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Research Article

Neutrophil (PMN) adhesion to the vascular endothelium is an important mechanism of myocardial reperfusion injury. The adhesion process is initially mediated by selectins (e.g., P- and L-selectin), and monoclonal antibodies directed against these adhesion molecules exert cardioprotective activity in ischemia/reperfusion models. The counterreceptors for these selectins are thought to be carbohydrate-containing moieties. In this connection, we studied the effect of a soluble sialyl Lewisx-containing oligosaccharide (SLex-OS) on PMN-endothelial interactions in a feline model of myocardial ischemia/reperfusion (MI/R). SLex-OS (10 mg/kg), administered 10 min before R, significantly reduced myocardial necrosis compared with its vehicle 270 min after reperfusion (6 +/- 1% vs. 35 +/- 4% of area at risk, P < 0.01). The cardioprotection was confirmed by significantly lower plasma creatine kinase activities in SLex-OS vs. vehicle-treated cats (P < 0.01). Cardiac contractility (dP/dt max) of cats receiving SLex-OS was significantly preserved after 270 min of R (97 +/- 2% vs. 78 +/- 5% of initial, P < 0.01). Furthermore, endothelium-dependent relaxation to acetylcholine in coronary artery rings isolated from MI/R cats treated with SLex-OS was significantly preserved (73 +/- 7% vs. 22 +/- 6% vasorelaxation, P < 0.01). In vitro PMN adherence to coronary vascular endothelium after 270 min of R was significantly attenuated in the SLex-OS-treated group compared with the vehicle group (14 +/- 5 vs. 91 +/- 12 […]

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Sialyl Lewis^x–containing Oligosaccharide Attenuates Myocardial Reperfusion Injury in Cats

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Abstract

Neutrophil (PMN) adhesion to the vascular endothelium is an important mechanism of myocardial reperfusion injury. The adhesion process is initially mediated by selectins (e.g., P- and L-selectin), and monoclonal antibodies directed against these adhesion molecules exert cardioprotective activity in ischemia/reperfusion models. The counterreceptors for these selectins are thought to be carbohydrate-containing moieties. In this connection, we studied the effect of a soluble sialyl Lewis^x-containing oligosaccharide (SLe^x-OS) on PMN–endothelial interactions in a feline model of myocardial ischemia/reperfusion (MI/R). SLe^x-OS (10 mg/kg), administered 10 min before R, significantly reduced myocardial necrosis compared with its vehicle 270 min after reperfusion (6±1% vs. 35±4% of area at risk, $P < 0.01$). The cardioprotection was confirmed by significantly lower plasma creatine kinase activities in SLe^x-OS vs. vehicle-treated cats ($P < 0.01$). Cardiac contractility (dP/dt max) of cats receiving SLe^x-OS was significantly preserved after 270 min of R (97±2% vs. 78±5% of initial, $P < 0.01$). Furthermore, endothelium-dependent relaxation to acetylcholine in coronary artery rings isolated from MI/R cats treated with SLe^x-OS was significantly preserved (73±7% vs. 22±6% vasorelaxation, $P < 0.01$). In vitro PMN adherence to coronary vascular endothelium after 270 min of R was significantly attenuated in the SLe^x-OS–treated group compared with the vehicle group (14±5 vs. 91±12 PMN/mm^2, $P < 0.01$). Our results indicate that a SLe^x-OS is cardioprotective and preserves coronary endothelial function after MI/R, indicating an important role of sialyl Lewis^x in PMN accumulation, endothelial dysfunction, and myocardial injury in myocardial ischemia/reperfusion. (*J. Clin. Invest.* 1994. 93:1140–1148.) Key words: endothelial dysfunction • myocardial reperfusion injury • neutrophil adherence • selectins

Introduction

Reperfusion of an ischemic coronary vascular bed enhances myocardial injury despite the reestablishment of blood flow to the heart (1). This phenomenon is termed reperfusion injury. Adhesion of neutrophils (PMNs) to the vascular endothelium is one of the important early mechanisms which leads to reperfusion injury (2, 3). The adhesion process commences with PMN rolling owing to rapid expression of P-selectin on the endothelial surface as well as constitutively expressed L-selectin on unactivated PMNs (4). P-selectin tethers the neutrophils to the endothelial cell surface leading to PMN activation (i.e., shape change, shedding of L-selectin, and conformational change in CD11/CD18) (5, 6). Recently, our group has shown that monoclonal antibodies directed against either P-selectin or L-selectin prevent PMNs from adhering to coronary endothelium, preserve coronary endothelial function, and attenuate myocardial necrosis after myocardial ischemia and reperfusion (MI/R)[1] (7, 8).

The ligands for selectin-mediated adherence are presumably carbohydrate-containing structures (e.g., glycolipid or glycoprotein) (9, 10). In vitro studies have demonstrated that sialyl Lewis^x, Lewis^x or Lewis^a-containing carbohydrates inhibit selectin-mediated PMN adherence to endothelium (11, 12). However, the role of sialyl Lewis^x-containing carbohydrates in vivo in ischemia/reperfusion has not been studied.

Therefore, the main purposes of this study were to determine the effects of a soluble sialyl Lewis^x-containing oligosaccharide (SLe^x-OS) on (*a*) myocardial tissue injury, (*b*) cardiac contractility, (*c*) adherence of PMNs to the coronary vascular endothelium, and (*d*) coronary endothelial integrity in a well-established model of MI/R.

Methods

Experimental protocol and determination of myocardial necrosis. Adult male cats (2.6–3.4 kg) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). An intratracheal cannula was inserted through a midline incision, and the cats were placed on intermittent positive-pressure ventilation (small animal respirator, Harvard Apparatus Co., Dover, MA). A polyethylene catheter was inserted into the right external jugular vein for additional pentobarbital infusion in order to maintain a surgical plane of anesthesia and for administration of SLe^x-OS or its vehicle. A polyethylene catheter was inserted through the left femoral artery and positioned in the abdominal aorta for the measurement of mean arterial blood pressure (MABP) via a pressure transducer (Cobe Instruments, Lakewood, CO). After a midsternal thoracotomy, the anterior pericardium was incised and a 3-0 silk suture was placed around the left anterior descending (LAD) coronary artery 8–10 mm from its origin. A high-fidelity catheter (tip pressure transducer, model MPC 500, with transducer control unit, model TCB 500, Millar Instruments Inc., Houston, TX) was introduced into the left ventricle (LV) through the apical dimple. The catheter was positioned via observation

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1. *Abbreviations used in this paper:* ACh, acetylcholine; CK, creatine kinase; HR, heart rate; K-H, Krebs-Henseleit (solution); LAD, left anterior descending (coronary artery); LCX, left circumflex (coronary artery); LV, left ventricle; MABP, mean arterial blood pressure; MI/R, myocardial ischemia and reperfusion; SLe^x-OS, soluble sialyl Lewis^x-containing oligosaccharide.

of LV pressure and dP/dt wave form and was secured in place by a silk suture. Standard lead II of the scalar electrocardiogram (ECG) was used to determine heart rate (HR) and ST-segment elevation. ST-segment elevations were determined by analysis of the ECG recording at 50 mm/s every 20 min. The ECG, MABP, LVP, and dP/dt were continuously monitored on a model 78304 A unit (Hewlett Packard Co., Palo Alto, CA) and recorded on a recorder (Gould Inc., Cleveland, OH) every 20 min. The pressure-rate index (PRI), an approximation of myocardial oxygen demand, was calculated as the product of MABP and HR divided by 1,000.

