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Single Injection of P-Selectin or P-Selectin Glycoprotein Ligand-1 Monoclonal Antibody Blocks Neointima Formation After Arterial Injury in Apolipoprotein E-Deficient Mice

J. William Phillips, MD; Kurt G. Barringhaus, MD; John M. Sanders, BS; Sean E. Hesselbacher, BS; Ann C. Czarnik, BS; David Manka, PhD; Dietmar Vestweber, PhD; Klaus Ley, MD; Ian J. Sarembock, MB, ChB, MD

Background—Emerging data suggest that P-selectin, by controlling adhesion of white blood cells, may be important in limiting the response to vascular injury.

Methods and Results—We tested the hypothesis that transient inhibition of P-selectin with either anti-P-selectin monoclonal antibody (mAb) or anti-P-selectin glycoprotein ligand-1 (PSGL-1) mAb would reduce neointima formation in the setting of carotid denudation injury in atherosclerosis-prone apolipoprotein E^{-/-} mice. Neointima formation at 28 days was reduced significantly, by 50% or 80%, by a single injection on the day of injury of 100 or 200 μg P-selectin mAb RB 40.34 and by 55% by a single injection of 100 μg PSGL-1 mAb 4RA10 ($P \leq 0.005$). In addition, there was a significant reduction in neointimal macrophage content.

Conclusions—These findings demonstrate that transient P-selectin or PSGL-1 blockade at the time of arterial injury significantly limits plaque macrophage content and neointima formation in a dose-dependent manner after carotid denudation injury in apolipoprotein E^{-/-} mice. (*Circulation*. 2003;107:2244-2249.)

Key Words: antibodies ■ arteries ■ atherosclerosis ■ cell adhesion molecules ■ inflammation

Mechanical injury stimulates a cascade of events involving the interaction of platelets, leukocytes, endothelial cells, and arterial wall cells in a healing response to maintain vascular integrity. These cellular interactions are not only important in normal healing but also have been shown to play pivotal roles both in the development of spontaneous atherosclerosis and in neointima formation after arterial injury.^{1,2} The inflammatory cascade characterized by the expression of cellular adhesion molecules, such as P-selectin, plays a critical role in the early interactions of platelets, endothelial cells, and leukocytes that result in leukocyte rolling on the injured arterial wall, resulting in leukocyte recruitment to the injury site. P-selectin, found in storage granules of platelets and endothelial cells, initiates capture and rolling of circulating leukocytes at sites of inflammation and atherosclerosis.³⁻⁵ P-selectin is rapidly mobilized and expressed on activated platelets, and immobilized platelets are capable of supporting leukocyte rolling.^{6,7} This interaction is mediated to a great extent by binding of platelet P-selectin to P-selectin glycoprotein ligand-1 (PSGL-1) expressed on leukocytes.⁵ This function of supporting leukocyte capture and rolling by platelets is particularly important in the setting of arterial injury, in which endothelial denudation exposes the subendothelial basement membrane, allowing platelets to adhere and serve as a surface that supports leukocyte rolling.⁶⁻⁸ In the mouse

carotid wire injury model, reendothelialization occurs by 3 to 4 weeks, and the regenerating endothelium is known to express adhesion molecules, including P-selectin.^{9,10} The strategy of P-selectin blockade to limit leukocyte recruitment in the setting of arterial injury is therefore expected to be effective both early after injury, when blockade at the level of the platelet is critical, and possibly also later, when expression of P-selectin on regenerating endothelium might be important.

See p 2175

We recently reported a dramatic reduction in neointima formation together with a reduction in macrophage recruitment in the apolipoprotein E (apoE)^{-/-} P-selectin^{-/-} double-knockout mouse after carotid wire injury.¹¹ In a mouse model of femoral transluminal arterial injury without hyperlipidemia, absence of early leukocyte recruitment in P-selectin-deficient mice correlated with a reduction in neointimal formation.¹² Another group showed that there is a positive effect on remodeling in the pig carotid artery after double injury with administration of recombinant soluble PSGL-1 Ig through inhibition of platelet-neutrophil adhesion in the setting of normal cholesterol levels.¹³ We tested the hypothesis that transient P-selectin blockade with a blocking monoclonal antibody (mAb), RB 40.34, or blockade of its ligand,

