The Role of Platelet-Endothelial Cell Adhesion Molecule-1 in Atheroma Formation Varies Depending on the Site-Specific Hemodynamic Environment

Matthew Harrison,* Emily Smith,* Ewan Ross, Robert Krams, Dolf Segers, Christopher D. Buckley, Gerard B. Nash, G. Ed Rainger

Objective—Polymorphisms in the platelet-endothelial cell adhesion molecule (PECAM-1)-1 gene are linked to increased risk of coronary artery disease. Because PECAM-1 has been demonstrated to form a mechanosensory complex that can modulate inflammatory responses in murine arterial endothelial cells, we hypothesized that PECAM-1 contributes to atherogenesis in a shear-dependent and site-specific manner.

Approach and Results—ApoE−/− mice that were wild-type, heterozygous, or deficient in PECAM-1 were placed on a high-fat diet. Detailed analysis of the aorta at sites with differing hemodynamics revealed that PECAM-1−/− deficient mice had reduced disease in areas of disturbed flow, whereas plaque burden was increased in areas of steady, laminar flow. In concordance with these observations, bone marrow chimera experiments revealed that hematopoietic PECAM-1−/− resulted in accelerated atheroma formation in areas of laminar and disturbed flow, however endothelial PECAM-1−/− moderated disease progression in areas of high shear stress. Moreover, using shear stress–modifying carotid cuffs, PECAM-1−/− was shown to promote macrophage recruitment into lesions developing in areas of low shear stress.

Conclusion—PECAM-1 on bone marrow cells is proatherogenic irrespective of the hemodynamic environment, however endothelial cell PECAM-1 is antiatherogenic in high shear environments. Thus, targeting this pathway therapeutically would require a cell-type and context-specific strategy. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: ApoE−/− ■ atherosclerosis ■ carotid cuffs ■ low shear ■ platelet endothelial cell adhesion molecule-1

Platelet-endothelial cell adhesion molecule (PECAM)-1 is a type-1 transmembrane glycoprotein of the Ig superfamily that is expressed on platelets, most leukocyte subsets, and at endothelial cell (EC) junctions.1–5 PECAM-1 sustains both homophilic interaction (PECAM-1–PECAM-1) and heterophilic interactions with integrin αvβ3, CD38, and CD177 and is a highly efficient signaling molecule mediating both outside-in and inside-out signaling. Because of its identification within the plasma membrane of EC,7 and its cloning soon thereafter,8 and subsequent generation of PECAM-1 knockout mouse,9 the role of PECAM-1 in the vascular system, as well as during inflammatory and autoimmune responses, has been extensively studied.1–5,9,10 For example, antibody blockade of PECAM-1 function in in vitro adhesion assays limits leukocyte migration through EC monolayers, as well as the directionality of migrating neutrophils under flow conditions.2,11–14 PECAM-1 has also been shown to mediate leukocyte motility and extravasation through the basement membrane in vivo, in response to IL-1β but not TNF-α.5,15–17

A mechanosensory role for PECAM-1 was first demonstrated in EC that had been mechanically stimulated or exposed to osmotic stress.18,19 Antibody-conjugated magnetic bead ligation of PECAM-1 followed by exposure to a mechanical force resulted in tyrosine phosphorylation of the cytoplasmic tail within 30 seconds.18,19 Downstream kinases such as ERK1/2 were subsequently activated.19 EC-expressed PECAM-1 can also form a mechanosensory complex with VEGFR-2 and VE-cadherin, which responds to fluid shear stress.20 Disturbed flow profiles with relatively low and oscillatory shear stress are found in the inner curvature of the aortic arch or at bifurcations in the arterial system, and are associated with localized formation of atheroma. These areas also show activation of the proinflammatory transcription factor NF-xB in EC, compared with regions experiencing laminar flow with high levels of shear stress. Importantly, in PECAM-1−/− deficient mice, EC positioned in the inner curvature of the aortic arch were protected against NF-xB activation.20 However, cultured EC, which had been subjected to PECAM-1 small interfering RNA knock down, did not align in response to laminar high shear, but remained polygonal,20 a phenotype found in areas of arteries prone to atherogenesis. Generally, laminar shear stress suppresses...
the inflammatory responses of EC,\textsuperscript{21,22} and we have recently shown that this protective effect also requires expression of PECAM-1.\textsuperscript{23} Thus, taken together, these results indicate that PECAM-1 may play roles in the proinflammatory response believed to promote atherogenesis in regions of disturbed flow and in the protective effects of laminar, high shear stress.

The expression of PECAM-1 on EC, platelets, and leukocytes has led investigators to hypothesize that it may play a key role in cardiovascular disease. In humans, population studies have linked single nucleotide polymorphisms of the PECAM-1 gene and elevated levels of soluble PECAM-1 to severe coronary artery stenosis as well as myocardial infarction.\textsuperscript{24-27} In mice, the role of PECAM-1 in atherogenesis remains controversial. Recent studies in the \textit{Pecam-1}−/− mouse\textsuperscript{28,29} or \textit{Pecam-1}−/−ldlr−/− mouse\textsuperscript{30} on high-fat diet (HFD) have produced conflicting results. Although all the studies agree that PECAM-1 is proatherogenic in the inner curvature of the aortic arch, the site-specific effects of PECAM-1 in the descending aorta are inconsistent between models, with a net proinflammatory\textsuperscript{28} or antiinflammatory\textsuperscript{30} role of PECAM-1 in lesion formation being observed over the whole aorta.