After completing all surgical procedures, the cats were allowed to stabilize for 30 min, at which time baseline readings of ECG, MABP, LVP, and dP/dt were recorded. MI was induced by tightening the initially placed reversible ligature around the LAD so that the vessel was completely occluded. This was designated time point 0. 10 mg/kg of SLe^x-OS (i.e., a sialyl Lewis^x-containing carbohydrate, Cytel, San Diego, CA) or its vehicle Krebs-Henseleit (K-H) solution consisting of (in mM): NaCl 118; KCl 4.75, $CaCl_2 \cdot 2H_2O$ 2.54, KH_2PO_4 1.19, $MgSO_4 \cdot 7H_2O$ 1.19, $NaHCO_3$ 12.5, and glucose 10.0, pH 7.4, was given intravenously as a bolus 80 min after the coronary occlusion (i.e., 10 min before R). 10 min later (i.e., after a total of 90 min of ischemia) the LAD ligature was untied and the ischemic myocardium was reperfused for 4.5 h.

The cats were randomly divided into three major groups: four sham MI + R cats received SLe^x-OS (10 mg/kg in 1 ml/kg K-H solution), six MI + R cats received K-H solution (1 ml/kg), and six MI + R cats received SLe^x-OS (10 mg/kg in K-H solution). Sham MI + R cats were subjected to the same surgical procedures as MI + R cats except that the LAD coronary artery was not occluded.

To determine whether the effect of the SLe^x-OS was specific, we used the nonsialylated form of the oligosaccharide (Le^x-OS, 10 mg/kg) in three additional intact cats, and in isolated coronary artery rings from several additional cats.

Determination of myocardial necrosis. At the end of the 4.5-h reperfusion period, the ligature around the LAD was again tightened. 20 ml of 0.5% Evans blue was rapidly injected into the left ventricle to stain the area of myocardium which was perfused by the patent coronary arteries. The area-at-risk was determined by negative staining. Immediately after this injection, the heart was rapidly excised and placed in warmed, oxygenated K-H solution. The left circumflex (LCX) and the LAD coronary arteries were isolated and removed for subsequent study of coronary ring vasoactivity and PMN adherence. The right ventricle, great vessels, and fat tissue were carefully removed, and the LV 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 sectioned into small cubes (2 × 2 × 2 mm) and incubated in 0.1% nitroblue tetrazolium in phosphate solution at pH 7.4 and 37°C for 15 min. The tetrazolium dye forms a blue formazan complex in the presence of myocardial cells containing active dehydrogenases and their cofactors. The irreversibly injured or necrotic portion of the myocardium at risk which did not stain was separated from the stained portion of the myocardium (i.e., the ischemic but nonnecrotic area). The three portions of the myocardium (i.e., nonischemic, ischemic nonnecrotic, and ischemic necrotic tissues) were subsequently weighed. Results were expressed as 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.

In three additional cats receiving vehicle, the above described procedures were repeated except the area-at-risk was evenly divided before nitroblue tetrazolium staining into two portions. One portion was incubated with 250 μM SLe^x-OS and the other with K-H solution to determine whether SLe^x-OS altered the staining properties of the nitroblue tetrazolium. The area of necrotic tissue, expressed as a percentage of the area-at-risk, was 32.3±1.0% in the control area-at-risk samples and 33.4±1.8% (NS) in the area-at-risk samples incubated with SLe^x-OS. Thus, SLe^x-OS had no effect on nitroblue tetrazolium staining properties, and therefore could not artifactually alter the degree of myocardial necrosis.

Plasma creatine kinase (CK) analysis. Arterial blood samples (2 ml) were drawn immediately before ligation and hourly thereafter. The blood was collected in polyethylene tubes containing 200 IU of heparin sodium. Samples were centrifuged at 2,000 *g* and 4°C for 20 min and the plasma was decanted for biochemical analysis. Plasma protein concentration was assayed using the biuret method of Gornall et al. (13). Plasma CK activity was measured using the method of Rosalki (14) and expressed as international units per microgram protein. All assays were measured without prior knowledge as to the group of origin of each cat. In three cats receiving vehicle, the above described procedures were repeated except that aliquots of the plasma samples were incubated with 250 μM SLe^x-OS or an equal volume of K-H solution to determine whether SLe^x-OS altered the CK assay. The CK activities were 25.6±1.0 IU/μg protein in the K-H solution-treated samples and 26.4±1.8 IU/μg in the samples incubated with SLe^x-OS. These values were not significantly different, indicating that SLe^x-OS had no effect on the CK assay.

Cat PMN isolation and labeling. Peripheral blood (20 ml) was collected from the femoral artery at the beginning of the surgical procedure and PMNs were isolated by a procedure modified from Lafrado and Olsen (15) and described in detail previously (7, 8). After centrifugation to remove platelets the remaining blood was mixed with 6% dextran (average molecular weight 60,000–90,000, Sigma Chemical Co., St. Louis, MO) and PBS to allow erythrocytes to settle for 40–60 min. The leukocyte-enriched fraction was layered onto the Percoll/platelet-poor plasma gradient (Percoll/platelet-poor plasma density gradient of 80%, 62%, and 50%, Sigma Chemical Co.). The gradient was then centrifuged to separate the different cell populations. PMNs were collected from the 62%–80% interface and washed twice with PBS before being assayed for viability using trypan blue exclusion. PMN preparations obtained by this method were in general > 95% pure and > 95% viable.

Isolated autologous PMNs were then labeled with a fluorescent dye (Sigma Chemical Co.) according to the method of Yuan and Fleming (16). 1 ml of the diluent was added to a loose cell pellet containing about 10 million cells. One ml of PKH2-GL dye (4 mM) was added to the cell suspension, mixed, and then incubated for 5 min. 2 ml of PBS (containing 10% platelet-poor plasma in PBS) was added to stop the labeling reaction and another 5 ml of PBS was added to the suspension. Cells were then centrifuged at 400 *g* for 10 min 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 and function of cat PMNs (17).