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From the Department of Medicine, Cardiovascular Division (J.W.P., K.G.B., J.M.S., S.E.H., A.C.C., I.J.S.), the Cardiovascular Research Center (K.L., I.J.S.), and the Department of Biomedical Engineering (D.M., K.L.), University of Virginia Health System, Charlottesville; and the University of Münster, Münster, Germany (D.V.). Dr Manka is currently at the Center for Transgene Technology and Gene Therapy, Leuven, Belgium.

Correspondence to Ian J. Sarembock, MD, Cardiovascular Division, University of Virginia Health System, Box 800158, Charlottesville, VA 22908-0158. E-mail ijs4s@virginia.edu

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PSGL-1, with an mAb, 4RA10, would limit leukocyte entry and accumulation and neointima formation after carotid wire injury in the Western diet-fed hyperlipidemic, atherosclerosis-prone apoE^{-/-} mouse.

Methods

Animals

Animals used for flow cytometry were female C57/BL6 mice (The Jackson Laboratory, Bar Harbor, Me) weighing 18 to 22 g and 8- to 10-week-old female C57/BL6 apoE^{-/-14} mice weighing 18 to 20 g, which were used for all other experiments. Handling was in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. Protocol approval was obtained from the Animal Research Committee of the University of Virginia Health System.

Flow Cytometry

C57BL/6 mice (n=16) were given 100 µg of anti-P-selectin antibody-FITC, RB40.34 (Becton Dickinson Pharmingen) or 100 µg monoclonal rat IgG1, λ anti-KLH-FITC (isotype control) (Becton Dickinson Pharmingen) via intraperitoneal (IP) injection and killed with an overdose of sodium pentobarbital at 3, 6, 12, 24, 48, and 72 hours and 7 and 14 days.¹⁵ Blood samples were obtained at the time of death by direct cardiac puncture, drawn into a syringe containing buffered sodium citrate, and immediately placed in buffered sodium citrate Vacutainers (Becton Dickinson). Samples were then processed as described previously.¹⁶

Mouse Injury Model

The mouse carotid artery wire injury model of Lindner et al⁹ was used, with minor modification as described previously.¹¹ All mice were fed a Western atherogenic diet containing 21% fat by weight (0.15% by weight cholesterol and 19.5% by weight casein without sodium cholate) for 1 week before and 4 weeks after carotid injury. Before carotid injury, all mice were anesthetized by IP injection with a mixture of ketamine (80 mg/kg body wt; Ketaset, Aveco Inc) and xylazine (5 mg/kg; AnaSed, Lloyd Laboratories) diluted in an equal volume of 0.9% sodium chloride solution. Surgical procedures were performed by sterile surgical technique with a dissecting microscope (Zeiss). Via a midline neck incision, the left external carotid artery (LECA) was looped proximally and tied off distally with 6-0 silk suture (Ethicon). Additional 6-0 silk ties were looped around the common and internal carotid arteries for temporary vascular control during the procedure. A transverse arteriotomy was made in the LECA, and a 0.014-inch flexible angioplasty guidewire was introduced and advanced ≈1 cm to the aortic arch. Endothelial denudation injury of the left common carotid artery was performed by use of wire withdrawal injury and 3 passes along the common carotid artery with rotating motion to ensure uniform and complete endothelial denudation. Endothelial denudation has been confirmed by scanning electron microscopy in our laboratory.⁸ After wire removal, the LECA was tied off and the skin closed with 6-0 silk suture. At the time of death (28 days), animals were reanesthetized, and after an overdose of pentobarbital (210 mg/kg IP), a 24-gauge angiocatheter was placed in the left ventricle, and in-situ perfusion fixation was performed at physiological pressure (100 mm Hg) with phosphate-buffered paraformaldehyde (4%, 0.1 mol/L, pH 7.3). Both injured left and uninjured right carotid arteries were excised. Serial 5-µm sections were cut from the paraffin-embedded blocks and prepared for histomorphometry.