To investigate the site-specific effects of PECAM-1, we generated \textit{Pecam-1}−/− mice and placed them on a HFD to promote atherogenesis. The disease phenotype was dominated by the descending aorta, which delivered a net elevation in plaque burden in the whole aorta. However, a detailed analysis of disease burden at sites subject to different hemodynamic forces showed a reduction of plaque formation in the inner curvature of the aortic arch where low shear stress and complex patterns of flow are apparent. There was also a concomitant elevation in plaque burden in the descending aorta, which is subject to laminar high shear flow. Formation of radiation chimeras revealed that increased plaque burden in the descending aorta was primarily a result of loss of the EC pool of PECAM-1; the hematopoietic pool appeared to be more important for disease progression in the aortic arch. Experiments using shear stress–modifying carotid cuffs in \textit{Pecam-1}−/− and \textit{ApoE}−/− mice also showed a reduction in plaque burden in areas of low shear stress in mice deficient in PECAM-1. Interestingly, in this model, we also found a strong dependence on PECAM-1 for recruitment of monocytes into the atheromatous environment.

Results

Effects of PECAM-1 Ablation on the Hematology and Blood Chemistry of the \textit{Pecam1}−/− \textit{ApoE}−/− Mouse

Total white blood cell counts did not vary between \textit{ApoE}−/− deficient mice with a \textit{Pecam-1}−/− genotype or the control groups of \textit{ApoE}−/− mice, which were \textit{Pecam-1}−/− or \textit{Pecam-1}+/− on either dietary regimen (chow versus HFD; Tables I–III in the online-only Data Supplement). However, ablation of PECAM-1 did result in a small but significant increase in the percentage of circulating peripheral blood lymphocytes, which in chow-fed animals was associated with a significant reduction in the percentage of circulating monocytes (Table I in the online-only Data Supplement). In animals on HFD, there was a trend toward lower phagocyte (monocytes and PMN) numbers in the circulation, but this was not significant. Triglyceride and cholesterol levels were elevated significantly by HFD; although triglyceride levels were not sensitive to alterations in genotype, cholesterol levels in \textit{ApoE}−/− mice that were \textit{Pecam-1}−/− were significantly reduced compared with other genotypes in both dietary regimes. The loss of PECAM-1 did not alter the rate of weight gain over the duration of these experiments (Table II in the online-only Data Supplement).

Site-Specific Effect of PECAM-1 Ablation on Atheroma Formation in the Aorta of the \textit{Pecam1}−/− \textit{ApoE}−/− Mouse

In mice on a chow diet, plaque burden over the whole aorta was low (≈7%), and was not affected by genotype. A full analysis of the animals on this dietary regimen can be found in Figure I the online-only Data Supplement. \textit{ApoE}−/− animals fed a HFD showed an increase in plaque burden compared with chow-fed animals. Interestingly, the loss of PECAM-1 led to a net elevation (≈25%) in disease burden over the whole aorta (Figure 1A–1C). However, detailed analysis showed that the effects of PECAM-1 ablation were complex and site-specific. Thus, there was significant reduction in plaque burden in the aortic arch, particularly in the inner curvature (Figure 1D). In the same animals, the loss of PECAM-1 led to a significant elevation in disease burden in the thoracic and abdominal aortas (ie, the descending aorta; Figure 1E).

No effect of the loss of PECAM-1 was found on atheroma formation in the aortic sinus (Figure II the online-only Data Supplement). In the descending aorta, atheroma often occurs at the sites where branching arteries form areas of disturbed flow. In a more detailed analysis (Figure 2), we observed that plaques in the descending aorta were, in general, associated with branch points of smaller arteries, although in some instances, especially in the abdominal aorta, plaques were large enough to encompass an area of vessel wall that included number of branching arteries (data not shown). Interestingly, the number of plaques in the descending aorta did not vary with the loss of PECAM-1; however, the average size of the plaques was significantly larger in \textit{Pecam-1}−/− \textit{ApoE}−/− mice. Interestingly, the site-specific effects of the ablation of PECAM-1 on atheroma formation were maintained for an extended period. Thus, in \textit{Pecam-1}−/− \textit{ApoE}−/− mice fed a HFD for 24 weeks, there was a ≈23% increase in plaque burden over the whole aorta compared with the control group (Figure IIIA–IIIC in the online-only Data Supplement). Importantly, plaque burden remained significantly reduced in the arch of the aorta (Figure IIID in the online-only Data Supplement), whereas disease was more extensive in the descending aorta (Figure IIIE in the online-only Data Supplement) in the \textit{Pecam1}−/− \textit{ApoE}−/− mice. Taken together, these experiments show that the loss of PECAM-1 has either proatherogenic or antiatherogenic effects, which appear to be dependent on the local hemodynamic environment.

