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Recombinant soluble P-selectin glycoprotein ligand-1 protects against myocardial ischemic reperfusion injury in cats

Reid Hayward, Barry Campbell, Yong K. Shin, Rosario Scalia, Allan M. Lefer*

Department of Physiology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107, USA

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Abstract

Objective: Neutrophils (PMNs) contribute importantly to the tissue injury associated with ischemia and subsequent reperfusion of a vascular bed. The effects of a recombinant soluble human form of P-selectin glycoprotein ligand-1 (rsPSGL.Ig) on PMN–endothelial cell interactions were investigated in a well established model of feline myocardial-ischemia reperfusion injury. **Methods:** Cats were subjected to 90 min of myocardial ischemia followed by 270 min of reperfusion. **Results:** Administration of rsPSGL.Ig (1 mg/kg) just prior to reperfusion resulted in a significant reduction in myocardial necrosis compared to that in cats administered a low affinity mutant form of rsPSGL.Ig (1 mg/kg) (16±3 vs. 42±7% of area-at-risk, $P<0.01$). Cardioprotective effects were confirmed by significant ($P<0.05$) reductions in plasma creatine kinase activity in cats treated with rsPSGL.Ig. Inhibition of PMN–endothelial cell interactions was evidenced by a significant attenuation in cardiac myeloperoxidase activity ($P<0.01$) and reduced PMN adherence to ischemic–reperfused coronary endothelium ($P<0.001$). In addition, rsPSGL.Ig treatment significantly ($P<0.01$) preserved endothelium-dependent vasorelaxation in ischemic–reperfused coronary arteries. **Conclusion:** These results demonstrate that the administration of a recombinant soluble PSGL-1 reduces myocardial reperfusion injury and preserves vascular endothelial function, which is largely the result of reduced PMN–endothelial cell interactions.

Keywords: Cat; Cardiac necrosis; Creatine kinase activity; Endothelial function; Myeloperoxidase activity; Neutrophil adherence

1. Introduction

Myocardial ischemia resulting from coronary artery occlusion initiates a cascade of inflammatory cellular events. Although prolonged myocardial ischemia inevitably results in myocardial necrosis, reperfusion of the ischemic vascular bed results in an acute exacerbation of tissue injury [1]. Reperfusion-induced injury begins with endothelial dysfunction, which can be seen as early as 2.5 min post-reperfusion [2], and is the result of increased superoxide and reduced nitric oxide (NO) generation by the endothelium [3]. This endothelial dysfunction is manifested by a decreased relaxation of the coronary arteries to endothelium-dependent vasodilators, including acetylcholine (ACh) or the calcium ionophore A-23187 [2]. The ramifications of impaired endothelial NO production are important since NO is a key regulator of coronary vascular tone as well as a powerful anti-adherent agent [4–6]. In this connection, a significant increase in neutrophil (PMN) adherence to the vascular endothelium occurs 20 min following reperfusion [7]. At 3 h post-reperfusion, significant increases in myocardial myeloperoxidase activity occur, indicative of PMN infiltration, leading to marked myocardial necrosis at 4.5 h [2].

A substantial portion of the myocardial injury and endothelial dysfunction observed following reperfusion has been attributed to activated PMNs [8,9], which are capable of releasing proteases, cytokines and oxygen-derived free radicals [10]. The progression of PMN–endothelium interactions resulting from reperfusion of the ischemic myocardium begins with neutrophils rolling along the luminal surface of endothelial cells [11], mediated in large part by P- and L-selectin. P-selectin is rapidly translocated from Weibel-Palade bodies to the endothelial cell surface during the first 20 min of reperfusion [12], whereas L-selectin is constitutively expressed on non-stimulated leukocytes [13].

*Corresponding author. Tel.: +215-503-7760; fax: +215-503-2073.

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Rolling allows intimate interaction between PMNs and the endothelium, enabling firm adherence and subsequent emigration of PMNs into tissues at the site of inflammation.

The selectins are a family of calcium-dependent lectins that have been shown to bind several counterligands that may be important in reperfusion injury [13,14]. All selectins (P-, L- and E-selectin) bind sialylated and fucosylated oligosaccharides, such as sialyl Lewis^x (SLe^x), while soluble P- and L-selectin have been shown to bind both heparin and heparin-like molecules as low affinity ligands [13,15]. Another selectin ligand, P-selectin glycoprotein ligand-1 (PSGL-1) [16,17], is a highly extended homodimer with two disulfide-linked subunits [18] and is located on the microvilli of leukocytes where it is positioned to favorably interact with its counterligands under flow conditions [17,19]. Although PSGL-1 is the primary leukocyte-bound ligand for P-selectin [20], PSGL-1 also demonstrates E- [21] and L-selectin- [22] binding properties. However, E-selectin is not involved in the development of reperfusion injury in myocardial ischemia/reperfusion [23,24]. We hypothesized that administration of a recombinant soluble form of PSGL-1 (rsPSGL.Ig) would inhibit leukocyte–endothelium interaction, thus attenuating P-selectin-mediated endothelial dysfunction and subsequent myocardial reperfusion injury. Therefore, the purposes of this study were (a) to investigate whether a recombinant soluble human form of PSGL-1 exerts a cardioprotective effect in myocardial ischemia–reperfusion injury in vivo and (b) to elucidate the mechanisms by which soluble PSGL-1 may achieve these beneficial effects relative to PMN–endothelial cell interactions.

2. Methods

2.1. Experimental protocol

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institutes of Health. Adult male cats (2.6–3.6 kg) were anesthetized with sodium pentobarbital (30 mg/kg, iv). An intratracheal cannula was inserted through a midline incision, and cats were placed on mechanical ventilation (Harvard small animal respirator, Dover, MA, USA). A catheter was inserted into the jugular vein for administration of additional anesthesia and for administration of rsPSGL.Ig or the rsPSGL.Ig low affinity mutant. Another catheter was inserted into the right femoral artery for the measurement of mean arterial blood pressure (MABP) via a pressure transducer (Cobe Instruments, Lakewood, CO, USA). After a midsternal thoracotomy, the anterior pericardium was incised and a 3-0 silk ligature was placed around the left anterior descending (LAD) coronary artery, 8 to 10 mm from its origin. Standard lead II of the scalar electrocardiogram (ECG) was used to determine heart rate (HR) and S-T segment elevation. The ECG and MABP were continuously monitored on a model 78304 A unit oscilloscope (Hewlett Packard, Palo Alto, CA, USA) and recorded on an oscillographic recorder (Gould, model 2107-4490-00, Cleveland, OH, USA) every 20 min. The pressure-rate index (PRI), an index of myocardial oxygen demand, was calculated as (MABP$\times$HR)/1000.

