Absence of P-Selectin, but Not Intercellular Adhesion Molecule-1, Attenuates Neointimal Growth After Arterial Injury in Apolipoprotein E–Deficient Mice

David Manka, MS; Robert G. Collins, PhD; Klaus Ley, MD;
Arthur L. Beaudet, MD; Ian J. Sarembock, MB, ChB, MD

Background—We tested the hypothesis that apolipoprotein (apo)E-deficient (apoE−/−) mice with targeted disruption of the *intercellular adhesion molecule-1* (*ICAM-1*) or *P-selectin* gene (apoE−/− ICAM-1−/− or apoE−/− P-selectin−/− mice, respectively) are protected from neointima formation after arterial injury through inhibition of monocyte trafficking to sites of endothelial denudation.

Methods and Results—ApoE−/−, apoE−/− ICAM-1−/−, or apoE−/− P-selectin−/− mice were fed an atherogenic Western diet for 5 weeks and underwent wire denudation of the left common carotid artery after 1 week of feeding. The absence of P-selectin in apoE−/− mice inhibited neointima formation by 94% ($P<0.0001$) after arterial injury and reduced the intima-to-media ratio compared with the presence of P-selectin in apoE−/− mice. ICAM-1 deficiency did not protect against plaque formation after injury. Large numbers of macrophages were found in the neointima and media of apoE−/− and apoE−/− ICAM-1−/− mice. In contrast, almost no macrophages were found in the media or neointima of injured apoE−/− P-selectin−/− arteries.

Conclusions—These findings demonstrate that the complete absence of P-selectin, but not ICAM-1, markedly reduces plaque area and suggest that P-selectin is critical for monocyte recruitment to sites of neointima formation after arterial injury. **(*Circulation*. 2001;103:1000-1005.)**

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

Neointima formation after arterial injury represents a complex healing process that includes adhesive interactions between platelets, leukocytes, and the vessel wall. Distinct adhesion molecules regulate different stages of leukocyte trafficking in a multistep process at sites of inflammation and atherosclerosis.[1] P-selectin, found in the storage granules of resting platelets and endothelial cells, participates in the initial steps of capture and binding of circulating monocytes.[2] The second step of arrest and firm adhesion of leukocytes is thought to be mediated by members of the immunoglobulin superfamily, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). ICAM-1 is the most robustly expressed adhesion molecule on the endothelium overlying lesion-prone sites, and its expression is regulated by cytokines, shear stress, and hyperlipidemia.[3,4] In the present study, we assessed whether P-selectin or ICAM-1 is required for neointima formation after endothelial denudation in an atherogenic mouse model of arterial injury with targeted disruption of both the *apoE* and either the *P-selectin* or *ICAM-1* genes.

ApoE deficiency in mice fed a normal chow diet results in lipid accumulation in the subendothelial matrix as early as 3 weeks of age that precedes monocyte attachment to the endothelium of the aorta at 5 weeks.[5] After oxidation, intimal lipid may activate the endothelium to increase surface expression of ICAM-1 and recruit inflammatory cells, predominantly monocytes.[4–6] Once in the subendothelial space, monocytes differentiate to macrophages, scavenge lipid, and secrete cytokines, chemokines, and growth factors that result in an amplification of monocyte recruitment into atherosclerotic lesions.[7]

After arterial injury, the subendothelial basement membrane is exposed and supports the adhesion of platelets.[8] Immobilized activated platelets can support P-selectin–mediated rolling,[9,10] suggesting that platelet-monocyte interactions may participate in monocyte recruitment after arterial injury. Induced arterial lesions heal rapidly and demonstrate an almost complete reendothelialization by 3 weeks.[11] Regenerating endothelium is known to have an activated phenotype with expression of inflammatory adhesion molecules.[12] We tested the hypothesis that the absence of either P-selectin or ICAM-1 inhibits macrophage recruitment into the vessel wall and protects against neointima formation after arterial denu-

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From the Departments of Biomedical Engineering (D.M., K.L.) and Medicine (I.J.S.), Cardiovascular Division, University of Virginia Health Sciences Center, Charlottesville, and the Department of Molecular and Human Genetics (R.G.C., A.L.B.), Baylor College of Medicine, Houston, Tex.