Antibody Administration

Three hours before carotid injury, each mouse was given a single bolus of 100 or 200 µg of RB40.34 mAb, 100 µg 4RA10 mAb, or isotype control Ab (n=10 for the 100-µg RB40.34 group, n=14 for the 200-µg RB40.34 group, and n=28 for the 100-µg 4RA10 group) via IP injection with the operator blinded to treatment.^{17,18}

Quantitative Histopathology

The arterial segments were dehydrated in ethanol and xylene and embedded in paraffin. Sections (5 µm thick) were stained by the Movat method.¹⁹ Histomorphometric analysis was performed by individuals blinded to treatment. For quantitative histopathological comparisons, the mean of 10 sections from each injured vessel was taken. The areas of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were determined by planimetry using Image Pro Plus 3.0 (Media Cybernetics), and the lumen area, plaque area, medial area, intima to media (I/M) ratio, and overall vessel area were calculated. Neointimal area was calculated by subtracting lumen area from the IEL area, and medial area was determined by subtracting the EEL area from the IEL area. Arterial size was measured by tracing the circumference of the EEL.

Immunocytochemistry

Sections were stained for macrophages/foam cells WITH an anti-mouse macrophage mAb, F4/80 (Accurate Chemical and Scientific Corp). For quantitative immunocytochemical comparisons of macrophage content, sections were digitized, and the number of positively stained pixels was counted and normalized to total neointimal and medial area WITH Image Pro Plus 3.0 (Media Cybernetics).

Complete Blood Counts and Lipoprotein Levels

At the time of euthanasia, blood samples were drawn by cardiac puncture into EDTA-containing Microtainer tubes (Becton-Dickinson). Complete blood counts and automated differential leukocyte counts were performed by the University of Virginia Clinical Pathology Laboratory. For lipoprotein levels, blood samples at the time of death were drawn by cardiac puncture and placed in serum separator tubes (Becton-Dickinson). Lipid levels were determined by the University of Virginia Clinical Pathology Laboratory.

Statistical Analysis

Statistical analysis was performed with NCSS 97. Data are reported as the number of carotid arteries in each group, and plaque area and I/M ratio are expressed as the mean±SEM. Data were compared by 1-way ANOVA and Student's *t* test to evaluate 2-tailed levels of significance. A probability value of *P*<0.05 was considered significant.

Results

Flow Cytometry/Complete Blood Count/Lipoprotein Levels

Flow cytometry of platelets revealed binding of RB40.34 mAb after an IP injection at 3 hours after administration. There was more surface binding of RB40.34 mAb in the 200-µg dose group than the 100-µg dose group at 72 hours (Figure 1, A–D). RB40.34 mAb on platelets was no longer detectable at 14 days (Figure 1E). No significant differences were seen between RB40.34-treated and isotype-treated groups with respect to complete blood counts, platelet counts, or leukocyte counts (Table). Total cholesterol levels were not significantly different between the 100-µg P-selectin mAb-treated group versus the isotype control (1400±140 versus 1310±100, *P*=NS, n=6), the 200-µg P-selectin mAb-treated group versus the isotype control (1395±80 versus 1450±100, *P*=NS, n=6), or the 100-µg PSGL-1 mAb-treated group versus the isotype control (1180±110 versus 1250±130, *P*=NS, n=10). There were no significant differences in lipoprotein fractionations between any of the groups (data not shown).

Quantitative Histopathology

Neointima formation at 28 days was reduced significantly, by 50%, in the 100-µg P-selectin mAb-treated group versus the isotype control (20 000±4000 versus 42 000±9000 µm², *P*≤0.005, n=10, Figure 2A). In the 200-µg P-selectin mAb-