All cats were allowed to stabilize for 30 min, and baseline readings of the ECG and MABP were recorded. Myocardial ischemia was produced by tightening the reversible silk ligature that was previously placed around the LAD, to completely occlude the vessel. This was designated as time 0. Eighty min after coronary occlusion (i.e., 10 min prior to reperfusion), rsPSGL.Ig or the rsPSGL.Ig low affinity mutant was administered intravenously as a bolus. After the 90 min ischemic period, the LAD ligature was untied and the ischemic myocardium was allowed to reperfuse for 270 min. Twenty-one cats were randomly divided into three major groups: (a) sham myocardial ischemia/reperfusion (MI/R) cats receiving rsPSGL.Ig (1 mg/kg), (b) MI/R cats receiving the rsPSGL.Ig low affinity mutant (1 mg/kg) and (c) MI/R cats receiving rsPSGL.Ig (1 mg/kg). Sham MI/R cats were subjected to the same surgical procedures and observed for the same duration of time as MI/R cats except that no coronary artery was occluded.

2.2. Myocardial tissue analysis

Following the 6 h experimental period, the ligature around the LAD was again tightened. A 20-ml volume of 0.5% Evans blue (Sigma, St. Louis, MO, USA) was injected directly into the left ventricular lumen, thereby staining the area of myocardium that was perfused by the patent coronary arteries (i.e., left circumflex and right coronary arteries). The area-at-risk was thus determined by negative staining. The heart was then rapidly excised and placed in warmed, oxygenated Krebs-Henseleit (K-H) buffer 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. The left circumflex (LCX) and the LAD coronary arteries were removed and isolated for subsequent study of coronary ring vasoactivity and PMN adherence. The right ventricle, great vessels, and fat tissue were removed, and the left ventricle was sliced parallel to the atrioventricular groove in 2–3 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 further sectioned into small cubes (1 to 2 mm^3) and incubated in 0.1% nitroblue tetrazolium in phosphate-buffered saline (PBS) at pH 7.4 and 37°C for 10 min. The tetrazolium dye forms a blue formazan complex in the presence of viable, intact 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 non-necrotic area). All three portions of the left ventricular myocardium (i.e., nonischemic, ischemic non-necrotic and ischemic necrotic tissue) were subsequently weighed. Results were expressed as the area-at-risk indexed to the total left ventricular mass, and the area of necrotic tissue indexed to either the area-at-risk or the total left ventricular mass. The three portions of cat myocardium were stored at −70°C for later assay of myeloperoxidase activity.

2.3. 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 2000 *g* and 4°C for 20 min and the plasma was decanted for subsequent biochemical analysis. Plasma CK activity was measured using the method of Rosalki [25] and expressed as international units per μg of protein. Plasma protein concentration was assayed utilizing the biuret method of Gornall et al. [26]. All assays were conducted without prior knowledge as to the group of origin of each sample.

2.4. Autologous cat PMN isolation and labeling

Peripheral blood (20 ml) was drawn from the femoral artery cannulated at the beginning of the surgical procedure and anticoagulated with citrate–phosphate–dextrose solution (Sigma; 1.5:10, v/v anticoagulant to whole blood). PMNs were isolated by a procedure that was modified from Lafrado and Olsen [27]. After whole blood centrifugation, the pellet was mixed with 8 ml of 6% dextran (MW 60,000–90,000; Sigma) and PBS to allow the red blood cells to settle. The leukocyte-rich upper fraction was layered onto a Percoll/platelet-poor plasma (PPP) gradient (density gradients of 80, 62 and 50%). Following centrifugation at 700 g for 40 min, PMNs were collected from the 62 and 80% interface and washed in PBS. PMN preparations obtained by this method were generally >95% pure and >95% viable. Isolated PMNs were then labeled with Zynaxis PKH-2 fluorescent cell linker (Zynaxis Cell Science Inc., Malvern, PA, USA), based on the procedure of Yuan and Fleming [28]. A 2-ml volume of diluent and 10 μl of the fluorescent dye were added to a loose cell pellet containing approximately ten million cells. Following a seven-min incubation period, 200 μl of PPP were added to stop the reaction and 2 ml of PBS were added to underlay the suspension. The mixture was then centrifuged for 10 min at 400 g. The cells were resuspended in PBS, counted and utilized in the adherence assay.

2.5. Ex vivo PMN adherence to coronary artery endothelium

PMNs were isolated and fluorescently labeled as previously described. Segments from both the LAD and LCX were isolated from each cat and placed into warmed K-H buffer. Arteries were cut into rings of 2 to 3 mm length. The rings were then opened and placed with the endothelial surface facing upwards in a cell culture dish filled with 3 ml of oxygenated K-H solution and incubated in culture dishes with autologous labeled PMNs (1.2×10^6 cells) for 20 min at 37°C in a shaker bath, which stimulates shear forces. Following the 20 min incubation period, sections were washed in K-H buffer and placed on glass microscope slides. PMNs adhering to the endothelium were counted using epifluorescence microscopy (Nikon Diaphot, Nikon, Garden City, NY, USA). Five different fields of each endothelial surface were counted and the results were expressed as adherent PMNs/mm^2 of endothelial surface.

2.6. In vitro adherence of PMNs to thrombin-stimulated coronary endothelium

In three additional cats, peripheral blood (40 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. The LAD and LCX coronary arteries were isolated and placed in warm K-H solution. Fat and connective tissue were removed from the coronary vessels, and arteries were cut into rings of 2–3 mm in length. The rings were then opened, and placed, endothelial surface up, into a well with 2 ml of K-H solution. Coronary artery segments were incubated for 10 min with 2 U/ml of thrombin (Sigma) in a shaker bath to stimulate shear forces. The segments were then placed in fresh K-H solution. Labeled PMNs (1×10^6 PMNs/well) were added to unstimulated endothelial cells, thrombin-stimulated endothelial cells alone, thrombin-stimulated endothelial cells in combination with rsPSGL.Ig (20 μg/ml) or thrombin-stimulated endothelial cells in combination with a monoclonal antibody directed against P-selectin (PB1.3, 20 μg/ml) and incubated for 20 min and counted as described above.

