Critical Role of Platelet P-Selectin in the Response to Arterial Injury in Apolipoprotein-E–Deficient Mice

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Objective—Mice deficient in apolipoprotein-E ($apoE^{-/-}$) experience severe hypercholesterolemia that is exacerbated by a high-fat Western-type diet and atherosclerotic lesions spontaneously develop. In addition, we have reported that deficiency of P-selectin dramatically protects against neointimal lesion formation after arterial injury in $apoE^{-/-}$ mice. To define the mechanism, bone marrow transplantation (BMT) after lethal irradiation was used to generate $apoE^{-/-}$ chimeric mice deficient in platelet, but not endothelial, P-selectin.

Methods and Results—Mice underwent vascular injury and were euthanized 4 weeks later. Absence of platelet P-selectin (pPS) expression in $apoE^{-/-}$ mice after BMT was confirmed by flow cytometry and Western blot analysis. Lack of pPS in $apoE^{-/-}$ mice resulted in a 62% reduction in neointimal area ($45\,000\pm27\,000$ versus $17\,000\pm13\,000$ μm^2, $P<0.000001$) and a 30% reduction ($P<0.02$) in macrophage infiltration, compared with control $apoE^{-/-}$ BMT. Absence of pPS was also associated with a reduction in plaque neovascularization as compared with pPS-competent controls (0/8 versus 3/8, $P<0.05$).

Conclusions—Lack of pPS significantly attenuates macrophage recruitment and neointimal lesion formation, indicating that pPS on platelets lining the vessel wall plays a critical role in inflammation after wire-withdrawal injury of the carotid artery in $apoE^{-/-}$ mice. **(*Arterioscler Thromb Vasc Biol*. 2004;24:1124-1129.)**

Key words: inflammation ■ atherosclerosis ■ cell adhesion molecules ■ angiogenesis ■ carotid arteries

Initiated by endothelial dysfunction or injury, atherosclerotic neointimal lesions are composed largely of smooth muscle cell, macrophages, and foam cells, and may result in loss of lumen area. Smooth muscle cells are thought to migrate toward and subsequently proliferate at the site of injury.[1] Macrophage entry into the vessel at sites of injury is a hallmark of nascent atherosclerotic lesions and involves a complex interaction of adhesion molecules, chemokines, and cytokines. Similarly, the remodeling of the artery at the site of injury is an inflammatory response that becomes pathogenic when the balance shifts from a normal wound-healing event to a chronic inflammatory fibroproliferative process.[2]

P-selectin, found in the storage granules of resting platelets and endothelial cells, is rapidly translocated on activation to the cell membrane from storage sites in Weibel-Palade bodies (endothelium) and α-granules (platelets) and participates in the initial steps of capture and binding of circulating neutrophils and monocytes during leukocyte rolling.[3,4] After destruction of or damage to the endothelium, P-selectin and integrins expressed on platelets and platelet particles deposited on the provisional matrix at the site of injury may provide a surface for recruitment and subsequent migration of leukocytes, including monocytes, mediating the first steps of injury-induced neointimal hyperplasia.[3–8]

The role of individual adhesion molecules in the response to injury changes depending on the type of arterial insult. For instance, absence of ICAM-1 or P-selectin substantially protects against spontaneous atherosclerosis in the setting of hyperlipidemia alone.[9] Only absence of P-selectin, and not ICAM-1, however, protects against wire-injury–induced neointima formation, a more severe form of endothelial and arterial injury.[10] The lack of protection in ICAM-1–deficient mice after injury demonstrates that the response to wire injury is a complex process in which the role of a given adhesion molecule cannot be predicted based on its role in inflammation or spontaneous atherosclerosis alone.

We previously reported that P-selectin is a crucial mediator of monocyte trafficking into the vessel wall and neointimal lesion formation after wire injury in $apoE^{-/-}$ mice.[10,11] To define the mechanism, we used bone marrow transplantation (BMT) to generate chimeric apoE-deficient mice that lacked platelet, but not endothelial, P-selectin to elucidate the role of cell-type–specific P-selectin expression after arterial injury.