Effect of Losing Either Endothelial or Bone Marrow PECAM-1 on Plaque Burden

Because PECAM-1 is expressed widely in cells of hematopoietic origin, as well as EC, we generated bone marrow (BM) chimeras to differentiate the function of these
Site-Specific Effects of PECAM-1 in Atheroma

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Figure 1. Site-specific effects of platelet-endothelial cell adhesion molecule (PECAM)-1 in ApoE–/– mice on high-fat diet (HFD) for 13 weeks. ApoE–/– mice that were wild-type (WT), heterozygous, or deficient in PECAM-1 were placed on a HFD at 10 weeks of age for a total of 13 weeks. (A) Plaque burden and (B) ORO extraction over the whole aorta was analyzed. C, Representative images of the whole aorta. (D) Image analysis of plaque burden found in the aortic arch, inner and outer curvature, and (E) the whole descending aorta as well as its parts, thoracic and abdominal aorta. *P<0.05; and **P<0.01 Pecam-1–/– ApoE–/– mice compared with ApoE–/– mice. tP<0.05; and $P<0.01 Pecam-1–/– ApoE–/– mice compared with Pecam-1–/– ApoE–/– mice by unpaired Student t test; n=7 to 11.

Figure 3B). In contrast, the opposite construct (ie, reconstituting EC with BM from Pecam-1+/– ApoE–/– animals (Figure 3A–3C) showed an increase in disease across the whole aorta, with a decrease in disease burden in the aortic arch and increased disease in the descending aorta (Figure 3A–3C), which phenocopied the nonirradiated animals. Further analysis in chimeric animals indicated that differences in disease susceptibility were based on the presence of the different pools of PECAM-1. Thus, in the arch (Figure 3B), reconstitution of the Pecam-1+/– ApoE–/– animals with Pecam-1+/– ApoE–/– BM (Figure 3B) significantly exacerbated disease, when compared with Pecam-1+/– ApoE–/– mice receiving Pecam-1+/– ApoE–/– BM (Figure 3B) and Pecam-1+/– ApoE–/– mice receiving Pecam-1+/– ApoE–/– BM (Figure 3B). In contrast, the opposite construct (ie, reconstituting EC PECAM-1 in the double knockout mice) had no significant effect when compared with Pecam-1+/– ApoE–/– mice receiving Pecam-1+/– ApoE–/– BM. We interpret this to show a dominant effect of the BM pool in disease progression at this site. Interestingly, in the descending aorta (Figure 3C), reconstitution of Pecam-1+/– ApoE–/– with BM from Pecam-1+/– ApoE–/– mice (Figure 3C) had no significant effect on disease compared with Pecam-1+/– ApoE–/– mice receiving Pecam-1+/– ApoE–/– BM (Figure 3C). However, the opposite construct (ie, reconstituting the Pecam-1+/– ApoE–/– mice with Pecam-1+/– ApoE–/– BM) significantly reduced disease in comparison with Pecam-1+/–ApoE–/– mice receiving Pecam-1+/– ApoE–/– BM (Figure 3C). Thus, in the descending aorta, the presence of PECAM-1 in the BM pool had no significant effect on disease progression when compared with the Pecam-1+/– ApoE–/– mice receiving Pecam-1+/– ApoE–/– BM (Figure 3C). However, the EC pool had antiatherogeneic properties at this site and conferred rescue from disease. Interestingly, however, the BM pool significantly increased disease progression in the descending aorta when compared with the Pecam-1+/– ApoE–/– mice receiving Pecam-1+/– ApoE–/– BM.

PECAM-1 Is Proatherogenic in Regions of Low Shear Stress Generated by Restrictive Carotid Cuffs

We used surgically implanted shear-stress-modifying carotid cuffs to generate areas of defined low shear stress in the common carotid artery in which plaque was evident (Figure 4A). Extensive control studies for this model have been described in detail in Cheng et al. Briefly, these studies used nonflow-altering control cuffs (constant diameter) that were placed around the vessel touching the adventitial layer, to test for nonspecific effects. Short-lasting placement (7 days) did not change eNOS expression, and long-lasting placements (9 weeks; unpublished data) did not induce plaques or inflammatory responses. Analysis after 9 weeks of HFD showed that there was no disease in the common carotid artery of ApoE–/– control mice undergoing sham surgery (Figure 4B). In the ApoE–/– mouse, a large plaque developed in the low shear environment, no plaque was evident in the high shear region, and a small plaque was found downstream of the cuff in the region with oscillatory-like patterns of flow (Figure 4A and 4B), patterns in agreement with other reports using this methodology. In the common carotid artery of ApoE–/–Pecam-1+/– mice, a significant reduction in plaque burden...
was seen in the low shear region (Figure 4B). Cross sections of the carotid arteries were also examined, and ORO staining confirmed that lack of PECAM-1 dramatically diminished the deposition of lipid within the artery wall in the low shear region (Figure 5A and 5B). Interestingly, even though there was no change in plaque burden or in lipid deposition in the region subjected to oscillatory shear, collagen deposition was significantly reduced in this area (Figure 5C and 5D). Analysis of the common carotid artery for macrophage content also revealed interesting variations between hemodynamic environments. Thus, plaques in the low shear environment were rich in CD68+ cells, but ablation of Pecam-1 significantly reduced the presence of macrophages in the double knockout mouse (Figure 6). No significant differences were found in SMC actin content or CD3+ T-cells between mouse groups (data not shown).