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Correspondence to Ian J. Sarembock, MD, Cardiovascular Division, University of Virginia Health System, Box 158, Charlottesville, VA 22908. E-mail ijs4s@virginia.edu

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dation in atherosclerosis-prone apoE-deficient (apoE−/−) mice.

Methods

Animals

The animals used for these experiments were generated in the laboratory of Dr Beaudet (Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Tex) and are recently described.[13] The cellular adhesion molecule (CAM)-deficient mice with targeted disruption of either the *ICAM-1* or *P-selectin* gene were backcrossed onto a C57BL/6 background a minimum of 6 generations (98.5% congenic). These mice were then mated with apoE−/− mice from the Jackson Laboratory, which are in the same C57BL/6 background, and their progeny interbred to generate apoE−/− CAM−/− double-knockout mice (apoE−/− ICAM-1−/− or apoE−/− P-selectin−/− mice for apoE−/− mice with disruption of the *ICAM-1* or *P-selectin* gene, respectively). It should be noted that only 4 apoE−/− P-selectin−/− mice were used in the present study because of the difficulty of breeding these animals. Dong et al[14] recently observed splenomegaly and increased macrophage infiltration to the spleen in apoE−/− P-selectin−/− mice, resulting in higher mortality. The cause for these defects in both studies is unclear.

Mouse Injury Model

The recently described mouse carotid artery injury model of Lindner et al[11] was used with minor modification. The same operator injured all mice over a period of 2 months, and different genotypes were injured on the same day. 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 intraperitoneal injection with a solution composed 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. With use of a midline neck incision, the left external carotid artery was looped proximally and tied off distally with 6-0 silk suture (Ethicon). Additional 6-0 silk ties were looped round the common and internal carotid arteries for temporary vascular control during the procedure. A transverse arteriotomy was made in the left external carotid artery, and a 0.014-in 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 using wire withdrawal injury and 3 passes along the common carotid artery with rotating motion to ensure uniform and complete endothelial denudation. Endothelial denudation was confirmed by scanning electron microscopy.[8] After removal of the wire, the left external carotid artery was tied off, and the skin was closed with 2 suture clips. At the time of euthanasia (28 days), the 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 achieved 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.

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.[15] Histomorphometric analysis was performed by individuals blinded to treatment (injured versus noninjured) and genotype. For quantitative histopathologic comparisons, the mean of 10 sections was taken. The areas of the lumen, internal elastic lamina, and external elastic lamina 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.

Immunocytochemistry

Fifty sections were stained for macrophage/foam cells with the use of an anti-mouse macrophage monoclonal antibody (mAb) F4/80 (Accurate Chemical and Scientific Corp) or for smooth muscle actin (SMA)-positive cells with the use of mAb 1A4 (Dako Corp). For quantitative immunocytochemical comparisons of macrophage content, sections were digitized, and the number of positively stained pixels was counted by use of Image Pro Plus 3.0 (Media Cybernetics) and normalized to either total neointimal or medial area.

Statistical Analysis

Statistical analysis was performed with the use of NCSS 97 (Dr Jerry L. Hintze). Data are reported as the number of carotid arteries in each group, and plaque area and I/M ratio are expressed as mean±SD. Data were compared by 1-way ANOVA and the Student *t* test to evaluate 2-tailed levels of significance.

Results

The most striking result in the present study was the profound inhibition of neointimal growth 28 days after wire injury of the left common carotid artery in mice with mutations of both the *apoE* and *P-selectin* genes that were fed a Western diet (n=4 animals). It should be noted that the extent of vascular wall injury was moderately severe in these animals, with focal disruption of the internal elastica (note bold arrowheads in Figure 1F). Despite the extent of this injury, the absence of P-selectin in apoE−/− mice was dramatically protective against the development of a neointima compared with the presence of P-selectin (P-selectin+/+) in apoE−/− mice (n=11 animals) (plaque area 3000±4000 versus 40 000±10 000 μm^2, respectively, $P<0.0001$; Figure 1). Macrophage infiltration into sites of arterial injury was also markedly attenuated by P-selectin deficiency compared with wild type or ICAM-1 deficiency in an apoE null background (Figure 2). The neointima and also the media of apoE−/− P-selectin−/− mice were rich in cells expressing SMA. The percentage of the area stained by macrophages in the neointima or media of injured carotid arteries was zero in apoE−/− P-selectin−/− mice (Figures 2 and 3). In addition, there was a significant difference in the I/M ratio in the apoE−/− P-selectin−/− mice (0.05±0.07 versus 1.1±0.4 for apoE−/− mice, $P<0.0005$; Figure 3).