Received February 19, 2004; revision accepted March 25, 2004.

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This study was supported by National Institutes of Health Grants NIH HL-66264 (to I.J.S.), HL-58108 (to K.L.), HL-10447 (to S.B.F.), and the National Science Foundation Grant MCB-9904433 (to S.A.G.).

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Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000127619.04687.f4**

Methods

Mouse Injury Model

The mouse carotid arterial injury model was used with minor modification as described.[10,12] Endothelial denudation has previously been confirmed by scanning electron microscopy.[13] Injured left and uninjured right carotid arteries were excised. Serial 5-μm sections were cut from the paraffin-embedded blocks and prepared for histomorphometry. Wire injury was performed at 20 weeks, before sizeable lesions form spontaneously in the carotid artery, which was verified by analyzing the contralateral, noninjured carotid artery from each animal. In this way, this model is not a combination of spontaneous atherosclerosis and mechanical-injury–induced neointima.

BMT

Recipient mice received ≈1 to 2 million unfractionated donor bone marrow (BM) cells via tail vein injection as described.[14] Sixteen apoE-deficient female mice were lethally irradiated at 13 weeks of age. Eight were reconstituted with BM from a donor apoE/P-selectin$^{-/-}$/$^{+/+}$ female mouse (platelet P-selectin–positive, or pPS+ group) to control for lethal irradiation and BMT, and 8 were reconstituted with donor cells from an apoE/P-selectin$^{-/-}$/ $^{-/-}$ female mouse (platelet P-selectin-negative, or pPS− group). We were unable to perform the complementary set of experiments, namely lethally irradiating and reconstituting apoE/P-selectin$^{-/-}$/ $^{-/-}$ recipients with apoE/P-selectin $^{-/-}$/$^{+/+}$ BM because of breeding difficulties and thus inadequate numbers of double-knockout recipients, a characteristic of apoE/P-selectin$^{-/-}$/ $^{-/-}$ animals, consistent with observations by Dong et al.[15] These experiments would have provided additional controls for the effects of BMT and a lack of endothelial P-selectin alone. At 20 weeks of age, all 16 BMT mice underwent wire withdrawal injury of the left carotid artery after 1 week of Western diet feeding (0.15% by weight cholesterol and 19.5% by weight casein without sodium cholate). All animals were euthanized 4 weeks after injury and after 5 weeks of Western diet feeding at 24 weeks of age.

Flow Cytometry

Platelet-rich plasma (PRP) was prepared from mouse blood centrifuged for 5 minutes at 200*g*. Platelets were stimulated with thrombin and incubated with a fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse P-selectin monoclonal antibody (RB.40.34; BD PharMingen, San Diego, Calif) or an isotype-matched control antibody to detect pPS expression with flow cytometry.

Western Blotting

Platelets were recovered from equal volumes of PRP by centrifugation at 12 000*g* for 10 minutes, lysed in electrophoresis sample buffer, resolved on 7.5% acrylamide gels, and transferred to nitrocellulose membranes as described.[13] P-selectin was detected by enhanced chemiluminescence using antipeptide rabbit antiserum, recognizing the COOH-terminus of P-selectin and horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel ICN, Costa Mesa, Calif) as described.[16]

Complete Blood Counts and Lipoprotein Levels

Blood samples were collected by cardiac puncture at the time of euthanization into EDTA-containing microtainer tubes (for complete blood count, differential) or serum separator tubes (Becton-Dickinson, Franklin Lakes, NJ) for lipoprotein levels. Complete blood counts, automated differential leukocyte counts, and lipid levels were determined by the University of Virginia Clinical Pathology Laboratory.

