Administration of Recombinant P-Selectin Glycoprotein Ligand Fc Fusion Protein Suppresses Inflammation and Neointimal Formation in Zucker Diabetic Rat Model

Zhongmin Zhou, Marc S. Penn, Farhad Forudi, Xiaorong Zhou, Khaldoun Tarakji, Eric J. Topol, A. Michael Lincoff, Kai Wang

Objective—P-selectin–mediated leukocyte-endothelium and leukocyte-platelet interaction has been reported after vascular injury and has been correlated with neointimal hyperplasia, but its role in neointimal formation after arterial injury in diabetes has not been described.

Methods and Results—Using a Zucker diabetic rat balloon injury model, we examined the role of P-selectin in the vascular inflammatory process and neointimal formation after balloon injury. Immunohistochemistry revealed that P-selectin was intensely expressed and that CD45-positive leukocyte infiltration was significantly increased after arterial injury. A single preprocedural intravenous administration of a recombinant P-selectin–soluble glycoprotein ligand-Ig inhibited CD45-positive leukocyte accumulation and suppressed neointimal formation in the Zucker diabetic rat model.

Conclusions—These results suggest that reduction of P-selectin–mediated leukocyte activation with the use of recombinant P-selectin–soluble glycoprotein ligand-Ig decreases the inflammatory response and limits neointimal formation after balloon injury in diabetes. (Arterioscler Thromb Vasc Biol. 2002;22:1598-1603.)

Key Words: P-selectin diabetes mellitus restenosis balloon injury

Compared with nondiabetic patients, patients with diabetes mellitus have a greater incidence of restenosis after percutaneous coronary intervention (PCI), which is associated with increased target lesion revascularization and late morbidity and mortality.1–4 The inflammatory response to vascular injury is increasingly becoming recognized as a major contributor to restenosis in diabetes.5,6 This local leukocyte-platelet and leukocyte-endothelium reaction is related to the expression of cell adhesion molecules, the migration of leukocytes, cytokine release, and the secretion of growth factors at the site of injury. The selectin family is a key mediator for the earliest event in the inflammatory response, leading to leukocyte “rolling,” followed by migration. P-selectin is an integral membrane glycoprotein contained within platelet α-granules and Weibel-Palade bodies of endothelial cells. On activation, P-selectin is expressed on the cell surface, where it mediates the adherence of activated platelets to monocytes and neutrophils and the interaction between activated endothelial cells and leukocytes via its carbohydrate ligands and P-selectin glycoprotein ligand (PSGL)-1.7,8 Platelet and leukocyte activation and binding to endothelium have been reported after PCI and have been correlated with restenosis.9,10 It has recently been demonstrated that antagonism of P-selectin reduces neointimal formation in several animal models of restenosis; however, most studies of P-selectin–mediated inflammatory reaction have been performed with the subjects in a nondiabetic state, and little attention has been paid to P-selectin as a possible contributor to restenosis in diabetes mellitus. Recent studies have indicated that diabetes is associated with an enhanced inflammatory response and overproduction of multiple growth factors after arterial injury.11 Therefore, we hypothesized that administration of rPSGL-Ig, a recombinant soluble form of PSGL-1, will inhibit inflammation and suppress neointimal formation after vascular injury in diabetes by competing with the cell-associated PSGL-1 for its P-selectin binding site. Using a recently established model of enhanced neointimal hyperplasia in Zucker obese diabetic rats undergoing carotid artery injury,12 we examined the expression of P-selectin and leukocyte infiltration in the injured vessel wall. In addition, we evaluated the effects of rPSGL-Ig on inflammation and neointimal formation in diabetic rats. In the present study, we provide evidence that P-selectin is importantly involved in diabetes-associated enhanced neointimal hyperplasia consequent to arterial injury.

Methods

Two sequential series of experiments were performed. The first phase examined tissue P-selectin expression, leukocyte accumula-
tion, and the ability of rPSGL-Ig to attenuate leukocyte accumulation after balloon injury of carotid arteries of Zucker diabetic rats. The second phase investigated the effect of anti-P-selectin therapy on restenosis in the same model. Zucker obese rats (average weight 400 g) aged 9 to 12 weeks were used. Animal procedures were approved by the Cleveland Clinic Foundation Animal Research Committee and complied with all state, federal, and National Institutes of Health regulations.

