Endothelial Cell PECAM-1 Promotes Atherosclerotic Lesions in Areas of Disturbed Flow in ApoE-Deficient Mice

Brian L. Harry, **John M. Sanders**, **Ryan E. Feaver**, **Melissa Lansey**, **Tracy L. Deem**, **Alexander Zarbock**, **Anthony C. Bruce**, **Andrew W. Pryor**, **Bradley D. Gelfand**, **Brett R. Blackman**, **Martin A. Schwartz**, and **Klaus Ley**

Robert M. Berne Cardiovascular Research Center (B.L.H., J.M.S., R.E.F., T.L.D., A.Z., A.C.B., A.W.P., B.D.G., B.R.B., M.A.S., K.L.), Departments of Biomedical Engineering (B.L.H., R.E.F., B.D.G., B.R.B., M.A.S., K.L.), Molecular Physiology and Biophysics (M.L., K.L.), and Microbiology (M.A.S.), University of Virginia, Charlottesville; Department of Anesthesiology and Critical Care Medicine (A.Z.), University of Münster, Germany; and the Division of Inflammation Biology (A.Z., K.L.), La Jolla Institute for Allergy & Immunology, La Jolla, Calif

Abstract

Objective—Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) has recently been shown to form an essential element of a mechanosensory complex that mediates endothelial responses to fluid shear stress. The aim of this study was to determine the in vivo role of PECAM-1 in atherosclerosis.

Methods and Results—We crossed C57BL/6 *Pecam1*$^{-/-}$ mice with apolipoprotein E–deficient (*Apoe*$^{-/-}$) mice. On a Western diet, *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice showed reduced atherosclerotic lesion size compared to *Apoe*$^{-/-}$ mice. Striking differences were observed in the lesser curvature of the aortic arch, an area of disturbed flow, but not in the descending thoracic or abdominal aorta. Vascular cell adhesion molecule-1 (VCAM-1) expression, macrophage infiltration, and endothelial nuclear NF-κB were all reduced in *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice. Bone marrow transplantation suggested that endothelial PECAM-1 is the main determinant of atherosclerosis in the aortic arch, but that hematopoietic PECAM-1 promotes lesions in the abdominal aorta. In vitro data show that siRNA-based knockdown of PECAM-1 attenuates endothelial NF-κB activity and VCAM-1 expression under conditions of atheroprone flow.

Conclusion—These results indicate that endothelial PECAM-1 contributes to atherosclerotic lesion formation in regions of disturbed flow by regulating NF-κB–mediated gene expression.

Keywords

atherosclerosis; shear stress sensing; adhesion molecules; endothelium; macrophages

A therosclerosis is an inflammatory and degenerative disease of arterial walls characterized by monocyte recruitment, foam cell formation, and complex lesions with smooth muscle cell proliferation, a necrotic core, cholesterol crystals, and calcification.[1,2] Apolipoprotein E–deficient (*Apoe*$^{-/-}$) mice on a C57BL/6 background develop atherosclerotic lesions in the aorta and its major branches with a distribution similar to human atherosclerosis.[3] Disease progression can be accelerated by feeding a Western diet (21% fat).[4] Atherosclerosis

Correspondence to Klaus Ley, MD, Division of Inflammation Biology, La Jolla Institute for Allergy & Immunology, 9420 Athena Circle Drive, La Jolla, CA 92037. E-mail klaus@liai.org.

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preferentially develops in regions of disturbed flow (ie, branch points and bifurcations) that are characterized by oscillatory and low time-averaged shear stress.[5] The local hemodynamic environment promotes distinct proatherosclerotic ("atheroprone") or antiatherosclerotic ("atheroprotective") endothelial phenotypes.[6-10]

A minimal complex necessary for the endothelial cell shear stress response requires platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31), vascular endothelial cadherin (VE-cadherin), and vascular endothelial growth factor receptor 2 (VEGFR2).[11] In this cascade, PECAM-1 senses force exerted by blood flowing across endothelial cells, leading to transactivation of endothelial VEGFR2. VEGFR2 triggers conformational activation of integrins followed by stimulation of nuclear factor of kappa light chain gene enhancer in B cells (NF-κB), a transcription factor responsible for expression of inflammatory adhesion molecules, cytokines, and chemokines. Therefore, we hypothesized that the PECAM-1–dependent mechanosensory pathway may be involved in atherogenesis.