2.7. Isolated coronary artery ring studies

The LAD and LCX coronary arteries were isolated and placed into warmed K-H solution. Arteries were cut into rings of 2 to 3 mm length. The rings were then mounted on small stainless steel hooks, transferred to tissue baths, and connected to FT-03 force transducers (Grass, model 7, Instrument Co., Quincy, MA, USA). Isometric force changes were recorded on a Grass-7 oscillographic recorder. The baths were filled with 10 ml of K-H solution and

gassed with 95% O_2+5% CO_2 at 37°C. Coronary artery rings were stretched to give a preload of 0.5 g and equilibrated for 90 min. During this period, the K-H solution was replaced every 20 min. After equilibration, the rings were stimulated with 100 nM U-46619 (9,11-epoxymethano-PGH_2, Biomol Research Laboratories, Plymouth Meeting, PA, USA), a thromboxane A_2 mimetic, to generate about 0.5 g of developed force. Once the contraction reached a stable plateau, ACh, an endothelium-dependent vasodilator, was added to the bath in cumulative concentrations of 0.1, 1, 10 and 100 nM. After the response to the highest concentration stabilized, the rings were washed three times with fresh K-H buffer and allowed to equilibrate for 20 min to reach baseline tone. This procedure was repeated with another endothelium-dependent vasodilator, A-23187, (1, 10, 100 and 1000 nM), as well as with an endothelium-independent vasodilator, acidified $NaNO_2$ (0.1, 1, 10 and 100 μM), which was titrated to pH 2. NaCl solutions titrated to pH 2 exerted no detectable vasorelaxant effect. Relaxation was calculated as the percentage decrease from the peak U-46619-induced precontraction value.

2.8. Determination of tissue myeloperoxidase (MPO)

Cardiac activity of MPO, an enzyme occurring virtually exclusively in polymorphonuclear leukocytes, was determined using the method of Bradley et al. [29], as modified by Mullane et al. [30]. Tissue was carefully rinsed in 0.9% NaCl prior to homogenization in 0.5% HTAB [hexadecyltrimethyl ammonium bromide (Sigma), which was dissolved in 50 mM potassium phosphate buffer at pH 6.0] using a Polytron (PCU-2) homogenizer (Kinematica GMBH, Luzern, Switzerland). Homogenates were centrifuged at 12,500 *g* at 4°C for 30 min. The supernatants were then collected and reacted with 0.167 mg/ml of *o*-dianisidine dihydrochloride (Sigma) and 0.0005% H_2O_2 in 50 mM phosphate buffer at pH 6.0. The resultant change in absorbance was determined spectrophotometrically at 460 nm. One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide/min at 25°C.

2.9. Flow cytometric analysis of rsPSGL.Ig binding

Flow cytometric analysis of rsPSGL.Ig binding to freshly isolated cat platelets was performed according to standard procedures [31]. Blood (15–20 ml) was collected from the femoral artery of anesthetized cats before starting the MI/R experiments and was anticoagulated with sodium citrate phosphate buffer (Sigma). Platelet-rich plasma was obtained by centrifuging the blood at 300 *g* for 20 min. The platelet-rich plasma was then centrifuged at 2000 *g* for 10 min to form a platelet-rich pellet. This pellet was washed twice in calcium-free Tyrode's solution containing 0.2% bovine serum albumin (BSA). The final cell pellet was resuspended in RPMI 1630 medium (Gibco, Grand Island, NY, USA) containing 0.1% sodium azide, 0.1% BSA and 1 mM Ca^{2+}. The platelet suspensions containing 5×10^5 platelets/tube were incubated with either rsPSGL.Ig (20 μg/ml) or the low activity mutant rsPSGL.Ig (20 μg/ml) and maintained at 4°C for 30 min. The platelets were then washed in RPMI 1630 to remove any excess of primary antibody. A goat anti-human IgG F(ab′)2–fluorescein isothiocyanate (FITC) conjugate was used as the secondary antibody at a 1:100 dilution, and the cells were maintained at 4°C for 30 min. The stained platelets were washed twice with RPMI 1630 and finally were fixed in 1% paraformaldehyde, and then analyzed by flow cytometry (FACScan, Becton-Dickinson, San Jose, CA, USA).

2.10. rs.PSGL.Ig

rsPSGL.47mutFc (Genetics Institute, Inc., Cambridge, MA, USA) is derived from pED.47.Fc, a recombinant soluble form of PSGL-1 fused to human IgG_1 [32]. Polymerase chain reaction (PCR) was performed on the Fc portion of this plasmid using 5′ primer: TAAATAGCGGCCGCACACATGCCCACCGTGCCCA-GCACCTGAAGCCCTGGGGGCACCGTCAGTCTTCC-TC and 3′ primer: GCATGTGCAC-CGAGGCCCCAGATCA. The PCR product was digested with the restriction enzymes *Not*I and *Kpn*I and ligated to the large fragment of pED.47.Fc restricted with the same digest. This resulting vector, pED.47mutFc, was confirmed by DNA sequencing and then stably transfected and amplified in a DHFR-negative Chinese hamster ovary cell line that had previously been stably transfected with vector pMT4neo expressing both the cDNA encoding an α(1,3/1,4)fucosyltransferase [33] and a cDNA encoding core 2β-1,6-*N*-acetylyglucosaminyltransferase [34]. Analysis of several resulting clonal cell lines revealed a cell line having readily detectable core 2, fucosyltransferase activities and SLe^x modified 47mutFc: 'high affinity rsPSGL'. Another cell line was identified that lacked detectable fucosyltransferase activity and SLe^x-modified glycans on 47mutFc: 'low affinity rsPSGL' Each type of these secreted rsPSGL.47mutFc molecules was purified separately from serum-free Chinese hamster ovary cell conditioned medium, essentially as described [35]. The final purified material was formulated into 10 mM histidine, 1% (w/v) sucrose, 260 mM glycine, 0.005% Tween-80, pH 6.6, at room temperature (RT) and at a concentration of at least 3 mg/ml. Endotoxin was measured to be less than 10 EU/mg. Selectin binding activity was assessed via in vitro binding assays as described [32].