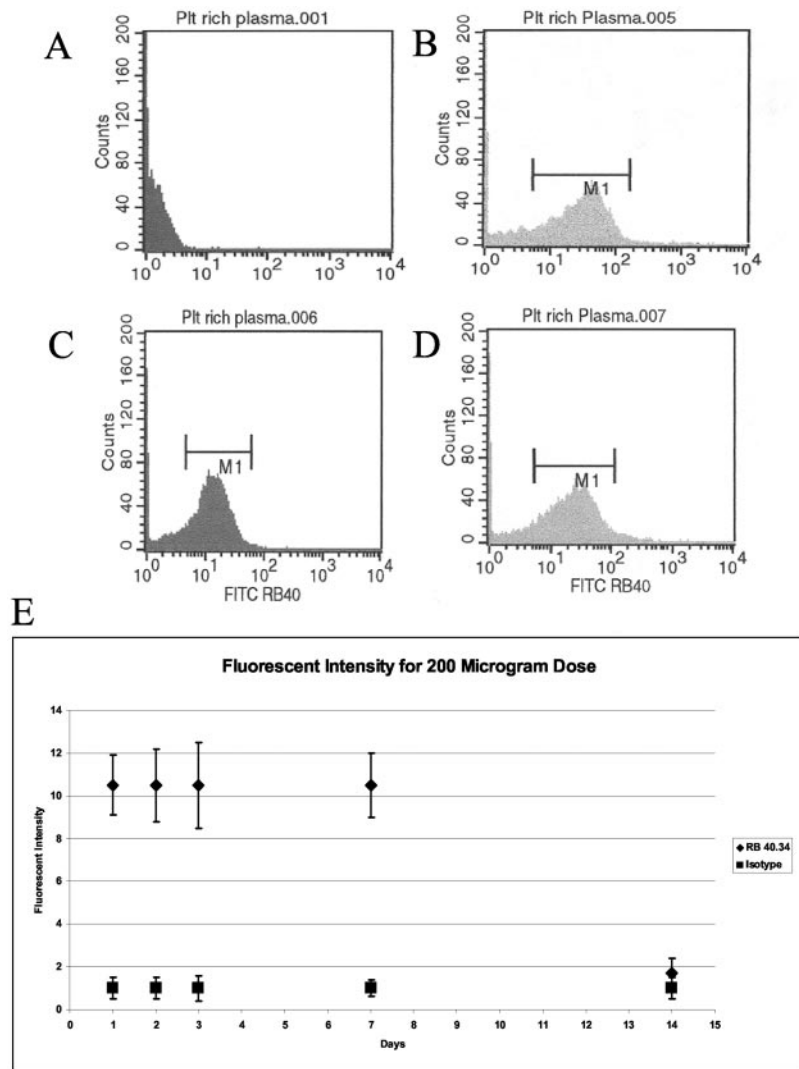


Figure 1. Flow cytometry. A, Platelets (Plt) from a mouse administered isotype control antibody. B, Platelets from a mouse 3 days after injection with 100 µg RB40.34 mAb. C, Platelets from a mouse 3 days after injection with 200 µg RB40.34 mAb. D, Platelets from a mouse 7 days after injection with 200 µg RB40.34 mAb. Note shift on histogram with P-selectin fluorescent labeling (B, C, and D) vs isotype control (A). E, Change in fluorescent intensity over time with 200 µg RB40.34 mAb vs isotype control.

treated group, there was a more pronounced, 80%, reduction in neointima formation compared with the 200-µg isotype control group (6000 ± 1800 versus $30\,000 \pm 8000 \mu\text{m}^2$, $P \leq 0.005$, $n = 14$, Figure 2A). To test the role of the most important leukocyte ligand for P-selectin, PSGL-1, a group of mice was treated with a single injection of 100 µg PSGL-1 mAb. Neointima formation was reduced significantly, by 58%, in the 100-µg PSGL-1 mAb-treated group versus the isotype control ($13\,000 \pm 3000$ versus $31\,000 \pm 4000 \mu\text{m}^2$, $P \leq 0.001$, $n = 28$, Figure 2A). Representative histological sections of vessels from the 100- and 200-µg P-selectin mAb-treated groups and the 100-µg PSGL-1 mAb-treated group are shown in Figure 2B-E. The I/M ratios were also reduced in the P-selectin antibody-treated groups compared with the isotype controls (100 µg: 0.47 ± 0.07 versus