PECAM-1 is also expressed on platelets and leukocytes[12] and has been implicated in leukocyte transmigration through endothelial cell monolayers in vitro[13] and in vivo.[14,15] In a model of peritonitis, *Pecam1*$^{-/-}$ mice[16] had no defect in leukocyte transmigration when investigated in the C57BL/6 background,[17] the background used in the present study.

Materials and Methods

Mice

Pecam1$^{-/-}$ mice backcrossed 5 times into the C57BL/6 background (Dr S. Albelda, University of Pennsylvania)[18] were crossed 3 times to C57BL/6 *Apoe*$^{-/-}$ mice. Double heterozygous offspring were used to generate *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice. Nine *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ and 10 *Apoe*$^{-/-}$ mice aged 10 weeks were fed a Western diet for 13 weeks (Harlan Teklad, TD88137). *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice were fertile and healthy under vivarium conditions. Their blood lipid profile was indistinguishable from that of *Apoe*$^{-/-}$ mice (supplemental Table I, available online at http://atvb.ahajournals.org). Anesthesia (16 μL/g, 1 part atropine sulfate 0.4 mg/mL, 1 part xylazine 20 mg/mL, 2 parts ketamine 100 mg/mL, and 16 parts 0.9% NaCl) was injected intraperitoneally before surgeries. Animal experiments and care were approved by the University of Virginia Animal Care and Use Committee according to AAALAC guidelines.

Tissue Acquisition

After carotid artery cannulation, the intact circulation of the mouse was flushed with PBS and perfusion-fixed with 4% paraformaldehyde (PFA) in PBS. The aorta was microdissected, immersed in 4% PFA/PBS for 2 days, cleaned of external fat by blunt dissection, and processed for en face preparation or paraffin embedding and sectioning.

En Face Preparation and Measurement of Atherosclerotic Lesion

Aortas were stained with oil red O and mounted en face.[19] Digital microphotographs of aortas were analyzed for lesion size in specific regions (supplemental Figure I) by finding percent stained surface area using ImageJ (NIH).

Bone Marrow Transplantation

Pecam1$^{-/-}$*Apoe*$^{-/-}$ mice were lethally irradiated and reconstituted with *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ bone marrow (n=3 recipients for each).[20] Mice began a 16-week Western diet 6 weeks after irradiation.

Histopathology and Immunoperoxidase

Paraffin-embedded aortas were sectioned from aortic valve to descending thoracic aorta and stained with the Modified Russell-Movat Pentachrome Method (Armed Forces Institute of Pathology) or antibodies against Mac2 (clone M3/38, Accurate Chemicals),[21] P-selectin (rabbit polyclonal, Dr S. Green, University of Virginia),[22] VCAM-1 (SC-1504, Santa Cruz Biotech), intercellular adhesion molecule-1 (ICAM-1) (ICAM-1, SC-1511), CD3 (SC-1127), or CD20 (SC-7735) (Santa Cruz Biotech).[23,24] Microwave antigen retrieval, Vectastain Elite Kit (Vector Labs), and diaminobenzidine (Dako Corp.) were used for localization of antigens. [23] Sections were counterstained with Harris hematoxylin (Richard-Allen Scientific).

En Face Immunofluorescence

Aortic rings 1 to 2 mm thick, with anatomic location and orientation noted, were each placed in 5 to 10 mL of an antigen retrieval solution (Antigen Unmasking Solution H-3300, Vector Labs) and processed per manufacturer instructions. Rings were permeabilized with 0.2% Triton X-100 for 5 minutes, washed twice with 1% IgG-free BSA solution, and incubated with Alexa-546 –preconjugated (Molecular Probes A20183) antip65 antibody (1 μg/100 μL, mouse monoclonal 3026, Chemicon) in 1% IgG-free BSA at 4°C with TOTO-3 (Molecular Probes). An Alexa-546 –preconjugated IgG3 corresponding to antip65 antibody was used as a control (data not shown). Rings were washed, opened with the endothelium exposed on a glass slide, and mounted with antifading mounting gel (GelMount, Fisher).

Immunofluorescence on Cross-Sections

Paraffin-embedded aortic sections were mounted on glass slides, deparaffinized, rehydrated, processed for antigen retrieval, blocked with 10% goat or donkey serum in PBS/FSGO, and incubated overnight at 4°C with Alexa-546-preconjugated antip65 or anti-VCAM-1 (above), respectively. Donkey antigoat Alexa-546 was added for VCAM-1 preparations. Sections were stained with TOTO-3 and mounted with antifading mounting gel.