Quantitative Histopathology and Immunohistochemistry

The arterial segments were dehydrated in ethanol and xylene and embedded in paraffin. Sections (5-μm thick) were stained using the Russell-Movat pentachrome stain.[17] For quantitative histopathological comparisons, beginning at the carotid bifurcation, sections were collected every 55 μm, and every fourth (every 220 μm) section was analyzed. The area and circumference of the lumen, internal elastic lamina, and external elastic lamina, along with maximum neointima thickness, were determined by planimetry using Image Pro Plus 3.0 (Media Cybernetics, Silver Springs, Md). From these measurements, the plaque area, medial area, intima-to-media (I/M) ratio and overall vessel area were calculated. Data were compared using NCSS 97 (Kaysville, Utah) by 1-way analysis of variance (ANOVA) followed by a 2-tailed Student's *t* test or Fisher's exact test for binary response variables to evaluate levels of significance at 95% confidence. Differences were determined to be significant when $P<0.05$.

For immunohistochemistry, serial sections were stained as described with primary antibodies for monocyte/macrophages (Mac-2 antigen Clone M3/38; Accurate Chemical), endothelial cells (PECAM-1 or CD31 goat polyclonal antibody M-20; Santa Cruz Biotechnology), α-smooth muscle actin (clone 1A4; Dako Corp), or platelets (rabbit antimouse thrombocyte antiserum, mouse adsorbed; Accurate Chemical & Scientific Corp, Westbury, NY). Tissue sections were stained with the same polyclonal antibody directed against P-selectin that was used for Western blotting. To assess plaque neovascularization in BMT and nonBMT apoE$^{-/-}$ mice, the section with the largest plaque of 15 sampled sections every 220 μm from the injured carotid artery of each animal was selected for staining with anti-PECAM-1 antibodies to visualize endothelial cells and vascular lumens within the neointima.

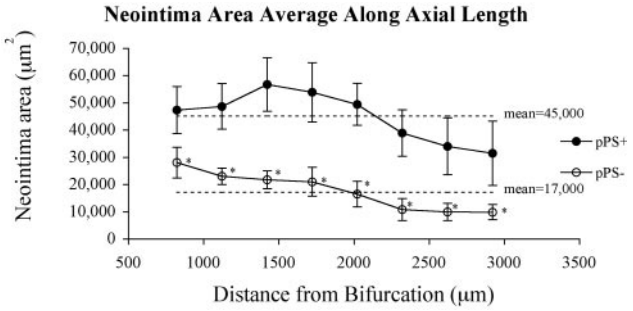

Figure 1. Neointimal lesion formation in the axial direction for each BMT treatment group after wire injury. Beginning 800 μm from the carotid bifurcation (origin), the neointimal area was calculated for each injured vessel. This measurement was repeated every 220 μm until 3000 μm from the carotid bifurcation. Data are represented as the mean neointimal area for 8 injured carotid arteries from each treatment group ±standard error of the mean. *$P<0.05$ compared with pPS+ control group at each position along the axis of the carotid artery. Animals were 24 weeks of age at the time of euthanization.

Results

Reconstitution of lethally irradiated apoE$^{-/-}$ mice with apoE/P-selectin$^{-/-}$/ $^{-/-}$ BM (pPS−) resulted in a complete absence of pPS, confirmed by flow cytometry and Western blot analysis of blood drawn from 3 of these animals (Figure I, available online at http://atvb.ahajournals.org). Lack of pPS resulted in a reduction in wire-injury–induced neointima lesion formation along the length of the carotid for every point examined compared with lethally irradiated apoE$^{-/-}$ control mice reconstituted with apoE/P-selectin$^{-/-}$/$^{+/+}$ BM (pPS+; Figure 1). There was no difference in plasma cholesterol levels between the 2 groups (data not shown). The percent reduction in neointima area varied from 40% to 70% ($P<0.05$) compared with control vessels at each 220-μm point along the axial length of the injured carotid arteries, with an average reduction of 62%; Table). There were no significant differences with respect to complete blood counts,

Neointima Formation After Vascular Injury in Bone Marrow Transplanted ApoE$^{-/-}$ Mice Positive or Negative for Platelet P-Selectin

	pPS+ (control)	pPS−	% Difference *vs* Control
Max NI thickness (μm)	110±70	60±40	−45*
NI area (μm^2)	45 000±27 000	17 000±13 000	−62*
Media area (μm^2)	64 000±32 000	39 000±15 000	−39*
I/M ratio	0.7±0.5	0.5±0.4	−30†
EEL length (μm)	1300±200	1100±100	−15*

Values are mean±1 SD 120 sections per treatment group (15 sections per animal × 8 animals per treatment group, sampled every 220 μm along the axial length of each injured carotid artery).