2.11. Statistical analysis

All values in the text and figures are presented as means±standard errors of the means of *n* independent experiments. All data were subjected to analysis of vari-

ance (ANOVA) followed by post-hoc analysis using Fisher's *t*-test. All data on S-T-elevation, white cell counts and PRI were analyzed by ANOVA incorporating repeated measures. Probabilities of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Confirmation of rsPSGL.Ig binding to cat platelets and vascular endothelium

In order to confirm that rsPSGL.Ig was able to bind to its high affinity ligand in the cat, we performed flow cytometry on freshly isolated cat platelets. The results clearly show that rsPSGL.Ig binds to isolated feline platelets (Fig. 1). Cat platelets stained 75±15% positive for rsPSGL.Ig in comparison to only 19±4% positive staining for the low affinity mutant ($P<0.01$) and 4±1% for the control lacking the primary antibody. Additionally, the mean channel fluorescence was 95±18 and 31±6 for rsPSGL.Ig and the rsPSGL.Ig low affinity mutant and only 3±1 for the control, respectively. In addition, in order to determine whether binding of rsPSGL.Ig to its ligand actually inhibits cat PMN-leukocyte interactions, we directly observed the effects of rsPSGL.Ig on neutrophil adherence to thrombin-stimulated cat coronary vascular endothelium in vitro (Fig. 2). When unactivated PMNs were added to normal unstimulated coronary artery segments (i.e., LAD, LCX), relatively few PMNs were found to be adherent. However, the addition of PMNs to thrombin-stimulated coronary endothelium resulted in a five-fold increase ($P<0.001$) in the number of adherent PMNs. Addition of rsPSGL.Ig (20 μg/ml) resulted in a 48% reduction in PMN adherence ($P<0.01$). This attenuation of PMN adherence was comparable to that resulting from administration of an equal concentration of a neutralizing monoclonal antibody directed against P-selectin, PB1.3 (57±6 vs. 51±10 PMNs/mm^2). These results therefore indicate that rsPSGL.Ig effectively binds to P-selectin and functionally inhibits PMN adherence to thrombin-activated vascular endothelium.

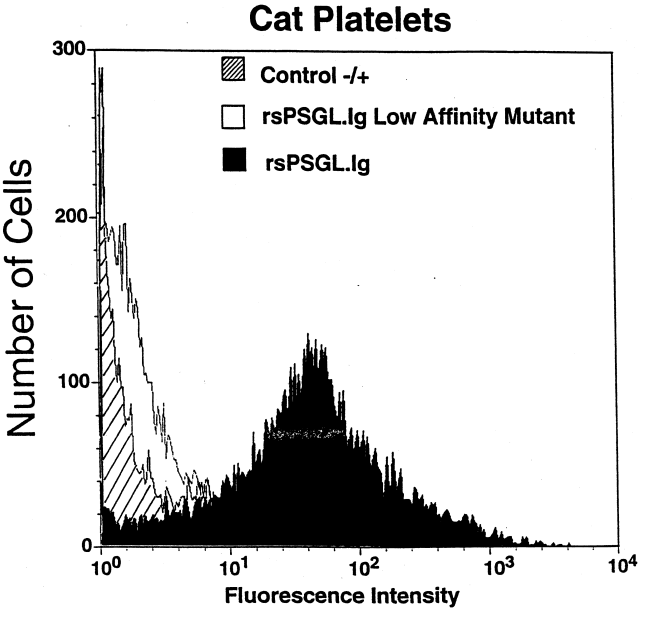

Fig. 1. Flow cytometry histogram of log fluorescence intensity vs. number of rsPSGL.Ig-positive cells. The two rsPSGL.Ig analogs were used at a concentration of 20 μg/ml. There was a high degree of cross-reactivity of rsPSGL.Ig with cat platelets.

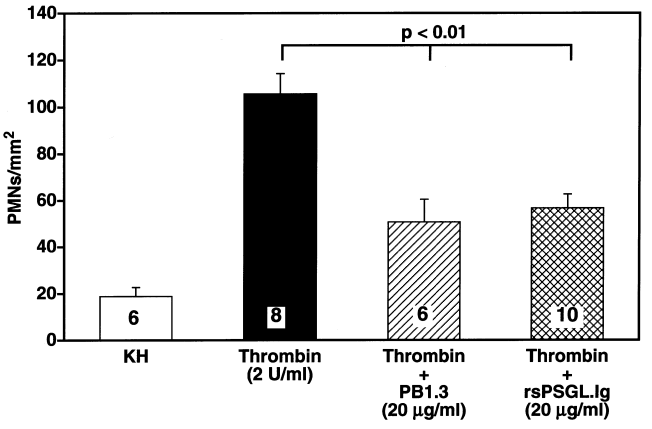

Fig. 2. In vitro effects of rsPSGL.Ig (20 μg/ml) or a monoclonal antibody against P-selectin (PB1.3, 20 μg/ml) on neutrophil adherence to thrombin-stimulated (2 U/ml) cat coronary arteries. Data are expressed as number of PMNs/mm^2. Bar heights represent means and brackets indicate ±SEM. Numbers at the bottom of each bar represent the number of coronary artery segments studied.

3.2. Electrophysiological, hemodynamic and leukocyte changes during the experimental protocol

Prior to coronary artery occlusion, all cats demonstrated comparable values in all of the observed variables. Immediately following occlusion of the LAD coronary artery, the portion of the left ventricle that had been perfused by the occluded artery became cyanotic and a substantial elevation in the S-T segment occurred. Reperfusion of the LAD was confirmed by the return of the ischemic region of the myocardium to normal coloration and a marked decline in the S-T segment elevation. All MI/R groups demonstrated substantial S-T segment elevation, with peak values occurring between 20–40 min after coronary occlusion. Similarly, in both groups of MI/R cats, the PRI decreased comparably after coronary occlusion and slowly increased following reperfusion (Table 1). No significant differences in PRI were observed among either of the MI/R groups throughout the entire data collection period. In order to determine whether rsPSGL.Ig exerted any neutropenic effects that could contribute to any observed protective effects, circulating white blood cell counts were determined immediately prior to coronary occlusion and at

Table 1
Time course of pressure-rate index (PRI) and white blood cell count (WBCC) during the 6 h protocol

Variable	Group	Time (h)			
		0	2	4	6
WBCC	Sham+rsPSGL.Ig	10,267±1325	6700±1782	10,650±1340	9833±999
	MI/R+rsPSGL.Ig low affinity mutant	10,667±708	7300±1703	11,800±2018	11,817±2033
	MI/R+rsPSGL.Ig	11,830±1543	9783±1374	11,200±1333	11,217±893
PRI	Sham+rsPSGL.Ig	31.8±2.3	29.3±2.7	29.2±3.7	30.9±2.9
	MI/R+rsPSGL.Ig low affinity mutant	25.9±2.4	20.3±1.3	20.3±1.3	20.8±1.4
	MI/R+rsPSGL.Ig	28.4±2.8	20.7±3.1	21.4±2.2	20.2±1.8

Values are expressed as means±SEM for seven cats in each group.
WBCC expressed as cells/μl; PRI expressed as [(heart rate×mean arterial blood pressure)/1000].