1.15 ± 0.08 , $P \leq 0.0001$; 200 µg: 0.22 ± 0.08 versus 0.56 ± 0.11 , $P \leq 0.05$). A similar reduction was observed in the 100-µg PSGL-1 mAb-treated group (0.2 ± 0.06 versus 0.6 ± 0.01 , $P \leq 0.01$). Macrophage content in the injured vessel wall was reduced dramatically in the 200-µg P-selectin antibody-treated group compared with isotype control ($0.3 \pm 0.1\%$ versus $16 \pm 4\%$, $P \leq 0.05$, Figure 3, A–C), with a more modest reduction seen in the 100-µg PSGL-1 mAb-treated group ($10.7 \pm 3\%$ versus $17.7 \pm 3\%$, $P \leq 0.05$, Figure 3, A, D, and E). There is a positive correlation between percent macrophage staining and neointimal area ($P \leq 0.05$) (Figure 4). Note the significant correlation between percent macrophage staining and neointimal area in both the treated and control groups, with a dramatic reduction in both macrophage staining and neointimal area in the 200-µg P-selectin antibody group. Although there was almost complete inhibition of macrophage content in the 200-µg P-selectin antibody group, there was nevertheless modest neointima formation, suggesting that macrophage-independent mechanisms of neointima formation in response to vascular injury exist. There was no difference in the extent of injury, defined by number of broken elastic laminae, between any of the treatment groups (data not shown).

Complete Blood Counts From P-Selectin Antibody (RB0.40.34)-Treated and Isotype-Treated Mice

	WBC	PLT	Neutrophils	Monocytes	Lymphocytes
RB.40.34	4.8	458.0	1.2	0.3	3.4
Isotype	5.0	521.3	1.4	1.4	1.4

WBC indicates white blood cells; PLT, platelets. Values are 1000/µL.