Confocal Imaging

Images of en face and paraffin-embedded sections were interrogated for each protein (Nikon C1 confocal microscope). All images were acquired at the same gain, aperture, and exposure settings.

Quantification of NF-κB

Intensity of nuclear NF-κB p65 was assessed by importing confocal images of stained aortic sections into MetaMorph Imaging (Molecular Devices). Average nuclear NF-κB intensity was measured for each TOTO-3-positive nucleus.

Western Blot

Dermal microvascular endothelial cells were harvested and plated for 48 hours in M199, 10% FBS, 1:250 endothelial cell growth supplement (ECGS, Sigma), and heparin (Sigma). Atheroprone waveform[25] was applied by a cone-and-plate viscometer for 16 hours with MCDB-131 (Gibco), 2% FBS, 1:1000 ECGS/heparin, and 4% dextran (Sigma). Samples were collected, run on 10% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane, and blotted for phospho-p65 (rabbit polyclonal 3031, Cell Signaling) and total p65 (rabbit polyclonal 3034).

In Vitro Flow Experiment, siRNA, and Luciferase Reporter Transfection

Passage two human umbilical vein endothelial cells (HUVECs) were isolated and plated at 50% confluence on 1% gelatin in M199 growth media (Biowhitaker), 10% FBS (Gibco), 5

μg/mL ECGS (Biomedical Technologies), 10 μg/mL heparin (Sigma), 2 mmol/L L-glutamine (Gibco), and 100U penicillin/streptomycin (Invitrogen). HUVECs were treated with 330pmol of control (D-001810, Dharmacon) or human PECAM-1 siRNA (L-017029—00) and 19.8 μL oligofectamine (Invitrogen) in 3 mL of OptiMEM-I media for 5 hours. High growth media (20% FBS) was used for 24 hours to grow to confluence. Cells were infected with 5 MOI adenovirus containing NF-κB-luciferase reporter (Vector Labs) for 16 hours. Cells were washed in DPBS and placed in reduced serum media (M199 supplemented with 2% FBS, 5 μg/mL ECGS, 10 μg/mL heparin, 2 mmol/L L-glutamine, 100U penicillin/streptomycin, and 2% dextran by weight to increase viscosity). Atheroprone or atheroprotective shear stress was applied for 24 hours using a cone-and-plate viscometer. Samples were collected in passive lysis buffer (Promega) for measurement of luciferase luminescence or SDS-MAPK sample buffer (Cell Signaling) for Western blot of human PECAM-1 and VCAM-1 (R&D Systems).

Statistics

Data are represented as mean±SEM and analyzed using a 2-tailed heteroscedastic Student t test or nonparametric Wilcoxon–Mann test. $P<0.05$ was considered significant.

Results

En Face Analysis of Atherosclerotic Lesions

We measured atherosclerotic lesion formation in specific aortic regions (supplemental Figure I) from $Pecam1^{-/-}Apoe^{-/-}$ and matched $Apoe^{-/-}$ mice on Western diet for 13 weeks. Oil red O staining revealed robust lesions at branch points of major and minor arteries from both groups and along the lesser curvature of the aortic arch of $Apoe^{-/-}$ mice (Figure 1A). Lesion size was reduced in $Pecam1^{-/-}Apoe^{-/-}$ mice by 26% in whole aortas ($P<0.05$), 28% in aortic arches ($P<0.02$), and 42% in lesser curvatures ($P<0.005$). Differences in the thoracic and abdominal aortas were not significant (Figure 1B). These findings suggest that PECAM-1 promotes atherosclerotic lesion development in a flow-dependent manner.

Bone Marrow Transplantation

To test whether PECAM-1 on endothelial or hematopoietic cells determines atherosclerotic lesion formation, $Pecam1^{-/-}Apoe^{-/-}$ mice were lethally irradiated, reconstituted with either $Apoe^{-/-}$ or $Pecam1^{-/-}Apoe^{-/-}$ bone marrow, and fed a Western diet for 16 weeks. $Pecam1^{-/-}Apoe^{-/-}$ mice reconstituted with $Apoe^{-/-}$ bone marrow developed more than twice as much lesion in the abdominal aorta compared to mice receiving $Pecam1^{-/-}Apoe^{-/-}$ bone marrow (129% increase, $P<0.05$, supplemental Figure II). This suggests that PECAM-1 on leukocytes and platelets promotes atherosclerosis in the abdominal aorta. Differences in the aortic arch were not significant between mice receiving $Apoe^{-/-}$ (22±1%) and $Pecam1^{-/-}Apoe^{-/-}$ bone marrow (25±3%, supplemental Figure II), suggesting that endothelial PECAM-1 determines atherosclerotic lesions in the aortic arch, an area of disturbed flow.