Preparation of rPSGL-Ig

rPSGL-Ig, a recombinant immunoglobulin Fc fusion protein form of P/SGL, is produced in Chinese hamster ovary cells that have been engineered to express the critical carbohydrate-modifying enzymes fucosyltransferase VII and core2 GlcNAc transferase. It consists of the first 47 amino acids from the N-terminal end of the extracellular domain of mature P/SGL-1, fused at the “hinge” region to human IgG1. Two “hinge-proximal” amino acids at positions 234 and 237 within the IgG Fc portion are mutated to alanine to reduce complement activation and Fc receptor binding (Anjali Kumar, and 237 within the IgG Fc portion are mutated to alanine to reduce complement activation and Fc receptor binding (Anjali Kumar, 2001). This manipulation of the compound gives it a longer half-life and also maintains the bivalent complement activation and Fc receptor binding (Anjali Kumar, 2001). This manipulation of the compound gives it a longer half-life and also maintains the bivalent complement activation and Fc receptor binding (Anjali Kumar, 2001). This manipulation of the compound gives it a longer half-life and also maintains the bivalent complement activation and Fc receptor binding (Anjali Kumar, 2001).

Arterial Injury

Injury to the carotid artery was performed by balloon deendothelialization. After induction of anesthesia with an intraperitoneal injection of xylazine (4.6 mg/kg) and ketamine (70 mg/kg), a midline cervical incision was made to expose the left carotid artery. The external carotid artery was ligated, and the internal carotid artery was ligated temporarily. A 2F Fogarty balloon catheter (Baxter Healthcare Corp) was introduced through the arteriotomy site of the cervical incision to expose the left external carotid artery. The balloon catheter was passed into the aortic arch, and the balloon was distended with saline until a slight resistance was felt on slight traction. After withdrawal into the common carotid artery, the balloon was rotated while pulling it back through the common carotid artery. This procedure was repeated 3 times.

Tissue Harvest and Preparation

For the first phase of the study, either rPSGL-Ig (1 mg/kg) or saline was randomly administered 15 minutes before balloon injury as an intravenous bolus. Twenty-eight animals were involved in this phase, including 4 euthanized before injury and 24 euthanized at days 1, 3, and 7 after injury (4 per time point per group). The excised carotid arteries were immediately embedded in OCT compound and rapidly frozen in liquid nitrogen and stored at −70 °C for later immunohistochemical analysis.

In the second phase of the study, 24 rats were randomized to receive either rPSGL-Ig (1 mg/kg) or saline 15 minutes before balloon injury. Animals were euthanized at 21 days after balloon injury. After anesthesia, a midline abdominal incision was performed, and the distal abdominal aorta was exposed. With the use of an 18-gauge intravenous catheter introduced at the aortic bifurcation, the aorta was flushed with 50 mL Ringer’s lactate solution at 120 mm Hg, followed by in vivo fixation with 200 mL of 5% Histochoice (Amresco) infused over 5 minutes at 120 mm Hg. Once the perfusion-fixation was started, the animals were euthanized with an overdose of Pentothal (Abbott Laboratories) through the tail vein. After 5 minutes of perfusion-fixation, the entire left carotid artery was harvested. The specimens were stored in 5% Histochoice for at least 24 hours.

P-Selectin Immunostaining

Cryostat samples from days 0, 1, 3, and 7 after balloon injury were immunohistochemically assessed for P-selectin antigen expression. Briefly, tissue cryosections were postfixed in 100% acetone at room temperature for 10 minutes. Endogenous peroxidase activity was blocked by 0.3% H2O2 for 10 minutes. Normal goat serum (5%) was applied for 30 minutes. The tissues were incubated with rabbit polyclonal anti-P-selectin antibody (PharMingen International) 10 μg/mL overnight at 4°C, followed by application of a biotinylated rabbit anti-mouse secondary antibody (1.25 μg/mL) for 30 minutes. P-selectin antigen expression was visualized with a diaminobenzidine chromogen substrate. Sections were counterstained with hematoxylin-eosin and mounted with Crystal Mount for light microscopic analysis. P-selectin expression was quantitatively analyzed by using computerized digital microscopic planimetry software (Image-Pro Plus, Version 4.0 for Windows, Media Cybernetics) by 2 observers blinded to treatment regimen.

