Decreased Atherosclerotic Lesion Formation in Human Serum Paraoxonase Transgenic Mice

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Background—Serum paraoxonase (PON1), an enzyme carried on HDL, inhibits LDL oxidation, and in human population studies, low PON1 activity is associated with atherosclerosis. In addition, PON1 knockout mice are more susceptible to lipoprotein oxidation and atherosclerosis. To evaluate whether PON1 protects against atherosclerosis and lipid oxidation in a dose-dependent manner, we generated and studied human PON1 transgenic mice.

Methods and Results—Human PON1 transgenic mice were produced by using bacterial artificial chromosome genomic clones. The mice had 2- to 4-fold increased plasma PON1 levels, but plasma cholesterol levels were unchanged. Atherosclerotic lesions were significantly reduced in the transgenic mice when both dietary and apoE-null mouse models were used. HDL isolated from the transgenic mice also protected against LDL oxidation more effectively.

Conclusions—Our results indicate that PON1 protects against atherosclerosis in a dose-dependent manner and suggest that it may be a potential target for developing therapeutic agents for the treatment of cardiovascular disease. (Circulation. 2002;106:484-490.)

Key Words: antioxidants ■ lipoproteins ■ free radicals ■ atherosclerosis ■ genes

Mounting evidence points to LDL oxidation as an important etiologic agent of atherosclerosis.1-3 LDL is believed to exit the lumen of arteries and become trapped in the subendothelial space, perhaps by the binding of apoB-100 to intimal proteoglycans. Once trapped, LDL may become oxidized directly by cellular byproducts of respiration or enzymatically by lipoxygenases, myeloperoxidase, NADPH oxidase, cytochrome P-450, and others.3-4 In in vitro assays, enzymatically by lipoxygenases, myeloperoxidase, NADPH oxidase, cytochrome P-450, and others.3,4 In in vitro assays, this minimally modified LDL is capable of inducing endothelial expression of adhesion molecules, chemokines, and cytokines. This evidence points to a mechanism whereby LDL oxidation is responsible in part for monocyte/macrophage recruitment and differentiation and, hence, the initiation of atherogenesis.

Substantial epidemiological evidence points to an inverse correlation between HDL levels and coronary artery disease.5 One plausible hypothesis explaining this phenomenon is based on the idea that HDL can exert a direct antiatherogenic effect at least in part through preventing LDL oxidation.1-3 Serum paraoxonase (PON1) is a 45-kDa glycoprotein that is expressed in the liver and has been found to be associated with HDL particles in the blood.6,7 PON1 was initially identified for its ability to hydrolyze organophosphate insecticides.8 More recently, PON1 has been shown to prevent LDL oxidation in vitro,9,10 and decreased levels of PON1 are associated with an increased risk of cardiovascular disease.10-12 In studies with PON1 knockout (KO) mice, PON1 has been shown to be both necessary and sufficient for the in vitro protective effects of HDL on LDL oxidation and monocyte transmigration.13 Furthermore, when both dietary and apoE-null models were used, PON1 KO mice exhibited an ≈2-fold increase in atherosclerosis.13,14

The present study aims to provide additional in vivo evidence showing that PON1 protects against atherosclerosis. In particular, we have addressed whether in an in vivo setting PON1 influences atherosclerosis in a dose-dependent manner.

Methods

Cloning and Microinjection of Human PON1 Gene

A clone of the human PON1 gene in a bacterial artificial chromosome vector (GenBank No. AC004022) was purchased from Genome Systems. Polymerase chain reaction (PCR) and restriction enzyme digestion confirmed that this clone contained leucine and glutamine at codons 55 and 192, respectively. This clone, designated pPON1, was purified by CsCl gradient centrifugation. The pPON1 bacterial artificial chromosome DNA was digested with Pmel and then run out on a pulse field gel to separate a 45-kb DNA fragment containing the human PON1 gene from the rest of the plasmid. The 45-kb DNA fragment was isolated from the gel, diluted to a concentration of 0.5 µg/ml, and microinjected into fertilized C57BL/6J (B6) mouse eggs (Jackson Laboratories, Bar Harbor, Me) to produce transgenic (Tg) mice.