1, 2, 4 and 6 h following coronary occlusion (Table 1). All groups of cats demonstrated the same time course of circulating leukocyte changes, exhibiting an initial decline of about 30% and a subsequent return to control values by the end of the reperfusion period. This reduction in circulating leukocytes is probably due to the trauma of the surgical intervention. No significant differences in circulating leukocyte counts were observed at any time among any of the groups.

3.3. Effect of rsPSGL.Ig on the extent of myocardial injury

To determine the ability of rsPSGL.Ig to protect against myocardial necrosis, we measured the amount of necrotic tissue both as a percentage of the area-at-risk and as a percentage of the total left ventricle (Fig. 3). There was no significant difference in mass of the areas-at-risk as expressed as a percentage of the total left ventricular mass (31±2 vs. 33±5%, P=NS), indicating that a comparable degree of ischemia occurred in both MI/R groups. In contrast, the necrotic area, when expressed as a percentage of the area-at-risk, was significantly ($P<0.01$) attenuated in cats treated with rsPSGL.Ig in comparison to cats treated with the rsPSGL.Ig low affinity mutant (16±3 vs. 42±6%, respectively). Similar results were obtained when the necrotic area was expressed as a percentage of the left ventricular mass. These results with the low affinity mutant are very similar to necrosis values obtained with prior saline vehicle-treated MI/R cats subjected to the same protocol. One additional MI/R cat was administered a saline vehicle instead of rsPSGL.Ig or the rsPSGL.Ig low affinity mutant. The above procedures were repeated except that half of the area-at-risk was incubated with 20 μg/ml rsPSGL.Ig in order to rule out the possibility that rsPSGL.Ig altered the staining properties of the nitroblue tetrazolium. The area of necrotic tissue computed as a percentage of the area-at-risk was 27.6% in the control area-at-risk sample and 28.4% in the area-at-risk sample incubated with rsPSGL.Ig, indicating that rsPSGL.Ig had no direct effect on the staining properties of nitroblue tetrazolium.

To confirm the preservation of ischemic tissue, the effects of rsPSGL.Ig on plasma CK activity, a biochemical marker of myocardial injury, were determined (Fig. 4). In

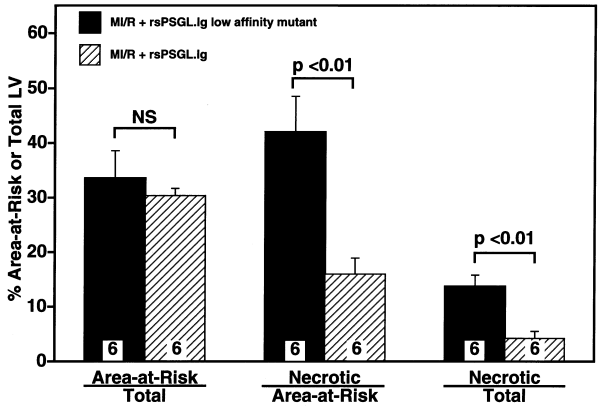

Fig. 3. Tissue wet weight of area-at-risk as a percentage of the total left ventricular wet weight and of the necrotic tissue, either as a percentage of the area-at-risk or of the total left ventricular wet weight for the two MI/R groups. Bar heights represent means and brackets indicate ±SEM. Numbers at the bottom of each bar represent the number of cats studied.

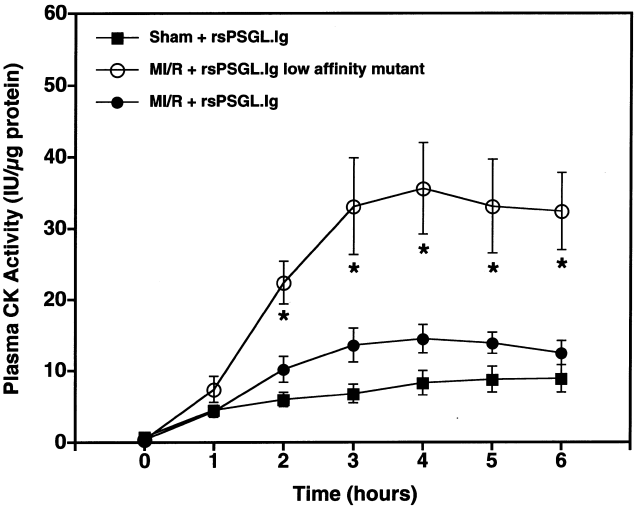

Fig. 4. Plasma CK activity expressed as international units/μg protein, measured hourly throughout the experiment for three groups comprising six cats each. All values are expressed as means±SEM. *$P<0.05$ vs. MI/R+rsPSGL.Ig low affinity mutant.

sham MI/R cats receiving rsPSGL.Ig, plasma CK activity increased slightly as a result of the surgical procedures, reaching a peak value of 8.9±1.9 IU/μg protein. In the two ischemic groups, plasma CK activity was slightly elevated during the period of myocardial ischemia. However, marked elevations in plasma CK activity were observed 30 min after reperfusion in the rsPSGL.Ig low affinity mutant-treated cats, reaching a peak value of 35.6±6.4 IU/μg protein at 4 h. In contrast, MI/R cats receiving rsPSGL.Ig developed significantly ($P<0.05$) lower plasma CK activities at 2 h and beyond in comparison to cats receiving the rsPSGL.Ig low affinity mutant. In three additional cats, a saline vehicle instead of rsPSGL.Ig or the rsPSGL.Ig low affinity mutant was administered and CK analysis was completed as previously described except that aliquots of the plasma samples were incubated with 20 μg/ml rsPSGL.Ig or an equal volume of K-H solution in order to determine if rsPSGL.Ig had any effect on the CK assay. The samples obtained at 6 h were 26±1 IU/μg protein in the control K-H solution samples and 27±2 IU/μg protein in those samples incubated with rsPSGL.Ig. These values were not significantly different, thus indicating that rsPSGL.Ig does not interfere with the CK assay.

