Prevention of Intimal Hyperplasia With Recombinant Soluble P-Selectin Glycoprotein Ligand-Immunoglobulin in the Porcine Coronary Artery Balloon Injury Model

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OBJECTIVES The role of P-selectin in the process of restenosis was evaluated using a recombinant immunoglobulin (Ig) chimera form of its ligand, soluble P-selectin glycoprotein ligand-Ig (rPSGL-Ig), as a competitive inhibitor for the natural ligand on leukocytes.

BACKGROUND Inflammation and coagulation activation after vascular injury may be an important factor in the development of restenosis. P-selectin has been shown to mediate leukocyte-endothelium and leukocyte-platelet interaction. These interactions are mediated through binding of P-selectin to P-selectin glycoprotein ligand-1 (PSGL-1) located on the surface of leukocytes.

METHODS Balloon injury was induced in the left anterior descending and right coronary arteries of 16 pigs at a balloon/artery diameter ratio of 1.5:1. Either rPSGL-Ig (1 mg/kg) or saline was randomly administered 15 min before balloon injury as an intravenous bolus. Four weeks after injury, morphometric analysis, immunohistochemistry and histological evaluation were performed on injured arterial segments.

RESULTS Increased luminal area was found in the rPSGL-Ig group compared with the placebo group (1.63 ± 0.57 mm² vs. 1.26 ± 0.32 mm², p = 0.044) owing to significantly reduced neointimal hyperplasia (cross-sectional area, 0.46 ± 0.45 mm² vs. 0.13 ± 0.11 mm², p = 0.013). Immunohistochemistry and histological evaluation showed a significant decrease in the presence of tumor necrosis factor-alpha, interleukin-1 beta, and infiltration of macrophages in the injured vessel segments in the rPSGL-Ig group.

CONCLUSIONS P-selectin antagonism using rPSGL-Ig decreases neointimal hyperplasia following balloon injury, by inhibiting the inflammatory and thrombotic responses at the site of balloon injury, which appears to play a pivotal role in the pathogenesis of restenosis. (J Am Coll Cardiol 2001;38:577–82) © 2001 by the American College of Cardiology

Restenosis remains the major limitation of percutaneous transluminal intervention. Recently, it has been recognized that local inflammatory and thrombotic reactions including platelet/leukocyte and leukocyte-endothelial cell complex formation induced by vessel injury due to balloon angioplasty play an important role in this complex process (1–5). This recruitment of inflammatory cells results in the release of growth factors and cytokines, which induce smooth muscle cell (SMC) migration and proliferation. This local leukocyte/platelet response is mediated by a group of complementary cell adhesion molecules, which consists of three families: the selectins, the integrins, and the immunoglobulin supergene family. Selectins, a family of calcium-dependent lectins including P-, L- and E-selectins, are known to be very important in leukocyte and platelet adhesion to the endothelium, with P-selectin mediating the initial response. P-selectin is stored in alpha granules of platelets and Weibel-Palade bodies of endothelial cells and is rapidly translocated to the platelet and endothelial cell surface after cell activation. P-selectin promotes rolling of circulating leukocytes on the endothelium and positions them for activation. In addition, P-selectin induces leukocyte-leukocyte and leukocyte-platelet interaction. These interactions are mediated through the binding of P-selectin to P-selectin glycoprotein ligand-1 (PSGL-1) located on the surface of leukocytes.

The rPSGL-Ig is a recombinant immunoglobulin chimera form of PSGL-1; it inhibits P-selectin–mediated platelet-neutrophil adhesion by acting as a competitive inhibitor for PSGL-1 on leukocytes. It has been recently shown that antagonism of P-selectin reduced the thrombotic and inflammatory response and resulted in increased luminal diameter after balloon injury in the rat and porcine carotid artery models (6–8). In a recent study (9), our group demonstrated that administration of rPSGL-Ig competitively binds P-selectin, decreases the degree of inflammatory response and attenuates the selectin-mediated endothelial dysfunction and myocardial injury following reperfusion of ischemic myocardium. In the current study, the effect of rPSGL-Ig on neointimal hyperplasia after balloon injury in the porcine coronary artery model was assessed.