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Mice, Diets, and Atherosclerotic Lesion Analysis
Mice on the B6 background were maintained either on a 6% fat chow diet or, in the case of atherosclerotic lesion analysis, on a high fat diet (Teklad) containing 15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate for 15 weeks. PON1 Tg mice on the B6 background were also backcrossed twice with the apoE KO mice on the B6 background (The Jackson Laboratory, Bar Harbor, Me) to obtain PON1 Tg/apoE KO mice and apoE KO littermates. These were maintained on a 6% fat chow diet. Lesions were analyzed as described.14

Southern Blot and PCR Analyses
A 32P-labeled human PON1 cDNA clone was used as a probe for Southern hybridization, and bands were visualized and quantified by using a PhosphorImager 445SI (Molecular Dynamics). For routine identification of PON1 Tg mice, PCR analysis was performed with the use of the primers 5'-GCTTGATTTTTCTCCTCCAT-3' and 5'-ATCTGTAATGTGCTAATCC-3', yielding a 194-bp product.

Northern Blot and Quantitative RT-PCR Analyses
Total RNA was subjected to Northern blot analysis as described.14 For reverse transcription (RT)-PCR analysis, first-strand cDNAs were synthesized with the use of 2 μg total RNA isolated from the thoracic portion of the aorta and by use of the Thermoscript RT-PCR system (Invitrogen). Quantitative PCR was then performed with the use of the primers 5'-GCTTGATTTTTCTCCTCCAT-3' and 5'-ATCTGTAATGTGCTAATCC-3', yielding a 194-bp product.
Atherosclerotic Lesion Formation on B6 Background
Atherosclerotic lesion formation was examined in the PON1 Tg and wild-type littermates on the B6 background that were fed a high fat diet for 15 weeks. Compared with the female non-Tg littermates, the female Tg mice exhibited a 60% decrease in atherosclerotic lesion size ($P < 0.13$, Figure 3A). The lack of a statistically significant result in the female group likely represents an effect of sample size. Compared with the male non-Tg littermates, the male Tg mice, on the other hand, exhibited a statistically significant 54% reduction in lesion size ($P < 0.001$, Figure 3B). When data from the female and male mice were combined, the PON1 Tg mice, compared with the wild-type littermates, exhibited a statistically significant (57%) decrease in atherosclerotic lesion size ($P < 0.01$, Figure 3C). Both the male and female PON1 Tg mice, compared with their wild-type littermates, exhibited similar plasma total cholesterol, VLDL/LDL cholesterol, HDL cholesterol, triglyceride, and glucose levels when they were maintained on either the chow or high fat diet (Table 1).

Atherosclerotic Lesion Formation on ApoE KO Background
Atherosclerotic lesion formation was examined in the PON1 Tg and wild-type littermates on the B6 background that were fed a high fat diet for 15 weeks. Compared with the female non-Tg littermates, the female Tg mice exhibited a 60% decrease in atherosclerotic lesion size ($P = 0.13$, Figure 3A). The lack of a statistically significant result in the female group likely represents an effect of sample size. Compared with the male non-Tg littermates, the male Tg mice, on the other hand, exhibited a statistically significant 54% reduction in lesion size ($P < 0.001$, Figure 3B). When data from the female and male mice were combined, the PON1 Tg mice, compared with the wild-type littermates, exhibited a statistically significant (57%) decrease in atherosclerotic lesion size ($P = 0.01$, Figure 3C). Both the male and female PON1 Tg mice, compared with their wild-type littermates, exhibited similar plasma total cholesterol, VLDL/LDL cholesterol, HDL cholesterol, triglyceride, and glucose levels when they were maintained on either the chow or high fat diet (Table 1).

Figure 2. Plasma PON1 activities and hepatic PON1 mRNA expression of human PON1 Tg and wild-type mice. A and B, Plasma PON1 activities of wild-type (open bars) and PON1 Tg (solid bars) mice, fed a low fat chow diet (A) or a high fat atherogenic diet for 15 weeks (B), were determined by the arylesterase activity assay. Values shown are mean ± SEM obtained from >10 mice from each genotype group. *$P < 0.0001$ for PON1 Tg vs wild-type mice. C, Liver RNA samples (20 µg) from wild-type (left) or Tg (right) mice fed chow or high fat diet for 15 weeks were subjected to Northern blot analysis with use of probes for mouse Pon1 (upper left) or human PON1 (upper right). To standardize for loading, a mouse GAPDH cDNA probe was later used to hybridize the same filters (bottom). Hybridized bands were then quantified by PhosphorImager (see Methods).

Figure 3. Decreased atherosclerotic lesion formation in PON1 Tg mice on C57BL/6J mouse background. Atherosclerotic lesions at aortic root were quantified in female wild-type (open bar, $n = 8$) and PON1 Tg (solid bar, $n = 8$) mice (A) or male wild-type ($n = 17$) and PON1 Tg ($n = 16$) mice (B) fed atherogenic diets for 15 weeks. Combined data from panels A and B are shown in panel C. Values shown are mean ± SEM. *$P < 0.01$ and **$P < 0.001$ for PON1 Tg mice vs wild-type mice.

