reperfusion and that constitutively expressed ICAM-1 may serve as the ligand for activated PMNs.

In a previous study, we have demonstrated that administration of a monoclonal antibody to the common β-chain of CD11/CD18 complex of PMN (i.e., MAb R15.7) 10 minutes before reperfusion significantly protected myocardial tissue from reperfusion injury and attenuated the endothelial dysfunction associated with ischemia/reperfusion. Our present result clearly shows that MAb RR1/1, a monoclonal antibody against ICAM-1 of the cat endothelial cells as demonstrated by using immunohistochemical technique, also exerts a significant protective effect on reperfusion injury. Compared with cats receiving a nonbinding control antibody, those cats treated with MAb RR1/1 exhibited a lower plasma CK activity after reperfusion, reduced area of cardiac necrosis, lower myeloperoxidase activity in ischemic tissue, and significantly preserved vasorelaxant responses of ischemia/reperfused LAD coronary rings to endothelium-dependent vasodilators. Furthermore, in vitro evidence showed that MAb RR1/1 significantly inhibits unstimulated PMN adherence to ischemic/reperfused coronary artery endothelium. These results indicate that ICAM-1 is an important ligand for PMN adherence to vascular endothelium and subsequent infiltration into myocardium in the cat, and PMN–ICAM-1 interaction play an important role in PMN accumulation in ischemic/reperfused myocardium. MAb RR1/1 may bind to basally expressed ICAM-1 in the early phase of reperfusion and upregulated ICAM-1 in the later phase of reperfusion, thus blocking ICAM-1–dependent PMN adherence and migration. Furthermore, MAb RR1/1 may also block ICAM-1 receptors on the ischemic/reperfused myocyte and thus inhibit infiltrated PMNs from adhering to cardiac tissues and prevent PMN-induced myocardial injury. Because collateral flow in the cat heart is very low (i.e., about 7% of control blood flow) and MAb RR1/1 had no hemodynamic effect, it is unlikely that MAb RR1/1 exerts its protective effect by influencing collateral flow.

Although MAb RR1/1 exerted a significant cardioprotective effect in this model of reperfusion injury, it did not exert as complete a protection against PMN accumulation and myocardial injury as does MAb R15.7, a monoclonal antibody against the common β-chain of the CD11/CD18 complex on the PMN surface (i.e., MAb R15.7). These differences may be due to the fact that PMNs adhere to the endothelium and migrate to the myocardiun via CD18-dependent but ICAM-1–independent mechanisms. Indeed, in this study, even at the highest concentration of MAb RR1/1 (i.e., 50 μg/ml), we only observed about 65% inhibition of PMN adherence, whereas addition of MAb against the common β-chain of the CD11/CD18 complex on the PMN surface (i.e., MAb R15.7) almost totally inhibited PMN adherence. Although ICAM-1 is the major ligand of CD11a/CD18, it is only one of the ligands for CD11b/CD18. Furthermore, CD11c/CD18 (i.e., p150,95), for which the endothelial ligand is unknown, may play a role in ischemia/reperfusion–induced PMN accumulation. Alternatively, the target for MAb R1/1...
may be less accessible to blood-borne antibodies (e.g., myocardial cells and other extravascular parenchymal cells).

In summary, we have demonstrated that 90 minutes of ischemia plus 4.5 hours of reperfusion induced a significant accumulation of PMN in ischemic myocardium, marked endothelial dysfunction, and significant myocardial injury. Administration of a monoclonal antibody against ICAM-1, a major endothelial ligand for CD-18-dependent PMN adherence, significantly inhibited PMN accumulation in ischemic myocardium and thus protected the myocardium from reperfusion injury and preserved endothelial function. These results suggest that ICAM-1-dependent PMN-endothelial cell interaction plays an important role in PMN adherence and accumulation associated with ischemia/reperfusion in the cat.

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