CD45 Immunostaining

To further characterize the involvement of inflammatory leukocytes at days 1, 3, and 7 after balloon injury and uninjured arteries, immunohistochemistry for CD45, a specific marker for leukocytes, was conducted on the cryostat sections. CD45-positive cells were immunolocalized by incubation with a mouse monoclonal antibody against CD45 (Chemicon International) overnight at 4°C, followed by application of a biotinylated rabbit anti-mouse secondary antibody (1.25 109 f−1 s−1 g/mL) for 30 minutes. Detection of CD45 was completed with diaminobenzidine chromogen substrate, which produces a brown cell surface stain on CD45-positive cells. Similar quantitative analysis was performed as described above.

Morphometry

The sections were cut into 4 or 5 segments at 5-mm intervals before embedding to allow identification of the most severely injured segment of the carotid artery (section at 5 μm). Slides were stained with hematoxylin-eosin and Movat. Morphometric analysis of the arterial segments was carried out by an observer who was blinded to the study groups and who used computerized digital microscopic planimetry software (Image-Pro Plus, Version 4.0 for Windows, Media Cybernetics). The section (from 4 or 5 sections) of each injured arterial segment exhibiting the most severe degree of luminal narrowing was assessed as the “lesion” point. The neointimal and medial boundaries were determined; the cross-sectional areas subtended by the luminal border, the internal elastic lamina (IEL), and the external elastic lamina (EEL) were measured; and the ratio of intimal to medial area was calculated.

Blood Chemistry Assay

Blood samples were collected at 21 days. Blood glucose was measured by an enzymatic method, total cholesterol was determined by a cholesterol oxidase enzyme assay, and triglyceride levels were measured by a glycerol triphosphate oxidase enzyme assay.

Statistical Analysis

All data were expressed as mean±SD. Statistical analysis was performed with the use of SPSS software (Version 7.0 for Windows, SPSS Inc). Continuous variables were compared by using unpaired t tests. A value of P≤0.05 was considered to be statistically significant.

Results

P-Selectin Expression

In the uninjured arteries, the endothelial monolayer remained intact, and P-selectin expression was negative. However, at days 1, 3, and 7 after balloon injury, P-selectin expression was significantly upregulated in luminal layer/surface and adventitial tissue, and rPSGL-Ig significantly reduced P-selectin expression by >50% compared with the control group (Figure 1 and Table 1).

CD45-Positive Leukocyte Accumulation

Consistent with the findings of P-selectin expression, no CD45-positive staining was found in uninjured arteries. After balloon injury, CD45 was intensely expressed in the arterial...
wall, with an increase in the number of CD45-positive cells at days 1, 3, and 7. The administration of rPSGL-Ig significantly attenuated the recruitment of CD45-positive leukocytes to the injured vessel walls by >50% (Figure 2 and Table 2).

Histological Analysis and Morphometry
Quantitative morphometric measurements are summarized in Table 3. There were no differences regarding the extent of vessel injury, IEL, EEL, and medial areas between the 2 treatment arms. In both groups, we did not find laceration of IEL or EEL. However, treatment with rPSGL-Ig significantly reduced neointimal area (0.06±0.05 versus 0.13±0.05 mm², P=0.019), representing >50% inhibition in the rPSGL-Ig treatment group. Luminal area was increased by rPSGL-Ig treatment (0.25±0.09 versus 0.19±0.05 mm², P=0.048; Figure 3 and Table 3). The IEL, EEL, and media were 0.36±0.07, 0.49±0.08, 0.12±0.02 mm², respectively, in normal uninjured carotid artery. There were no differences in IEL, EEL, and media between 2 treatment arms.

Metabolic Parameters
The serum glucose, cholesterol, and triglyceride levels were 285±63 mg%, 165±25 mg/dL, and 340±168 mg/dL, respectively, in rPSGL-Ig–treated animals and 293±81 mg%, 177±40 mg/dL, and 334±103 mg/dL, respectively, in the control animals. There were no differences in serum metabolic parameters between the rPSGL-Ig–treated and control groups.