PMN adherence to stimulated coronary artery endothelium. In three additional cats, peripheral blood (80 ml) was collected from the femoral artery and anticoagulated with citrate-phosphate-dextrose solution. PMNs were isolated and fluorescently labeled as described above. Hearts from each control cat were removed and placed in warm, oxygenated K-H solution. Both LAD and LCX coronary segments were isolated and placed into warmed K-H solution. Fat and connective tissue was removed from coronary vessels, and arteries were cut into rings of 2–3 mm in length. The arteries were then opened, and placed with the endothelial surface up into a cell culture dish filled with 3 ml of K-H solution. In order to stimulate endothelial cells to increase their adhesiveness for PMNs coronary, rings were incubated for 10 min with 2 U/ml of thrombin (Sigma Chemical Co.). The coronary ring segments were then placed in fresh K-H solution. Labeled PMNs (400,000 PMNs/ml) were added to the thrombin-stimulated endothelial cells alone or in combination with increasing concentrations of SLe^x-OS or Le^x-OS (i.e., 50, 100, 250, and 500 μM) and incubated for 20 min in a metabolic shaker bath at 37°C. After the incubation period, the coronary rings were removed, placed onto glass microscope slides. PMNs were counted using epifluorescence microscopy (Axioplan, Carl Zeiss, Inc., Thornwood, NY). Adherent neutrophils were counted on five fields from each vessel segment and expressed as PMNs/square millimeter.

PMN adherence to ischemic-reperfused coronary artery endothelium. Three additional cats were subjected to 90 min of myocardial ischemia and 20 min of reperfusion, to upregulate P-selectin on the vascular surface (8). The ischemic-reperfused LAD and the nonischemic LCX coronary artery segments were removed and prepared for PMN adherence as described above. The rings were placed in cell culture dishes, and unstimulated PMNs (400,000 PMNs/ml) were added simultaneously with either 250 μM of SLe^x-OS or an equal amount of K-H solution in a shaker bath for 20 min at 37°C. Coronary artery segments were then removed from the culture dishes, and PMN adherence to the endothelium was assessed by fluorescence microscopy as described above.

In the MI/R groups, coronary vessels were isolated at the end of the 270-min R period and incubated in culture dishes with autologous fluorescence-labeled neutrophils for 20 min at 37°C. These autologous neutrophils were isolated from the cats before induction of ischemia and are unstimulated. Adherent neutrophils were determined from each vessel segment and expressed as PMNs per square millimeter.

Isolated coronary ring studies. Both LAD and LCX coronary segments were isolated from the heart and placed into warmed K-H solution as described previously. Coronary vessels were cleaned from connective tissue and cut into rings of 2–3 mm in length. The rings were then mounted on stainless steel hooks, transfered to 10-ml tissue baths, and connected to FT-03 force displacement transducers (Grass Instrument Co., Quincy, MA) in order to record changes in force on an oscillographic recorder (model 7, Grass Instrument Co.). The baths were filled with 10-ml of K-H solution and gassed with 95% O_2 and 5% CO_2 at 37°C. Coronary rings were initially stretched to give a preload of 0.5 g of force and equilibrated for 90 min. During this period, the K-H solution in the tissue baths was replaced every 15 min. After equilibration, the rings were then stimulated with 100 nM U-46619 (9,11-epoxymethano-PGH_2, Biomol Research Laboratories, Plymouth Meeting, PA), a TXA_2 mimetic, to generate about 0.5 g of developed force. Once the contraction reached a stable plateau, acetylcholine (ACh), an endothelium-dependent vasodilator, was added to the bath in cumulative concentrations of 0.1, 1, 10, and 100 nM. After the response stabilized, the rings were washed three times and allowed to equilibrate for 20 min to reach baseline once again. The procedure including addition of U46619 was repeated with another endothelium-dependent vasodilator, A23187, (1, 10, 100, and 1,000 nM) and an endothelium-independent vasodilator, acidified $NaNO_2$, (0.1, 1, 10, and 100 μM), that was titrated to pH 2. Titration of K-H solution to pH 2 produced no detectable vasorelaxation in cat coronary artery rings. Relaxation was calculated as percent relaxation from the U46619-induced contraction. To determine whether SLe^x-OS exerted any direct vasoactive properties, we studied its effect on coronary rings of vehicle-treated cats. Incubation of eight U46619-contracted coronary rings with 250 μM SLe^x-OS did not show any vasorelaxation over a 20-min observation period (1.5±0.7%). Additionally, the ACh-induced vasorelaxant response of the contracted coronary rings was not affected by 250 μM SLe^x-OS.

Histological analysis of neutrophil accumulation and myocardial necrosis. Two additional cats, each receiving vehicle or 10 mg/kg OS that were subjected to 90 min of ischemia and 270 min of reperfusion in order to determine the presence of neutrophils in the ischemic region. The base of the heart was cross-clamped to arrest blood flow and the heart was excised and prepared for histological studies. Fixative was injected into the left ventricle, and the clamped heart was placed in fixative for 7–8 d to preserve the contents of the blood vessels and interstitium in situ. Slices, cut from the nonischemic or ischemic-reperfused region were dehydrated in a graded series of acetone solutions at 4°C. Tissue blocks were embedded in Immunobed (Polysciences Inc., Warrington, PA) at 4°C for 12 h. 5-μm-thick sections were cut using glass knives and transferred to coated slides (Vectabond, Vector Laboratories Inc., Burlingame, CA). Slides were stained with 1% toluidine blue in 1% sodium borate solution. The sections were examined using a Zeiss microscope at ×400.

Sialyl Lewis^x-containing oligosaccharide (SLe^x-OS). The SLe^x-containing oligosaccharide, NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ-O-CH_2CH_3, and its nonsialylated form employed in this study was synthesized by Cytel Corp. Both oligosaccharides had a purity of > 95% and was readily soluble in K-H solution (pH 7.4).

Initial studies indicated that 1 mg/kg SLe^x-OS dissolved in K-H solution (pH 7.4) exerted no myocardial protective effects in three cats subjected to MI and R. However, at 3 mg/kg, SLe^x-OS resulted in a partial cardioprotective effects (i.e., 20±4% necrosis/area-at-risk) in three cats subjected to MI/R. Thus, 10 mg/kg was selected as the dose for the full study.

Statistical analysis. All values in the text and figures are presented as means±standard errors of the mean of n independent experiments. All data were subjected to ANOVA followed by Fisher's t test. Probabilities of 0.05 or less were considered to be statistically significant.

Results

Confirmation of PMN accumulation in reperfused feline myocardium. Accumulation of PMNs in the ischemic region during R has been thought to be one of the major mechanisms responsible for R injury. We histologically analyzed the reperfused and nonreperfused regions of hearts treated with either 10 mg/kg SLe^x-OS or vehicle. In the nonischemic myocardium (i.e., area not at risk) there was no PMN adherence to coronary endothelium nor was there any myocardial cell damage visible in either group. However, MI cats receiving only vehicle, exhibited a marked increase in PMN accumulation in the ischemic region. A representative photomicrograph is shown Fig. 1. Adherence of PMNs to the coronary endothelium is related to a marked myocardial cell injury. In contrast, SLe^x-OS–treated ischemic cats exhibited significantly lower PMN accumulation in the area-at-risk and markedly reduced myocardial necrosis. These results indicate that accumulation of PMNs occurs in ischemic-reperfused myocardium and this is markedly inhibited by SLe^x-OS (Fig. 1).