Involvement of NF-κB Activation

To investigate the mechanism by which PECAM-1 promotes atherosclerosis at sites of disturbed flow, we stained aortas en face for NF-κB p65. Endothelial cells lining the lesser curvature of $Apoe^{-/-}$ but not $Pecam1^{-/-}Apoe^{-/-}$ mice demonstrated robust nuclear p65 localization (Figure 2A). This suggests that PECAM-1 absence reduces nuclear NF-κB. We also compared p65-stained aortic arch cross-sections from $Apoe^{-/-}$ and $Pecam1^{-/-}Apoe^{-/-}$ mice (Figure 2B). $Pecam1^{-/-}Apoe^{-/-}$ mice demonstrated a 77% and 59% reduction in NF-κB staining in the lesser and greater curvatures, respectively, compared to $Apoe^{-/-}$ controls ($P<0.01$, Figure 2C).

To determine whether regional flow patterns affect PECAM-1 regulation of NF-κB activity, we cultured human umbilical vein endothelial cells (HUVECs) under atheroprone or atheroprotective flow patterns derived from the human circulation[25] or static conditions. Atheroprone flow increased VCAM-1 expression, which was reduced by siRNA targeting PECAM-1 ($P<0.001$, Figure 3A, supplemental Figure III). NF-κB activity measured using a luciferase reporter increased 3.6-fold in HUVECs treated with control siRNA under atheroprone compared to atheroprotective flow ($P<0.001$, Figure 3B). NF-κB activity under atheroprone flow decreased by nearly 50% after siRNA-based PECAM-1 knockdown to 20 ±13% of control levels ($P<0.001$). siRNA treatment did not alter NF-κB activity under static conditions or atheroprotective flow. PECAM-1 knockdown also attenuated the VCAM-1 protein expression under atheroprone flow ($P<0.001$, supplemental Figure III). Collectively, these data indicate that PECAM-1 is necessary for NF-κB activation and VCAM-1 expression under atheroprone flow.

Cross-Sectional Analysis of Atherosclerotic Lesions

We serially sectioned the entire aortic arch from aortic valve to descending thoracic aorta (Figure 4A). Foam cell-containing and complex lesions were seen in the aortic root of *Apoe*$^{-/-}$ and *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice (supplemental Figure IV). Lesions of the lesser curvature were strikingly absent or diminished in thickness and size in *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice (Figure 4B). VCAM-1 was expressed in atherosclerotic endothelial and smooth muscle cells of *Apoe*$^{-/-}$ mice,[27] but this was visibly reduced in *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice (Figure 4C). *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice expressed VCAM-1 on 19±5% of the endothelial circumference of aortic cross-sections compared to 51±6% in *Apoe*$^{-/-}$ mice (n=13 to 15 cross-sections, 3 mice per group). This difference was significant in the ascending aorta ($P<0.05$) but much less pronounced in the more distal segments of the aortic arch and the descending aorta (data not shown). VCAM-1 is relatively specific for supporting the adhesion of mononuclear leukocytes. Its expression correlates well with the occurrence of atherosclerotic lesions.[31]

Macrophage presence by Mac2 staining was also dramatically reduced in *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice (supplemental Figure V). Foam cells were present on 41±1% of the endothelial circumference of Movat-stained aortic cross-sections in *Apoe*$^{-/-}$ mice compared to 10±1% in *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice ($P<0.00001$, *n*=18 to 19 cross-sections, 3 mice per group). Maximum thickness of foam cell regions reached 116±4 μm in *Apoe*$^{-/-}$ mice, but only 45±3 μm in *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice ($P<0.0003$). Differences in ICAM-1, P-selectin, (supplemental Figure V), T cells (CD3) and B cells (CD20, data not shown) were not observed.

