Adhesion of Monocytes to Arterial Endothelium and Initiation of Atherosclerosis Are Critically Dependent on Vascular Cell Adhesion Molecule-1 Gene Dosage

Hayes M. Dansky, Courtenay B. Barlow, Chris Lominska, John L. Sikes, Catherine Kao, Jonathan Weinsaft, Myron I. Cybulsky, Jonathan D. Smith

Abstract—Vascular cell adhesion molecule-1 (VCAM-1/Vcam1) is a cytokine-inducible member of the immunoglobulin gene superfamily that is expressed by arterial endothelial cells in regions predisposed to atherosclerosis and at borders of atherosclerotic plaques. To determine whether VCAM-1 expression regulates atherosclerotic lesion formation, we crossed Vcam1 domain 4–deficient (D4D) mice, which partially circumvent the embryonic lethality of Vcam1 null mice, with apolipoprotein E null (Apoe−/−) mice, which spontaneously develop hypercholesterolemia and atherosclerosis. In the Apoe−/− background, mice homozygous for the Vcam1 D4D allele had markedly reduced arterial VCAM-1 expression, monocyte adherence in the aortic root, and fatty streak formation. Heterozygous Vcam1 D4D mice revealed a Vcam1 gene-dosage effect and had intermediate, yet significant, reductions in these parameters. Our data demonstrate that VCAM-1 plays a pivotal role in the initiation of atherosclerosis in Apoe−/− mice. (Arterioscler Thromb Vasc Biol. 2001;21:1662-1667.)

Key Words: vascular cell adhesion molecule-1 ■ apolipoprotein E ■ hypercholesterolemia ■ gene targeting ■ monocytes

Atherosclerosis begins as a focal process at specific regions of the vascular system, so-called lesion-prone areas, where hemodynamic flow is altered. The arterial endothelium expresses numerous adhesion molecules, such as P-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), at these lesion-prone areas before lesion development and at the borders of atherosclerotic lesions. Whereas ICAM-1 is also abundantly expressed at lesion-prone areas in wild-type mice with normal cholesterol levels, VCAM-1, a cytokine-inducible member of the immunoglobulin gene superfamily, is specifically upregulated in arterial endothelial cells at lesion-prone areas in hypercholesterolemic mice and rabbits. This specific upregulation of VCAM-1 at lesion-prone areas in hypercholesterolemic animals suggests that VCAM-1 may regulate monocyte adhesion in early atherogenesis.

To circumvent the embryonic lethality of Vcam1 null mice, mutant mice were generated with a targeted disruption of the exon encoding the fourth immunoglobulin domain of VCAM-1, which codes for an α4 integrin binding site. Domain 4–deficient (D4D) mice (Vcam1D4D/D4D) express only a 6-immunoglobulin domain form of Vcam1, with VCAM-1 mRNA and protein levels <10% of those found in wild-type mice. Reduced expression of VCAM-1 protein resulted in decreased embryonic survival of Vcam1D4D/D4D mice. The frequency of embryonic survival was strain dependent, ranging from 29% in 129-C57BL/6 hybrids to 6% of expected in C57BL/6 Vcam1D4D/D4D mice. When Vcam1D4D/D4D of mixed genetic background were bred with LDL receptor–deficient (Ldlr−/−) mice, the aortic surface area occupied by atherosclerosis was reduced by 40% compared with that of Ldlr−/− mice. In the present study, Vcam1D4D/D4D mice were bred with hypercholesterolemic apoE null (Apoe−/−) mice to determine whether relative deficiency in VCAM-1 would also attenuate lesion formation in the apoE-deficient background. Mice homozygous for the Vcam1 D4D allele (Apoe−/− and Vcam1D4D/D4D) had markedly reduced arterial VCAM-1 expression, monocyte adherence, and an 84% decrease in aortic root lesion area. We also demonstrate a significant Vcam1 gene-dosage effect on these parameters.

Our data indicate that endothelial VCAM-1 plays a critical role in monocyte entry into the subendothelial space in early atherogenesis.