In contrast, deficiency of ICAM-1 was not protective against lesion formation after injury in apoE−/− ICAM-1−/− mice (n=12 animals, Figure 1). In apoE−/− ICAM-1−/− mice and apoE−/− ICAM-1–positive (ICAM-1+/+) mice (Figure 3), the plaque area (50 000±20 000 versus 40 000±10 000 μm^2, respectively; $P=0.16$) and also the I/M ratio (0.9±0.05 versus 1.1±0.4, respectively; $P=0.52$) were similar. Macrophage infiltration into injured apoE−/− ICAM-1−/− arteries was also not significantly different compared with apoE−/− ICAM-1+/+ arteries in either the neointima or media (percentage of area stained by macrophages was 17±4% versus 13±15% [$P=0.24$] and 12±6% versus 12±10% [$P=0.48$], respectively). The neointima of both apoE−/− and apoE−/− ICAM-1−/− mice showed significant infiltration of SMA-positive cells, whereas the cells of the vessel wall showed an apparent decrease in SMA expression (Figure 2D and 2F).

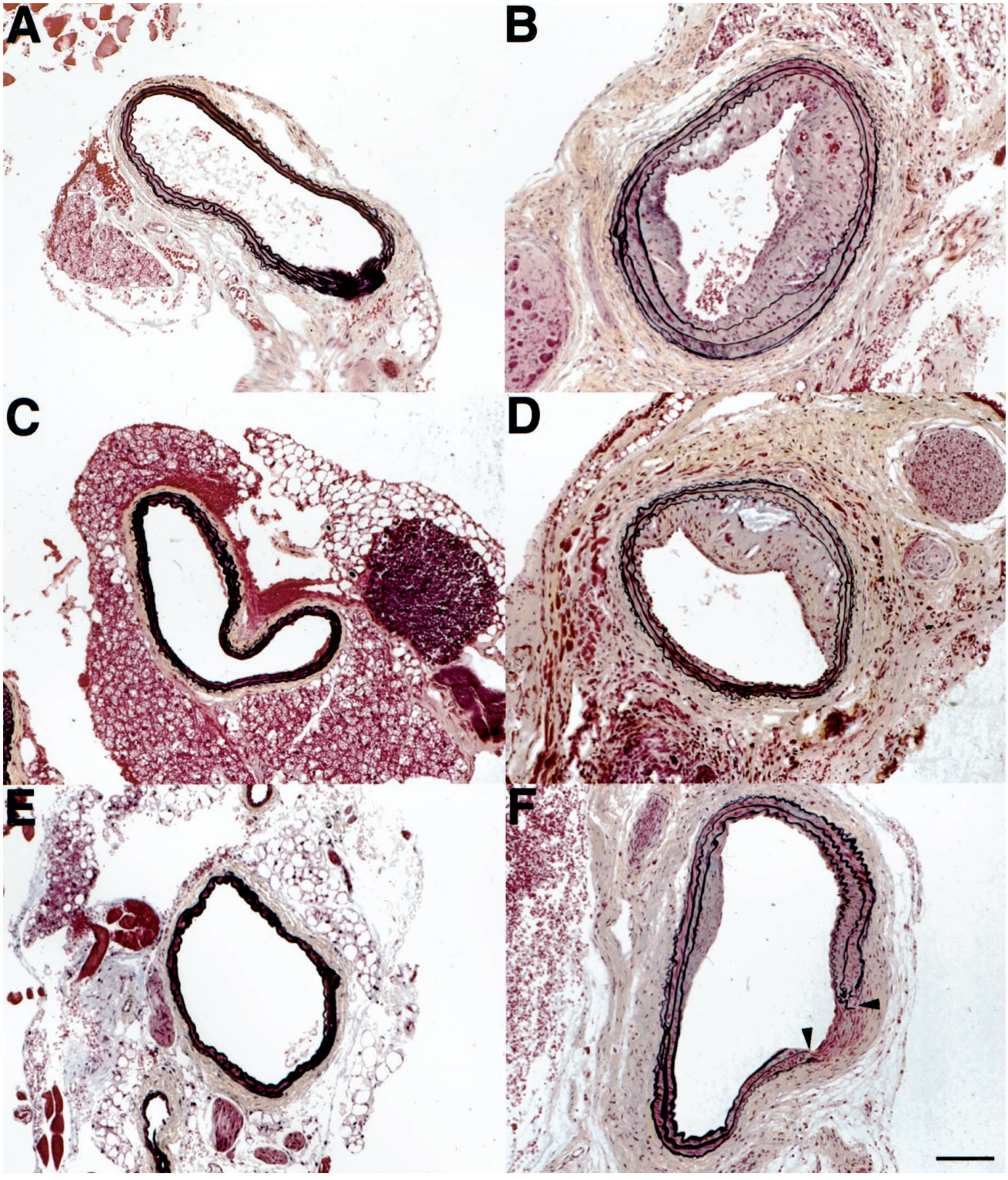

Figure 1. Movat staining of noninjured right carotid artery (RCA) (A, C, and E) and injured left carotid artery (LCA) (B, D, and F) in mice 4 weeks after denudation and 5 weeks on Western diet. A, Noninjured apoE−/− RCA with no plaque growth. B, Injured apoE−/− LCA with robust neointima formation and significant medial thickening. C, Noninjured apoE−/− ICAM-1−/− RCA with minimal plaque formation. D, Injured apoE−/− ICAM-1−/− LCA with significant neointima formation and medial thickening. E, Noninjured apoE−/− P-selectin−/− RCA with no plaque growth. F, Injured apoE−/− P-selectin−/− LCA with minimal plaque formation despite rupture of all 3 elastic laminae (arrowheads). Original magnification ×100. Bar=100 μm.

Discussion

These compelling results (a 94% reduction in plaque size and complete absence of monocytes in the arterial wall) are the first report emphasizing the critical role of P-selectin in intimal hyperplasia and monocyte recruitment after arterial injury. Our data are consistent with earlier studies reporting