NI indicates neointima; I/M, intima-to-media area ratio; EEL, external elastic lamina; pPS, platelet P-selectin-positive; pPS−, platelet P-selectin-negative.

*$P<0.001$.

†$P=0.016$

platelet counts, leukocyte counts, total plasma cholesterol levels, or lipoprotein fractionations between the 2 groups (data not shown).

Because of a phenomenon known as the Glagov effect, atherosclerotic vessels remodel outward with increasing plaque burden, which initially tends to preserve the cross-sectional area of the lumen.[18] Accordingly, the overall vessel caliber, measured as the circumference of the external elastic lamina, was larger in the pPS+ group as compared with the pPS− group, which had smaller neointimal areas (1300±200 versus 1100±100 μm, $P=0.016$). Similarly, the average medial area in injured arteries of the pPS+ group was larger than in the pPS− group (64 000±32 000 versus 39 000±15 000 μm^2, $P<0.00001$).

A striking cellular feature of all lesions examined from both the pPS+ (Figure 2) and pPS− (Figure 3) groups was the abundance of large, foamy Mac-2–positive monocyte/macrophages in the neointima cells. The foam cells accounted for all subendothelial cells of the neointima in the pPS− animals, and most neointimal cells of the control pPS+ group. These foam cells have been observed by us in spontaneous atherosclerosis and injury-induced neointimal lesions of apoE$^{-/-}$ mice, but in a lesser proportion.[10,12] Overall, the subendothelial neointimal lesions examined from the pPS− group were composed almost exclusively of foam cells, with only a thin endothelial cell layer. Also absent from the subendothelial neointima in the pPS− group were SMA-positive cells and necrotic zones with a fibrofatty nodule detected in only 1 of 8 animals. These features were previously reported to be ubiquitously present in neointimal lesions in non-BMT apoE$^{-/-}$ mice by our group 4 weeks after carotid wire injury and by other groups in advanced spontaneous atherosclerotic lesions of the innominate artery.[19] Although more complex than the pPS− group, lesions from the pPS+ animals were less diverse, although just as large, compared with those in non-BMT apoE$^{-/-}$ mice previously reported.[10,12] This suggests that BMT after lethal irradiation alone affects the growth and composition of atherosclerotic lesions in apoE$^{-/-}$ mice, confirming a similar observation in low-density lipoprotein receptor knockout mice (LDLr$^{-/-}$).[20]

All sections analyzed from both treatment groups had completely re-endothelialized along the luminal surface (Figures 2 and 3). Lack of pPS resulted in a 30% reduction in medial macrophage infiltration compared with pPS+ animals (60%±30% versus 90%±10%, $P<0.02$, respectively). Areas of medial erosion, defined here as a lack of nuclei and absence of SMA staining (Figures 2 and 3), were also quantified. The percent of the media that was eroded in pPS− animals was 50% less than the pPS+ group (80%±20% versus 40%±30%, $P<0.003$). In the pPS− group, areas of pPS expression along the luminal surface did not colocalize