Discussion
An increased propensity to restenosis after PCI is a major challenge in the clinical management of diabetic patients with cardiovascular diseases. In the present study, we have shown marked expression of P-selectin and CD45-positive leukocytes in balloon-injured diabetic rat carotid arteries. We have demonstrated that rPSGL-Ig, administered as a single preprocedural intravenous bolus, significantly reduces the neointimal hyperplasia and results in increased luminal area in a diabetic rat carotid artery injury model; these occurrences are accompanied by reduced P-selectin expression and CD45-positive leukocyte infiltration. The results of the present study suggest an important role of P-selectin in diabetes, implying that the enhanced inflammatory response in diabetes after vascular injury may contribute to increased restenosis in diabetic patients.

The process of restenosis is multifactorial. Although the mechanism remains incompletely defined, inflammation and thrombosis induced by vessel injury have recently been recognized as major contributors to restenosis. The interaction between platelets/leukocytes and endothelium/leukocytes promotes mutual activation and leads to the secretion of inflammatory cytokines, thus stimulating vascular smooth muscle cell migration and proliferation and resulting in neointimal formation. This process is known to be mediated by a group of adhesion molecules, with P-selectin initiating it. P-selectin is stored in α-granules of inactivated platelets and Weibel-Palade bodies of endothelium. After a stimulus, such as vessel injury, P-selectin is rapidly expressed in the outer membrane of activated platelets and endothelium and mediates leukocyte recruitment to the activated platelets and endothelium through PSGL-1 in leukocytes. It has been shown that P-selectin upregulates tissue factor in monocytes and leads to leukocyte accumulation in the area of vascular injury associated with thrombosis and inflammation. Furthermore, several studies have shown that interaction between monocytes and P-selectin through PSGL-1 induces the expression of the inflammatory cytokines, such as tumor necrosis factor-α, monocyte chemoattractant protein-1, and interleukin-8.

In nondiabetic models of arterial injury in a normal or atherosclerotic background, P-selectin has been shown to be intensely expressed on activated platelets covering the denuded segment and on endothelial cells of the inflamed adventitial small vessels after balloon injury in rats. P-selectin–deficient mice form less compact platelet layers on the denuded arterial surface in vivo, accumulate fewer leukocytes into the vascular wall, and are substantially protected from neointimal formation. However, in wild-type mice, a carpet of platelets develops on the damaged vascular surface immediately after injury, and leukocytes adhere to the platelets. Monoclonal antibodies to P-selectin significantly inhibit shear-induced platelet

### Table 1. % Area Occupied by P-Selectin Positive Cells After Balloon Injury

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<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
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<tr>
<td>Placebo</td>
<td>24.10±1.55</td>
<td>28.67±3.48</td>
<td>18.18±1.65</td>
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<tr>
<td>rPSGL-Ig</td>
<td>9.55±3.42*</td>
<td>12.26±3.86*</td>
<td>5.60±1.78†</td>
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*P<0.05, †P<0.01 compared with the placebo group.
aggregation. Administration of various types of P-selectin inhibitors has been found to be beneficial in reducing platelet-neutrophil interactions and restenosis in nondiabetic animal models. Studies have also shown that anti–P-selectin therapy beneficially affects remodeling after balloon injury in normal rat and swine models. Our laboratory has recently demonstrated that P-selectin antagonism using rPSGL-Ig decreases neointimal hyperplasia by inhibiting the inflammatory response at the site of injury in a normal porcine coronary artery balloon injury model.

Patients with diabetes mellitus account for 15% to 25% of those undergoing PCI procedures. There are 2 major types of diabetes: type I diabetes is mediated by autoimmune processes that result in insulin deficiency, and type II diabetes is manifested by insulin resistance. Some key processes known to activate macrophages (and, therefore, the release of cytokines) are enhanced in diabetes. These include oxidation and glycoxidation of proteins and lipids; advanced glycation end product microcirculation and that this inflammatory response to vascular injury in diabetes. Acute hyperglycemia has been shown to increase soluble P-selectin levels and platelet and leukocyte activation in diabetes have also been reported. Siminiak et al found that the activation and accumulation of leukocytes to the injured arterial wall determined the enhanced inflammatory reaction in response to vascular injury in diabetes. Acute hyperglycemia has been shown to increase soluble P-selectin in patients with diabetes mellitus. Furthermore, Jilma et al have demonstrated that acute increases in glucose is associated with upregulation of the endothelial cell adhesion molecule P-selectin. However, the role of P-selectin in the vascular response to balloon-induced injury in diabetes has not yet been reported. The present study provides some insight into the contribution of P-selectin to the balloon injury–induced recruitment of leukocytes in Zucker diabetic rats. Although no P-selectin expression was found before injury, we identified the early intense expression on the injured luminal side and on the adventitial side after vascular balloon injury. rPSGL-Ig downregulated P-selectin expression in the injured vessel segment, accompanied by reduced CD45-positive leukocyte infiltration. The present study also demonstrated that treatment with rPSGL-Ig significantly suppressed neointimal formation 21 days after balloon injury. Our findings suggest that P-selectin–mediated leukocyte recruitment into damaged arterial walls is a major contributor to neointimal formation in diabetes. The findings of the present study are in part attributed to blockade of the P-selectin–mediated inflammatory response at the site of injured arterial segments and to the decreased release of inflammatory cytokines. Another potential mechanism is that rPSGL-Ig might reduce platelet accumulation on the injured luminal surface and limit the platelet contribution to neointimal formation after injury in diabetes. There are no differences in blood lipid profiles between rPSGL-Ig–treated and control rats, suggesting that the beneficial effects of rPSGL-Ig treatment on neointimal formation in

