Basic Science Reports

Prominent Role of P-Selectin in the Development of Advanced Atherosclerosis in ApoE-Deficient Mice

Zhao Ming Dong, MD, PhD; Allison A. Brown, BA; Denisa D. Wagner, PhD

Background—Adhesive interactions between leukocytes and endothelial cells are characteristic of the development of atherosclerotic lesions, but the receptors involved remain to be defined. P-selectin is an adhesion receptor expressed on activated endothelial cells or platelets and was shown to be involved in fatty streak formation in LDL receptor−deficient mice on an atherogenic diet. The main purpose of this study is to examine the role of P-selectin in the spontaneous development of advanced atherosclerosis in apoE-deficient mice.

Methods and Results—We intercrossed P-selectin−deficient mice with mice lacking apoE and compared lesion development in apoE-deficient mice with P-selectin (apoE−/− P+/+) and without P-selectin (apoE−/− P−/−) that were fed normal mouse chow. At 4 months of age, apoE−/− P−/− mice had 3.5-fold smaller aortic sinus lesions than apoE−/− P+/+ mice. These were limited to fatty streaks in the apoE−/− P−/− mice, whereas 70% of apoE−/− P+/+ lesions contained smooth muscle cells. Significantly more of the aortic sinus circumference was covered by lesions in the apoE−/− P+/+ animals. The P-selectin genotype affected macrophage recruitment, because twice as many mononuclear cells were present in the P-selectin−positive lesions. At 15 months, the lesions progressed to the fibrous plaque stage in both genotypes and spread throughout the aorta, but this process was delayed in apoE−/− P−/− mice. In the aortic sinus, the lesions of the apoE−/− P−/− mice were 2.6-fold smaller and less calcified.

Conclusions—P-selectin appears to be a key adhesion receptor mediating leukocyte recruitment into lesions and promoting advanced atherosclerosis in apoE-deficient mice. (Circulation. 2000;101:2290-2295.)

Key Words: selectins, atherosclerosis, lipoproteins, leukocytes

Atherosclerosis is an inflammatory disease of large and medium-size arteries. Lesion development involves leukocyte, especially monocyte, adhesion to the vascular endothelium and migration into the intima, where it becomes a macrophage or foam cell.1 Experimental evidence shows that in human and animal atherosclerosis, one of the observed changes in the endothelium is an increase in the expression of leukocyte adhesion receptors, such as P-selectin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1.2 However, the role of these adhesion molecules in mediating monocyte infiltration and promoting the late stages of fibrous plaque lesion development in atherogenesis has not been clearly defined.

P-selectin is a member of the selectin family of adhesion receptors and is expressed on activated endothelium and platelets.3 It binds to monocytes, neutrophils, T cells, and platelets and mediates the first step (rolling) in leukocyte extravasation.4,5 Soluble P-selectin is proposed to be a marker of endothelial dysfunction in hypercholesterolemic patients.6 P-selectin polymorphism is reported to be associated with human atherosclerosis.7 To test the role of P-selectin in the formation of atherosclerotic lesions, we have intercrossed P-selectin−deficient mice with mice lacking the LDL receptor (LDLR−/−), which are susceptible to atherosclerosis when fed an atherogenic diet. After 8 weeks on this diet, male mice lacking both LDLR and P-selectin showed a 2-fold reduction in fatty streak lesion size in the aortic sinus. Such reduction was not observed in the females. At 37 weeks on the diet, the lesions in the LDLR−/− animals progressed to the fibrous plaque stage, and lesion size and distribution were no longer dependent on P-selectin.8 The atherogenic “Paigen” diet9,10 includes an artificially high cholesterol content (10 to 20 times that of a Western-type diet) plus the unnatural dietary constituent cholic acid. This diet could produce unintended pathophysiological processes in these animals. We have shown that it induced leukocyte rolling in the LDLR−/− mice.8 E-selectin, which has an overlapping function with P-selectin,3 could be upregulated during the proinflammatory state of mice on this diet. Furthermore, expression of VCAM-1 on the endothelium in experimental atherosclerosis has been found to be positively correlated to cholesterol levels.11 VCAM-1 has also been found to mediate leukocyte arrest as well as rolling.12,13 Therefore, the atherogenic diet could reduce the role of P-selectin in both early and late stages of lesion development in the LDLR−/− mice because of an excessive induction of other adhesion molecules with similar functions.
For this reason, we were interested in evaluating the role of P-selectin in atherosclerosis in an animal model that develops atherosclerosis spontaneously. ApoE<sup>−/−</sup> mice have elevated cholesterol levels (300 to 600 mg/dL)<sup>10</sup> and develop fibrous plaque lesions with features characteristic of those seen in humans.<sup>14</sup> We therefore compared the lesion development in apoE-deficient mice with and without P-selectin maintained on normal chow diet.