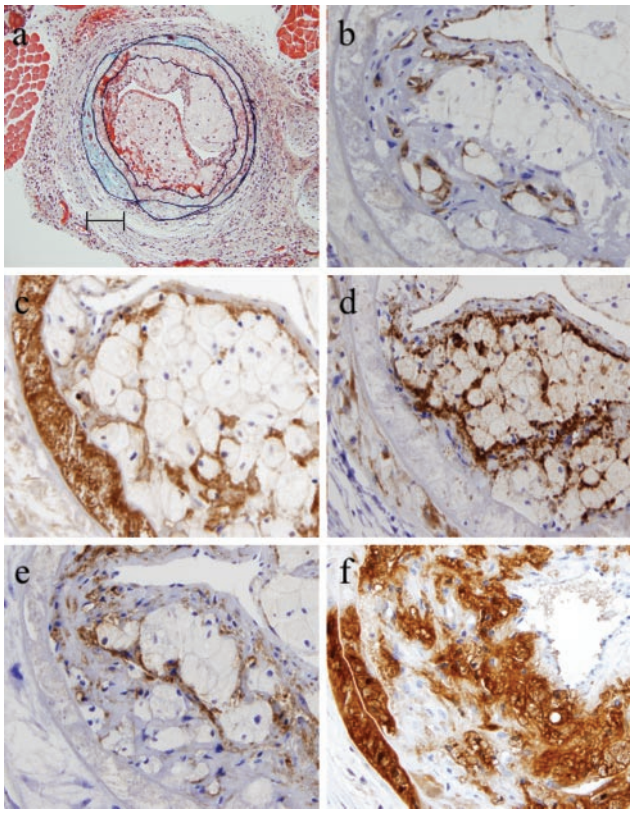

Figure 2. Immunohistochemical staining with Movat pentachrome stain (a) or staining for endothelial cells (b), platelets (c), P-selectin (d), α-smooth muscle actin-positive cells (e), and macrophages (f) of the carotid artery of a lethally irradiated pPS+ control mouse. Note the complex lesion composed of plaque neovessels, large foamy neointimal cells, smaller SMA+ cells, and a cellular cap, as well as extensive medial necrosis and medial macrophage infiltration. 400× original magnification for all but the Movat stain (100×). Bar=100 μm.

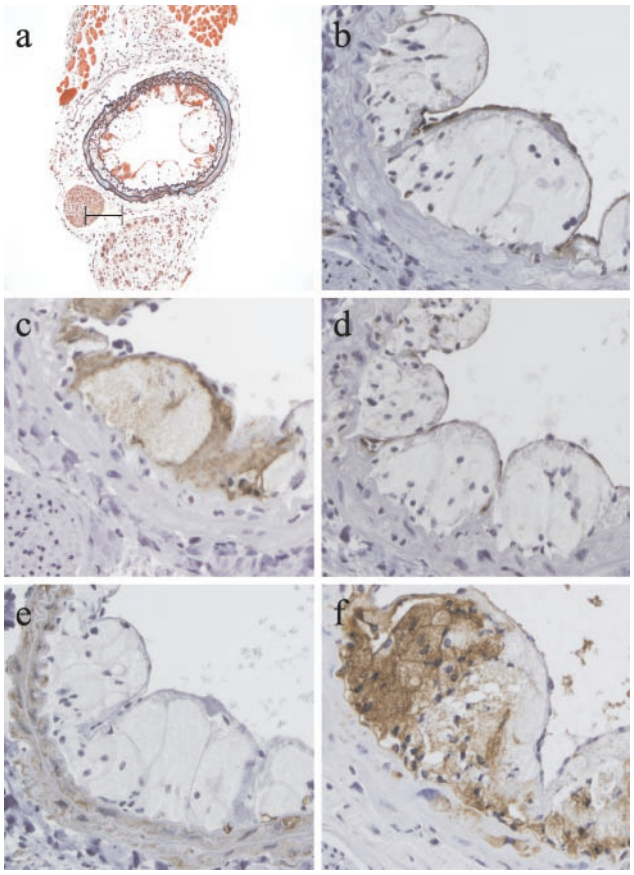

Figure 3. Immunohistochemical staining with Movat pentachrome stain (a), or staining for endothelial cells (b), platelets (c), P-selectin (d), α-smooth muscle actin-positive cells (e), and macrophages (f) of the carotid artery of a lethally irradiated pPS− mouse. Note the simple lesion lacking SMA+ cells and a cellular cap, characterized by large foamy neointimal cells, as well as decreased medial necrosis and medial macrophage infiltration compared with control animals (Figure 2). 400× original magnification for all but the Movat stain (100×). Bar=100 μm.

with positive platelet staining, nor with subendothelial neointimal platelets, consistent with a regenerated endothelium expressing P-selectin (Figure 3). This suggests that regenerated endothelial cells in this model predominantly originate from adjacent intact endothelium and not BM-derived endothelial progenitor cells, although this hypothesis was not specifically tested. In the pPS+ group, pPS and platelet staining often colocalized throughout the neointima and media on serial sections (Figure 2).