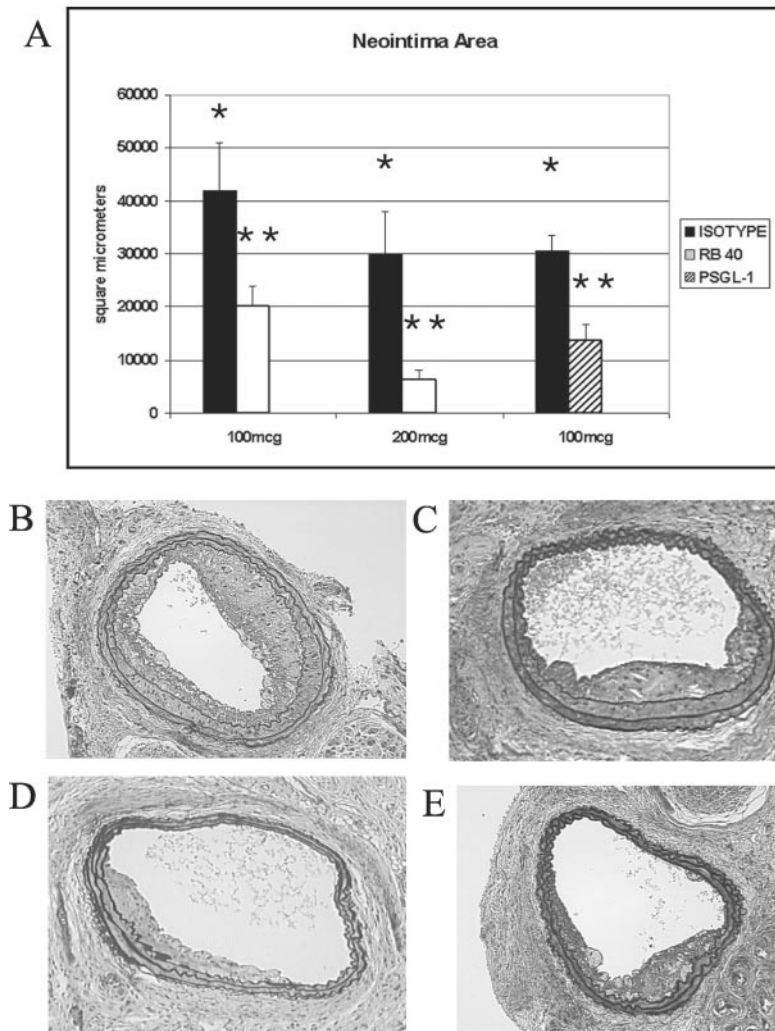


Figure 2. Quantitative histomorphometry. A, Quantitative histomorphometry of plaque area in injured carotid arteries 4 weeks after wire denudation and 5 weeks on a Western diet. Note 50% reduction in plaque size in apoE^{-/-} mice administered 100 µg P-selectin antibody, ** $P < 0.005$ vs apoE^{-/-} mice given 100 µg isotype antibody. Note more dramatic 80% reduction in plaque area in apoE^{-/-} mice given 200 µg P-selectin antibody group, ** $P < 0.005$ vs isotype control. Note 58% reduction in plaque size in apoE^{-/-} mice injected with 100 µg PSGL-1 antibody, ** $P < 0.001$ vs isotype antibody. B, Movat-stained injured left carotid artery (LCA) treated with isotype antibody with robust neointima formation and significant medial thickening. C, Movat staining of an injured LCA treated with 100 µg P-selectin antibody showing moderate neointimal growth. D, Movat staining of an injured LCA treated with 200 µg P-selectin antibody with minimal neointimal growth. E, Movat staining of injured LCA treated with 100 µg PSGL-1 antibody with moderate neointimal growth. * $P = NS$ for controls across treatment groups. Magnification $\times 200$.

Discussion

These experiments demonstrate a significant and dose-dependent reduction in neointima formation after carotid wire injury in the cholesterol-fed, atherosclerosis-prone apoE^{-/-} mouse by use of a strategy of transient blockade of P-selectin by a mAb. Blocking the major P-selectin ligand, PSGL-1, caused a similar reduction in neointima formation. These results build on previous work from our laboratory showing a dramatic reduction in neointima formation after carotid wire injury in the P-selectin-knockout mouse on the apoE^{-/-} background.¹¹ In this series of experiments, we show that similar benefits are achieved with transient blockade of P-selectin before arterial injury by a single injection of antibody against either P-selectin or its ligand, PSGL-1. These data reinforce the important role of early inflammatory events in modulating the response to vascular injury. Our data are consistent with previous studies in P-selectin-knockout mice in limiting both spontaneous atherosclerosis and neointima formation after carotid ligation.^{20–22} However, the present data are the first to demonstrate this benefit in the setting of both hypercholesterolemia and vascular injury. Previous arterial injury studies using pharmacological strategies have not been performed in the setting of hypercholesterolemia

and demonstrate a modest benefit on arterial remodeling without a significant reduction in neointima formation.¹³

After a single IP injection of the P-selectin antibody RB40.34, flow cytometry data demonstrate that the antibody is available in the serum to bind to activated platelets. In addition, we demonstrate that the mAb remains present for ≥ 7 days but is no longer detectable at 14 days after administration. This observation is important in this model for several reasons. After wire injury, the endothelial surface is denuded and covered with platelets.^{8,12} It has been shown that after arterial injury, the endothelium is almost completely regenerated by 3 weeks.⁹ In addition, regenerating endothelium has been shown to actively express inflammatory adhesion molecules.¹⁰ In the setting of endothelial denudation, as occurs in this model, platelets are capable of supporting P-selectin-mediated rolling in the absence of an intact endothelium.^{6,7,12} In addition, platelet-endothelial interactions in mouse venules are also P-selectin/PSGL-1 dependent.^{17,23} Blocking P-selectin prevents not only neutrophil and monocyte rolling on platelets and endothelium but also platelet-endothelial interactions.^{17,24} The blocking antibody thus will block both P-selectin expressed on platelets at the sites of denuded endothelium early after injury and P-selectin expressed on the regenerating endothelium. Our intervention is