When fed a chow diet, the PON1 Tg/apoE KO mice, compared with their apoE KO littermates, exhibited 2.5-fold higher plasma PON1 (arylesterase) activities (Table 2), whereas plasma total cholesterol, HDL cholesterol, VLDL/LDL cholesterol, triglyceride, and glucose levels were the same between the 2 groups (Table 2). Atherosclerotic lesion sizes of PON1 Tg/apoE KO mice at 6.5 months of age, on a chow diet, were 22% smaller than those of their apoE KO littermates ($P < 0.01$) (Figure 4A). Aortic MCP-1 expression was also reduced by 44% in the PON1 Tg/apoE KO mice compared with their apoE KO littermates ($P = 0.05$) (Figure 4B).
In Vitro Oxidation of LDL

Previously, we observed that PON1-deficient HDL lacked the ability to prevent LDL oxidation in a cell culture system of the arterial wall.13 In the present study, we examined the ability of HDLs isolated from mice of the human PON1 Tg mice and wild-type mice on the B6 background as well as on the apoE KO mouse background to prevent LDL oxidation induced by copper in vitro. For HDL isolated from mice on the B6 background, we found that the PON1 Tg HDL, compared with the wild-type HDL, exhibited 3.2-fold higher arylesterase activity (152 000 versus 48 000 mOD·min⁻¹·mg protein⁻¹). At a dose of 0.25 mg HDL/mL, compared with the LDL plus wild-type HDL group, the LDL plus PON1 Tg HDL group exhibited statistically significant 33% (P<0.01, Fisher’s PLSD) and 14% (P<0.001) decreases in lipid hydroperoxide levels after 3 and 6 hours of incubation, respectively (Figure 5A). At a higher dose of 0.5 mg HDL/mL, compared with the LDL plus wild-type HDL group, the LDL plus PON1 Tg HDL group exhibited statistically significant 62% (P<0.01, Fisher’s PLSD) and 19% (P<0.0001) reduction in lipid hydroperoxide levels after 3 and 6 hours of incubation, respectively (Figure 5B). Thus, PON1 Tg HDL prevents LDL oxidation more effectively than does the wild-type HDL.

We also examined HDLs isolated from mice on the apoE KO background for their ability to protect against LDL oxidation. We found that the PON1 Tg/apoE KO HDLs, compared with the apoE KO HDLs, exhibited 3.7-fold higher arylesterase activity (139 000 versus 37 000 mOD·min⁻¹·mg protein⁻¹). At a dose of 0.25 mg HDL/mL and after 3 hours of incubation, the LDL plus PON1 Tg/apoE KO HDL group, compared with the LDL plus apoE KO HDL group, exhibited a significant 15% (P<0.001, Fisher’s PLSD) decrease in lipid hydroperoxide level (Figure 6A). After 6 hours of incubation and at the same dose of 0.25 mg HDL/mL, compared with the LDL group, neither the LDL plus PON1 Tg/apoE KO HDL group nor the LDL plus apoE KO HDL group exhibited any significant reduction in lipid hydroperoxide level (Figure 6A). At a higher dose of 0.5 mg HDL/mL, the LDL plus PON1 Tg/apoE KO HDL group, compared with the LDL plus apoE KO HDL group, exhibited statistically significant 21% (P<0.0001, Fisher’s PLSD) and 14% (P<0.01) reduction in lipid hydroperoxide levels after 3 and 6 hours of incubation, respectively (Figure 6B). Thus, on the apoE KO mouse background, PON1 Tg HDL was also more effective in preventing LDL oxidation than was wild-type HDL.

**Discussion**

Both biochemical11–13 and epidemiological16–19 studies suggest that LDL oxidation plays a major role in the development of atherosclerosis. However, human clinical trials and animal studies that used antioxidant supplementation to treat cardiovascular disease have been inconclusive.20–22 Alternative targets for the prevention and/or treatment of atherosclerosis

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### TABLE 1. Plasma Lipid and Glucose Levels of PON1 Tg and Wild-Type Mice Fed Chow or High-Fat Diet

<table>
<thead>
<tr>
<th>Sample, n</th>
<th>Cholesterol, mg/dL</th>
<th>VLDL/LDL Cholesterol, mg/dL</th>
<th>HDL Cholesterol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
<th>Glucose, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, chow diet</td>
<td>15</td>
<td>97±4</td>
<td>19±3</td>
<td>77±2</td>
<td>30±6</td>
</tr>
<tr>
<td>Male, chow diet</td>
<td>14</td>
<td>90±4</td>
<td>18±3</td>
<td>72±2</td>
<td>22±8</td>
</tr>
<tr>
<td>Female, high-fat diet</td>
<td>19</td>
<td>105±2</td>
<td>19±2</td>
<td>86±2</td>
<td>42±6</td>
</tr>
<tr>
<td>Male, high-fat diet</td>
<td>23</td>
<td>107±2</td>
<td>18±1</td>
<td>88±1</td>
<td>35±3</td>
</tr>
</tbody>
</table>

Data are mean±SEM.

