Site-Specific Effects of PECAM-1 on Atherosclerosis in LDL Receptor–Deficient Mice


Objective—Atherosclerosis is a vascular disease that involves lesion formation at sites of disturbed flow under the influence of genetic and environmental factors. Endothelial expression of adhesion molecules that enable infiltration of immune cells is important for lesion development. Platelet/endothelial cell adhesion molecule-1 (PECAM-1; CD31) is an adhesion and signaling receptor expressed by many cells involved in atherosclerotic lesion development. PECAM-1 transduces signals required for proinflammatory adhesion molecule expression at atherosusceptible sites; thus, it is predicted to be proatherosclerotic. PECAM-1 also inhibits inflammatory responses, on which basis it is predicted to be atheroprotective.

Methods and Results—We evaluated herein the effect of PECAM-1 deficiency on development of atherosclerosis in LDL receptor–deficient mice. We found that PECAM-1 has both proatherosclerotic and atheroprotective effects, but that the former dominate in the inner curvature of the aortic arch whereas the latter dominate in the aortic sinus, branching arteries, and descending aorta. Endothelial cell expression of PECAM-1 was sufficient for its atheroprotective effects in the aortic sinus but not in the descending aorta, where the atheroprotective effects of PECAM-1 also required its expression on bone marrow–derived cells.

Conclusion—We conclude that PECAM-1 influences initiation and progression of atherosclerosis both positively and negatively, and that it does so in a site-specific manner. (Arterioscler Thromb Vasc Biol. 2008;28:1996-2002)

Key Words: PECAM-1 ■ CD31 ■ atherosclerosis ■ LDL-receptor ■ inflammation
effects in LDLR-deficient mice, but that the former dominate in the inner curvature of the aortic arch whereas the latter dominate in the aortic sinus, branching arteries, and descending aorta. On the basis of these findings, we conclude that PECAM-1 influences initiation and progression of atherosclerosis both positively and negatively, and that it does so in a site-specific manner.

Methods

Animals and Diet
Mice were maintained in a facility free of well defined pathogens under the supervision of the Biological Resource Center at the Medical College of Wisconsin (MCW). Animal protocols were approved by the MCW Institutional Animal Care and Use Committee. Mice were housed in groups of 4 per cage, maintained under alternating 12-hour light and dark cycles, and had free access to food and water. LDLR−/−, PECAM−/−, and PECAM−/−/LDLR−/− mice21 backcrossed for more than 10 generations onto a C57BL/6J background, were crossed with LDL receptor−/− mice (Jackson Laboratory, Bar Harbor, Maine). Subsequently, PECAM−/− mice and LDLR−/− mice were backcrossed to the C57BL/6J strain to enrich homozygosity (PECAM−/−/LDLR−/−). Male and female PECAM−/−/LDLR−/− mice gained similar amounts of weight over 24 weeks on the HFD (Table 1).

Plasma Lipid Analyses
Plasma aliquots (100 μL) of blood collected by cardiac puncture from anesthetized mice were stored at −80°C. Levels of total cholesterol, HDL cholesterol, and triglycerides, which were determined in individual aliquots by the clinical laboratory at Children’s Hospital of Wisconsin using Vitros 5.1 fractional shortening (FS) Chemistry System (Ortho-Clinical Diagnostics), did not differ significantly between age- and gender-matched mice. Results are expressed as mean ± SE. No statistically significant differences were found between the groups.

Lesion area was assessed using Adobe Photoshop software and expressed as a percent of the total surface area encompassed by the aortic arch, inner curvature of the arch, thoracic aorta, abdominal aorta, or total aorta as indicated.

Immunohistochemistry
Serial cryostat sections of aortic sinus adjacent to oil red O−stained sections were stained with Sirius Red to detect collagen or antimouse CD68 (1:100, AbD Serotec) and a tyramide amplification plus kit (Perkin Elmer) to identify macrophages. Sections stained with anti-CD68 were counterstained with DAPI to visualize nuclei. Quantification of macrophage and collagen content was determined by computer-assisted image analysis and expressed as percentage of lesion area.