METHODS

rPSGL-Ig. PSGL-1 is the natural ligand for P-selectin and binds P-selectin via an anionic amino-terminal peptide...
sequence. Also, PSGL-1 appears to account for all of the high-affinity P-selectin binding sites on neutrophils (10,11). rPSGL-Ig, a recombinant immunoglobulin chimera form of PSGL-1, is produced in Chinese hamster ovary cells, which have been engineered to co-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 PSGL-1, fused at the “hinge” region to human IgG1. Two “hinge-proximal” amino acids at positions 234 and 237 within the IgGFc portion are mutated to alanine to reduce both complement activation and Fc receptor binding. This manipulation of the compound gives it a long half-life, ≈11 days in pigs (unpublished data) and also maintains the bivalent presentation of the native molecule as well as high P-selectin affinity and reduced L-selectin and E-selectin binding.

**Animal preparation.** Juvenile domestic farm pigs weighing 22 to 25 kg were treated with 325 mg of aspirin orally one day before surgery through 28 days follow-up. General anesthesia was induced by intramuscular injection of ketamine 22 mg/kg and maintained with inhaled isoflurane. The carotid artery and jugular vein were dissected from the surrounding tissue through a midline cervical incision. An 8F sheath was introduced into the carotid artery over a guide wire, and a heparin bolus of 300 IU/kg was administered. Either rPSGL-Ig (1 mg/kg) or saline was randomly administered 15 min before balloon injury as an intravenous (IV) bolus.

Following baseline coronary angiography, balloon injury was performed to one segment of the right coronary artery (RCA) and left anterior descending coronary artery (LAD) of each animal, based on the angiographic estimation of vessel diameter and a balloon/artery ratio of 1.5:1. Three inflations were made at a pressure of 8 atm for 30 s at 1-min intervals. Following arterial injury, coronary angiography was repeated to confirm vessel patency. The carotid artery was repaired or ligated. The wound was closed with layered interrupted sutures. Animals were treated with prophylactic antibiotics and acetaminophen for postoperative pain. Throughout the study period all animals were fed a standard laboratory diet.

After 28 days, follow-up angiography was performed using the same method as described above. Following angiography, animals were euthanized with an overdose of IV sodium pentobarbital and potassium chloride. All experiments conformed to the position of the American Heart Association on research animal use and care and were conducted with the approval of the Animal Research Committee of the Cleveland Clinic Foundation.

**Histological analysis and morphometry.** The animal heart was removed immediately after death and perfusion-fixed with IV sodium pentobarbital and potassium chloride. Quantitative measurements were performed by an observer blinded to the treatment regimen, using computerized digital microscopic planimetry software (Image-Pro Plus, Version 4.0 for Windows, Media Cybernetics, Silver Spring, Maryland). Morphometric parameters were measured in four to six sections of injured arterial segments per vessel and averaged. The fracture length (FL) of internal elastic lamina (IEL) was measured. The ratio of neointimal area/fracture length of IEL (IA/FL) was applied to provide the normalized value of intimal area related to the extent of vessel injury.

**Immunohistochemical assay.** Immunohistochemical analysis was performed to determine the extent of inflammation and SMC proliferation and to assess for the presence of the cytokines interleukin-1 beta (IL-1 beta) and tumor necrosis factor-alpha (TNF-alpha). The following antibodies were used in this study: anti-proliferating cell nuclear antigen (PCNA) (Dako, Carpinteria, California), anti-fibronectin (Dako, California), anti-IL-1 beta (R&D System, Minneapolis, Minnesota), and anti-TNF-alpha (R&D System). As described before (4), the amount of each antigen in the samples was assessed semiquantitatively by an independent observer. Immunohistochemical staining was performed using a Jung Histostainer (Leica, Chicago, Illinois), with processing occurring at 30°C. A 1% hydrogen peroxide solution in methanol for 5 min was used to remove any endogenous peroxidase present in the tissue section.

For mouse monoclonal primary antibodies, a blocking solution comprising a 1:10 dilution of normal rabbit serum (Dako) in phosphate-buffered saline (PBS) was added for 10 min before application of the primary antibody. The required dilutions of antibody were prepared using 1% bovine serum albumin (BSA) in PBS. Incubation occurred at 30°C for 60 min, and a 1:200 dilution of biotinylated rabbit anti-mouse polyclonal antibody (Dako) was added for...
a further 30 min. The antibody was labeled using an Elite avidin/biotin/peroxidase complex (Vector Laboratories, Burlingame, California) applied for 30 min. Then 3,3-diaminobenzidine was added as a chromogen (DAB Kit, Vector Laboratories).