The more robust platelet staining in the media of pPS+ versus pPS− animals (Figures 2 and 3) may be caused by differences in heterotypic platelet–monocyte aggregation between the 2 genotypes. Huo et al[21] found that injection of apoE$^{-/-}$ mice with activated wild-type, but not P-selectin–deficient, platelets caused monocytes, decorated with adherent platelets, to disappear from the circulation and adhere to atherosclerotic lesions. In addition, serial injection of apoE$^{-/-}$ mice with activated wild-type platelets over a 12-week period resulted in a 39% increase in the size of atherosclerotic lesions compared with injection with activated P-selectin–deficient platelets. A similar mechanism may be responsible in this study for the increased platelet staining in injury-induced neointimal formation in pPS+ animals, whereby activated pPS+ platelets are carried deep into the media by infiltrating monocytes.

In pPS+ animals, plaque neovessels (INVs) were detected 4 weeks after wire injury in 3 of 8 animals (Figure II, available online at http://atvb.ahajournals.org). No such INVs were detected in the pPS− group. The sections that were INV-positive (INV+) had neointima thicknesses of 160, 160, and 200 μm (mean=170 μm; Figure II). The mean neointima thickness of INV-negative (INV−) pPS+ sections was 140±50 μm, suggesting that neovascularization may be triggered by a large neointimal thickness. INVs were also observed in nonirradiated, non-BMT apoE$^{-/-}$ mice after wire injury with similar frequency (data not shown), suggesting that plaque neovascularization is not a result of lethal irradiation and/or BMT.

None of the sections from pPS− animals was positive for INVs (Figures 3 and IIC). The neointima thickness for the sections examined for INVs ranged from 50 to 160 μm (mean=90 μm), with 3 greater than 100 μm. Among sections examined for the presence of INVs, the average neointima thickness in the pPS− group was 40% less ($P<0.0001$) versus those in the pPS+ group (both INV-positive and INV-negative), but there was no significant difference compared with INV− pPS+ sections ($P=0.06$; Figure II).

All INV− sections (n=13) were 0 to 330 μm (in the axial direction) from an adjacent section with neointima thickness <100 μm. In contrast, INV+ sections (n=3) had a plaque thickness of at least 160 μm and were 550 to 1000 μm from a section with neointima thickness <100 μm. This indicates that the 3-dimensional neointimal mass is an important factor in initiating or supporting plaque neovessel growth. Carotid sections taken from 3 of 8 pPS+ and 1 of 8 pPS− animals contained red blood cells within the plaque, consistent with intraplaque hemorrhage (Figure II). In 2 of 3 pPS+ animals, hemorrhage occurred in close proximity to INVs (385 μm and 770 μm, respectively), suggesting that some of these vessels had ruptured within the plaque.

Discussion

We have previously reported that targeted disruption of the P-selectin gene dramatically attenuates neointimal hyperplasia and macrophage infiltration 4 weeks after injury in apoE$^{-/-}$ mice.[10] More recently, we have demonstrated that transient P-selectin blockade using a single injection of P-selectin or P-selectin glycoprotein ligand-1 monoclonal antibody blocks neointima formation after arterial injury in apolipoprotein E-deficient mice.[11]

Chimeric apoE$^{-/-}$ mice deficient in platelet, but not endothelial, P-selectin were generated by BMT to determine the effect of cell-type–specific absence of P-selectin on neointima lesion formation after arterial injury. Lack of pPS significantly attenuated macrophage infiltration and neointima formation after arterial injury (62% reduction, $P<0.000001$ compared with control BMT mice). This was less than the previously reported 94% reduction in neointima area observed with complete absence of endothelial and pPS.[10] It was also <80% observed by a single injection on the day of injury of 200 μg of the anti-P-selectin monoclonal antibody RB

40.34 and slightly higher than the 55% after a single injection of 100 μg of anti-PSGL-1 monoclonal antibody 4RA10. We believe that this mechanistic finding is important and suggests that P-selectin in the regenerated endothelium also plays an important role in the response to vascular injury.