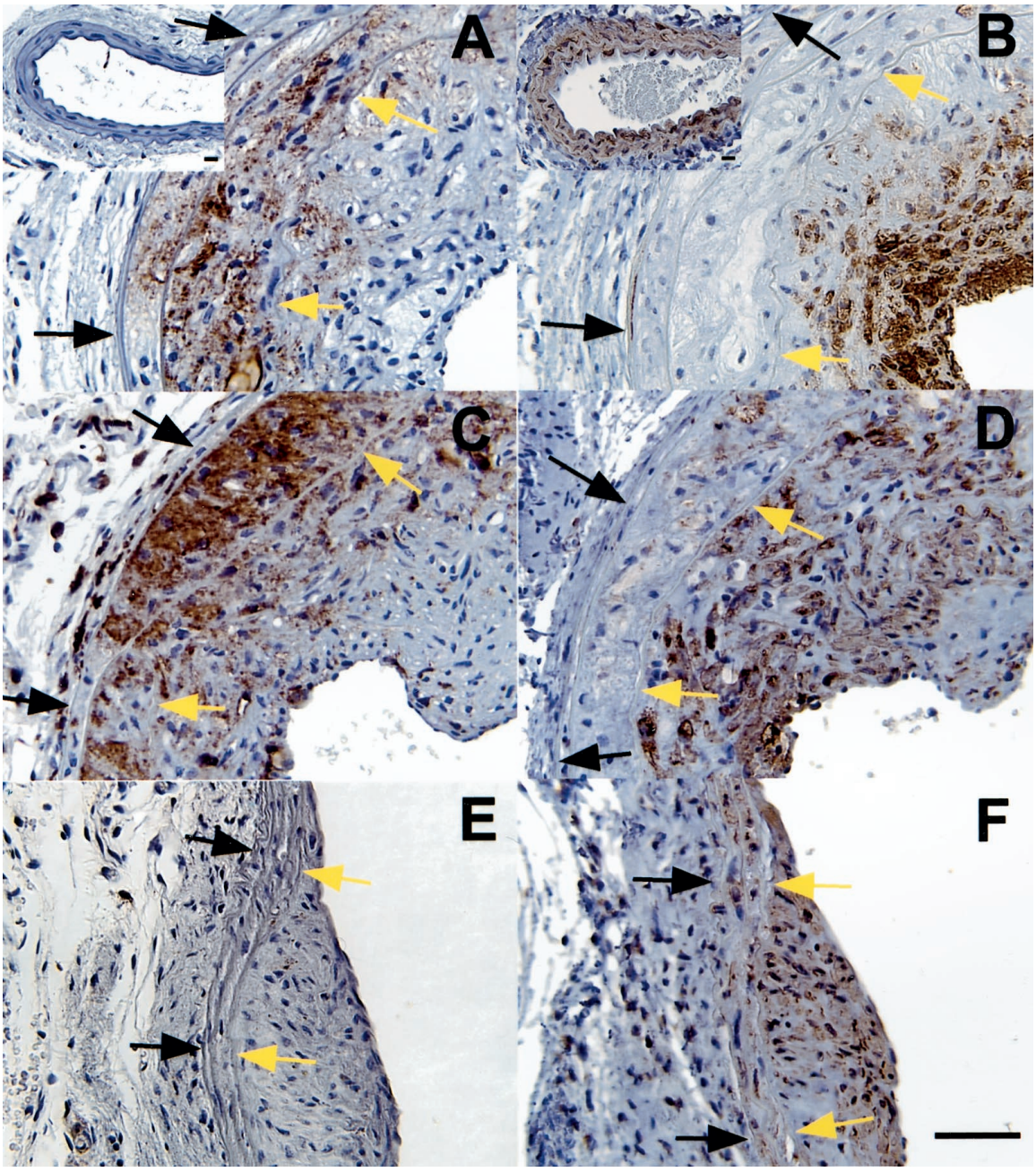

Figure 2. Immunocytochemical staining for macrophages with mAb F4/80 (A, C, and E) and for SMA-positive cells with mAb 1A4 (B, D, and F) of injured left carotid arteries (LCA) in mice 4 weeks after denudation and 5 weeks on Western diet. A, Injured apoE−/− LCA with significant macrophage infiltration in media and neointima. Inset, Noninjured right carotid artery control from same animal with no macrophage infiltration. B, Injured apoE−/− LCA with significant infiltration of SMA-positive cells primarily in neointima but not media. Inset, Noninjured right carotid artery control from same animal with consistent SMA staining throughout media. C, Injured apoE−/− ICAM-1−/− LCA with significant macrophage infiltration into media and neointima, as in panel A. D, Injured apoE−/− ICAM-1−/− LCA with significant number of SMA-positive cells in neointima but only minimal staining in media. E, Injured apoE−/− P-selectin−/− LCA with no macrophage infiltration into either media or neointima. F, Injured apoE−/− P-selectin−/− LCA with consistent SMA staining throughout both media and neointima. Original magnification ×400. Bar=50 μm. Black arrows indicate external elastica, and yellow arrows indicate internal elastica, which define boundaries of media.

on the role of P-selectin in both the spontaneous development of atherosclerosis and neointima formation after carotid ligation.[13,16,17] Our results suggest that the protective effect of the absence of P-selectin on neointima formation after injury is much more dramatic than on the spontaneous progression of atherosclerosis. Similarly, Kumar et al[16] found that the absence of P-selectin reduced neointima formation by 76% in a model of altered flow after common carotid arterial ligation by completely blocking inflammatory cell recruitment to the neointima, media, and adventitia.

Restenosis can be described as accelerated atherosclerosis in response to arterial injury.[18] In agreement with De Geest et al,[19] we found that wire denudation of the left carotid artery in apoE null mice fed a Western diet resulted in robust atherosclerotic plaque