Ex Vivo Micro-CT Imaging and Quantification of Plaque Volume
Mice were maintained in a facility free of well defined pathogens under the supervision of the Biological Resource Center at the Medical College of Wisconsin (MCW). Animal protocols were approved by the MCW Institutional Animal Care and Use Committee. Mice were housed in groups of 4 per cage, maintained under alternating 12-hour light and dark cycles, and had free access to food and water. LDLR−/−, PECAM−/−, and PECAM−/−/LDLR−/− mice21 backcrossed for more than 10 generations onto a C57BL/6J background, were crossed with LDL receptor−/− mice (Jackson Laboratory, Bar Harbor, Maine). Subsequently, PECAM−/− mice and LDLR−/− mice were backcrossed to the C57BL/6J strain to enrich homozygosity (PECAM−/−/LDLR−/−). Male and female PECAM−/−/LDLR−/− mice gained similar amounts of weight over 24 weeks on the HFD (Table 1).

Preparation of Mouse Aortas and Quantification of Atherosclerosis
The heart and aorta of each animal were perfused, dissected out, and subjected to quantification of atherosclerosis as previously described.20,21 Briefly, to quantitate atherosclerosis in the aortic sinus, branching arteries, and descending aorta, the heart was embedded in optimal cutting temperature (OCT) medium and frozen, after which serial sections (10 μm) were taken from the aortic sinus and valve region.22 Images were obtained of the sections after staining with oil red O (neutral lipid; counterstaining with hematoxylin), and lesion area was quantified using SPOT image analysis software (Diagnostic Instruments). The percent lesion area in each section was calculated as follows: lesion area/total area surrounded by aortic wall × 100. The mean percent atherosclerotic lesion area for each animal was determined by averaging 4 to 6 sections from each mouse, using 60 to 80 μm intervals between sections. To quantify atherosclerosis in the aorta by the en face method, the entire aortic tree was dissected free of fat and other tissue. The aorta was stained with oil red O and digitally scanned.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pecam−1+/− Idlr−/− Male</th>
<th>Pecam−1+/− Idlr−/− Female</th>
<th>Pecam−1−/− Idlr−/− Male</th>
<th>Pecam−1−/− Idlr−/− Female</th>
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<tbody>
<tr>
<td>Cholesterol, mg/dL</td>
<td>1335±280 (n=6)</td>
<td>1605±167 (n=8)</td>
<td>1048±120 (n=9)</td>
<td>1291±152 (n=9)</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>302±97 (n=5)</td>
<td>354±62 (n=8)</td>
<td>120±25 (n=9)</td>
<td>184±19 (n=9)</td>
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<tr>
<td>HDL Cholesterol, mg/dL</td>
<td>335±36 (n=7)</td>
<td>374±29 (n=7)</td>
<td>340±25 (n=9)</td>
<td>367±16 (n=9)</td>
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<tr>
<td>Body Weight, g</td>
<td>37±2.8 (n=10)</td>
<td>44±3.8 (n=8)</td>
<td>33±1.6 (n=14)</td>
<td>34±1.7 (n=11)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. No statistically significant differences were found between the groups.
Results

PECAM-1 Deficiency Renders LDLR-Deficient Mice More Susceptible to Development of Atherosclerotic Lesions in the Aortic Sinus