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### TABLE 2. Plasma Arylesterase Activity, Glucose, and Lipid Levels of PON1 Tg/ApoE KO Mice and ApoE KO Mice Fed a Chow Diet

<table>
<thead>
<tr>
<th>Sample, n</th>
<th>Arylesterase Activity, mOD₂₇₀·min⁻¹·µL</th>
<th>Total Cholesterol, mg/dL</th>
<th>VLDL/LDL Cholesterol, mg/dL</th>
<th>HDL Cholesterol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
<th>Glucose, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE KO</td>
<td>18</td>
<td>247±13</td>
<td>486±47</td>
<td>461±48</td>
<td>25±3</td>
<td>57±4</td>
</tr>
<tr>
<td>PON1 Tg/ApoE KO</td>
<td>18</td>
<td>610±42*</td>
<td>472±24</td>
<td>445±23</td>
<td>27±2</td>
<td>56±5</td>
</tr>
</tbody>
</table>

Data are mean±SEM.

*P<0.0001 vs corresponding value for apoE KO mice.
are antioxidative enzymes on HDL, such as PON1. We have previously shown that mice lacking PON1 had increased atherogenesis, which is consistent with predictions from in vitro studies. To determine whether high levels of PON1 can protect against LDL oxidation and decrease atherosclerosis in vivo, we have generated and characterized human PON1 Tg mice. Our results, indicating protection against both early and late stages of atherogenesis, are significant in that they demonstrate a dose-dependent effect of PON1. Hence, normal levels of PON1 are limiting with respect to their antiatherosclerotic activity. The present study is also the first to demonstrate that the human PON1 protein exhibits antioxidative and antiatherogenic functions in vivo.

We observed that the human PON1 transgene was expressed in a liver-specific manner (Figure 1), similar to the expression pattern of the endogenous mouse Pon1 gene, suggesting that the transgene constructs contain the necessary cis-regulating elements for liver-specific expression. Interestingly, expression of the human PON1 mRNA, unlike the endogenous mouse Pon1 mRNA, was not decreased by the high fat diet (Figure 2C). It is unclear whether this represents a true difference between human and mouse regulation of PON1 or if it is simply due to the construct that we used. On the basis of Northern blot analysis (Figure 2C), we conclude that the high fat diet–induced reduction of plasma total PON1 activities in the PON1 Tg and wild-type mice (Figure 2B versus 2A) is probably caused by the decreased mouse Pon1 mRNA levels.

We observed that HDL isolated from the PON1 Tg mice, compared with the wild-type HDL, exhibited ~3-fold higher PON1 activity and was more effective at protecting against LDL oxidation (Figures 5 and 6). Several antioxidative functions of PON1 have been elucidated and include a phospholipase A2–like activity that hydrolyzes biologically active oxidized phospholipids (such as phosphatidylcholine...
Human epidemiological studies have revealed an association between low PON1 levels and an increased risk for coronary artery disease. In the present study, we observed that a moderate increase in PON1 levels protects against atherosclerotic lesion formation in the PON1 Tg mice, compared with the wild-type mice, at both the early fatty streak stage (B6 model) and the intermediate-advanced-lesion stage (apoE KO model). Thus, PON1 may be a potential therapeutic agent for the prevention and/or treatment of atherosclerosis. Consistent with its ability to reduce cardiovascular mortality, a recent study has demonstrated that moderate alcohol consumption increases plasma HDL, apoA-I, and PON1 levels in volunteers. Also, cigarette smoking is associated with reduced serum PON1 activity. Interestingly, flavonoids, such as glabridin (found in licorice) and quercetin (found in red wine), have been shown to scavenge reactive oxygen species and preserve the antioxidative functions of PON1 in vitro. Supplementation of flavonoids protects LDL against oxidation and attenuates atherosclerosis in animal models. Therefore, it will be interesting to examine whether a combined supplementation of flavonoids (or other antioxidants) and PON1 will exert a synergistic effect on protection against LDL oxidation and atherosclerosis in animal models. Further analysis of the regulation of PON1 levels in humans may yield promising avenues for atherosclerosis therapy.

Acknowledgments
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