**Methods**

**Mice, Genotype Analysis, and Diet**
P-selectin–deficient (P<sup>−/−</sup>) mice<sup>15</sup> were backcrossed 4 times to C57BL/6J. ApoE-deficient (apoE<sup>−/−</sup>) mice backcrossed 8 times to C57BL/6J were purchased from Jackson Laboratory.<sup>16</sup> P<sup>−/−</sup> mice were interbred with apoE<sup>−/−</sup> mice. Littermates from the F2 generation of this intercross were used to establish apoE<sup>−/−</sup> P<sup>−/−</sup> matings used in our study. Polymerase chain reaction analysis was performed for genotyping for P-selectin<sup>17</sup> and cholesterol measurement for apoE<sup>−/−</sup>.<sup>16</sup> Mice were maintained on a normal chow diet containing 5% fat (wt/wt) (Prolab 3000; PMI Feeds).

**Cholesterol Determination**
Mice were fasted overnight, and blood was collected through the retro-orbital venous plexus in polypropylene tubes containing EDTA. Cholesterol in total plasma was determined with a kit (352-50, Sigma).

**Blood Counts**
Blood was obtained as above. Complete blood counts were determined with an automatic cell counter (Coulter).

**Quantification of Aortic Sinus Lesions**
The sections containing aortic sinus were prepared as described,<sup>8</sup> with modifications. The heart and attached aorta were fixed in 4% paraformaldehyde for 30 minutes before being embedded in OCT compound. Four consecutive 10-μm sections were collected for each slide. Ten slides were made from each animal. Odd-numbered slides were stained with oil red O and hematoxylin, counterstained with light green, and examined for lesion size and calcium deposits. Even-numbered slides were kept for immunohistochemical analysis. The area of the lesion was measured with a Leica Q500 MC image analysis program (Leica Inc). Values reported represent the mean lesion area from 5 sections for each animal.

**Quantification of Lesions in Entire Aorta**
The procedure for quantification of the lesions was described in Reference 18.

### Characteristics of ApoE-Deficient Mice With and Without P-Selectin on Chow Diet

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Sex</th>
<th>Genotype</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Spleen Weight, g</th>
<th>Cholesterol, mg/dL</th>
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<tr>
<td>4</td>
<td>M</td>
<td>P&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>9</td>
<td>30.8±0.67</td>
<td>0.113±0.007*</td>
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<td>0.197±0.017</td>
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<tr>
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<td>P&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>12</td>
<td>23.1±0.53</td>
<td>0.105±0.005*</td>
<td>429±30</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>P&lt;sup&gt;+/+&lt;/sup&gt;</td>
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<td>35.4±1.84</td>
<td>0.145±0.018*</td>
<td>386±32</td>
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<tr>
<td></td>
<td></td>
<td>P&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6</td>
<td>32.1±1.13</td>
<td>0.540±0.101</td>
<td>324±49</td>
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</tbody>
</table>

Mice were maintained on standard low-fat mouse chow for 4 or 15 months, at which time plasma was collected and analyzed for total cholesterol. Values are mean±SEM. n represents the numbers of animals evaluated.