Effect of SLe^x-OS on PMN adherence to stimulated coronary endothelium in vitro. We studied the effect of the SLe^x-OS on PMN adherence to activated coronary endothelium in vitro. To upregulate P-selectin on the endothelial surface, we stimulated the coronary vascular endothelium with thrombin (2 U/ml) for 10 min. Only a few PMNs adhered to unstimulated endothelium. However, addition of PMNs to thrombin-stimulated coronary endothelium resulted in a significant ($P < 0.001$), five- to ninefold increase in PMN adherence. Addition of SLe^x-OS (0, 50, 100, 250, and 500 μM) inhibited PMN adherence to the stimulated endothelium in a concentration-dependent manner (Fig. 2). At a concentration of 250 μM, a maximal inhibitory effect was obtained (i.e., ~ 60% of thrombin induced adherence). Increasing the concentration of SLe^x-OS above 250 μM did not inhibit PMN adherence any further. In contrast the nonsialylated form exhibited no inhibition of PMN adherence to the thrombin-stimulated coronary vasculature even up to concentrations of 500 μM. These results indicate that SLe^x-OS effectively inhibits PMN adherence to the thrombin-activated vascular endothelium.

Effect of SLe^x-OS on PMN adherence to ischemic-reperfused coronary artery endothelium. Since MI/R upregulates P-selectin, we examined the effect of SLe^x-OS on adhesive properties of unstimulated PMNs to coronary artery endothelium after 90 min of ischemia and 20 min of reperfusion. Fig. 3 summarizes the results of six to eight coronary artery segments taken from three different cats. After 90 min of MI and 20 min of R, unstimulated PMN adherence to coronary artery endothelium was significantly enhanced from unstimulated control en-

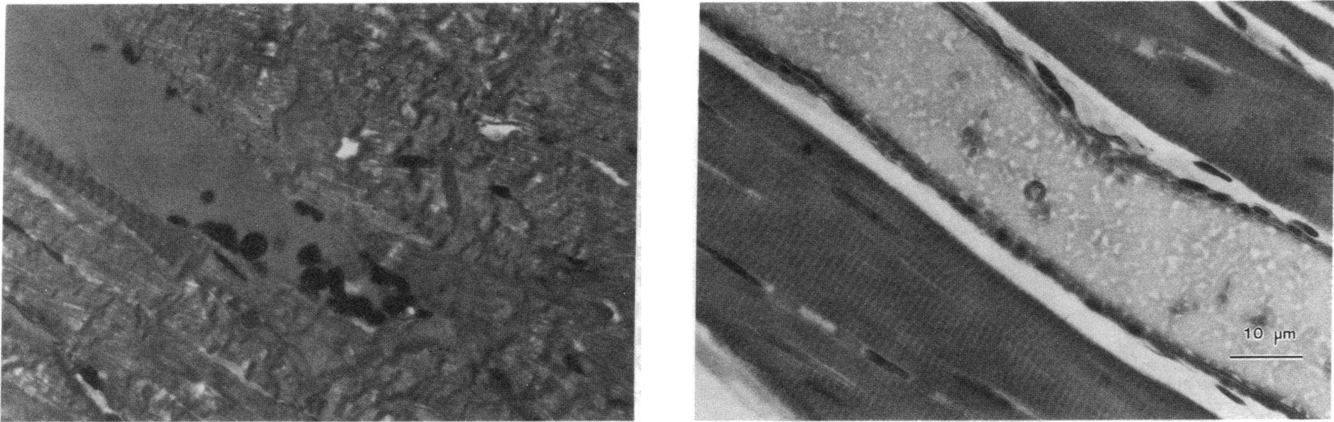

Figure 1. Photomicrographs of ischemic/reperfused (I + R) myocardium of cats treated with either vehicle (*left*) or 10 mg/kg SLe^x-OS (*right*). *Left panel:* Ischemic tissue from a cat subjected to 90-min I + 270-min R and administered with vehicle 10 min before reperfusion. I + R myocardial tissue exhibits numerous PMNs sequestered inside a coronary venule. Cardiac myocytes are injured, lack striations, and show myofibrillar contraction bands. *Right panel:* Ischemic tissue from a cat subjected to 90-min I + 270-min R and treated with 10 mg/kg SLe^x-OS 10 min before reperfusion. I + R myocardial tissue exhibits no PMN adhesion inside a coronary venule and there are no visible signs of cardiac myocyte injury (scale bar, 10 μm).

dothelium ($P < 0.001$). This increased PMN adhesion was significantly inhibited by 250 μM of SLe^x-OS ($P < 0.001$). These results demonstrate the antiadhesive properties of SLe^x-OS on PMN adherence to ischemic-reperfused coronary endothelium.

Effect of SLe^x-OS on myocardial injury after reperfusion. To ascertain the effects of SLe^x-OS on the degree of actual myocardial salvage of ischemic tissue after R, we measured the amount of necrotic cardiac tissue expressed as a percentage of either the area-at-risk or of total LV mass. There was no significant difference in the wet weights of the areas-at-risk expressed as a percentage of total LV between the two ischemic groups (Fig. 4), indicating that a comparable degree of MI occurred in both groups. In contrast, the necrotic area, expressed either as percentage of the area-at-risk or as percentage of the total LV mass, was significantly attenuated ($P < 0.001$) in cats treated with SLe^x-OS. About 35% of the jeopardized myocardium became necrotic in the vehicle group and a similar amount of necrosis developed in the nonsialylated oligosaccharide (i.e., Le^x-OS) treated group (35±4% vs. 31±4%, respectively). These values are not significantly different from each other. However, in the regular SLe^x-OS–treated ischemic-reperfused group, the amount of necrotic tissue was < 10% ($P < 0.001$). Therefore, SLe^x-OS (10 mg/kg) significantly protected against necrotic injury in the MI/R cats (Fig. 4).