**Discussion**

PECAM-1 is expressed by EC and hematopoietic cells, such as leukocytes and platelets. It is well known to act as a transducer of mechanical signals in EC, and also to regulate migration of leukocytes through endothelium. In the past decade, evidence that PECAM-1 is important in cardiovascular disease has emerged. Polymorphisms of PECAM-1 have been linked to coronary artery disease and myocardial infarction in various ethnic groups. However, the functional basis of such pathogenesis is unclear, and to attempt to clarify this, a number of studies have now been conducted in whole-body PECAM-1 knockouts superimposed on murine models of atherosclerosis (the LDLR−/− or ApoE−/− mice). Unfortunately, even separate studies conducted on mice with the same genetic background have used different dietary regimen and assessed disease at different time points, which makes comparative interpretation problematic.
Unfortunately, even these interesting variations are not consistent between models, or even between studies using the same model. Thus, to understand the role of PECAM-1 in atherogenesis more thoroughly, we generated Pecam-1–/– ApoE–/– mice and evaluated 2 models: natural variation in disease in different regions of the arterial tree, which experience different patterns of blood flow; and placement of a carotid cuff to generate disease in defined areas of low shear stress and oscillating shear stress, which would be free of disease otherwise.

Figure 4. Restriction of blood flow using carotid cuffs demonstrates the proinflammatory role platelet-endothelial cell adhesion molecule (PECAM)-1 has in regions of low shear. At 10 weeks of age, ApoE–/– mice that were wild-type (WT) or deficient for PECAM-1 were placed on high-fat diet (HFD) for 2 weeks, when restrictive carotid cuffs were placed around the left common carotid artery (LCCA). The mice were continued on HFD for a further 9 weeks. A, Schematic diagram of the carotid arteries and cuff placement, and pictures of the carotid arteries from PECAM-1 WT or deficient ApoE–/– mice. B, Analysis of the plaque burden found in the low shear (LS), high shear (HS), and oscillatory shear (OS) regions induced by the placement of the carotid cuff. *P<0.05, Pecam-1–/– ApoE–/– mice compared with ApoE–/– mice by unpaired Student t test; n=3.

Figure 5. Cross section of left common carotid artery (LCCA) after the placement of carotid cuffs demonstrates that platelet-endothelial cell adhesion molecule (PECAM)-1 has proinflammatory effects on plaque formation and collagen deposition. A, Cross sections of LCCA stained with ORO (red) and hematoxylin (blue). B, Analysis of plaque burden as percentage of cross section. C, Cross sections of LCCA stained with Sirius Red for collagen deposition (dark pink) and hematoxylin (blue). D, Analysis of plaque burden as percentage of cross section. *P<0.05; and **P<0.01, Pecam-1–/– ApoE–/– mice compared with ApoE–/– LCCA cross sections by unpaired Student t test; n=5.
Effects of PECAM-1 Ablation on Disease Burden in the Whole Aorta

Using the first model to assess natural variation in disease, mice were subjected to a HFD-feeding regimen for either 13 or 24 weeks. On general analysis, ApoE–/– mice lacking PECAM-1 in EC and leukocytes showed elevated levels of plaque in a simple analysis of total disease burden in the whole aorta. This is in agreement with the study utilizing the ldlr–/–/Pecam-1–/– mouse by Goel et al., however it contradicts the reported decrease in total burden arising from PECAM-1 ablation in the ApoE mouse.24,25 When we used BM chimeras to discriminate between the function of the EC and hematopoietic pools (BM), the EC pool had a strong regulatory role on disease progression so that, in the presence of EC PECAM-1, disease was significantly reduced compared with animals completely lacking in PECAM-1, however the presence of BM PECAM-1 significantly increased disease compared with animals either completely lacking or completely expressing PECAM-1. Importantly, as the aorta is a complex vessel that is subjected to variation in the hemodynamic environment in a site-specific manner, such a crude analysis of plaque burden is insufficiently stringent to describe the effects of PECAM-1 ablation on the anatomical patterning of atherosclerosis.

Effects of PECAM-1 Ablation on Disease Burden in Areas of Low Shear Stress

In the aortic arch, which is an anatomic site subject to low shear and oscillatory flow, we observed that total ablation of PECAM-1 in ApoE–/– mice fed a HFD for 13 or 24 weeks resulted in decreased plaque burden. More detailed analysis of the aortic arch at the sites of the inner and outer curvature revealed a decrease in plaque formation at the inner curvature, but no significant difference at the outer curvature. These observations are in broad agreement with all previous reports, irrespective of the genotype of the models used (ie, ApoE–/–/Pecam-1–/– or ldlr–/–/Pecam-1–/– mice).28,30 Here, the use of BM chimeras revealed that the presence of BM PECAM-1 significantly increased disease burden compared with both PECAM-1 null mice and mice expressing PECAM-1 on both the BM and EC pools. In contrast, however, abolition of EC PECAM-1 had no significant effect on disease. We interpret these data to show a strong proatherogenic role for the hematopoietic (BM) pool of PECAM-1 in the aortic arch.