3.4. Effects of rsPSGL.Ig on endothelial dysfunction

Since endothelial dysfunction is an early consequence of reperfusion of an ischemic vessel, we assessed endothelial function by comparing the vascular response of coronary artery rings to the endothelium-dependent vasodilators, ACh and A-23187, and to the endothelium-independent vasodilator, $NaNO_2$. Fig. 5 summarizes the vasorelaxation responses of LAD and LCX rings to ACh, A-23187 and $NaNO_2$. Both LAD and LCX coronary artery rings isolated from sham MI cats exhibited full relaxation to all vasodilators. In LAD rings obtained from MI/R cats receiving the rsPSGL.Ig low affinity mutant, the ACh- and the A-23187-induced relaxation was significantly reduced whereas the relaxation to $NaNO_2$ was completely maintained. However, the ACh- and A-23187-induced relaxation of LAD rings isolated from cats treated with rsPSGL.Ig were significantly preserved ($P<0.01$). Thus, rsPSGL.Ig significantly protected against the endothelial dysfunction that results from MI/R. In three additional control cats, coronary arteries were removed and placed in warmed K-H solution and prepared as previously described. Endothelium-dependent relaxation was tested with ACh, and endothelium-independent relaxation was tested with $NaNO_2$ in 12 rings. These rings relaxed 94±5% to ACh and 97±2% to $NaNO_2$. Subsequently, these same rings were again precontracted with U-46619 and then administered rsPSGL.Ig at 20 and 40 μg/ml. Vasorelaxation was determined to be 1.3±0.7 and 2.5±1.2% to 20 and 40 μg/ml rsPSGL.Ig, respectively. Those values are not significantly different from pre-rsPSGL.Ig values. Therefore, the ability of rsPSGL.Ig to preserve endothelial dysfunction is not due to any direct vasodilator properties of this substance.

3.5. Effects of rsPSGL.Ig on PMN accumulation in ischemic myocardial tissue

Accumulation of PMNs in the ischemic–reperfused myocardium is considered to be one of the primary contributory mechanisms to reperfusion injury. MPO activity in the three different portions of the myocardium was measured as a marker for PMN accumulation (Fig. 6). It is evident from the MPO activity in the nonischemic region (i.e., area-not-at-risk) of both MI/R groups that there were very few resident neutrophils in this portion of the myocardium. However, MI/R cats receiving the rsPSGL.Ig low affinity mutant exhibited a marked increase in MPO activity in the necrotic myocardium, whereas this increase in MPO activity associated with MI/R was significantly attenuated in those cats receiving rsPSGL.Ig. These results indicate that the infiltration of neutrophils into the ischemic–reperfused myocardium was markedly inhibited by rsPSGL.Ig.

3.6. Effect of rsPSGL.Ig on PMN adherence to ischemic–reperfused coronary endothelium

One of the first steps in PMN-mediated reperfusion injury is an increase in the adhesiveness of the vascular endothelium. Therefore, we measured the adherence of PMNs following the in vivo administration of rsPSGL.Ig or the rsPSGL.Ig low affinity mutant (Fig. 7). When unstimulated autologous PMNs were incubated with nonischemic LCX coronary artery segments for 20 min, relatively few adhered to the endothelium regardless of the group of origin (<25 PMNs/mm^2). In MI/R cats receiving the rsPSGL.Ig low affinity mutant, unstimulated PMNs incubated with the ischemic–reperfused LAD coronary artery segments resulted in a significant increase in PMN adherence ($P<0.001$). When autologous unstimulated PMNs were incubated with LAD segments isolated from cats treated with rsPSGL.Ig, there was a significant ($P<0.001$) attenuation in PMN adherence, to values comparable to those observed in sham MI/R cats, indicating that rsPSGL.Ig significantly reduced MI/R-induced endothelial adherence of PMNs.

4. Discussion

The recruitment of leukocytes from the circulation to sites of tissue injury is mediated by three groups of cell adhesion molecules, which orchestrate the initial contact

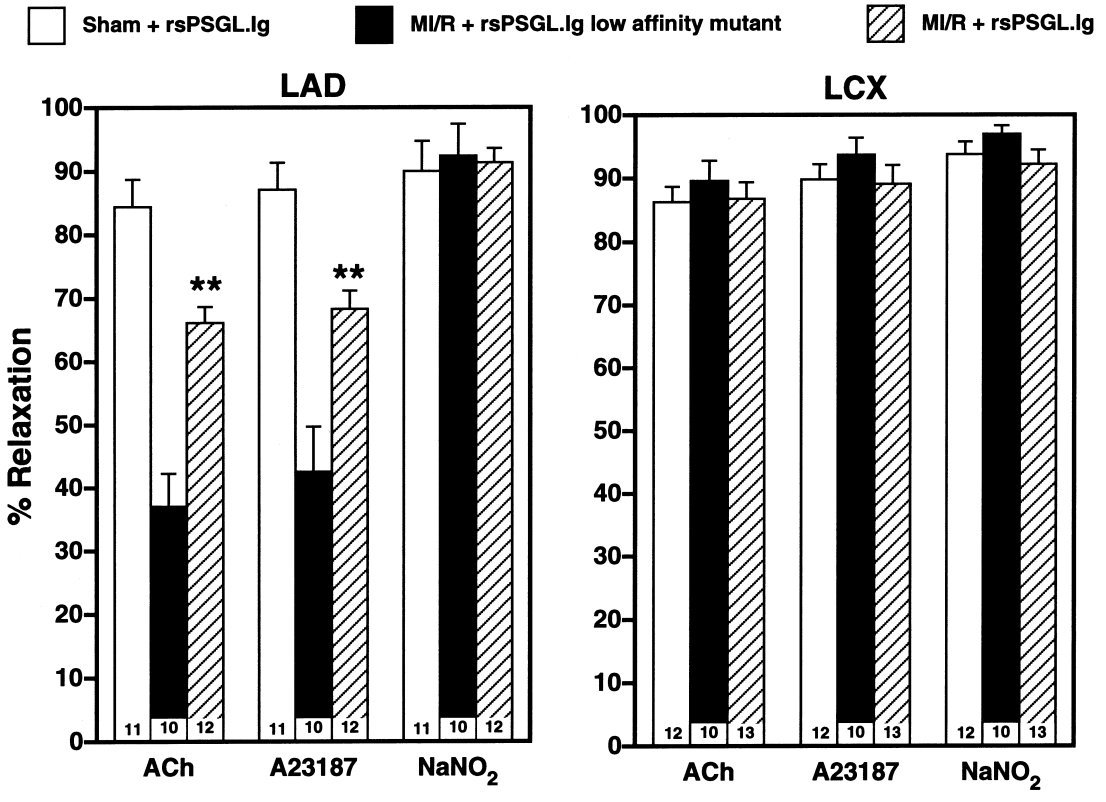

Fig. 5. Summary of the responses of ischemic–reperfused LAD and non-ischemic LCX coronary rings to the highest concentration of vasodilators: 100 nM ACh, 1 μM A-23187 and 100 μM $NaNO_2$. Bar heights represent means and brackets indicate ±SEM. Numbers at the bottom of each bar represent the number of coronary rings studied. $^{**}P<0.01$ vs. MI/R+rsPSGL.Ig low affinity mutant.