To confirm the preservation of ischemic tissue, we determined the effects of SLe^x-OS on CK activity, a biochemical marker of myocardial injury. In sham MI/R cats receiving SLe^x-OS, the plasma CK activity increased slightly throughout the 6-h observation period reaching a final value of 4.1±1.3 IU/μg protein. In the two ischemic groups, plasma CK activity increased slightly during the period of MI. However, a marked

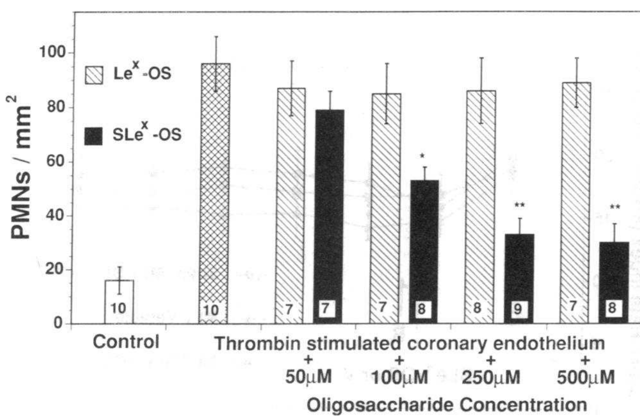

Figure 2. Effect of SLe^x-OS and Le^x-OS (0, 50, 100, 250, and 500 μM) on PMN adhesion in vitro on coronary artery endothelium-stimulated with thrombin (2 U/ml). Oligosaccharides were added simultaneously with the PMNs and incubated for 20 min. Data are expressed as numbers of PMNs/mm^2. Bar heights are means; brackets indicate±SEM, and numbers at the bottom of the bars are numbers of coronary rings studied (*$P < 0.05$, **$P < 0.01$ compared to Le^x-OS).

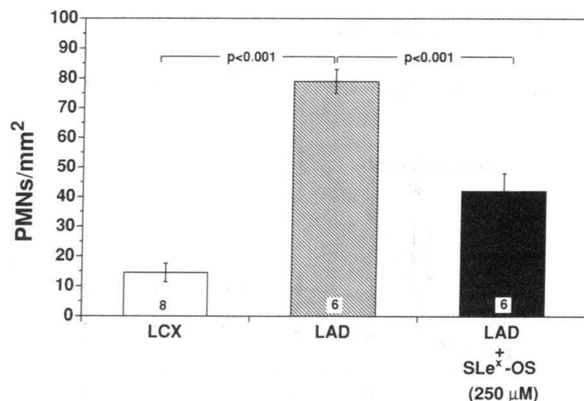

Figure 3. Effects of in vitro administration of SLe^x-OS on unstimulated neutrophil adherence to coronary endothelium after 90 min of ischemia and 20 min of reperfusion ex vivo. Data are expressed as numbers of PMNs/mm^2. Bar heights are means; brackets indicate±SEM, and numbers at the bottom of the bars are numbers of coronary rings studied.

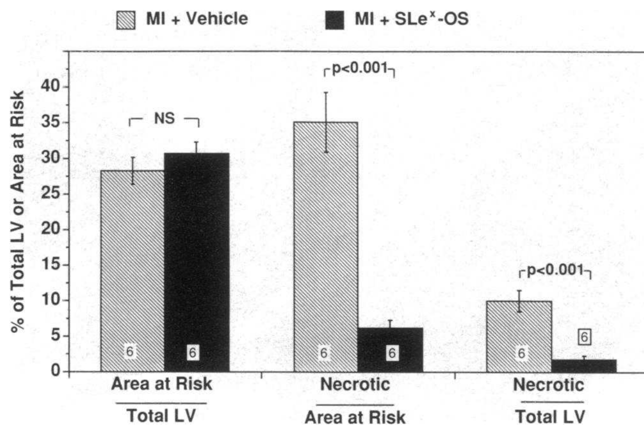

Figure 4. Tissue wet weight of area-at-risk as a percentage of the total left ventricular wet weight, and of necrotic tissue as a percentage of area-at-risk and of the total left ventricle for the two MI + R groups. Height of bars are means; brackets represent±SEM for six cats.

washout of CK into the circulating blood occurred within the first 30 min after R which progressed markedly during the remaining 4 h of reperfusion in cats receiving only the vehicle. In contrast, MI/R cats treated with SLe^x-OS had significantly lower plasma CK activities compared with ischemic cats receiving only K-H solution. The effect was sustained over the entire R period suggesting SLe^x-OS significantly attenuated myocardial R injury (Fig. 5).

Cardiac electrophysiologic and hemodynamic changes. In the sham MI cats, we observed that an intravenous bolus administration of 10 mg/kg SLe^x-OS had no detectable effect on any of the measured hemodynamic, electrocardiographic, or biochemical variables. Thus, SLe^x-OS at the dose regimen employed appeared to be devoid of any significant side effects that could interfere with this study. In the two groups of MI/R cats, there were no significant differences in any of the variables observed before coronary occlusion. However, several minutes after LAD occlusion, the ST-segment of the ECG became significantly elevated, peaking by 1 h after coronary occlusion. There was no significant difference in peak ST-segment elevation between the two MI/R groups (i.e., 0.19±0.04 mV in vehicle group vs. 0.18±0.03 mV in SLe^x-OS group), indicating that the ischemic insult was similar in both ischemic groups. After reperfusion, the ST-segment decreased to nearly control values, indicating that the coronary perfusion had been effective. During reperfusion, there was a noticeable increase in the incidence of premature ventricular contractions in all cats. Two cats in the MI/R + vehicle group and one cat in the MI/R + SLe^x-OS group developed ventricular fibrillation which was successfully converted to a normal sinus rhythm by using cardiac defibrillation (DC electronic defibrillator, Sanborn Co., Waltham, MA). In both MI/R groups, PRI decreased significantly one hour after coronary occlusion ($P < 0.05$) and gradually returned to baseline after R. There were no significant differences between the two MI/R groups at any of the hourly PRI readings, suggesting that SLe^x-OS did not appear to alter myocardial oxygen demand (Fig. 6).

Effects of SLe^x-OS on number of circulating white blood cells (WBC). In order to determine whether SLe^x-OS exerted any neutropenic effects that could contribute to its cardioprotection, we counted circulating WBC at the beginning and during the experimental period. Peripheral WBC were counted 10 min before coronary occlusion, 10 min before R (i.e., just before infusion of SLe^x-OS), and 60 and 270 min after R. WBC counts did not change significantly over the course of the experiment in either the vehicle or the SLe^x-OS treated group (Table I). Although differential WBC counts were not done in all cats and at all time points, all the PMN values analyzed remained in the 55–65% of total WBC range. Furthermore, there were no significant differences between these groups at any timepoint (Table I). These results clearly indicate that administration of SLe^x-OS does not produce leukopenia in cats. Therefore, any protective effects of SLe^x-OS in cat MI and R could not be attributed to changes in the number of circulating PMNs.