Methods

Mice

Because of the high incidence of embryonic lethality of Vcam1D4D/D4D mice on the C57BL/6 genetic background, all studies were performed with littermate-controlled outbred mice. Apoe−/− mice on an outbred C57BL/6 genetic background were first crossed to Vcam1D4D/D4D mice on the same outbred background, and progeny were bred back to Apoe−/− mice to obtain Apoe−/−, Vcam1D4D/D4D mice. Apoe−/−, Vcam1D4D/D4D mice were then intercrossed to obtain the 3 possible Vcam1 genotypes: Vcam1−/−, Vcam1D4D/D4D, and Vcam1D4D/D4D. Because of

Received June 13, 2001; revision accepted July 25, 2001.
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TABLE 1. Plasma Lipids and Body Weight Measurements in Apoe−/− Mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Genotype</th>
<th>Weight, g</th>
<th>TC, mg/dL</th>
<th>HDL-C, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Apoe−/−, Vcam1+/−</td>
<td>32±3 (14)</td>
<td>483±174 (12)</td>
<td>38±5 (12)</td>
</tr>
<tr>
<td></td>
<td>Apoe−/−, Vcam1+/D4</td>
<td>31±3 (36)</td>
<td>618±199 (35)</td>
<td>37±7 (35)</td>
</tr>
<tr>
<td></td>
<td>Apoe−/−, Vcam1+/D4D</td>
<td>35±2 (6)</td>
<td>542±179 (4)</td>
<td>36±9 (4)</td>
</tr>
<tr>
<td>Female</td>
<td>Apoe−/−, Vcam1+/−</td>
<td>26±2 (15)</td>
<td>509±143 (9)</td>
<td>30±3 (12)</td>
</tr>
<tr>
<td></td>
<td>Apoe−/−, Vcam1+/D4D</td>
<td>26±2 (24)</td>
<td>455±162 (25)</td>
<td>32±1 (25)</td>
</tr>
<tr>
<td></td>
<td>Apoe−/−, Vcam1+/D4D</td>
<td>25±2 (10)</td>
<td>502±152 (5)</td>
<td>34±3 (6)</td>
</tr>
</tbody>
</table>

TC indicates total plasma cholesterol; HDL-C, HDL cholesterol. Values are mean±SD, with the number of mice assayed in parentheses. No statistical differences among genotypes were obtained by ANOVA.
Effect of VCAM-1 on monocyte adherence and atherosclerotic lesion area in apoE−/− mice. A, Quantification of CD11a+ mononuclear cells bound to the aortic root endothelium of male Vcam1+/+ (n=5), Vcam1+/−D4D (n=16), and Vcam1−/−D4D (n=8) apoE−/− mice. Values are mean±SD. B and C, Aortic root atherosclerotic lesion areas in male (B) and female (C) apoE−/− mice according to Vcam1 genotype.

VCAM1+ mice and ApoE−/−, Vcam1+/−D4D mice than in ApoE−/−, Vcam1−/−D4D mice (Figure 2A). Linear regression analysis revealed a significant correlation between the areas of CD11a+ cells and Vcam1 genotype (r=0.24, P<0.01), suggesting an overall Vcam1 gene-dosage-dependent effect on mononuclear cell adherence. This suggests that VCAM-1 plays an important role in monocyte adherence to arterial endothelium during early atherogenesis. This is consistent with the observation that antibody blockade of VCAM-1
A Vcam1 gene-dosage–dependent effect on atherosclerotic plaque formation was noted in mice of both sexes (Figure 2). In males, there was a 54% decrease in median lesion area in Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mice and a 74% decrease in Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mice compared with the median lesion area in Apoe<sup>−/−</sup>, Vcam1<sup>+/+</sup> mice (Figure 2B). In females, there was a 55% decrease in median lesion area in Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mice and an 89% decrease in Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mice (Figure 2C). Because there were no significant differences in lesion size between male and female mice of a given Vcam1 genotype, male and female data were combined. In the combined sex groups, the median lesion areas were 37×10<sup>4</sup>, 17×10<sup>4</sup>, and 6×10<sup>4</sup> µm<sup>2</sup> for Apoe<sup>−/−</sup>, Vcam1<sup>+/+</sup> mice, Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mice, and Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mice, respectively (P<0.001 for Vcam1<sup>+/D4D</sup> versus Vcam1<sup>+/+</sup>, P<0.01 for Vcam1<sup>+/D4D</sup> versus Vcam1<sup>+/+D4D</sup>, and P<0.05 for Vcam1<sup>+/D4D</sup> versus Vcam1<sup>+/D4D</sup>). Thus, the presence of 1 and 2 Vcam1 D4D alleles was associated with significant (56% and 84%, respectively) reductions in lesion areas. These data reveal a robust Vcam1 gene-dosage effect on lesion cross-sectional area.