Discussion

Endothelial cells have long been known to respond to steady[32,33] and transient shear stress.[34,35] The latter has been hypothesized to determine the localization of atherosclerotic lesions in vivo.[36] PECAM-1 plays a key role in the flow-dependent mechanotransduction events leading to atherosclerosis,[11] but this was not found in all experimental systems[37] and has not been directly investigated in vivo. Oscillatory or low time-averaged shear stress promotes the development of atherosclerotic lesions, whereas areas with high shear stress are protected.[38] Cheng et al manipulated the shear stress pattern using a circumferential cuff,[38] which also alters local blood pressure and may disturb the adventitia, an area that contains inflammatory cells and participates in the process of atherosclerosis.[24] The data from *Pecam1*$^{-/-}$*Apoe*$^{-/-}$ mice described here suggest that PECAM-1 on the endothelium is critically important for atherosclerotic lesion development in the aortic arch of *Apoe*$^{-/-}$ mice. This may be attributable to the involvement of PECAM-1 in the mechanosensory complex containing PECAM-1–VE-cadherin–VEGFR2.[11,39] Our data suggest that causative factors for atherosclerotic lesion development may vary for different locations in the vasculature.

Several possible explanations may account for residual atherosclerotic lesions in *Pecam1*^{−/−}*Apoe*^{−/−} mice. First, although shear stress–induced phosphatidylinositol-3-OH kinase p85 and AKT phosphorylation were completely absent in *Pecam1*^{−/−} endothelial cells, shear stress applied to *Pecam1*^{−/−} endothelial cells activated $\alpha_V\beta_3$ integrin to a small extent.[11] Second, other unidentified mechanisms in addition to the PECAM-1 pathway may exist for sensing shear stress. Third, rheological factors such as increased residence time of monocytes and inflammatory cytokines at atheroprone sites may promote atherosclerosis.[40,41] Fourth, prolonged permeability of inflamed endothelium in *Pecam1*^{−/−} mice[42] may enhance monocyte recruitment and the action of inflammatory factors. Finally, PECAM-1 may prime the endothelium in response to atheroprone flow, but its impact may diminish over time as other factors contribute.[43]

PECAM-1 regulates transendothelial migration of monocytes[44] and neutrophils.[13] In a model of acute lung inflammation, the importance of PECAM-1 for neutrophil recruitment was demonstrated in vivo.[15] Intravital microscopy showed that PECAM-1–specific antibody hampered leukocyte movement across the basement membrane but not transendothelial migration.[45,46] However, the role of PECAM-1 in transendothelial migration depends on the mouse strain, because C57BL/6 mice showed no discernible defect in two models of inflammation.[17] Because we investigated *Apoe*^{−/−} mice on a C57BL/6 background, it is unlikely that PECAM-1 absence significantly curbed atherosclerosis by inhibiting transmigration.

Bone marrow transplantation experiments performed here demonstrate that PECAM-1 expressed on leukocytes or platelets promotes atherosclerosis in the abdominal aorta, but not the aortic arch, even in the absence of PECAM-1 on the endothelium. This suggests a putative role for either homophilic binding between leukocyte and platelet PECAM-1 via immunoglobulin-like domain 1 or heterophilic binding via immunoglobulin-like domains 1, 2, and 6.[44] As one possible mechanism, elevated very low density lipoprotein leads to increased human endothelial expression of CD38,[47] which may be a PECAM-1 ligand.[48]

The present findings show that the net effect of removing *Pecam1* from the mouse genome is atheroprotective in regions of the aorta exposed to atheroprone flow patterns. We believe dynamic fluid shear stress generates tension between adjacent endothelial cells and activates PECAM-1, which leads to nuclear translocation of NF-κB and expression of inflammatory and adhesive mediators, such as VCAM-1, that promote atherosclerosis. NF-κB regulation by PECAM-1 might depend on the underlying extracellular matrix on which the endothelial cells are sitting. Indeed, endothelial cells on fibronectin-rich, but not collagen-rich, matrices activate NF-κB in response to atheroprone flow.[49]

PECAM-1 may also regulate atherosclerosis by mechanisms not investigated here. *Pecam1*^{−/−} mice have increased bleeding times,[50] and altered hemostatic function may reduce atherosclerosis. Shear stress sensing is also involved in regulating nitric oxide production,[51,52] preventing apoptosis, and maintaining anticoagulant properties, which might be affected following knockout of the *Pecam1* gene.