Our results are in agreement with other groups demonstrating a beneficial effect of P-selectin antagonism[11,22,23] or absence of P-selectin[24] on mechanical injury-induced neointima formation in other animal models. In the former studies, a single bolus of P-selectin antagonist was injected immediately before injury, resulting in 30% to 80% reductions in neointima area 4 weeks later.[11,22,23] It is important to note that in the current study, absence of pPS alone was not as effective as complete P-selectin deficiency on platelets and endothelial cells previously observed.[10] Taken together, these results suggest that early and sustained inhibition of P-selectin is necessary to maximally attenuate inflammation, and the role of P-selectin–mediated monocyte recruitment on regenerated endothelium may be important, especially at later times after injury. Burger and Wagner have also reported that pPS facilitates spontaneous atherosclerosis development in apoE$^{-/-}$ mice. Wild-type recipients receiving P-selectin–deficient platelets had lesions that were 30% smaller than those receiving transplants with wild-type platelets.[25]

Smyth et al observed that platelets were able to adhere to the injured femoral artery wall in P-selectin$^{-/-}$ mice but were severely impaired in their ability to recruit leukocytes.[24] The current study further elucidates the relative contribution of pPS to monocyte recruitment and neointima formation and emphasizes the critical role of P-selectin in wire-injury–induced neointimal lesion formation. Absence of pPS alone significantly reduced monocyte infiltration at 28 days by 30%, associated with a 62% reduction in neointima area. We were unable to perform BMT of apoE$^{-/-}$ BM into apoE$^{-/-}$ pPS$^{-/-}$ animals. Previously, we found that when macrophage infiltration was completely absent in P-selectin$^{-/-}$ mice, neointima formation was reduced by 94%. Our current study further highlights the central role of monocytes in injury-induced neointima formation, in agreement with Rogers et al who found a positive correlation between macrophage infiltration and neointimal growth after mechanical injury.[26] The P-selectin ligand PSGL-1 is also found on neutrophils, however, which also adhere to injured vessel walls.[22,24,27] It is possible, therefore, that the beneficial effect of pPS deletion is caused in part by reduced neutrophil recruitment after injury. Neutrophil adhesion is an early event, however, and we did not specifically study it in these experiments.

Lack of pPS in apoE$^{-/-}$ mice was associated with an absence of plaque neovessels and a decrease in monocyte infiltration. Monocyte/macrophages augment angiogenesis by increasing endothelial cell mitogens and adhesion molecule expression.[28] In the present study, absence of pPS may have prevented the accumulation of a critical mass of macrophages necessary to enable plaque angiogenesis. Lack of pPS also resulted in a significantly reduced neointimal thickness, which would serve to ameliorate intraplaque hypoxia, a potent, if not necessary, stimulus for angiogenesis. It is unlikely that pPS directly stimulates angiogenesis in atherosclerotic lesions.

In summary, these results emphasize the critical role of pPS in wire-injury–induced neointimal lesion formation in apoE-deficient mice. P-selectin expression on platelets adhering to the vessel wall appears to support monocyte recruitment in lieu of an endothelium after arterial injury. As healing progresses, however, the regenerating endothelium participates in P-selectin–mediated recruitment of platelets and monocytes, resulting in neointimal growth. In fact, the beneficial effect of a lack of pPS may be greater earlier in the healing process before re-endothelialization is complete. In addition, absence of pPS was associated with a reduction in plaque neovascularization as compared with pPS-competent controls. Our findings provide additional mechanistic insights into the role of P-selectin in the response to vascular injury and the rationale for considering early and sustained antiinflammatory treatments using P-selectin blockade in the prevention of intimal growth after vascular injury.

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