To determine whether the mechanosensory or antiinflammatory role of PECAM-1 dominates in atherosclerosis, we tested the effect of PECAM-1 deficiency on development and progression of atherosclerosis in LDL receptor–deficient mice.\(^{28}\) We first evaluated the time course over which atherosclerotic lesions develop in the aortic sinus (Figure 1A and 1B). Both pecam-1\(^{-/-}\)ldlr\(^{-/-}\) and pecam-1\(^{-/-}\)ldlr\(^{-/-}\) mice developed similarly small atherosclerotic lesions in the aortic sinus after 8 weeks on the HFD (fractional lesion areas [FLA] \(-4.6\pm0.7\% \ [n=7] \) and \(3.7\pm0.5\% \ [n=8], \) respectively). However, after 16 weeks on the HFD, atherosclerotic lesions in the aortic sinuses of pecam-1\(^{-/-}\)ldlr\(^{-/-}\) mice were significantly larger than those in pecam-1\(^{-/-}\)ldlr\(^{-/-}\) mice (FLA \(-36.4\pm2.0\% \ [n=8] \) and \(25.9\pm1.7\% \ [n=8], \) respectively) and this difference persisted through 24 weeks on the HFD (FLA \(-41.8\pm1.2\% \ [n=14] \) and \(36.3\pm1.6\% \ [n=20], \) respectively). Differences in aortic sinus lesion areas between pecam-1\(^{-/-}\)ldlr\(^{-/-}\) and pecam-1\(^{-/-}\)ldlr\(^{-/-}\) mice were statistically significant when both genders were evaluated together and when each gender was evaluated separately (data not shown).

The composition of atherosclerotic lesions, along with lesion size, is critically important in atherogenesis, cell and matrix components of the lesions were also characterized by analyzing macrophage and collagen content, respectively. After 24 weeks on the HFD, macrophage content in atherosclerotic lesions of the aortic sinus was significantly greater in pecam-1\(^{-/-}\)ldlr\(^{-/-}\) mice than in pecam-1\(^{-/-}\)ldlr\(^{-/-}\) mice (Figure 1C and 1D), whereas collagen deposition was similar between the 2 groups of mice (Figure 1E and 1F). These results indicate that PECAM-1 inhibits development of, and macrophage accumulation in, atherosclerotic lesions in the aortic sinus of LDLR-deficient mice.

PECAM-1 Deficiency Renders LDLR-Deficient Mice More Susceptible to Development of Atherosclerotic Lesions in the Descending Aorta

We next assessed the effect of PECAM-1 deficiency on development of atherosclerotic lesions in the aorta of LDLR-deficient mice by en face staining with oil red O. We found that lesion area in the aortic arch as a whole did not differ significantly between pecam-1\(^{-/-}\)ldlr\(^{-/-}\) and pecam-1\(^{-/-}\)ldlr\(^{-/-}\) mice after 24 weeks on the HFD (Figure 2). However, lesion areas in both the thoracic and abdominal aorta, and thus the

Figure 1. PECAM-1 suppresses atherosclerotic lesion development in the aortic sinuses of LDLR-deficient mice. A, Each symbol represents the mean percent atherosclerotic lesion area calculated from 4 to 6 oil red O–stained sections spanning the aortic sinus of an individual pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (filled squares) or pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (open circles) mouse maintained on a HFD for the indicated time. Horizontal lines indicate mean percent lesion area for each group of animals. Asterisks denote statistically significant differences between groups (**P<0.01, ***P<0.001). B, Representative oil red O–stained aortic sinus sections from pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (top) and pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (bottom) mice fed a HFD for 24 weeks. C, Quantitative analysis of macrophage content in aortic sinus lesions of pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (filled bars) or pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (open bars) mice fed a HFD for 24 weeks. Results are expressed as the mean percent of atherosclerotic lesion area occupied by CD68\(^{+}\) macrophages ±SE. Asterisks denote statistically significant differences between groups (**P<0.01). D, Representative anti-CD68–stained aortic sinus sections from pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (top) and pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (bottom) mice. E, Quantitative analysis of collagen deposition in aortic sinus lesions of pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (filled bars) or pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (top) and pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (bottom) mice. F, Representative Sirius Red–stained aortic sinus sections from pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (top) and pecam-1\(^{-/-}\)ldlr\(^{-/-}\) (bottom) mice.
Micro-CT has been recently used to visualize the powerful imaging technique for visualization of small specimens in the aortic arch in more detail using another method. We used microcomputed tomography (micro-CT), which is a cated aortic section, including the aortic arch, thoracic aorta, abdominal aorta, and total aorta (aortic arch + thoracic aorta + abdominal aorta), for each group of animals. Asterisks denote statistically significant differences between groups (***P<0.001).