Effect of SLe^x-OS on coronary endothelial function. Since endothelial dysfunction is an early and critical event in R injury, we assessed endothelial function by comparing the vasorelaxant activity of isolated coronary artery rings to the endo-

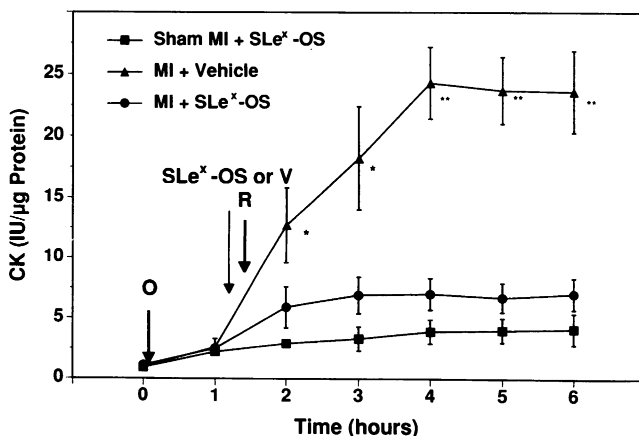

Figure 5. Plasma CK activity expressed as IU/µg protein measured hourly throughout the experiment for all three groups. All values are means±SEM for six cats in each ischemic group and four cats in the sham MI group (*$P < 0.05$, **$P < 0.01$ compared to MI + SLe^x-OS). V, vehicle; O, occlusion; R, reperfusion.

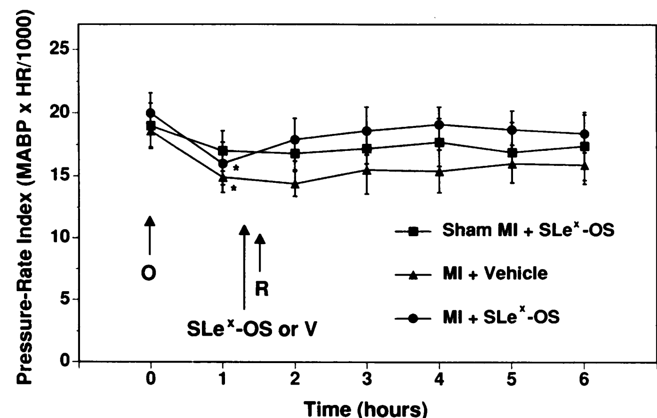

Figure 6. PRI expressed as mmHg × (beats/min)/1,000 during the 6-h observation period. All values are means±SEM for four sham MI cats and six cats in each MI group. PRI decreased significantly (*$P < 0.05$) in the MI groups 1 h after coronary occlusion. No significant differences occurred among the three groups at any time after addition of SLe^x-OS. V, vehicle; O, occlusion; R, reperfusion.

Table I. Circulating WBC Counts in Cats during MI/R

Ischemia group	Time (min) Initial	10 before R	60 after R	270 after R
MI/R + vehicle	6520±231	7045±217	7221±174	6958±253
MI/R + SLe^x-OS	7210±226	7012±312	7493±224	7138±179

All values are means of circulating WBCs/μl ± SEM for four to five cats each group.

thelium-dependent vasodilators, ACh and A-23187, and to the endothelium-independent vasodilator, $NaNO_2$. Coronary rings isolated from sham MI cats exhibited full relaxation to the endothelium-dependent vasodilators ACh and A23187 as well as to the endothelium-independent vasodilator, $NaNO_2$. In the rings obtained from MI/R cats receiving only K-H solution, the ACh- and the A23187-induced relaxation was significantly reduced whereas $NaNO_2$-induced relaxation was unchanged. In contrast, the ACh- and the A23187-induced relaxation of the rings obtained from cats treated with SLe^x-OS was significantly preserved. Fig. 7 summarizes the vasorelaxant responses to ACh, A-23187, and $NaNO_2$ in isolated cat coronary artery rings. Thus, SLe^x-OS significantly protected against the loss of endothelium-dependent relaxation observed in coronary artery rings after MI/R.

Effect of SLe^x-OS administration in vivo on PMN adherence to ischemia-reperfused coronary endothelium ex vivo. An initial step in PMN-mediated R injury is the increased adhesion of neutrophils to the vascular endothelium. When unstimulated autologous PMNs were added alone to nonischemic-reperfused control LCX coronary arteries for 20 min, only a few PMNs adhered to the endothelial surface. However, similar PMNs added to the ischemia-reperfused LAD coronary arteries 270 min after reperfusion in the cats receiving vehicle, resulted in a dramatic increase in the number of PMNs adhering to the reperfused endothelium (Fig. 8). In contrast, when autologous unstimulated PMNs were incubated with the ischemia-reperfused LAD coronary arteries isolated from the cats treated with SLe^x-OS, the number of PMNs adhering to the coronary endothelium was significantly reduced ($P < 0.01$) (Fig. 8). However, using the nonsialylated analog of the SLe^x-OS did not significantly inhibit adherence of PMNs to the ischemic-reperfused LAD endothelium (95±7 vs. 91±12 PMNs/mm^2 in cats given vehicle). These results indicate a significant attenuation of neutrophil adherence by SLe^x-OS in MI/R. Moreover, this effect was sustained for the entire 270-min post-R period, suggesting that this SL^x-OS significantly prevents the inflammatory response from developing in the ischemic reperfused myocardium.

Effect of SLe^x-OS on cardiac function. LV pressure and the first derivation of LV pressure, dP/dt max, an index of myocardial contractility, were measured by a catheter tip manometer inserted in the LV cavity. All three groups showed comparable initial values for these cardiac variables. In the sham MI group there were no significant changes in dP/dt max over the 6-h experimental period. However, in both MI/R groups, myocardial contractility, measured as dP/dt max, decreased upon occlusion of the LAD to ~ 60%. In cats given only vehicle, contractility decreased further within the first 15 min of reperfusion, and recovered very slowly thereafter. However, in SLe^x-OS–treated MI-R cats, dP/dt max recovered to initial values after reperfusion. After 4.5 h of R dP/dt max of the cats receiving vehicle was significantly lower than that of SLe^x-OS–treated cats ($P < 0.01$) (Fig. 9). These results indicate that SLe^x-OS not only prevented myocardial necrosis after R of the ischemic myocardium, but this myocardial salvage was also manifested as a preservation of mechanical performance of the heart.