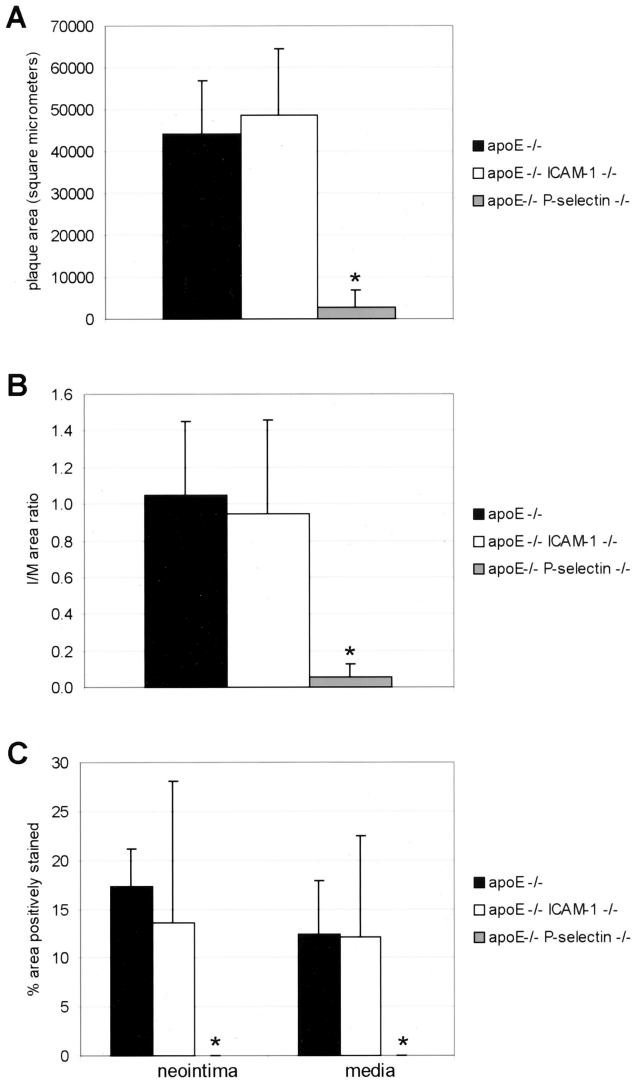

Figure 3. A, Quantitative histomorphometry of plaque area in injured carotid arteries 4 weeks after denudation and 5 weeks on Western diet. Note marked reduction in plaque size in apoE−/− P-selectin−/− group. *$P<0.0001$ vs apoE−/−. B, I/M ratio in injured carotid arteries 4 weeks after denudation and 5 weeks on Western diet. Again, note significant reduction in this ratio in apoE−/− P-selectin−/− animals as a result of small neointima. *$P<0.0005$ vs apoE−/−. C, Quantitative immunocytochemistry of macrophage infiltration into media or neointima of injured carotid arteries 4 weeks after denudation and 5 weeks on Western diet. Note marked reduction in percent area occupied by macrophages in apoE−/− P-selectin−/− animals. *$P<0.0001$ vs apoE−/−.

formation that is similar to that observed months later in noninjured arteries.[20] From this perspective, the model described in the present study may help provide insights into the pathogenesis of the response to arterial injury, including restenosis. However, restenosis occurs in patients that have undergone balloon angioplasty to open preexisting occlusive atherosclerotic lesions in which the lumen narrows again after treatment. An important difference between restenosis in humans and in our model of arterial injury in mice is that the apoE−/− mice used in the present study were too young to have developed spontaneous atherosclerosis in the carotid arteries before wire injury. Despite this, the model can provide insight into the response to arterial injury in an atherosclerotic background that can be genetically manipulated.

Deficiency of either ICAM-1 or P-selectin has been shown to protect against atherosclerosis in mice.[17] In the aortas of apoE null mice, P-selectin deficiency had a greater effect than did ICAM-1 deficiency on the reduction of atherosclerotic plaque size in lesion-prone sites.[13] Our data demonstrate that ICAM-1 deficiency does not protect against neointimal growth after arterial injury, whereas P-selectin deficiency dramatically reduces neointima formation.

Collins et al[13] have previously described the lipid profiles of these apoE−/− ICAM-1−/− and apoE−/− P-selectin−/− mice on a chow diet. The ICAM-1– or P-selectin–deficient apoE−/− mice had severely elevated total cholesterol levels (471±123 and 495±134 mg/dL, respectively) similar to levels in apoE−/− mice (487±152 mg/dL, control), with no differences between genotypes or sexes. No differences were observed in HDL levels (≈40 mg/dL) between genotypes or sexes. This indicates that the beneficial effect of P-selectin disruption on neointimal formation after injury was not due to lower levels of atherogenic lipids.