For polyclonal antibodies, a similar procedure was followed, except that normal swine serum instead of rabbit serum was used as a blocking agent and biotinylated swine anti-rabbit polyclonal antibody was used as the link. Following staining, the sections were counterstained with hematoxylin, dehydrated and cleared in xylene. The negative control sections were treated in an identical manner to the test tissue, except that 1% BSA in PBS was added instead of primary antibody.

Statistical analysis. Statistical analysis was performed using SPSS software (Version 7.0 for Windows, SPSS, Chicago, Illinois). Data are presented as mean ± SD. Continuous variables were compared using unpaired t tests. A value of p ≤ 0.05 is considered to be statistically significant.

RESULTS

A total of 19 animals underwent balloon injury (9 in the rPSGL-Ig group, 10 in the control group). Two of them died during the procedure due to ventricular fibrillation (one in the control group, the other in the rPSGL-Ig group), and one additional death occurred one day after balloon injury in the control group. The surviving 16 animals (control group = 8; rPSGL-Ig group = 8) finished the study without complications, providing 15 lesions for analysis in the control group (7 in RCA; 8 in LAD) and 15 lesions in the rPSGL-Ig group (7 in RCA; 8 in LAD).

Histological analysis and morphometry. Qualitative histological examination 28 days after balloon injury showed that less macrophage infiltration occurred in the rPSGL-Ig group compared with the placebo group (Fig. 1). Quantitative morphometric measurements are summarized in the Table 1. There were no differences with regard to the extent of vessel injury (as measured by the extent of FL), IEL and external elastic lamina areas between treatment arms. However, treatment with rPSGL-Ig significantly reduced neointimal area by over 70% (0.46 ± 0.45 mm² vs. 0.13 ± 0.11 mm², p = 0.013), resulting in increased luminal area in the rPSGL-Ig group compared with the placebo group (1.63 ± 0.57 mm² vs. 1.26 ± 0.32 mm², p = 0.044) (Fig. 2). This difference still existed when intimal area was normalized to the extent of injury (IA/FL, 0.53 ± 0.53 vs. 0.16 ± 0.13; p = 0.014).

Immunohistochemistry assay. No qualitative differences were seen in the appearance of fibronectin staining between the two groups. In contrast, staining for TNF-alpha, IL-1 beta and PCNA was more evident among animals receiving placebo instead of rPSGL-Ig (Fig. 3). Only 3 of 15 lesions from the rPSGL-Ig group showed mild staining for TNF-alpha, whereas 13 of 15 lesions from the placebo group had mild-to-moderate degrees of staining (5/15 mild; 8/15 moderate). Similarly, only 5 of 15 lesions from the rPSGL-Ig group had positive staining for IL-1 beta (4/15 mild; 1/15 moderate), whereas 12 of 15 showed mild-to-moderate staining (5/15 mild; 7/15 moderate). None of 15 lesions from the rPSGL-Ig group showed the positive staining of PCNA, whereas 9 of 15 lesions from the placebo group had mild-to-moderate staining (4/15 mild; 5/15 moderate).

Table 1. Morphometric Results

<table>
<thead>
<tr>
<th>Group</th>
<th>Placebo (n = 15)</th>
<th>rPSGL-Ig (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal area (mm²)</td>
<td>1.26 ± 0.32</td>
<td>1.63 ± 0.57*</td>
</tr>
<tr>
<td>IA (mm²)</td>
<td>0.46 ± 0.45</td>
<td>0.13 ± 0.11*</td>
</tr>
<tr>
<td>IEL area (mm²)</td>
<td>1.72 ± 0.48</td>
<td>1.77 ± 0.55</td>
</tr>
<tr>
<td>Fracture IEL length (mm)</td>
<td>0.86 ± 0.12</td>
<td>0.84 ± 0.11</td>
</tr>
<tr>
<td>Medial area (mm²)</td>
<td>0.72 ± 0.21</td>
<td>0.83 ± 0.26</td>
</tr>
<tr>
<td>EEL area (mm²)</td>
<td>2.44 ± 0.59</td>
<td>2.59 ± 0.67</td>
</tr>
<tr>
<td>IA/FL</td>
<td>0.52 ± 0.46</td>
<td>0.16 ± 0.13*</td>
</tr>
</tbody>
</table>

*p < 0.05.