between leukocytes and the endothelium, followed by the firm adherence of leukocytes to the endothelium, and the subsequent extravasation of leukocytes into the target tissue [36]. These three groups of cell adhesion molecules are the selectin family (i.e., P-, L- and E-selectin), the β_2-integrin family (i.e., CD11/CD18), and the immunoglobulin superfamily (i.e., intercellular adhesion molecule-1, platelet endothelial cell adhesion molecule-1). Leukocyte rolling is the first step and is a prerequisite for firm adherence since integrin-mediated adherence is relatively ineffective at physiological shear rates [11]. Several investigators have demonstrated that inhibition of the rolling phase of leukocyte recruitment attenuates the inflammatory response [4,19]. Consistent with such find-

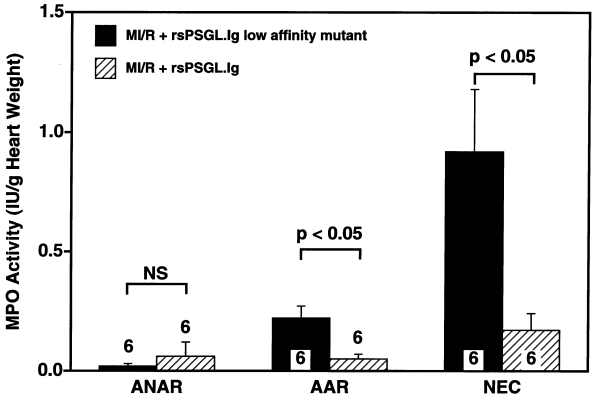

Fig. 6. Cardiac MPO activity in the area-not-at-risk, area-at-risk and necrotic area, in units/100 mg tissue wet weight, for the two MI/R groups. Bar heights represent means and brackets indicate ±SEM. Numbers at the bottom of each bar represent the number of cats studied.

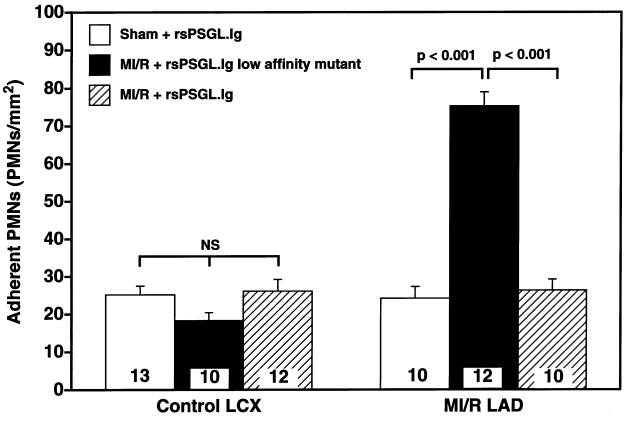

Fig. 7. Effects of in vivo administration of rsPSGL.Ig or its low affinity mutant on the in vitro adherence of unstimulated PMNs to non-ischemic–reperfused LCX coronary endothelium and ischemic–reperfused LAD coronary endothelium. Bar heights represent means and brackets indicate ±SEM. Numbers at the bottom of each bar represent the number of coronary artery segments studied.

ings, we now demonstrate that the administration of a soluble form of PSGL-1, which has selectin-binding capabilities, significantly attenuates both endothelial dysfunction as well as cellular injury associated with myocardial ischemia/reperfusion.

To our knowledge, this is the first study demonstrating the ability of rsPSGL.Ig to provide beneficial effects following MI/R. Our results show that the administration of rsPSGL.Ig significantly preserved the integrity of the ischemic–reperfused myocardium. Myocardial cell preservation was evidenced by a 62% reduction in the amount of necrotic myocardium in cats treated with rsPSGL.Ig in comparison to cats receiving the rsPSGL.Ig low affinity mutant. To further substantiate this cardioprotective effect, plasma CK activity was attenuated by 63% in those MI/R cats treated with rsPSGL.Ig. These impressive cardioprotective effects could not be explained by differences in the amount of myocardium rendered ischemic or to the degree of the ischemic insult, since no significant differences were detected in the areas-at-risk or in the peak S-T segment elevation between the two MI/R groups. The cardioprotective effects of rsPSGL.Ig also do not appear to be the result of differences in myocardial oxygen supply or demand. Collateral blood flow to the ischemic cat myocardium is only 7 to 8% of total coronary flow [37]. In addition, no significant differences were observed in the PRI of the two MI/R groups and the direct administration of rsPSGL.Ig at concentrations similar to those in vivo failed to exert any direct coronary vasoactive effects in isolated cat coronary artery rings. Thus, the protective effects of rsPSGL.Ig were not mediated by reducing the myocardial oxygen demand of the ischemic–reperfused myocardium (i.e., PRI) nor by increasing the oxygen supply to the reperfused myocardium (i.e., vasodilation).

In addition to attenuating myocardial cell necrosis, rsPSGL.Ig significantly preserved endothelial function in coronary arteries subjected to ischemia and reperfusion. Endothelial dysfunction has been shown to occur as early as 2.5 min following reperfusion, with progressively more severe dysfunction occurring during the subsequent 4.5 h of reperfusion [2]. The hallmark of endothelial dysfunction is a reduced ability of the endothelium to release NO [38]. The inability of the endothelium to release NO results in an impaired ability of the vessel to relax to endothelium-dependent vasodilators and leads to an increased leukocyte–endothelium interaction [4,7]. Activated neutrophils that have adhered to the endothelium release cytotoxic substances such as proteases, eicosanoids, cytokines and oxygen-derived free radicals [9,10], each of which can mediate tissue injury and exacerbate endothelial dysfunction. Following rsPSGL.Ig treatment, endothelial function was significantly preserved, as measured with both a non-receptor-mediated endothelium-dependent vasodilator (i.e., A-23187) and a receptor-mediated endothelium-dependent vasodilator (i.e., ACh). Therefore, since coronary endothelial dysfunction precedes myocardial cellular injury and is most likely a contributing factor to cardiac necrosis, preservation of normal endothelial cell function could substantially enhance myocardial cell integrity and function following reperfusion of the ischemic myocardium [38].