*P<0.01.

**Histological Analysis**
Calcium deposits were identified by the hematoxylin stain.<sup>18,19</sup> Mononuclear cells/macrophages were identified by their cellular and nuclear appearance and counted on hematoxylin-stained sections.

**Immunohistochemical Analysis**
To identify macrophages in spleens, frozen sections 10 μm thick were fixed in cold acetone for 5 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 minutes and then incubated with a biotin-conjugated rat anti-mouse F4/80 antibody (BioSource International). Antibodies were visualized by an avidin/biotin peroxidase–linked detection system (Vector Laboratories). To quantify smooth muscle cells, 2 sections on even-numbered slides for each mouse were stained with a mouse monoclonal antibody against human α-actin directly coupled to horseradish peroxidase (No. U7033, dilution 1:2; Dako Corp).<sup>18</sup> The α-actin–positive area was measured with an ocular micrometer.

**Statistical Analysis**
Data are presented as mean±SEM. Student’s t test was performed with software for Macintosh. Because a relatively small number of animals were available for the 15-month group, the Mann-Whitney U test (2-tailed) was selected. Pearson r product-moment correlations were calculated.

**Results**

**Comparison of ApoE-Deficient Mice With and Without P-Selectin**
We bred P<sup>−/−</sup> mice with apoE<sup>−/−</sup> mice to generate apoE<sup>−/−</sup> P<sup>−/−</sup> and apoE<sup>−/−</sup> P<sup>+/−</sup> mice. At 4 months of age on normal chow, male and female apoE<sup>−/−</sup> P<sup>+/−</sup> and apoE<sup>−/−</sup> P<sup>−/−</sup> mice had similar body weights and comparable blood levels of cholesterol (Table). However, beyond ≈12 months of age, most male apoE<sup>−/−</sup> P<sup>−/−</sup> mice started to lose body weight (wasting), whereas this did not happen to female apoE<sup>−/−</sup> P<sup>−/−</sup> mice. Therefore, at 15 months of age, apoE<sup>−/−</sup> P<sup>−/−</sup> male mice had significantly lower body weight (34.7±1.8 versus 49.3±4.6 g, n=7, P=0.0086) and plasma cholesterol (407±26 versus 756±51 mg/dL, P=0.0008) than apoE<sup>−/−</sup> P<sup>+/−</sup> control male mice. ApoE<sup>−/−</sup> P<sup>−/−</sup> female mice maintained body weights and cholesterol levels similar to those of apoE<sup>−/−</sup> P<sup>+/−</sup> control female mice at that age (Table). Because body weight and cholesterol are significant factors affecting atherosclerotic lesion development,<sup>20,21</sup> we excluded the older male mice from this study. Unlike mice deficient in P-selectin only,<sup>22</sup> apoE<sup>−/−</sup> P<sup>−/−</sup> mice consistently displayed splenomegaly. Splenic weights, normalized to body weights, from
apoE−/− P−/− and apoE−/− P+/− mice were increased ≈2-fold at 4 months of age and 4-fold at 15 months of age in favor of the apoE−/− P+/− mice (Table). Microscopic examination of spleen sections from apoE−/− P−/− mice disclosed hyperplasia with an increased amount of white pulp and expanded red pulp (not shown), with larger infiltrates of F4/80-positive macrophages (Figure 1). Weights of livers, peripheral blood total leukocyte counts, and platelet counts were comparable between apoE−/− P+/− and apoE−/− P−/− mice.