Discussion

Carbohydrates such as sialyl Lewis^x are counterreceptors for selectin adhesion molecules that mediate interactions between PMNs and the endothelium. To our knowledge, this is the first

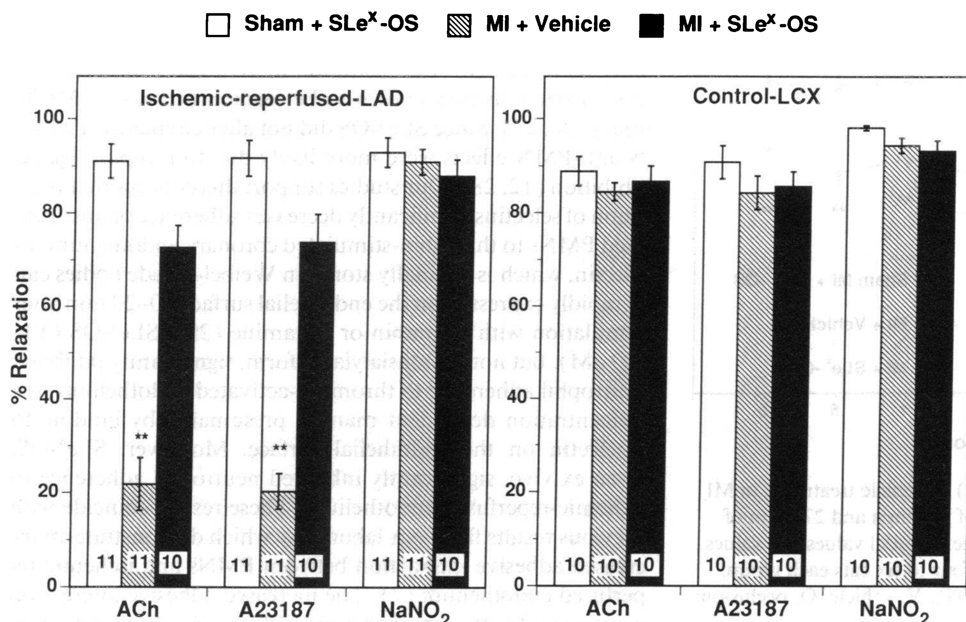

Figure 7. Summary of responses of ischemia-reperfused LAD rings and nonischemic LCX coronary rings to 100 nM ACh, 1 μM A23187, and 100 μM $NaNO_2$. Bar heights are means; brackets indicate±SEM for 10–11 rings (**$P < 0.01$ compared to sham).

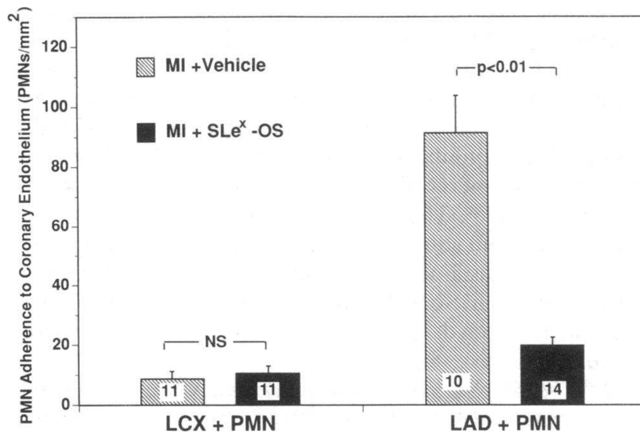

Figure 8. Effects of SLe^x-OS or vehicle administration in vivo on unstimulated PMN adherence to nonischemic-reperfused LCX coronary endothelium and ischemic-reperfused LAD coronary endothelium. Data are expressed as numbers of PMNs/mm^2. Bar heights are means; brackets indicate±SEM, and numbers at the bottom of the bars are numbers of coronary rings studied.

report of a selectin-binding oligosaccharide exerting beneficial effects in vivo in a MI/R state. Our data clearly demonstrate cardioprotective properties of a SLe^x-OS in MI/R, characterized by its ability to decrease myocardial necrosis compared to vehicle controls or the nonsialylated form of the oligosaccharide. The cardioprotection exerted by SLe^x-OS was characterized by a 70% reduction in necrotic myocardium compared to vehicle or to a nonsialylated analogue and by a markedly attenuated plasma CK activity. This degree of necrosis, although small, is still higher than that observed after 90 min of ischemia in cats without R (17). The reduction in necrotic tissue exerted by SLe^x-OS cannot be attributed to differences in the severity of ischemia since all MI/R groups had equivalent areas of myocardium at risk and similar elevations of ST-segment.

Comparable pressure-rate indices in both groups rule out the possibility that the cardioprotective effect of the oligosaccharide was related to alterations in myocardial oxygen demand. Since cats have very low (i.e., ~ 7%) coronary collateral blood flow, it is unlikely that the protective effect could be explained by variations in collateral flow to the ischemic myocardium (i.e., alterations in oxygen supply) (18–20). In addition, SLe^x-OS exerted no detectable vasoactive effects on coronary rings and since SLe^x-OS was administered to the cats at the end of the ischemic period (i.e., 10 min before R), SLe^x-OS administration should not have been able to exert any direct effects on collateral blood flow.

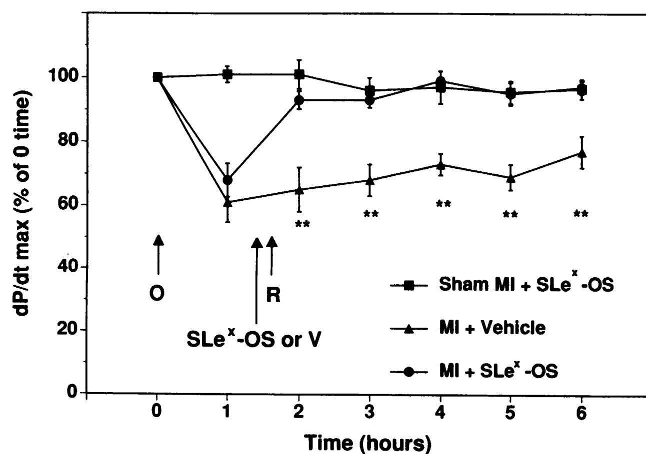

Figure 9. Effects of SLe^x-OS (10 mg/kg) or vehicle treatment in MI + R cats on dP/dt max during 90 min of ischemia and 270 min of reperfusion. Data expressed as percentage of initial values. All values are means, brackets indicate±SEM for four to six cats each group. (**$P < 0.01$ compared to MI + SLe^x-OS). V, vehicle; O, occlusion; R, reperfusion.

SLe^x-OS treatment not only exerted significant myocardial protection but also protected endothelial function. Endothelial dysfunction occurs shortly after the onset of R (i.e., 5 min) and is characterized by a reduction in basal release of nitric oxide (21, 22). Moreover, this loss of basal NO corresponds with increased PMN adherence to the vascular endothelium within 20 min (23). Adhered and activated neutrophils release a variety of cytotoxic mediators (i.e., hydrogen peroxide (H_2O_2), superoxide anion (O^-), hydroxyl radical ($OH-$), and elastase) which lead to enhanced tissue injury (3, 24). These mediators may aggravate endothelial dysfunction, resulting in increased PMN adhesion to the vascular endothelium and subsequent myocardial necrosis 2–4 h after R. However, SLe^x-OS preserved endothelial function, as measured by coronary vascular relaxation to ACh and A23187. Additionally, administration of the oligosaccharide in vivo resulted in diminished PMN adherence to the coronary endothelium evident after 4.5 h of R. These protective effects of SLe^x-OS might be explained by inhibition of PMN rolling along the endothelium, thereby inhibiting tight PMN adhesion, diapedesis, and migration of PMNs to myocardial tissue.