EEL = external elastic lamina; IA = neointimal area; IEL = internal elastic lamina; rPSGL-Ig = recombinant P-selectin glycoprotein ligand-immunoglobulin.
DISCUSSION

We investigated the efficacy of rPSGL-Ig in reducing restenosis in a porcine coronary artery balloon injury model. The findings demonstrate that rPSGL-Ig, when used by a single preprocedural IV bolus, significantly reduces the extent of neointimal hyperplasia and results in increased luminal area. Therapy with rPSGL-Ig was also associated with reduced immunostaining for inflammatory markers and inflammatory cellular infiltration. The results of this study highlight the important role of inflammation in the pathogenesis of restenosis following percutaneous coronary recanalization in this experimental model.

In the past decade, extensive research has aimed at reducing restenosis after percutaneous revascularization procedures. Although the mechanism of restenosis remains incompletely defined, the role of inflammation and thrombosis induced by vessel injury has recently been recognized (12). These inflammatory reactions following percutaneous revascularization procedures are characterized by damage to endothelial, subendothelial structures, and medial regions, with rupture of the IEL followed by the recruitment of inflammatory cells. Inflammatory cells induce release of growth factors and cytokines, which are potent mitogens for SMC migration and proliferation, possibly leading to restenosis. It has been shown that TNF-alpha and interleukin-1 (IL-1) are actively involved in the process of inflammation and that neointimal formation is associated with increased endothelial and SMC fibronectin synthesis induced by TNF-alpha and IL-1 after an immune inflammatory reaction (13–16). All of these processes are mediated by adhesion molecules, including P-selectin, which is the initial stimulus to leukocyte recruitment to the endothelium.

Figure 2. Photomicrographs of porcine coronary arteries 28 days after injury (Movat staining, 2.5 ×). (A) Representative light microscopy cross section of injured coronary artery from recombinant P-selectin glycoprotein ligand-immunoglobulin group, showing reduced neointimal hyperplasia with larger luminal area. (B) Representative light microscopy cross section of injured coronary artery from the placebo group, showing extensive neointimal hyperplasia with smaller luminal area. L = lumen. N = neointima, M = media.

Figure 3. Immunohistochemical photomicrographs of porcine coronary arteries 28 days after injury (40 ×). Brown stain denotes presence of tumor necrosis factor-alpha (TNF-alpha), interleukin (IL)-1 beta, and proliferating cell nuclear antigen (PCNA). (A) TNF-alpha immunostaining of injured coronary artery from recombinant P-selectin glycoprotein ligand-immunoglobulin (rPSGL-Ig) group. (B) TNF-alpha immunostaining of injured coronary artery from the placebo group. (C) IL-1 beta immunostaining of injured coronary artery from rPSGL-Ig group. (D) IL-1 beta immunostaining of injured coronary artery from the placebo group. (E) PCNA immunostaining of injured coronary artery from rPSGL-Ig group. (F) PCNA immunostaining of injured coronary artery from the placebo group.
The physiological ligand for P-selectin is PSGL-1, and it serves as the only high-affinity counter-receptor for P-selectin (17). It has been demonstrated that P-selectin expression on the endothelial surface is markedly upregulated following vessel injury and is associated with increased inflammatory cell adherence to coronary artery endothelium (18). Administration of a monoclonal antibody to P-selectin significantly attenuated inflammatory cell accumulation and endothelial dysfunction (18). Ikeda et al. (19) reported that treatment with sialyl Lewisx-containing oligosaccharide (SLex-Os) suppressed P-selectin expression and inhibited the recruitment and accumulation of platelets and leukocytes on the damaged endothelium in a canine thrombosis model. This finding was supported by the study from Kumar et al. (20), which demonstrated that administration of rPSGL-Ig in a porcine thrombosis model leads to faster thrombolysis and reduced reocclusion, probably by preventing interaction of leukocytes with platelets and the injured arterial wall.