Reduction in Lesion Size in ApoE-Deficient Mice Without P-Selectin

The extent of the lesions in the aortic sinus has been shown to correlate well with that of the entire aorta in apoE−/− mice. Mean lesion areas per cross section were measured in oil red O-stained tissue sections. At 4 months of age on normal chow, apoE−/− P−/− male mice had lesions 3.6-fold smaller than the apoE−/− P+/− males (0.039±0.001 versus 0.142±0.036 mm², n=7 to 14, P=0.002), and apoE−/− P−/− female mice had lesions 3.4-fold smaller than the apoE−/− P+/− females (0.077±0.013 versus 0.258±0.03 mm², n=12 or 13, P<0.0001). The combined data from males and females are shown in Figure 2. In addition, at this time point, 74% of the aortic sinus circumference in apoE−/− P+/− mice was covered by lesions, compared with 30% found in apoE−/− P−/− mice (P<0.0001, n=19 to 27). Thus, apoE−/− P−/− mice had more lesion-free aortic sinus surface as well as smaller lesions than apoE−/− P+/− mice.

With increasing age, lesions grew in size in both apoE−/− P+/− and apoE−/− P−/− mice. However, a significant difference in lesion size between the 2 genotypes persisted at 15 months, with 2.6-fold smaller lesions in apoE−/− P−/− mice and no overlap in lesion size between the 2 groups (Figure 2). Among all the 15-month-old animals combined, the cholesterol levels did not correlate with their lesion size (r=0.2, P=NS), indicating that the differences in lesion size were not linked to minor cholesterol variations but rather to the P-selectin genotype. At this time point, the lesions spread throughout the entire aorta. The percentage of surface area occupied by the lesions was determined from the subclavian branch to the iliac bifurcation and was significantly higher in apoE−/− P+/− aortas than apoE−/− P−/− aortas (Figure 3).

P-Selectin

Figure 1. Immuno-histochemical staining of macrophages in spleens. Spleens were taken from apoE−/− P+/− (A) and apoE−/− P−/− (B) female mice at 4 months of age. Spleen sections were stained with a rat anti-mouse F4/80 monoclonal antibody and then with hematoxylin. The red pulp of apoE−/− P−/− spleens (B) was expanded, with large numbers of F4/80-positive macrophages revealed in brown color. Bar=78 μm.

Figure 2. Atherosclerotic lesion size in aortic sinus of apoE-deficient mice with (apoE−/− P+/−) and without (apoE−/− P−/−) P-selectin. Mice were maintained on a standard low-fat mouse chow diet for 4 or 15 months. Sections of aortic sinus were stained with oil red O, hematoxylin, and light green, and the area of the lesion was measured. Values reported represent mean lesion area from 5 sections for each animal. At 4 months, male and female mice were combined. At 15 months, only female mice were used. ApoE−/− P+/− vs apoE−/− P−/− at 4 months, P<0.003 by t-test; at 15 months, P<0.003 by Mann-Whitney U test.

Figure 3. Atherosclerotic lesion coverage of entire aortas of apoE-deficient mice with (apoE−/− P+/−) and without (apoE−/− P−/−) P-selectin. Female mice were maintained on a chow diet for 15 months. Aortas were collected between subclavian and ilial branches and stained with Sudan IV, and percentage of area covered by lesion (percent coverage) was determined. ApoE−/− P+/− vs apoE−/− P−/−, P<0.005 by Mann-Whitney U test; n=6 to 8.
Comparison of Lesion Composition in ApoE-Deficient Mice With and Without P-Selectin