PECAM-1 and Atherosclerosis

PECAM-1 Affects Atherosclerotic Lesion Development in the Aortic Arch in a Site-Specific Manner

We observed a distinct pattern of plaque development in aortic arches of pecam-1<sup>−/−</sup>/ldlr<sup>−/−</sup> relative to pecam-1<sup>+/−</sup>/ldlr<sup>−/−</sup> mice by en face staining with oil red O. The pecam-1<sup>−/−</sup>/ldlr<sup>−/−</sup> mice had significantly larger lesions along the inner curvature of the arch but smaller lesions in branching arteries compared to the pecam-1<sup>+/−</sup>/ldlr<sup>−/−</sup> mice (Figure 3A and 3B). We therefore decided to evaluate the effect of PECAM-1 deficiency on development of atherosclerotic lesions in the aortic arch in more detail using another method. We used microcomputed tomography (micro-CT), which is a powerful imaging technique for visualization of small specimens. Micro-CT has been recently used to visualize the artery wall and early atherosclerotic lesions both with and without OT as a contrast agent. Micro-CT images revealed striking differences in plaque localization in pecam-1<sup>−/−</sup>/ldlr<sup>−/−</sup> relative to pecam-1<sup>+/−</sup>/ldlr<sup>−/−</sup> mice. Specifically, in pecam-1<sup>−/−</sup>/ldlr<sup>−/−</sup> mice, lesions were less extensive in the aortic branches and more extensive along the inner curvature of the aortic arch (Figure 3C). In contrast, pecam-1<sup>−/−</sup>/ldlr<sup>−/−</sup> mice developed substantial atherosclerosis in branching arteries but few lesions along the inner curvature of the arch (Figure 3). By visualizing the specimen in 3 dimensions, it is apparent that plaque was extensively distributed along the inner curvature of the arch and only minimally present along the lateral wall of the innominate artery (IA) and left common carotid artery (LCCA) of pecam-1<sup>−/−</sup>/ldlr<sup>−/−</sup> mice (supplemental Figure II), whereas plaque was broadly distributed along the lateral walls of all 3 branching arteries and restricted to small patches along the inner curvature of the arch in pecam-1<sup>−/−</sup>/ldlr<sup>−/−</sup> mice (supplemental Figure III). We also used OT in micro-CT to quantify plaque volume over defined areas of vessel wall within discrete regions of the aortic arch. We found that pecam-1<sup>−/−</sup>/ldlr<sup>−/−</sup> mice had sig-
significantly more plaque volume than did pecam-1+/−ldlr−/− mice along the inner curvature of the arch; however, this was offset by the presence of significantly greater plaque volume in the branching arteries of pecam-1+/−ldlr−/− relative to pecam-1+/−ldlr−/− mice, such that plaque burden in the total aortic arch as a whole was similar in pecam-1+/−ldlr−/− and pecam-1+−ldlr−/− mice (Figure 3D). Overall, these results indicate that PECAM-1 expression in LDLR-deficient mice is proatherosclerotic along the inner curvature of the aortic arch but atheroprotective in aortic branches.