Microscopic examination of atherosclerotic lesions revealed that the majority of the lesions in mice with 2, 1, or 0 wild-type Vcam1 alleles were fatty streaks composed of macrophage foam cells (Figure 3). Lesions in Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mice were limited to very small nascent fatty streak lesions (Figure 3E and 3F), and fatty streak lesions of progressively increased size were noted in Apoe<sup>−/−</sup>, Vcam1<sup>+/+D4D</sup> mice (Figure 3C and 3D) and in Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mice (Figure 3A and 3B). The extent of VCAM-1 immunostaining was also assessed in aortic root fatty streak lesions of 20-week-old Apoe<sup>−/−</sup> mice. In Vcam1<sup>+/+</sup> mice, VCAM-1 staining was observed on the endothelium and even more prominently within the intimal lesions (Figure 4A). In Vcam1<sup>+/D4D</sup> mice, aortic function reduced mononuclear cell adhesion by 75% in isolated-perfused carotid arteries from Apoe<sup>−/−</sup> mice.14

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**Figure 3.** Representative oil red O staining for lipid in aortic root sections from male 16-week-old chow-fed Apoe<sup>−/−</sup>, Vcam1<sup>+/+</sup> mice (A and B), Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mice (C and D), and Apoe<sup>−/−</sup>, Vcam1<sup>+/+D4D</sup> mice (E and F). Images of the aortic root were taken at low and intermediate magnification (×4 objective on the left and ×20 objective on the right, respectively).

**Figure 4.** Immunohistochemical staining for VCAM-1 in aortic root lesions (×20 objective) from Apoe<sup>−/−</sup> mice. VCAM-1 staining (red color) was abundant on endothelial cells and on cells within the lesions from an Apoe<sup>−/−</sup>, Vcam1<sup>+/+D4D</sup> mouse (A). Less abundant staining was found in lesions from an Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mouse (B), and no staining was visualized in lesions from an Apoe<sup>−/−</sup>, Vcam1<sup>+/D4D</sup> mouse (C).

**Discussion**

The pathogenesis of early atherosclerosis in Apoe<sup>−/−</sup> mice involves the accumulation of lipoprotein aggregates in the subendothelial space, upregulation of specific endothelial adhesion molecules and chemokines, monocyte recruitment, and subsequent foam cell formation. Deficiencies of P-selectin, E-selectin, and ICAM-1 have been shown to decrease atherosclerosis in hypercholesterolemic mice, although a gene-dosage–dependent effect has not been documented for any of these deficiencies, and results in ICAM-1–deficient mice have not been consistent. Embryonic demise of Vcam1 null mice has made it difficult to study the role of VCAM-1 in atherogenesis with the use of mutant mouse models. Cybulsky et al created Vcam1 D4D mice that...
had dramatic decreases in VCAM-1 expression and an improved survival rate compared with those in Vcam1 null mice. The effect of very low levels of VCAM-1 expression on survival was strain dependent, with very low rates of survival of C57BL/6 D4D homozygous mice and better survival rates of D4D homozygous mice on a mixed genetic background. To generate sufficient numbers of mice for atherosclerosis studies, we bred C57BL/6-129 hybrid Vcam1/D4D mice onto the apoE-deficient background to determine the effect of decreased VCAM-1 expression on atherosclerosis.