Polymorphisms in the human *PECAM1* gene correlate with the incidence of coronary atherosclerosis,[53] coronary artery disease,[54] and myocardial infarction.[55] A 53G>A polymorphism in the 5′ untranslated region of *PECAM1* reduced shear stress response in vitro. Compared with 53G homozygotes, carriers of the 53A allele showed less focal progression of disease of coronary atherosclerosis in the LOCAT study and a similar trend in diffuse progression of disease in the REGRESS study.[53] Another study reports that a 373C>G polymorphism, which may affect homophilic interactions of PECAM-1 via a Leu125Val exchange in the first immunoglobulin-like domain,[56] is associated with coronary artery disease

in Asian Indians.[55] Polymorphisms in the sixth immunoglobulin-like domain (Asn563Ser), which is important for heterophilic interactions with integrins,[57] and the cytoplasmic domain (Gly670Arg), which participates in signal transduction,[58] have been recognized as risk factors for myocardial infarction in the Japanese.[55]

In conclusion, the present data identify the PECAM-1–dependent endothelial shear stress response as a key factor in atherosclerotic lesion development in the aortic arch of the *Apoe*$^{-/-}$ mouse, a model that shares many similarities with the human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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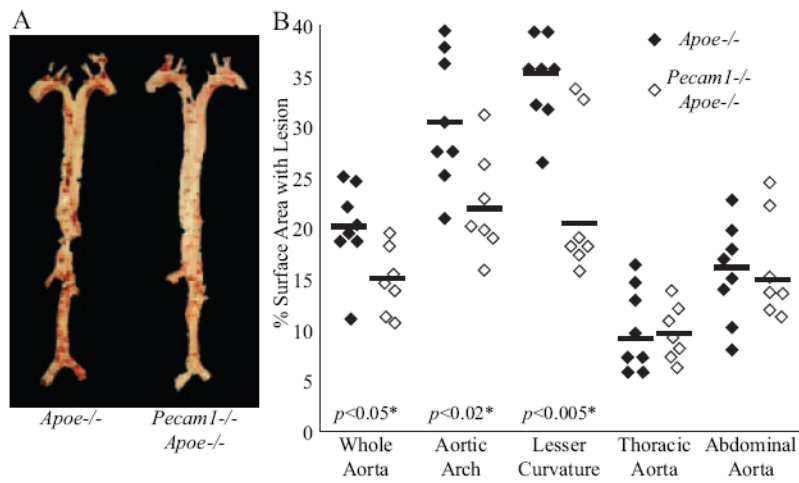
Figure 1.
PECAM-1 promotes atherosclerosis in regions of disturbed flow. A, Representative oil red O–stained aortas from $Apoe^{-/-}$ and $Pecam1^{-/-}Apoe^{-/-}$ mice. B, $Pecam1^{-/-}Apoe^{-/-}$ mice (2 females, 5 males) develop less lesion than $Apoe^{-/-}$ mice (1 female, 7 males) on the whole aorta, aortic arch, and lesser curvature, but not the thoracic and abdominal aorta.