In addition to analysis of the aorta, we also used shear-modifying carotid cuffs to generate an area of low flow and an atherosclerotic plaque in the carotid artery. This model demonstrated broadly similar results on ablation of PECAM-1. Thus, plaque burden was significantly reduced in ApoE–/– Pecam-1–/– compared with ApoE–/– animals. Interestingly, analysis of the content of these plaques demonstrated a much reduced representation of macrophages in the cellular infiltrate. These data, taken together with the demonstration of the importance of the hematopoietic pool of PECAM-1 in our chimera studies, imply that monocyte PECAM-1 may be important in supporting the trafficking of these cells into plaques that develop in areas of low shear stress.

The above observations made in vivo are well supported by previous studies of the effects of flow on EC, and the roles of PECAM-1 in endothelial responses and in leukocyte migration. First, exposure of EC to increasing levels of shear stress in vitro leads to progressive inhibition of their response to inflammatory cytokines; EC cultured at low shear stress are less prone to recruit leukocytes than those cultured under static conditions, but more prone than those cultured at high shear stress.21,22 Interestingly, oscillatory shear stress, which is often associated with areas of low shear stress in the aorta, is itself proinflammatory, inducing increased expression of adhesion molecules and ability to bind monocytes.38,39 Acute exposure to oscillatory flow itself can lead to nuclear translocation of NF-κB, a response which is reduced when PECAM-1 is absent.20,40 In addition, NF-κB activation in EC, which can be observed in the aortic arch of mice, was reduced in Pecam-1–/– mice.20 Thus, there is strong evidence that loss of PECAM-1 should protect from atheroma in such regions. The literature on the effects of PECAM-1 on leukocyte migration is well established.12,36,37 In the case of monocytes, especially, lack of PECAM-1 would be expected to reduce migration and hence be protective against atheroma, although the magnitude of this effect would depend on level of EC activation, which is the primary driver of leukocyte recruitment.
Effects of PECAM-1 Ablation on Disease Burden in the Descending Aorta

Examination of the descending aorta revealed that PECAM-1 total knockout mice exhibited an increase in plaque deposition within both the thoracic and abdominal aorta, leading to a net increase in deposition within the descending aorta, a phenotype previously reported in the ldlr−/− model. However, other studies in Apoe−/−/Pecam-1−/− models either report no change in disease burden, or failed to investigate disease burden at this anatomical site. Although the descending aorta is generally thought of as an atheroprotected environment because of the levels of high shear lamina flow that are present, atheromatous disease does develop, and this generally occurs at the points where the intercostal, mesenteric, and renal arteries branch from the aorta. At these points, blood flow is disturbed, and areas of low shear stress and complex flow are present, which appear to act as foci for the development of disease. When we conducted a more detailed analysis of the descending aorta, we observed that the number of disease foci was the same in Apoe−/− and Apoe−/−/Pecam-1−/− animals. However, the average size of plaques was significantly increased in the absence of PECAM-1. Moreover, the distribution of disease was consistent with development of atheroma at arterial branch points. These data strongly indicate that atherosclerosis develops at branch points in the descending aorta in the presence of PECAM-1; however, in its absence, the burden of atheroma at these foci of disease is exacerbated, so that individual plaques occupy a greater surface area of the aorta.

When BM chimeras were used to discriminate between the function of the EC and hematopoietic (BM) PECAM-1 in the descending aorta, it was clear that both the BM and EC pools of PECAM-1 played a role in regulating disease burden. Within the descending aorta, as with the aortic arch, the BM pool of PECAM-1 appears to be proinflammatory, leading to a net increase in plaque burden in comparison with both the PECAM-1 null mice and the mice expressing PECAM-1 on both the BM and EC pools. However, in addition, and at variance with the analysis in the aortic arch, the EC pool of PECAM-1 also played a strong role in regulating disease burden within the descending aorta. Indeed, EC PECAM-1 effectively rescued PECAM-1 null mice from the additional burden of atheroma. In other words, in the presence of EC PECAM-1, disease progression was suppressed within the descending aorta. The data from the whole-body PECAM-1 null mice and from our chimeric studies strongly imply that the EC pool of PECAM-1 provides antiatherogenic signals. As discussed above, exposure of EC to increasing levels of shear stress in vitro leads to progressive inhibition of their response to inflammatory cytokines. Importantly, we recently showed that the inhibitory effect of laminar shear stress in such assays was lost, when the experiments were conducted on human EC in which PECAM-1 expression was reduced by treatment with small interfering RNA duplexes, or when ECs isolated from the Pecam-1−/− mouse were compared with those of wild-type animals. Thus, loss of endothelial PECAM-1 would be expected to make disease worse in any situation of laminar flow, although these effects via EC would be more marked the greater the local shear stress. Bearing in mind the observations of our own studies and those in the ldlr−/−, it is appropriate to speculate that this aspect of PECAM-1 function is an important regulator of disease progression, with high shear-induced signaling into ECs limiting the extent of plaque formation.