Although the exact mechanisms involved in the protective effects of rsPSGL.Ig are not completely known, our data clearly show that the beneficial effects are due at least in part to an inhibition of PMN-endothelial cell interactions. PMN adherence to the coronary endothelium was significantly attenuated in both in vitro thrombin-stimulated as well as in vivo I/R-stimulated vascular segments treated with rsPSGL.Ig. Our PMN adherence data are in agreement with those of others who demonstrated that antibodies directed against PSGL-1 significantly attenuate P-selectin binding to PSGL-1 [18,20,39]. In addition, we measured cardiac MPO activity as a specific marker of PMN accumulation in the I/R myocardium [30]. Our finding of reduced MPO activity in the necrotic area following rsPSGL.Ig further supports the concept that rsPSGL.Ig attenuates PMN–endothelial cell interactions. These data, taken together, suggest that the protective effects of rsPSGL.Ig are dependent upon inhibition of the initial PMN–endothelial cell interactions, thereby inhibiting firm PMN adherence, extravasation and migration of PMNs into the myocardium. Since rsPSGL.Ig did not significantly alter total leukocyte count, the beneficial effects of rsPSGL.Ig cannot be attributed to a leukopenia that would pari passu provide cardioprotective effects following MI/R [40].

PSGL-1 is a highly extended homodimer of two disulfide-linked subunits, each of which displays N-linked glycans and sialylated O-linked glycans that present SLe^x. However, expression of PSGL-1 protein is not sufficient to allow binding to its primary high affinity ligand, P-selectin. Functional binding of PSGL-1 to P-selectin requires a number of posttranslational modifications by α(1,3/1,4)fucosyltransferase, which include sialylation, sulfation and fucosylation of the primary protein structure [16,41]. All three selectins demonstrate a high affinity for sialylated, fucosylated saccharide structures such as SLe^x and, although there are several leukocyte-bound structures that present SLe^x, it appears as though P-selectin must bind with PSGL-1 for PMNs to efficiently roll along the endothelium under physiological flow conditions [17,42]. During MI/R, an increase in oxygen-derived free radicals generated by endothelial cells leads to the upregulation of surface-expressed P-selectin, which can be sustained for several hours [24,43]. The increased translocation of P-selectin to the endothelial cell surface is largely responsible for a substantial portion of the PMN–endothelial cell interaction in the setting of MI/R [44]. Thus, PSGL-1 would bind only to surface-expressed P-selectin in the ischemic–reperfused area. Our in vitro adherence experiments suggest that the anti-adherent effects of rsPSGL.Ig are primarily due to inhibition of P-selectin. In those

experiments, the addition of rsPSGL.Ig attenuated PMN adherence to thrombin-stimulated endothelium to a comparable degree as an anti-P-selectin neutralizing monoclonal antibody (i.e., PB1.3).

Although it appears that the primary cardioprotective effects of rsPSGL.Ig are the result of an inhibition of P-selectin-mediated PMN–endothelial cell interactions, other selectins may be involved. PSGL-1 is capable of binding L- and E-selectin [22,41,45,46], which theoretically may provide significant cardioprotective effects in MI/R. However, inhibition of E-selectin-mediated leukocyte–endothelial cell interactions by rsPSGL.Ig in our model of feline MI/R is highly unlikely. E-selectin expression requires de novo protein synthesis and may take 4–6 h for the protein to be expressed on the endothelial surface. Moreover, E-selectin is not upregulated to a significant extent by myocardial ischemia/reperfusion in cats [24]. This is consistent with studies showing that an E-selectin mAb provided no beneficial effects in primates subjected to MI/R [23]. Takada et al. [35] reported that the administration of soluble PSGL-1 reduced PMN infiltration into the I/R kidney and inhibited the induction of E-selectin mRNA. These results were not observed following the treatment of soluble PSGL-1 with mocharhagin, a protease that causes PSGL-1 to lose P-selectin-binding capabilities while maintaining E-selectin-binding capabilities. Thus, by inhibiting P-selectin-mediated PMN–endothelial cell interactions, soluble PSGL-1 may limit the locally high concentrations of cytokines necessary to induce E-selectin expression in endothelial cells. This suggests that the protective effect of soluble PSGL-1 is primarily mediated via P-selectin-dependent mechanisms, even during longer periods of reperfusion.

In contrast to E-selectin, L-selectin-mediated PMN–endothelial cell interactions have been shown to be important in the development of myocardial reperfusion injury during the first 4.5 h of reperfusion [47]. L-selectin not only is capable of mediating leukocyte–endothelium interaction but also leukocyte–leukocyte interaction. Although PSGL-1 has been shown to bind with lower affinity to L-selectin than P-selectin [45], the interaction of L-selectin and PSGL-1 could initiate leukocyte–leukocyte interactions, which represents the first step in PMN aggregation during inflammatory states [22]. Unstimulated PMNs have been shown to roll under flow conditions on PSGL-1 immobilized on glass surfaces, and treatment of the immobilized PSGL-1 with an L-selectin mAb inhibits not only PMN rolling on immobilized PSGL-1 but also PMN rolling on adherent PMNs [48,49]. Even though activated PMNs shed their L-selectin, PSGL-1 remains intact. Thus, adherent activated PMNs that have shed L-selectin are capable of interacting with flowing PMNs if the adherent PMNs are expressing PSGL-1 and activated integrins, while flowing PMNs express L-selectin and the integrin counter-receptor [22].

The extent of cardioprotection afforded by rsPSGL.Ig treatment is in agreement with other studies investigating the roles of P- and L-selectin in MI/R. Monoclonal antibodies directed against L-selectin have been shown to significantly attenuate PMN rolling [50,51] and reduce myocardial necrosis by 60% following MI/R in cats [47]. In a similar fashion, a mAb against P-selectin reduced PMN adherence to coronary endothelium and decreased myocardial necrosis by more than 50% [44,52]. The use of soluble SLe^x oligosaccharides appears to result in even greater myocardial protection in the feline MI/R model [53].

In conclusion, we have demonstrated significant cardioprotective effects after the administration of a recombinant soluble human form of PSGL-1 in a 6-h model of MI/R in cats. These impressive effects are probably due to the fact that PSGL-1 has about four orders of magnitude greater avidity for binding to P-selectin than that of previously identified ligands [54]. The necrotic myocardium was significantly reduced in cats treated with rsPSGL.Ig in comparison to cats administered a low affinity mutant rsPSGL.Ig. Similarly, PMN accumulation was significantly reduced in the ischemic myocardium, and coronary rings isolated from cats given rsPSGL.Ig demonstrated preserved endothelial function. All of these findings are consistent with the ability of rsPSGL.Ig to attenuate PMN–endothelial cell interactions. These data demonstrate the significant role PSGL-1 plays in the development and progression of inflammation in disease states such as myocardial reperfusion injury. Moreover, these data suggest that PSGL-1 or a related molecule may have significant clinical value in the treatment of reperfusion injury.

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