TABLE 2. % Area Occupied by CD45 Positive Leukocytes After Balloon Injury

<table>
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<th></th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
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<tbody>
<tr>
<td>Placebo</td>
<td>49.43±5.89</td>
<td>57.32±18.18</td>
<td>68.58±2.22</td>
</tr>
<tr>
<td>rPSGL-Ig</td>
<td>16.51±6.76*</td>
<td>15.46±4.16†</td>
<td>32.08±10.43†</td>
</tr>
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*P<0.05, †P<0.01 compared with the placebo group.

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Figure 2. Photomicrographs of representative CD45 immunostaining showing arteries from control and rPSGL–treated rats at 1, 3, and 7 days after balloon injury. A, Section from control rat at day 1 showed intense CD45 immunostaining. B, Section from control rat at day 3 showed CD45 staining around lumen. C, By day 7, control rat sections still had intense CD45 staining, observed around lumen and extending into media. D through F, Much less CD45 immunostaining was observed in rPSGL–treated rat at days 1, 3, and 7.

Figure 3. Photomicrographs of carotid arteries 21 days after balloon injury (Movat, ×5). A, Representative light microscopy of cross section of injured carotid artery from rPSGL treatment group showing reduced neointimal hyperplasia with large luminal area. B, Representative light microscopy of cross section of injured carotid artery from control group showing extensive neointimal hyperplasia with smaller luminal area.
The present study has several limitations. First, the inflammatory markers were not compared between the obese diabetic and lean nondiabetic rat models, and the effect of rPSGL-Ig on neointimal formation was not assessed for lean nondiabetic rats in our experiment. The present study was not designed to demonstrate that P-selectin antagonism inhibits neointimal formation only in diabetes. Several studies using rPSGL-Ig and other compounds to block P-selectin have already shown beneficial effects on neointimal formation after arterial injury in nondiabetic animal models; we believe that the beneficial effects of rPSGL-Ig on diabetes shown in the present study have very important clinical implications because diabetes has been shown to be associated with increased inflammation and neointimal formation. Second, the most appropriate control for rPSGL-Ig is somewhat problematic. The “ideal” control would be an inactive mutant rPSGL-Ig molecule. This reagent has proven to be very difficult to produce because the activity of the rPSGL-Ig molecule. This reagent has proven to be very low-affinity rPSGL-Ig (not “dead” rPSGL-Ig) have indeed shown the effect to be similar to the effect with saline control. An IgG1 isotype control would be even less suitable because it would not reflect the FC receptor or fixing complement. Therefore, we believe that the overall interpretation of the data and the value of our observation are not severely limited by the use of the saline control.

To summarize, P-selectin appears to be involved in the vascular inflammatory response to balloon injury in diabetes. Given the higher occurrence and limitations of currently available therapy for restenosis in diabetes, P-selectin antagonism may represent an alternative promising strategy.

**Acknowledgment**

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**References**


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<tr>
<td>Luminal area, mm²</td>
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<tr>
<td>Neointimal area, mm²</td>
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<tr>
<td>Media area, mm²</td>
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<tr>
<td>Neointimal area/media</td>
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<tr>
<td>IEL area, mm²</td>
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<tr>
<td>EEL area, mm²</td>
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<td>Proximal reference</td>
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*P<0.05, †P<0.01 compared with the placebo group.


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