Through histological analysis and immunohistochemical staining, we characterized the stages of the atherosclerotic lesions in the apoE⁻/⁻ P⁺/+ and apoE⁻/⁻ P⁻/⁻ mice. At 4 months of age, 70% (13/19) of apoE⁻/⁻ P⁺/+ mice had already developed fibrofatty or early fibrous plaque lesions containing smooth muscle cells (Figure 4A, A and C), and 30% (6/19) remained at the fatty streak stage. In contrast, all (27/27) of the apoE⁻/⁻ P⁺/+ mice developed only a fatty streak lesion at this stage (Figure 4B). No smooth muscle cells were found in these lesions by α-actin staining (Figure 4D). The fibrofatty lesions of apoE⁻/⁻ P⁺/+ mice contained a mixture of foam cells and spindle-shaped cells, presumably smooth muscle cells (Figure 4A). The early fibrous plaques encompassed small necrotic cores, together with a few foam cells that were covered by a well-formed fibrous cap containing α-actin-positive smooth muscle cells (Figure 4C). We stained aortic sinus sections of the 4-month-old apoE⁻/⁻ mice with hema-toxylin and counted all mononuclear cells in the lesions. We found that the lesions in P⁺/+ mice had twice as many mononuclear cells (173±27 per section, n=14) as the lesions of P⁻/⁻ mice (88±15 per section, n=19). This difference was significant (P<0.006) and showed that the absence of P-selectin inhibited the recruitment of monocytes/macrophages to the lesion.

Furthermore, at 15 months of age, the fibrous plaques in apoE⁻/⁻ P⁺/+ appeared more advanced, with larger necrotic cores, more abundant fibrous tissue, and more calcification (Figure 4E) than in apoE⁻/⁻ P⁻/⁻ mice. Although the lesions in the latter also progressed to the fibrous plaque stage at this age, they were less calcified and contained less fibrous tissue (Figure 4F). The calcification frequencies of fibrous plaque lesions were 6 of 8 in the apoE⁻/⁻ P⁺/+ and 1 of 6 in the apoE⁻/⁻ P⁻/⁻ mice. The area of the lesion covered by α-actin–positive smooth muscle cells tended to be less in apoE⁻/⁻ P⁻/⁻ mice (6.5% versus 11.8%; P=0.058).

Discussion

P-selectin is located in the membranes of α-granules in platelets and the Weibel-Palade bodies in endothelial cells. On stimulation, P-selectin is rapidly translocated to the cell surface, where it mediates adhesion to carbohydrate ligand(s) on leukocytes. P-selectin is involved in many acute inflammatory conditions mediated by neutrophils and because it regulates monocyte and T-lymphocyte emigration from the blood stream, it may also be involved in chronic inflam-
matory conditions, such as atherosclerosis. P-selectin has been detected on the surface of the endothelium in all stages of atherosclerotic lesion development in humans and in animal models of atherosclerosis. Many atherogenic factors, such as oxidized LDL and inflammatory cytokines, induce P-selectin expression. Ramos et al demonstrated that anti-P-selectin antibodies inhibit monocyte rolling on endothelium of the carotid artery isolated from apoE-deficient mice. However, these studies could not distinguish whether the increased expression of P-selectin is a cause or a consequence of the atherosclerotic process. The availability of mice with a deficiency in P-selectin makes it possible to address these issues directly. Using C57BL/6 mice fed the atherogenic diet, Nageh and colleagues observed a 50% to 70% reduction in fatty streak size in the P−/− mice. Unfortunately, because normal mice are resistant to the development of fibrous plaque, this study could not evaluate the role of P-selectin in the more advanced stages of lesion development. As described in the introduction, using LDLR-deficient mice fed the atherogenic diet, we found that the absence of P-selectin partially reduces fatty streak formation but does not affect the more advanced stages of atherosclerosis. The present study is the first to demonstrate that P-selectin is also important for lesion growth at the fibrous plaque stage.