Conclusion

Our findings, and those of a previous studies, including one conducted in the ldlr−/−/Pecam-1−/− mouse, suggest that the flow characteristics of the aortic arch (ie, oscillatory disturbed flow) induce a proinflammatory environment in which PECAM-1 expressed on hematopoietic cells (BM) promotes the migration of leukocytes into tissue at sites of inflammation, which result in increased disease burden. The presence or absence of endothelial PECAM-1 at this site appears to have little influence on the progression of disease. In regions with high shear stress, such as the descending aorta, a proinflammatory environment is also produced and, similar to the situation in the aortic arch, PECAM-1 on leukocytes promotes their migration into developing lesions. However, an additional regulatory role for EC PECAM-1 is also evident. Thus, EC PECAM-1 appears to send antiinflammatory signals into (or to promote the antiinflammatory properties of) ECs, thereby moderating the burden of disease at this anatomic site. Thus, targeting EC PECAM-1 therapeutically would be expected to worsen disease. However, concentrating on inhibiting the proinflammatory properties of PECAM-1 in BM-derived cells could potentially lessen the disease profile in both high shear stress and oscillating flow regions of the aorta.

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Disclosures

None.

References

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Supplemental Figure I: Site specific effects of PECAM-1 in the aortic arch and branches of Pecam1<sup>+/−</sup>ApoE<sup>−/−</sup> mice on a chow diet. ApoE<sup>−/−</sup> mice that were WT, heterozygous or deficient in PECAM-1 were maintained on a chow diet from weaning until sacrifice at 23 wks. (A) Plaque burden and (B) representative images of the aortic arch. (C) Analysis of plaque burden found in the aortic arch, inner and outer curvature and (D) the descending aorta as well as its parts, the thoracic and abdominal aorta. * p<0.05, ** p<0.01 Pecam1<sup>+/−</sup>ApoE<sup>−/−</sup> mice compared to ApoE<sup>−/−</sup> mice. § p<0.05, §§ p<0.01 Pecam1<sup>+/−</sup>ApoE<sup>−/−</sup> mice compared to Pecam1<sup>+/−</sup>ApoE<sup>−/−</sup> mice by unpaired students t test.
Supplemental Figure II. PECAM-1 deficiency does not affect plaque development in the aortic sinus of ApoE−/− mice on HFD. 7 µm sections of the aortic sinus were cut sequentially and stained with ORO and hematoxylin. (A) Representative images of the aortic sinus and (B) plaque burden analysis of the sinus cross-section.
Supplemental Figure III: Site specific effects of PECAM-1 in Pecam1<sup>-/-</sup>ApoE<sup>-/-</sup> mice on HFD for 24 wks. ApoE<sup>-/-</sup> mice that were WT, heterozygous or deficient in PECAM-1 were placed on a HFD at 10 wks of age for a total of 24 wks. (A) Plaque burden and (B) ORO extraction over the whole aorta was analysed. (C) Representative images of the whole aorta. (D) Image analysis of plaque burden found in the aortic arch, inner and outer curvature and (E) the whole descending aorta as well as its parts, thoracic and abdominal aorta. * p<0.05, ** p<0.01 and Pecam1<sup>-/-</sup>ApoE<sup>-/-</sup> mice compared to ApoE<sup>-/-</sup> mice by unpaired students t test, n=6.
Table I. Blood counts and chemistry of mice on chow diet (23 wks):
Males and females combined. *p<0.05 compared to Pecam1+/+ApoE−/− mice.

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<th>Total WBC (K/µl)</th>
<th>PBL (%)</th>
<th>Mono (%)</th>
<th>PMN (%)</th>
<th>Total TG (mmol/L)</th>
<th>Total cholesterol (mmol/L)</th>
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<td>8.5 ± 0.8</td>
<td>*78.6 ± 2.4</td>
<td>*2.6 ± 0.5</td>
<td>18.8 ± 2.1</td>
<td>0.73 ± 0.07</td>
<td>11.01 ± 1.54</td>
</tr>
</tbody>
</table>

Table II. Blood counts, blood chemistry and weight profiles of mice on western Diet (13 wks)
Males and females combined. †p<0.05 compared to Pecam1+/+ApoE−/− compared to Pecam1−/−ApoE−/− mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Total WBC (K/µl)</th>
<th>PBL (%)</th>
<th>Mono (%)</th>
<th>PMN (%)</th>
<th>Start weight (10 wks)</th>
<th>End weight (23 wks)</th>
<th>Total TG (mmol/L)</th>
<th>Total cholesterol (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecam1+/+ApoE−/−</td>
<td>6.8 ± 0.5</td>
<td>60.8 ± 2.1</td>
<td>12.9 ± 2.1</td>
<td>26.3 ± 2.1</td>
<td>26.1g ± 1.2</td>
<td>35.6g ± 1.7</td>
<td>1.22 ± 0.17</td>
<td>26.32 ± 4.24</td>
</tr>
<tr>
<td>Pecam1+/−ApoE−/−</td>
<td>0.5 ± 0.5</td>
<td>4.8 ± 2.1</td>
<td>2.1 ± 1.4</td>
<td>6.4 ± 2.1</td>
<td>1.2 ± 0.7</td>
<td>1.7 ± 0.2</td>
<td>0.17 ± 0.07</td>
<td>4.24</td>
</tr>
<tr>
<td>Pecam1−/−ApoE−/−</td>
<td>8.8 ± 0.9</td>
<td>62.0 ± 1.4</td>
<td>12.0 ± 1.4</td>
<td>26.0 ± 1.4</td>
<td>24.7g ± 0.7</td>
<td>37.8g ± 0.2</td>
<td>1.33 ± 0.20</td>
<td>*16.08 ± 2.44</td>
</tr>
<tr>
<td>Pecam1−/−ApoE−/−</td>
<td>9.1 ± 1.0</td>
<td>†70.6 ± 1.4</td>
<td>10.2 ± 1.4</td>
<td>19.2 ± 1.4</td>
<td>23.6g ± 0.7</td>
<td>34.8g ± 0.2</td>
<td>1.09 ± 0.20</td>
<td>25.87 ± 2.44</td>
</tr>
<tr>
<td>Pecam1−/−ApoE−/−</td>
<td>0.9 ± 2.2</td>
<td>0.9 ± 0.9</td>
<td>2.2 ± 2.2</td>
<td>1.5 ± 0.5</td>
<td>3.8 ± 0.2</td>
<td>0.20 ± 0.20</td>
<td>2.45 ± 0.20</td>
<td>2.45</td>
</tr>
</tbody>
</table>
### Table III. Blood counts and chemistry of mice on western diet (24 wks)