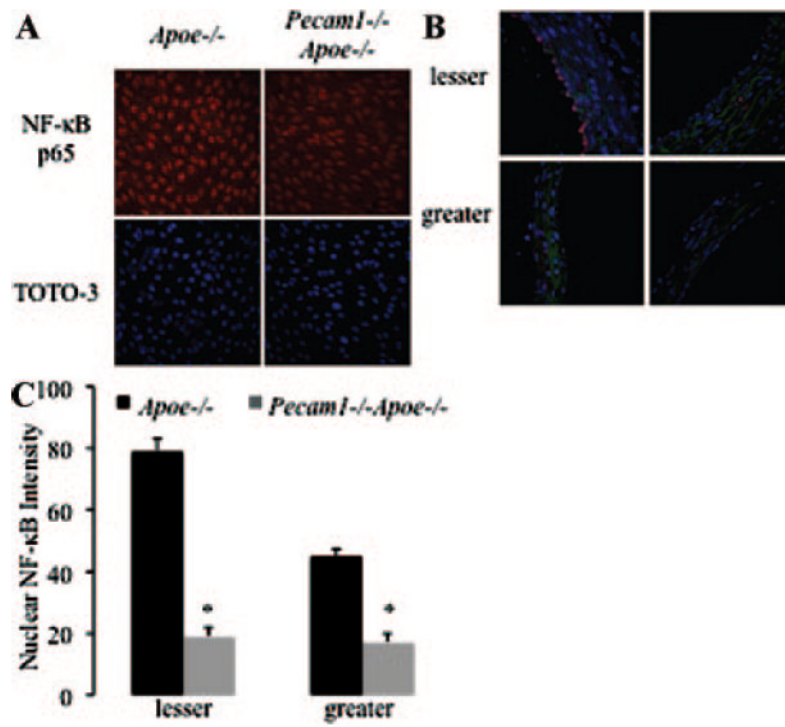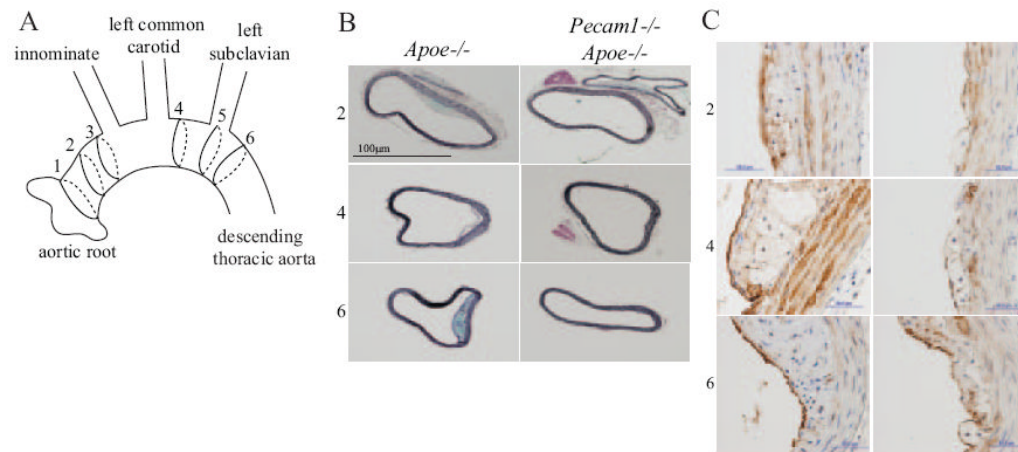
Figure 2.
Nuclear NF-κB translocation is PECAM-1–dependent. A, En face aortic arches stained for NF-κB p65 and TOTO-3 (nuclear stain). B, Aortic arch cross-sections stained for NF-κB p65 and TOTO-3. C, Quantification of nuclear NF-κB staining in the lesser and greater curvatures (n=44 to 84 nuclei, 11 sections, 2 mice per group, *P<0.01).
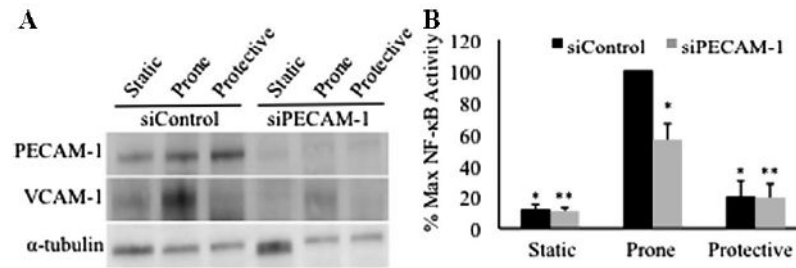
Figure 3.
PECAM-1–dependent activation of NF-κB under atheroprone shear stress. Ad-NF-κ B-Luc–transfected endothelial cells cotransfected with control siRNA (siControl) or siRNA to PECAM-1 (siPECAM-1) were exposed to static conditions, atheroprone ("prone") flow, and atheroprotective ("protective") flow. A, siPECAM-1 attenuates PECAM-1 and VCAM-1 expression. B, NF-κB activity increases under atheroprone flow in the presence of PECAM-1. (*$P<0.001$ vs atheroprone siControl, ** $P<0.001$ vs atheroprone siPECAM-1, n=5).

Figure 4.
PECAM-1–deficient aortas have decreased lesion and VCAM-1. A, Aorta was interrogated at various positions: (1) root, (2–3) proximal to innominate artery, (4) between innominate and left subclavian arteries, (5) at left subclavian artery, (6) descending thoracic aorta. B, Movat stain, greater and lesser curvatures are on the left and right of each image, respectively. C, VCAM-1 protein expression in $Apoe^{-/-}$ (left) and $Pecam1^{-/-}Apoe^{-/-}$ (right) mice.