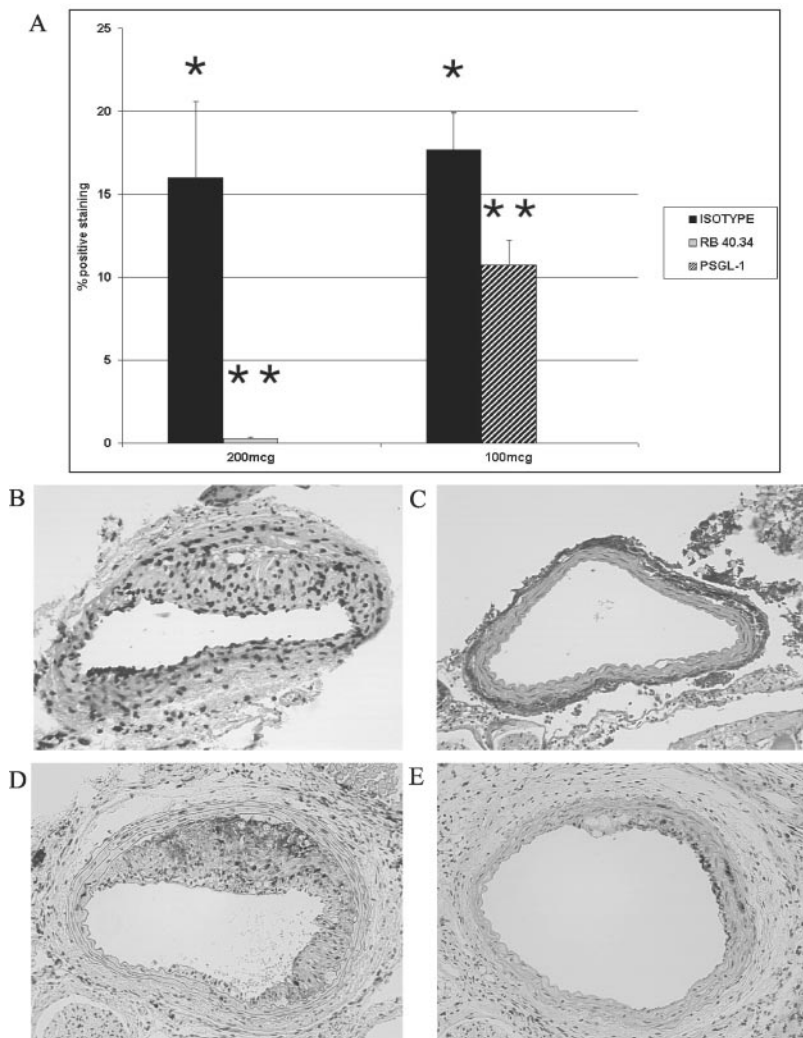


Figure 3. Macrophage content. A, Quantitative immunocytochemistry of macrophage infiltration into media and neointima of injured carotid arteries 4 weeks after denudation and 5 weeks on a Western diet. Note marked reduction in percent area occupied by macrophages in apoE^{-/-} 200- μ g P-selectin antibody group vs isotype control. A significant reduction in macrophage staining was also observed in 100- μ g PSGL-1 antibody-treated mice vs apoE^{-/-} mice given 100 μ g isotype antibody. ** P <0.05. Representative immunostaining for macrophages using F4/80 anti-mouse macrophage mAb in 200- μ g isotype control (B), 200- μ g P-selectin antibody (C), 100- μ g isotype control (D), and 100- μ g PSGL-1 antibody groups, respectively (E). Note positive staining in media and neointima in isotype controls. In 200- μ g P-selectin antibody-treated group, staining is visible only in adventitia. * P =NS for controls across treatment groups.

also likely to limit chemokine deposition by platelets, which in turn will prevent monocyte recruitment.²⁵