<table>
<thead>
<tr>
<th>Mice</th>
<th>Total WBC (K/µl)</th>
<th>PBL (%)</th>
<th>Mono (%)</th>
<th>PMN (%)</th>
<th>Start weight (10 wks)</th>
<th>End weight (34 wks)</th>
<th>Total TG (mmol/L)</th>
<th>Total cholesterol (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecam1+/+ ApoE+/+</td>
<td>10.3 ± 0.9</td>
<td>58.0 ± 6.1</td>
<td>11.4 ± 1.7</td>
<td>30.6 ± 4.9</td>
<td>24.5g ± 1.3</td>
<td>33.8g ± 4.0</td>
<td>0.89 ± 0.16</td>
<td>27.72 ± 2.91</td>
</tr>
<tr>
<td>Pecam1−/− ApoE−/−</td>
<td>11.5 ± 1.0</td>
<td>67.6 ± 1.0</td>
<td>10.1 ± 2.2</td>
<td>22.3 ± 3.0</td>
<td>25.1g ± 1.3</td>
<td>41.7g ± 4.0</td>
<td>1.07 ± 0.10</td>
<td>25.66 ± 3.56</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>1.0 ± 0.2</td>
<td>2.7 ± 0.8</td>
<td>0.8 ± 2.0</td>
<td>3.0 ± 2.2</td>
<td>0.7 ± 0.7</td>
<td>2.2 ± 0.3</td>
<td>0.10 ± 0.10</td>
<td>3.56 ± 2.1</td>
</tr>
</tbody>
</table>

### Table IV. Blood counts and chemistry of mice undergoing carotid surgery and on western diet (11 wks)

<table>
<thead>
<tr>
<th>Mice</th>
<th>Total WBC (K/µl)</th>
<th>PBL (%)</th>
<th>Mono (%)</th>
<th>PMN (%)</th>
<th>Start weight (10 wks)</th>
<th>End weight (21 wks)</th>
<th>Total TG (mmol/L)</th>
<th>Total cholesterol (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecam1+/+ ApoE+/+</td>
<td>12.5 ± 0.7</td>
<td>67.6 ± 9.6</td>
<td>9.6 ± 2.3</td>
<td>23.0 ± 2.2</td>
<td>28.0g ± 0.9</td>
<td>35.4g ± 1.8</td>
<td>1.23 ± 0.13</td>
<td>25.55 ± 3.19</td>
</tr>
<tr>
<td>Pecam1−/− ApoE−/−</td>
<td>10.7 ± 1.3</td>
<td>67.2 ± 13.0</td>
<td>10.1 ± 2.2</td>
<td>19.8 ± 2.2</td>
<td>25.1g ± 1.8</td>
<td>36.3g ± 2.0</td>
<td>0.65 ± 0.14*</td>
<td>24.68 ± 2.15</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>1.3 ± 0.2</td>
<td>2.5 ± 2.0</td>
<td>0.8 ± 2.2</td>
<td>2.2 ± 2.0</td>
<td>2.0 ± 2.0</td>
<td>5.3 ± 2.0</td>
<td>0.14* ± 0.10</td>
<td>2.15 ± 0.15</td>
</tr>
</tbody>
</table>

Males and females combined. *p<0.05 compared to Pecam1+/+ ApoE+/+ mice
Methods.

Animals

$\text{Pecam-1}^{-/-}\text{ApoE}^{-/-}$ mice on a C57BL6 background were generated from $\text{ApoE}^{-/-}$ (Taconic) and $\text{Pecam-1}^{-/-}$ animals. All mice were maintained at the Biomedical Services Unit at the University of Birmingham, according to Home Office regulations. Mice were genotyped using the DNeasy blood and tissue kit (Qiagen), according to manufacturer’s instructions. Age and sex matched $\text{Pecam-1}^{-/-}\text{ApoE}^{-/-}$, $\text{Pecam-1}^{+/+}\text{ApoE}^{-/-}$ and $\text{Pecam-1}^{+/+}\text{ApoE}^{-/-}$ mice were kept on chow diet between weaning and 10 wks. At 10 wks, the mice were either retained on a chow diet or placed on a HFD (21.4% cocoa butter [w/w] and 0.2% cholesterol [w/w]; Special Diet Services, UK) for 11, 13 or 24 wks as indicated. All protocols and procedures conform to the European Commission Directive 86/609/EEC and approval was granted by the University of Birmingham’s ethical review board. During all surgical procedures adequacy of anaesthesia was monitored by continuous monitoring of physiological parameters and by performing a reflex response test.