Vcam1 gene dosage affected endothelial VCAM-1 expression and the subsequent monocyte adherence to lesion-prone areas of the arterial wall. Decreased VCAM-1 expression in Vcam1<sup>Ind/Ind</sup> mice resulted in an overall 84% decrease in fatty streak formation in the aortic root. Cybulsky et al. reported a 40% decrease in the percentage of aortic surface area occupied by lesions in Ldr<sup>−/−</sup>, Vcam1<sup>+/−</sup> D4D homozygous mice. There are several potential differences that may explain the varying magnitude of reduction of atherosclerosis between these 2 studies. The varying lipoprotein profiles in Ldr<sup>−/−</sup> and Apoe<sup>−/−</sup> mice may have different downstream effects on lipoprotein deposition and endothelial activation. The high-fat high-cholesterol diet used in the study by Cybulsky et al might have induced a VCAM-1–independent inflammatory response that may not be present in the chow-fed Apoe<sup>−/−</sup> mice used in the present study. In addition, Cybulsky et al measured atherosclerosis as the percentage of the entire aorta occupied by lesions, whereas in the present study, atherosclerosis was assessed in cross sections through the aortic root. Taken together, these studies demonstrate that arterial expression of VCAM-1 plays an important role in atherosclerotic lesion formation in the context of varying lipoprotein profiles and at multiple sites in the vasculature. The interanimal variation in aortic root lesion size within each genotype may have been partly due to genetic heterogeneity. Despite this variation, the Vcam1 genotype had a highly significant effect on atherosclerosis.

The reduction in endothelial VCAM-1 expression most likely led to a decrease in monocyte adhesion and fatty streak formation in the Vcam1<sup>Ind/Ind</sup> mice. An alternative explanation for these findings is that decreased VCAM-1 expression affected the number of circulating monocytes and, by this mechanism, attenuated atherogenesis. There are several reasons why we do not favor this alternative explanation. It has been previously shown that the D4D mutation does not affect myeloid differentiation in vivo. In addition, leukocyte number and monocyte counts were not affected by the D4D mutation in the present study or in the study by Cybulsky et al. An additional explanation is that VCAM-1 may be involved in T-cell–dependent humoral immune responses, and the immune system has been shown to play a modifying role in atherosclerosis. However, complete ablation of cellular and humoral immunity in chow-fed Apoe<sup>−/−</sup> mice had a lesser effect in reducing atherosclerosis than that observed in Vcam1<sup>Ind/Ind</sup> mice in the present study.

Atherosclerotic lesion formation was not abolished in the present study. Although we observed an 84% reduction in lesion area in the Vcam1<sup>Ind/Ind</sup> mice, it is possible that the residual VCAM-1 expression was responsible for the remaining lesion formation. It is unclear whether a total deficiency in VCAM-1 would result in abolition of atherosclerosis. The use of temporal or tissue-specific conditional Vcam1 knockout mice could be used to study whether lesions develop in the total absence of VCAM-1 expression. Recently, 2 conditional Vcam1 knockout mouse models have been created. However, the observed impairment of immune responses in these conditional Vcam1 knockout mice may confound the interpretation of atherosclerosis studies. In conclusion, VCAM-1 plays a pivotal role in early atherosogenesis. Future studies are necessary to determine whether VCAM-1 expression plays a role in fibroproliferative lesion progression and whether therapeutic targeting of VCAM-1 will attenuate atherosclerosis in humans.

Acknowledgments
This work was supported in part by an Established Investigatorship from the American Heart Association (J.D.S. and M.I.C.), by a grant from the National Institutes of Health (J.D.S.), and by a grant from the Heart and Stroke Foundation of Ontario (M.I.C.). H.M.D. is a recipient of a new investigator development award from the American Heart Association Heritage Affiliate. The authors wish to thank Jie Tang and Jonathan Beslow for their technical assistance in the immunohistochemical protocols.

References


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doi: 10.1161/hq1001.096625

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