The reduction in neointima formation seen in these experiments may result from blockade of P-selectin or PSGL-1 in the initial cascade of neointima formation by preventing monocyte binding to platelets. Direct observations by our group with intravital microscopy have shown that antibody blockade of P-selectin inhibits monocyte rolling and adhesion to the endothelium of known lesion-prone sites near the carotid bifurcation in apoE-null mice.³ Weyrich et al²⁶ reported that platelet adhesion through P-selectin activates monocytes and results in the release of chemokines that may increase the efficiency of rolling and firm adhesion and further promote recruitment of other inflammatory cells. Once activated, macrophages release growth factors and cytokines that stimulate smooth muscle cell migration into the developing lesion, where they undergo phenotypic transformation from contractile to secretory cells.¹ Once in the secretory state, the production of extracellular matrix serves to amplify plaque growth and luminal narrowing and may become the dominant component of atherosclerotic lesions. These complex interactions lead to remodeling of the artery at the site of injury that can become pathological when the

balance shifts from a normal wound-healing event to a chronic inflammatory-fibroproliferative process.²⁷ In this study, we demonstrate a significant correlation between percent macrophage staining and neointimal area in both the treated and control groups, with a dramatic reduction in both macrophage staining and neointimal area in the 200- μ g P-selectin antibody group. Thus, effective early blockade of P-selectin favorably modulates this complex injury response.

In summary, our findings demonstrate prolonged *in vivo* availability of a single bolus of the blocking mAb, RB40.34, to bind to activated platelets. Transient P-selectin inhibition with this blocking mAb or with the PSGL-1 blocking mAb 4RA10 at the time of injury significantly limits neointima formation and macrophage accumulation in a dose-dependent manner after carotid denudation injury at 28 days in the atherosclerosis-prone apoE^{-/-} mouse on a Western diet. These observations, in concert with our earlier findings,¹¹ confirm a pivotal role of P-selectin-mediated inflammatory events in the response to vascular injury.

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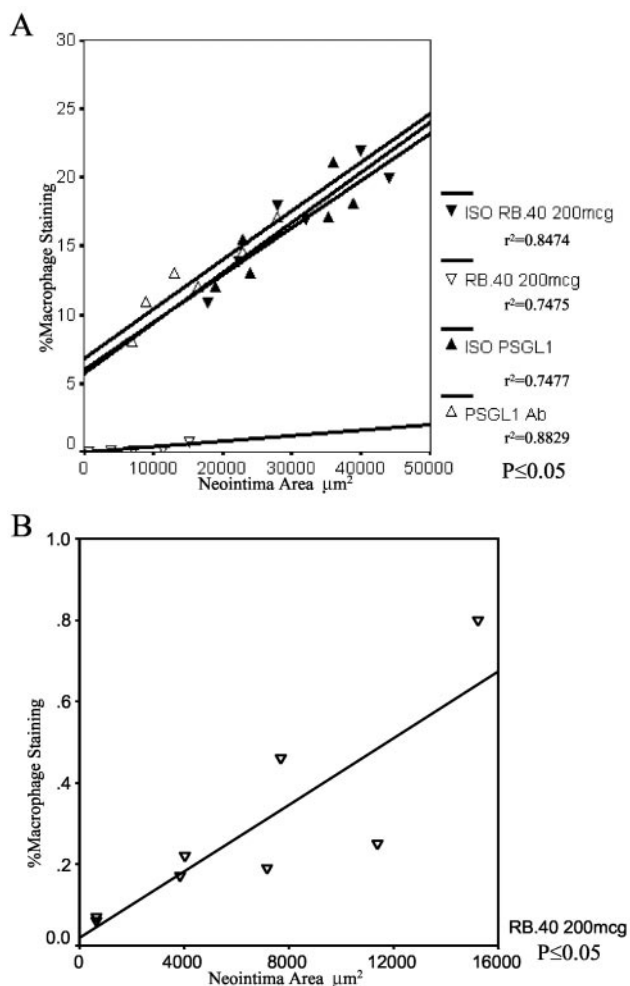


Figure 4. Correlation between percent macrophage staining and neointimal area. Positive correlation of percent macrophage staining vs neointimal area for 100- μ g PSGL-1 antibody, isotype control, and 200- μ g P-selectin antibody-treated groups vs isotype control (A). Note significant correlation between percent macrophage staining and neointimal area in both treated and control groups, with a dramatic reduction in both macrophage staining and neointimal area in 200- μ g P-selectin antibody group ($P \leq 0.05$). Bottom, Correlation for 200- μ g P-selectin antibody group on a different scale to allow representation of all data points (B).

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