Adenovirus-Mediated Expression of Human Paraoxonase 3 Protects Against the Progression of Atherosclerosis in Apolipoprotein E–Deficient Mice

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Objective—We have previously reported that human paraoxonase 3 (PON3) is an HDL-associated protein capable of preventing LDL oxidation in vitro. The objective of the present study was to determine whether elevated levels of human PON3 in mice could protect against the progression of atherosclerosis in vivo.

Methods and Results—Twenty-six week-old apolipoprotein E–deficient mice were injected with 3×10¹¹ particles of adenovirus expressing either GFP alone (AdGFP) or together with human PON3 (AdPON3). Three weeks after injection, lesion area was significantly lower in AdPON3-treated mice compared with AdGFP controls. Serum from AdPON3 mice contained significantly lower levels of lipid hydroperoxides and exhibited an enhanced potential to efflux cholesterol from cholesterol-loaded macrophages. In addition, LDL was less susceptible to oxidation, whereas HDL was more capable of protecting against LDL oxidation. Exogenous human PON3 was not detected in the serum or HDL and more surprisingly we demonstrate that endogenous mouse PON3 is not associated with HDL, suggesting that the antioxidant function of PON3 is at the cellular level in mice.

Conclusions—This study demonstrates for the first time that PON3 enhances the antiatherogenic capacity of serum and protects against the progression of atherosclerosis in vivo. (Arterioscler Thromb Vasc Biol. 2007;27:1368-1374.)

Key Words: paraoxonase ■ atherosclerosis ■ lipoproteins ■ oxidative stress ■ antioxidants

Atherosclerosis is a chronic inflammatory disease characterized by the narrowing, thickening, and accumulation of fatty deposits in the artery wall. Increasing evidence suggests that the oxidation of LDL plays an integral role in the initiation and progression of this process. Oxidized LDL has been reported to trigger a number of putatively atherogenic events including the induction of proinflammatory molecules that lead to an increase in recruitment of inflammatory cells to the artery wall. HDL, on the other hand, is antiatherogenic, and its protective effects have been ascribed primarily to its ability to shuttle excess cholesterol from peripheral tissues to the liver in the reverse cholesterol transport pathway and also to its capacity to protect against LDL oxidation. These protective effects of HDL have been attributed to various proteins associated with HDL in the circulation including apolipoprotein AI, lecithin cholesteryl-acyl transferase (LCAT), and serum paraoxonases (PONs).

The PON gene family consists of 3 members, PON1, PON2, and PON3, that share approximately 79% to 90% sequence identity at the amino acid level among mammalian species. Whereas PON1 and PON3 have been reported to associate with HDL in the circulation, PON2 appears to remain intracellular, associated with the membrane fractions of cells. Interestingly, PON1 is the only member of the PON family that exhibits paraoxonase activity. All 3 PON proteins do, however, have the capacity to hydrolyze various lactones. Although their physiological function is unknown, studies suggest that the PON proteins may possess antiatherogenic properties. PON1 knockout mice develop significantly larger atheromas in the aortic root when compared with their wild-type counterparts. In addition, transgenic mice over-expressing PON1 exhibit significantly less aortic lesions relative to wild-type controls. In humans, PON1 activity toward paraoxon has been shown to be an independent risk factor for coronary heart disease. Although much less is known about PON2 and PON3 relative to PON1, recent studies suggest that PON2 and PON3 may also possess similar antiatherogenic capacities. PON2 has been reported to possess antioxidant properties and to prevent LDL oxidation in vitro. PON2-deficient mice have been generated and, similar to PON1 knockout mice, PON2-deficient mice develop significantly larger atheromatous lesions than their wild-type counterparts. Similar to PON1 and PON2, we and others have previously reported...
that PON3 can protect against LDL oxidation in vitro.\textsuperscript{6,7,13} The objective of the present study was to determine whether an elevation in human PON3 could protect against atherosclerosis in vivo. Several studies have demonstrated that first generation adenoviral vectors are capable of inducing transgene expression/protein activity for 3 to 4 weeks after injection in a number of mouse models, including the apolipoprotein E-null mouse.\textsuperscript{14–17} Therefore, in this study, we used adenoviral vectors to study the effects of PON3 overexpression in a mouse model of established atherosclerosis. The results of this study suggest that PON3 protects against the progression of atherosclerosis in vivo.

Methods

Generation of Recombinant Adenovirus

Recombinant adenovirus was generated using the AdEasy system previously described.\textsuperscript{18} Briefly, shuttle vector containing full-length human PON3 cDNA with GFP or GFP alone was cotransformed with adenoviral backbone plasmid pAdEasy-1 for homologous recombination in E.coli BJ5183 cells. Positive recombinants were linearized and transfected into 293 cells for virus packaging and propagation. Adenoviruses expressing PON3 and GFP (AdPON3) or GFP alone (AdGFP) were purified by CsCl banding and stored at \(-80^\circ\)C until use.