**Endothelial Cell PECAM-1 Expression Protects Against Development of Atherosclerosis in the Aortic Sinuses of LDLR-Deficient Mice**

PECAM-1 is expressed on both endothelial cells and bone marrow–derived leukocytes and platelets, and is capable of transducing signals that affect the function of cells from both of these compartments.30 It was therefore important to determine whether the effects of PECAM-1 on atherosclerosis required its expression on endothelial cells, bone marrow–derived cells, or both. We created bone marrow chimeric ldlr−/− mice that selectively expressed PECAM-1 either on endothelium or bone marrow–derived cells (supplemental Figure I), and measured atherosclerotic lesion sizes after feeding these mice a HFD for 24 weeks. Unfortunately, the bone marrow transplantation experiments did not recapitulate decreased lesion formation in the lesser curvature of the arch in pecam-1−/− mice. The lesion area along the inner curvature of the arch of ldlr−/− mice was comparable in pecam-1+/− recipients of pecam-1−/− marrow and pecam-1−/− recipients of pecam-1+/− marrow (supplemental Figure IV); therefore, we could not use bone marrow chimeric mice to determine whether endothelial or bone marrow–derived leukocyte or platelet expression of PECAM-1 enhances lesion development in this region. However, like untransplanted pecam-1−/−ldlr−/− relative to pecam-1+/−ldlr−/− mice, pecam-1−/− recipients of pecam-1−/− bone marrow developed larger lesions in the aortic sinus (Figure 4A) and total aorta (Figure 4B) relative to pecam-1+/− recipients of pecam-1+/− marrow. Thus, bone marrow chimeric mice could be used to determine the effects of endothelial versus hematopoietic cell expression of PECAM-1 on lesion development in the aortic sinus and total aorta of ldlr−/− mice.

In the aortic sinus, lesions that developed in pecam-1+/− recipients of pecam-1−/− marrow were similar in size to those of pecam-1+/− recipients of pecam-1+/− marrow, and signifi-
cantly smaller than the lesions that developed in pecam-1−/− recipients of pecam-1−/− marrow (Figure 4A). Also, lesions that developed in the aortic sinuses of pecam-1+/− recipients of pecam-1+/− marrow were similar in size to those of pecam-1−/− recipients of pecam-1−/− marrow and significantly larger than the lesions that developed in pecam-1−/− recipients of pecam-1+/− marrow (Figure 4A). These results indicate that endothelial cell expression of PECAM-1 inhibits development of atherosclerotic lesions in the aortic sinuses of ldlr−/− mice.

Interestingly, mice that were missing PECAM-1 from either endothelial cells, bone marrow–derived leukocytes or platelets, or both exhibited a trend toward increased lesion sizes in the thoracic and abdominal aorta relative to mice that expressed PECAM-1 on both endothelial and bone marrow–derived cells; however, these differences did not reach statistical significance until lesion sizes in the total aorta were compared (Figure 4B). These results indicate that expression of PECAM-1 on both endothelial and bone marrow–derived leukocytes or platelets is required for PECAM-1 to inhibit development of atherosclerotic lesions in the aortas of ldlr−/− mice.
Discussion
The major finding of this study is that PECAM-1 expression affects development of atherosclerosis differently at different lesion-prone sites of the vasculature. Specifically, PECAM-1 is proatherosclerotic in the inner curvature of the aortic arch, but atheroprotective in the aortic sinus, branching arteries, and descending aorta. The atheroprotective effect of PECAM-1 in the aortic sinus requires PECAM-1 expression only on endothelial cells, whereas PECAM-1 expression on both endothelial cells and bone marrow-derived cells is required for its atheroprotective effects in the descending aorta.

The proatherosclerotic effect of PECAM-1 in the inner curvature of the arch is consistent with its role as part of a mechanostimulatory complex on endothelial cells that activates NF-κB in response to low shear stress and induces expression of adhesion molecules that enable recruitment of inflammatory cells into the lesion. A mechanostimulatory function for PECAM-1 is supported by the findings in many, but not all, studies that PECAM-1 facilitates responses of cultured endothelial cells to osmotic and fluid shear stresses. Previous studies have established that PECAM-1 is rapidly phosphorylated on cytoplasmic tyrosine residues in cultured endothelial cells exposed to fluid shear or osmotic stress; however, whether PECAM-1 tyrosine phosphorylation is required for its mechanostimulatory function is not yet known.