A possible explanation for the differential effect of deficiency of these adhesion molecules may lie in the distinct cellular localization of ICAM-1 and P-selectin after denudation. ICAM-1 expressed on endothelial and vascular smooth muscle cells may not be accessible to its integrin receptors on monocytes after denudation injury. Fibrin and adherent platelets are deposited on the artery wall within 30 minutes after injury.[8] In lieu of a functional endothelium, platelets express P-selectin and support leukocyte rolling,[9,10] a necessary prerequisite for subsequent leukocyte transmigration into the subendothelial space.[1] Consistent with the paradigm of the leukocyte adhesion cascade, our data suggest that deficiency of P-selectin prevents monocyte infiltration at the site of arterial injury by blocking a critical step in the chronic inflammatory process that results in atherosclerosis. Direct observations have shown that antibody blockade of P-selectin or its leukocyte ligand P-selectin glycoprotein ligand-1 inhibits monocyte rolling and adhesion to the endothelium of a known lesion-prone site near the carotid bifurcation in apoE null mice.[21]

P-selectin–dependent adhesion of platelets has also been shown to activate monocytes and neutrophils. Platelet-dependent activation of monocytes may work synergistically during leukocyte rolling to increase the efficiency of rolling and firm adhesion on a mural thrombus expressing platelet P-selectin, eg, by inducing the secretion of monocyte chemotactic protein-1 and interleukin-8 in monocytes.[22] Specific blockade of P-selectin adhesion with a $F(ab')_2$ molecule that recognizes P-selectin has been shown to impair leukocyte binding to mural thrombi and was associated with decreased fibrin deposition in a baboon arteriovenous shunt model.[23] P-selectin binding of platelets to monocytes has been shown to increase tissue factor expression on monocytes, which eventually leads to the conversion of fibrinogen to fibrin in the blood coagulation cascade.[24] However, this finding is controversial.[22] In a canine model of acute coronary arterial thrombosis, inhibition of P-selectin–dependent adhesive interactions by the tetrasaccharide sialyl Lewis^x or analogues has been shown to inhibit platelet aggregation associated with mural thrombi after endothelial injury.[25,26]

ICAM-1 binding to its integrin receptors on leukocytes containing the CD18 β-chain subunit (Mac-1 or lymphocyte function–associated antigen-1) mediates firm adhesion and

extravasation into the vessel wall of monocytes in the leukocyte adhesion cascade during inflammation, especially to cytokine-activated leukocytes. VCAM-1 on the endothelium has been implicated in atherogenesis and, like ICAM-1, mediates firm adhesion of leukocytes to the vessel wall by binding its integrin ligand on leukocytes, very late antigen-4.[27] Collins et al[13] found that the absence of ICAM-1 protected against spontaneous atherosclerosis in the aortas of apoE-deficient mice (30% reduction in plaque area) that was greater than partial protection by E-selectin deficiency in females and less than the 40% reduction seen in both males and females deficient in P-selectin. We have previously demonstrated that periluminal cells in the media express VCAM-1 in addition to ICAM-1 2 weeks after carotid artery denudation.[8] Inasmuch as blockade of either rolling or firm adhesion severely impairs leukocyte accumulation during inflammation, the participation of both ICAM-1 and VCAM-1 during firm adhesion in atherosclerotic inflammation may explain why the absence of only ICAM-1 fails to protect against neointima formation after arterial injury, whereas the absence of P-selectin is dramatically protective.

There may be a 4-fold effect of P-selectin deficiency on neointima formation in our model. The absence of P-selectin may (1) reduce monocyte recruitment to mural thrombi, (2) reduce monocyte rolling and adhesion to platelet monolayers on a denuded vessel wall, (3) prevent fibrin deposition by adherent leukocytes, and (4) reduce monocyte recruitment on the regenerating endothelium. These effects are not necessarily mutually exclusive and may play a greater role after arterial injury when compared with other models of spontaneous atherosclerosis in which similar protective effects of deficiency in either ICAM-1 or P-selectin on lesion size have been observed. The absence of ICAM-1 alone may not protect against neointima formation in deendothelialized carotid arteries of apoE−/− mice because of the redundancy in firm adhesion by VCAM-1–expressing vessel wall–associated cells.

In conclusion, the present data show that the complete absence of P-selectin protects apoE−/− mice from neointima formation in response to arterial injury. This >90% reduction in lesion size and I/M ratio was associated with a complete absence of macrophages in the vessel wall, suggesting that P-selectin–mediated trafficking of inflammatory cells is a critical component of the response to arterial injury that results in the formation of a neointima. This finding suggests that specific blockade of P-selectin may be a useful intervention aimed at preventing restenosis after balloon angioplasty.

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