If we consider the many differences between the LDLR-deficient and apoE-deficient mouse atherosclerosis models, it is not surprising to find that P-selectin plays a more prominent role in the apoE−/− mice than in the LDLR−/− mice. On a normal chow diet, LDLR−/− mice reach a total plasma cholesterol of 200 mg/dL, which is only 2-fold higher than that of wild-type mice, and do not develop any gross aortic lesions up to 13 months of age. In contrast, apoE−/− mice on chow food achieve cholesterol levels of 400 to 600 mg/dL and develop fatty streak and fibrofatty (intermediate lesion) and fibrous plaque starting at 2.5, 4, and 5 months of age, respectively. The increased lipoproteins, which may serve as atherogenic stimuli and inducers of adhesion molecules on endothelium in LDLR−/− mice, are the IDL and LDL, whereas the lipoproteins in apoE−/− mice are chylomicron remnants and VLDL. To induce lesion development in LDLR−/− mice, a high-fat/high-cholesterol diet such as the Paigen atherogenic diet is required. This diet, which contains cholic acid, can stimulate an inflammatory response and could induce expression of adhesion molecules such as E-selectin. Accordingly, we have found that the size of the fibrous plaque formed in the aortic sinus of LDLR-deficient mice on the atherogenic diet for 37 weeks is identical in the presence and absence of P-selectin but is reduced significantly, by 40%, when both P- and E-selectin are absent. On the basis of our present study in the apoE-deficient mouse, in which absence of P-selectin alone inhibited advanced lesion progression, we suspect that the profile and relative importance of adhesion molecules that promote lesion development in different animal models of atherosclerosis and different subgroups of human patients with this disease can vary.

One mechanism that may be responsible for decreased lesion progression in the absence of P-selectin in apoE-deficient mice is the inhibition of leukocyte, especially monocyte, infiltration into the lesions. Indeed, we observed 2-fold fewer mononuclear cells in the lesions of 4-month-old apoE-deficient mice lacking P-selectin. Monocyte attachment to endothelial cells of the aortic sinus in apoE-deficient mice was observed before obvious fatty streak formation. Macrophages loaded with fluorescent microspheres injected intravenously into 40-week-old apoE-deficient mice adhered to all stages of atherosclerotic plaques, including mature calcified fibrous lesions. Macrophages in the subendothelium may at first protect against atherosclerosis but later also contribute to lesion progression. It is also possible that the absence of P-selectin modifies systemic immune response in the animals, which could affect lesion progression. Atherosclerotic lesions, even in the apoE-deficient mouse model, are infiltrated with T helper (Th) 1 cells secreting proinflammatory cytokines. Th1 cells but not the anti-inflammatory Th2 cells can bind to P-selectin and thus efficiently enter Th1-dominated inflammatory sites. P-selectin deficiency could cause an imbalance in the recruitment of the Th1 versus Th2 cells, which could have a significant effect on the lesion progression and maturation. Indeed, we observed that the absence of P-selectin caused a delay in lesion maturation, with a smaller proportion of smooth muscle cells and reduced calcification (Figure 4). Platelets may also contribute to lesion development, and P-selectin mediates platelet interaction with both endothelial cells and leukocytes.

In this study, we unexpectedly found that apoE−/− mice had splenomegaly, with an increased number of macrophages. The cause of the large spleen in these animals is not clear. It is not due to infections, because these mice had a normal blood leukocyte count. We hypothesize that the monocytes, which were prevented from migrating into the intima in the absence of P-selectin, homed to spleen, became macrophages, and finally caused hyperplasia in this organ. Macrophage homing to spleen in apoE−/− mice appears not to depend on leukocyte adhesion molecules. Excessive, chronic accumulation of macrophages in spleen may cause these cells to become transformed. We had found on autopsy that 2 of 4 apoE−/− P−/− mice developed histiocytic sarcoma (a malignant tumor of macrophages) before 1 year of age. In addition, P-selectin has a protective role in experimental glomerulonephritis. Consistent with this finding, we also found on autopsy that 3 of 4 apoE−/− P−/− mice spontaneously developed glomerulonephritis. These diseases are the likely cause of wasting observed in the apoE−/− P−/− mice, especially males, leading to a higher mortality at 1 year (35% in apoE−/− P−/− mice versus <10% in apoE−/− P+/− or mice deficient in P-selectin only). Although anti–P-selectin therapy may be a powerful tool in inhibiting atherosclerotic lesion progression, possible side effects should be considered in evaluating its therapeutic potential.

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References

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