Hematological and serum lipid analysis

Blood was collected from the tail vein into EDTA coated tubes. Total WBC counts were determined using a hemocytometer and differential white cell counts determined using Diff-Quik® (Dade Behring, Newark, USA). Blood was also taken by cardiac puncture, whereby mice were anaesthetised with 4% isoflurane via inhalation, anaesthesia was maintained with 2.5% isoflurane, and cardiac puncture was performed and mice were sacrificed via terminal IP injection of 200 mg/kg pentobarbital. Blood was centrifuged at 14,000 rpm for 10 min. Serum was collected and stored at -20 °C. Serum triglyceride (Serum Triglyceride determining kit; Sigma, Poole, UK), and total cholesterol levels (Cayman Chemicals, Ann Arbor, MI, USA) were analysed.
**Aorta isolation**

Mice were sacrificed via terminal IP injection of 200 mg/kg Pentobarbital. Aortas were fixed in situ by the perfusion of 2% paraformaldehyde (Sigma) through the left ventricle of the heart. The whole aorta was excised, cut longitudinally and stained with Oil Red O (ORO; Sigma). Digital photographs were taken and analyzed for lesion size, as a percentage of a specific region (whole aorta, inner and outer curvature, aortic arch, aortic branches, thoracic and abdominal aorta), using ImageJ software (NIH). Detailed analysis of plaque burden in the descending aorta was conducted in order to evaluate the total number and average size of plaques in an area of the aorta which has several branch points (intercostals, mesenteric and renal arteries). Digital images were analysed in ImageJ software (NIH) via threshold analysis, individual plaque particles were analysed via a criteria of size (over 0.5mm²) and circularity (0.04mm). Plaque burden over the whole aorta was also analyzed by ORO extraction from weighed aortas into a chloroform:methanol mix (2:1 vol) and read as absorbance (nm) at 490 nm².

**Carotid Cuff surgery**

Carotid cuff surgery was performed as described. Briefly, mice were placed on HFD at 10 wks old. At 12 wks mice were anaesthetised with 4% isoflurane via inhalation, anaesthesia was maintained with 2.5% isoflurane, 0.1mg/kg buprenorphine was used for analgesia, and a carotid cuff with an internal diameter of 400µm diminishing to 200µm was placed around the left carotid artery and secured by suture. Mice were kept on the HFD for a further 9 wks until sacrificed via terminal IP injection of 200 mg/kg pentobarbital for tissue collection. The carotid artery was frozen in OCT medium or fixed in 2% paraformaldehyde. Tissue sections were assessed using immunohistochemistry (see below) or stained with ORO.
Flow and shear stress values in cuffs have previously been measured with Doppler\textsuperscript{4} and with MRI\textsuperscript{5}. The measurement in those papers indicate that blood velocity is reduced by 30\% upstream of the device and to near zero downstream of the device. Shear stress values are 10 Pa before cuff placement and decrease to 7 Pa upstream of the device and slightly higher than zero downstream of the device. In the throat of the device shear stress can increase to 30 Pa.

**Generation of bone marrow chimeras**

Ten week old female recipient mice were lethally irradiated with two doses of 5 gy three hours apart. Donor mice were sacrificed via cervical dislocation, femurs were then dissected and BM was flushed from the femurs of male donors under aseptic conditions using sterile PBS (Gibco), and $10^7$ cells injected into the tail vein of recipient mice. Mice were immediately placed on HFD. Efficiency of reconstitution was determined using isolated leukocyte DNA of female mice using real-time PCR primers targeted against the Y6 gene as previously described\textsuperscript{6}. Irradiation had no significant effects on circulating WBC counts, leukocyte differentials, or serum triglycerides and total cholesterol levels when compared to non-irradiated mice (data not shown).

**Immunohistochemistry**

Immunohistochemistry was performed on frozen or paraformaldehyde (2\%; Sigma) fixed tissue. 7-8 µm sections were cut and air dried, fixed in 2\% paraformaldehyde or acetone and stored at -80\°C. Sections were stained with ORO, hematoxylin and eosin (H&E; Sigma), and Sirius Red (Sigma). For antibody staining, PE-Cy2 conjugated anti-smooth muscle cell actin (Sigma), anti-CD68 (Clone FA-11; abcam, Cambridge, UK) with HRP- conjugated anti-
rat IgG (abcam) as a secondary, and anti CD3 (145-2C11; abcam) with biotinylated anti-hamster secondary, HRP-conjugated Streptavidin IgG (Invitrogen) as a secondary antibody were used.

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance between groups was set at p<0.05 using unpaired t tests unless otherwise stated.

References

5. van Bochove GS, Straathof R, Krams R, Nicolay K, Strijkers GJ. Mri-determined carotid artery flow velocities and wall shear stress in a mouse model of vulnerable and stable atherosclerotic plaque. MAGMA. 2010;23:77-84