One explanation, therefore, for the findings of reduced neointimal hyperplasia in the current study is that rPSGL-Ig administration abolished the activation of inflammatory cells after vessel injury, thus decreasing the release of inflammatory cytokines. In our study, local tissue level of the inflammatory mediators TNF-alpha and IL-1 beta as well as macrophage infiltration, were decreased by rPSGL-Ig treatment. Both TNF-alpha and IL-1 beta appear to play a pivotal role in the development of vascular stenosis following vessel injury (21,22). It has been reported that neutralization of TNF-alpha activity in a rabbit model reduced the severity of coronary artery lesions, which was associated with less inflammation and decreased accumulation of fibronectin in the vessel wall (13).

The inflammatory response induced by percutaneous transluminal revascularization is characterized by disruption of endothelial and subendothelial structures. Dysfunctional coronary endothelial cells will exhibit decreased basal nitric oxide (NO) release and promote further adhesion of leukocytes through the CD11b/CD18 mechanism. The restoration of NO release through adenovirus-mediated NO synthase transfer and NO donors has been demonstrated to significantly reduce neointimal formation after vessel injury in different animal models (23–27). It has been shown that restored NO production inhibited cytokine-induced endothelial expression of vascular selectins, decreased adhesion, migration, and activation of inflammatory cells, and reduced vascular SMC migration and proliferation (27–32). rPSGL-Ig has been shown to preserve the morphological integrity and function of the endothelium through the inhibition of leukocyte-endothelial cell interaction (33), thus potentially maintaining NO release. Gries et al. (34) recently reported that NO inhibits P-selectin expression and platelet aggregation both in vitro and in vivo. Thus, reduced neointimal formation found in the rPSGL-Ig group of the current study may relate in part to preserved endothelial integrity due to antagonism of P-selectin and maintained NO release. This mechanism is supported by recent kidney ischemia/reperfusion injury studies by Takada et al. (35,36). In these studies, a P-selectin inhibition-mediated decrease in leukocyte accumulation of reperfused ischemic rat kidneys resulted in a reduction of inflammatory cytokine message and in suppression of the elevated endothelin message found in nontreated control animals.

Conclusions. This study has several limitations. First, the most appropriate control for rPSGL-Ig is somewhat problematic. The “ideal” control would be a nonactive (nonfucosylated) chimera. This reagent has proven to be very difficult to produce because the activity of the “control” material is quite variable from batch to batch, with some batches as active as the rPSGL-Ig. The sponsor is unable to produce sufficient quantities of inactive reagent for large animal studies. Moreover, studies with the low-activity form of rPSGL-Ig have demonstrated results similar to those with saline control (33,37–39). A IgG1 isotype control would be even less suitable because it would not reflect the mutations in the rPSGL-Ig chimera that prevent it from binding the Fc receptor or fixing complement, nor would it represent the rPSGL portion of the molecule. Therefore, we believe that the overall interpretation of the data and the value of our observations are not severely limited by the use of the saline control group.

Second, we did not directly assess the effect of rPSGL-Ig in the injured vessel segments. However, other investigators have demonstrated (8) that P-selectin was upregulated in rat carotid arteries after balloon injury. Third, we observed abundant PCNA staining in control vessels 28 days after injury, whereas some prior studies have suggested that cellular proliferation is no longer apparent 14 days after injury. The explanation for this variation between ours and others is unknown, but we did use appropriate positive and negative controls for PCNA staining, and the results were reproducible in our laboratory.

Fourth, another limitation of this study was the use of injury to a normal porcine coronary artery rather than balloon dilation of a pre-existing coronary lesion, which more accurately resembles the human scenario with regard to the coronary disease process of inflammatory cell infiltration and cytokine expression. However, it should be noted that the post-injury response of this model did result in both infiltration of inflammatory cells and cytokine expression with distinct histopathological similarities to human restenosis, including a prominent inflammatory and cytokine response. The extrapolation of the findings of a restenotic study from any animal models should be made with caution because no single model has yet been shown to reliably predict restenosis in humans. Animals studies may, however, help to further the understanding of the pathophysiology of the human disease process.
role of P-selectin-mediated inflammation in the pathogenesis of restenosis.

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REFERENCES