The atheroprotective effect of PECAM-1 in the aortic sinus, branching arteries, and descending aorta indicates that PECAM-1 normally inhibits development of atherosclerosis in these regions of the vasculature. Our studies of bone marrow chimeric mice revealed that the cells on which PECAM-1 must be expressed to inhibit lesion development vary by vascular region. Specifically, in both the aortic sinus and descending aorta, the atheroprotective effect of PECAM-1 required its expression on endothelial cells; in the aortic sinus, PECAM-1 expression on endothelial cells alone was sufficient for its inhibitory function. Two functions of the endothelium that impact lesion development in atherosusceptible regions include maintenance of the vascular permeability barrier and insurance of nitric oxide (NO) bioavailability. PECAM-1 has been shown to support maintenance of vascular integrity in at least 4 different models of inflammation, including intradecal injection of histamine, autoimmune encephalomyelitis, collagen-induced arthritis, and lipopolysaccharide (LPS)-induced endotoxemia. PECAM-1 deficiency has also been shown to affect NO bioavailability, either as a consequence of decreased production of NO or increased production of reactive oxygen species. Thus, either increased vascular permeability or decreased NO bioavailability could contribute to the increased atherosclerosis observed in the aortic sinuses and descending aortas of mice with PECAM-1−/− deficient relative to PECAM-1+/− mice.

The inhibitory effect of PECAM-1 on lesion development in the aorta as a whole, in contrast, required its expression not only on endothelial cells but also on hematopoietic cells. The hematopoietic cells thought to play crucial roles in atherosclerotic lesion development include monocytes, T lymphocytes, and platelets. There is ample evidence that PECAM-1 inhibits platelet responsiveness, and PECAM-1 is also capable of interfering with both macrophage-mediated phagocytosis of viable cells and T cell receptor–mediated signaling pathways. Indeed, loss of PECAM-1 from circulating T cells correlated with occurrence of atherothrombotic plaque complications in humans and mice. Furthermore, in vivo administration of PECAM-1/IgG fusion proteins reduced lesion sizes in atherosusceptible mice coincident with blunted T cell activation, increased numbers of circulating regulatory T cells, and decreased infiltration of T cells into accumulating plaque. Collectively, these studies support the conclusion that interactions between PECAM-1–expressing endothelial cells and either platelets, monocytes, or T cells may decrease atherosclerosis in aortas of PECAM-1−/− relative to PECAM-1+/− mice. Studies in which the PECAM-1 gene is knocked out in specific types of hematopoietic cells are needed to determine the extent to which PECAM-1 expression by any one of these cell types normally interferes with development of atherosclerosis.

Finally, our findings indicate that PECAM-1 has both proatherosclerotic and atheroprotective effects on the vasculature; however, each of these opposing effects dominates in a different region of the vasculature. Thus, the inner curvature of the arch is more strongly influenced by the mechanostimulatory and therefore proatherosclerotic function of PECAM-1, whereas other atherosusceptible regions of the vasculature are more strongly influenced by its antiinflammatory and therefore atheroprotective effects. A possible explanation for the differential sensitivity of these regions to the mechanosensory versus antiinflammatory roles of PECAM-1 is that PECAM-1 might influence the type or magnitude of hemodynamic shear stress to which different regions of the vasculature are exposed, which can be addressed by comparing the hemodynamic properties of PECAM-1−/− versus PECAM-1+/− aortas. Alternatively, PECAM-1 might contribute in different ways to the responses of cells in different regions of the vasculature, even if they are exposed to the same shear stresses. This possibility is consistent with the concept that site-specific responses to systemic factors modulate how atherosclerosis develops in different atherosusceptible regions. In either case, by demonstrating that PECAM-1 both promotes and impedes development of atherosclerotic lesions in site-specific ways, our findings provide a more complete understanding of the factors that interact in complex ways to control initiation and progression of atherosclerosis.

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Disclosures
P.J.N. is a consultant for Novo Nordisk and serves on the scientific advisory board of the New York Blood Center.
References


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