One important component of the myocardial salvage afforded by SLe^x-OS is very likely caused by its anti-PMN effects. Clearly, PMNs are involved in feline MI/R since we observed significant numbers of PMNs in nontreated ischemic myocardial tissue, and very few in SLe^x-OS–treated tissue (Fig. 1). Moreover, Ma et al. (25) has shown that a CD18 specific monoclonal antibody protected to a comparable degree in this same model of MI/R. These anti-PMN properties, however, cannot be attributed to changes in circulating WBC counts since both MI/R groups had comparable and normal WBC counts throughout the MI/R periods. These data eliminate the possibility that SLe^x-OS administration in vivo had leukopenic effects, a phenomenon known to be cardioprotective in MI/R injury (26, 27). Since SLe^x-OS did not alter circulating PMNs, its anti-PMN effects were more likely due to receptor–ligand inhibition (12, 28). Our studies support this concept that inhibition of selectins significantly decreases adherence of unstimulated PMNs to thrombin-stimulated coronary endothelium. P-selectin, which is normally stored in Weibel-Palade bodies can be rapidly expressed on the endothelial surface 10–20 min after stimulation with thrombin or histamine (29). SLe^x-OS (50–500 μM), but not its nonsialylated form, significantly inhibited neutrophil adherence to thrombin-activated endothelium in a concentration dependent manner presumably by binding to P-selectin on the endothelial surface. Moreover, SLe^x-OS, given ex vivo, significantly inhibited neutrophil adherence to ischemic-reperfused endothelium. These results coincide with previous results from our laboratory which demonstrate an increased adhesive interaction between PMNs and ischemic reperfused endothelium (7). The increased adhesive interaction is associated with a marked burst of free radical generation by

coronary endothelial cells (22) that leads to the rapid expression of P-selectin on the endothelial surface (7, 29). P-selectin can be sustained on the endothelial surface several hours following exposure to oxygen radicals (29, 30) and is responsible for a significant degree of PMN–endothelium adhesion that occurs following reperfusion of an ischemic vasculature (8). In this regard, SLe^x-OS exerted a marked inhibition of PMN adherence to ischemic reperfused coronary endothelium, consistent with its effect on thrombin stimulated endothelium. Thus, the SLe^x-OS effectively attenuated PMN–endothelial interaction providing strong evidence that this inhibitory effect was directly related to a P-selectin–carbohydrate interaction although inhibition of L-selectin or E-selectin could also be involved.

The precise molecular interaction of carbohydrates and selectins in vivo is still unclear. In vitro assays have demonstrated a great variety of synthetic and natural oligosaccharide structures related to sialyl Lewis^x and sialyl Lewis^a, including phosphorylated mono- and polysaccharides, and sulfated polysaccharides as binding sites for selectins (31, 32). The C-type lectin domain of the three members of the selectin family recognize the carbohydrate ligands. Lewis^x and sialyl Lewis^x may recognize P- and L-selectin (33) and fucosylation or sialylation of the carbohydrates enhance their recognition of the selectins, but this varies in different species. The spectrum for carbohydrate binding to P-selectin is similar to that of E-selectin, although P-selectin appears to bind to a wider variety of carbohydrates (34, 35). More recently, it has been shown that L-selectin also binds to sialyl Lewis^x (12) and may present carbohydrate ligands to E- and P-selectin (36, 37). However, the carbohydrates on the surface of L-selectin are < 5% of the total carbohydrate pool on the PMN surface (34). Moreover, the carbohydrate–selectin interaction is also influenced by the multivalency of the oligosaccharide branches, ambient temperature, availability of calcium, and local shear forces (9). Based on these in vitro studies, the oligosaccharide-binding properties seem to be less restrictive and of lower affinity than the well-characterized binding properties between proteins and antibodies.

Our data support the hypothesis that the cardioprotective properties of the oligosaccharide observed in myocardial R is selectin mediated. Recently, the important role of P- and L-selectin has been shown in the same model of MI/R as that studied in this report (6, 7). Upregulation of P-selectin on the vascular endothelium is seen after 20 min of reperfusion (7), and L-selectin is constitutively expressed on PMNs that bind to ligands on the reperfused endothelium (6). In contrast E-selectin requires 4–6 h to be expressed on the vascular endothelium (31, 38). Therefore, E-selectin is probably less important during the early stages of MI/R since most of the cardiac necrosis occurs within the first 4–6 h after R. In this regard, a monoclonal antibody directed against E-selectin exerted no cardioprotection in a primate model of MI/R (39). However, E-selectin may be more important in the later stages of the fully developing infarct (i.e., at 24–48 h). Thus, SLe^x-OS presumably binds to L- and P-selectin thereby inhibiting PMN adherence and subsequent activation after ischemia and R. Since selectin-induced PMN–endothelium interaction is a prerequisite for further adhesive interactions mediated by CD11/CD18-ICAM-1 mechanisms (5), carbohydrate blockade of the selectin interaction results in cardioprotection of the reperfused myocardium due to interruption of the initial PMN activation sequence.

Initial prevention of endothelial dysfunction and PMN adherence is associated with decreased myocardial necrosis in MI/R (3, 8, 21, 22). We have extended the cardioprotective effects of SLe^x-OS to include improved cardiac function throughout the R period. This is the first known study to demonstrate this important finding. Specifically, cardiac contractility (dP/dt max) recovered rapidly to initial values during the first 30 min of reperfusion. Diminished contractile function after R is known as myocardial stunning (40) and is most likely related to free radical release at the onset of reperfusion (41). Since SLe^x-OS exerted no direct hemodynamic effects, it is possible that SLe^x-OS preserves contractile function by inhibiting PMN–endothelial interaction rather than by a direct cardiotonic effect of the oligosaccharide.

There are several approaches which can be employed to block the effects of adhesion molecules expressed on either endothelial cells or neutrophils. These include (*a*) monoclonal antibodies directed against adhesion molecules, (*b*) soluble forms of cell adhesion molecules, and (*c*) soluble carbohydrates which mimic ligands to adhesion molecules. All of these may be available as therapeutic tools to inhibit PMN–endothelium interaction in inflammatory disorders. Among these three approaches, carbohydrates might be the ideal therapeutic compound due to their low antigenicity and potential efficacy after oral administration (42).

In conclusion we have demonstrated that a SLe^x-OS inhibits PMN–endothelium interactions in vitro and in vivo. Furthermore, in vivo administration of SLe^x-OS attenuates myocardial injury, preserves endothelial function, and sustains normal cardiac performance. These in vivo results demonstrate the important role of selectins in inflammatory states such as ischemia-reperfusion injury. Although the selectins are intimately involved in the early PMN–endothelial interaction, these interactions are not sufficient to explain the full effects of myocardial necrosis, since subsequent PMN-endothelial cell effects of CD-18 and ICAM-1 are also very important in sustaining adherence of PMNs to the endothelium and in promoting transendothelial migration. (43).

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