RT-PCR

Organs from injected animals were collected and RNA extracted using RNeasy kit (Qiagen). First strand cDNA was synthesized using oligo dT primers and Omniscript RT (Invitrogen). Two microliters of first strand cDNA was subject to PCR analysis using GFP specific primers: forward, CAC CCT CGT GAC CAC CCT GA; reverse, GGA TGC AGG GAT GAT GTT CT. The PCR conditions were: 95°C for 4 minutes, 35 cycles of 94°C for 0.5 minutes, 61°C for 1 minute, 72°C for 1 minute, and 72°C for 10 minutes followed by 4°C hold.

Quantitative RT-PCR Analysis

Total RNA was isolated from various tissues and reverse transcribed into first strand cDNA as described above. Real-time RT-PCR was then performed using the QuantiTect SYBR Green PCR kit (Qiagen) in an ABI Prism 7700 cycler. The primers used for specific cDNA synthesis were: human PON3: 5'-GCC ACC AGA GAC CAC TAT TTC ACC A-3' and 5'-ATC ACC TTC AGT TGA GTT AAA GCC ACC AGA GAC CAC TAT TTC ACC A-3'; mouse GAPDH: 5'-AAG TGC AGG GAT GAT GTT CT-3'. The PCR conditions were: 95°C 15 minutes, 40 cycles of 94°C 15 sec, 60°C for 30 sec and 72°C 30 sec, and 4°C hold. The data were first expressed as ratios of human PON3 to mouse GAPDH.

Western Blot Analysis

Western blot analysis was performed as previously described.\textsuperscript{19} Briefly, 10 \(\mu\)g of protein extract or 0.25 \(\mu\)L of serum was fractionated by SDS PAGE and electroblotted onto nitrocellulose membranes. Membranes were incubated with either anti-human PON3,\textsuperscript{3} anti-mouse PON3 (kindly provided by Dr Luvisi, UCLA), or anti-mouse apoAI (Abcam) antibodies diluted 1:1000, 1:1000, and 1:20,000, respectively. Two hours later, membranes were washed and incubated with anti-rabbit–HRP secondary antibody for 1 hour. Human and mouse PON3 and mouse apoAI proteins were detected using ECL plus.

Lactonase, Paraoxonase, and LCAT Activities

Lactonase Activity

PON3 lactonase activity was measured as previously described.\textsuperscript{6} Briefly, 2 \(\mu\)g of 293 cell membrane extract\textsuperscript{8} or 10 \(\mu\)g of liver cell membrane extract was incubated at 25°C with 1.2 mmol/L lovastatin substrate for 30 minutes or 3 hours, with 293 cells or liver membrane extracts, respectively. The reaction was quenched and isocratically eluted in 78:22:0.2, \(\nu/\nu\) acetonitrile/water/acetic acid using high performance liquid chromatography.

Paraoxonase Activity

Serum PON1 activity was assessed using paraoxon as the substrate. PON1 activity was determined by measuring the increase in absorbance at 405 nm over a period of 12 minutes. A unit of PON1 activity was defined as the formation of 1 nmol of 4-nitrophenol per minute per ml of serum applied.

LCAT Activity

Serum LCAT activity was assessed using a kit from Roar Biomedical and measured by taking the ratio of two emission intensities: 470 nm (which represents the intact substrate) and 390 nm (which represents the LCAT hydrolyzed monomer).

Experimental Design

Twenty-six week-old female apoE\textsuperscript{-/-} mice (Jackson Laboratory, Bar Harbor, Me) fed a normal chow diet (Harlan Teklad) were injected via the tail vein with 3\(\times10^6\) viral particles of AdGFP (\(n=14\)) or AdPON3 (\(n=15\)). Three weeks after injection, apoE\textsuperscript{-/-} mice were euthanized, the serum isolated for lipid/lipoprotein analysis, and the hearts harvested for lesion analysis. The Animal Research Committee at UCLA approved this protocol.

Lipid Analysis

Serum was isolated from fasted mice 3 weeks after treatment with adenovirus. Triglycerides (TG), total cholesterol (TC), and HDL cholesterol (HDL-C) were measured with standard kits (Thermo). VLDL/LDL cholesterol (VLDL/LDL-C) was determined by subtracting HDL-C from TC.

Cholesterol Efflux

Cellular cholesterol efflux was performed as previously described.\textsuperscript{19} Briefly, human monocyte-macrophages at 5\(\times10^5\) cells/mL were incubated overnight with medium containing 10% lipoprotein-deficient serum. The cell were washed and further incubated with 1 \(\mu\)Ci/mL \(\nu\)-labeled cholesterol and 50 \(\mu\)g/mL of acetylated-LDL. 48 hours later, cells were washed and equilibrated in 1% essential fatty-acid free albumin. Efflux potential of sera was determined by incubating diluted sera (5% serum) with labeled cells for 4 hours. Cholesterol efflux was expressed as the percentage of total radioactive counts released to the medium.

Lipid Hydroperoxides

Lipids from the serum of AdGFP or AdPON3-treated mice were extracted with chloroform/methanol (2:1), and lipid hydroperoxides were quantified spectrophotometrically by the method described by Auerbach and coworkers.\textsuperscript{21}

LDL Induced Monocyte Chemotactic Activity and HDL Antiinflammatory Properties

LDL and HDL fractions were isolated from serum by fast-protein liquid chromatography. LDL-induced monocyte chemotactic activity and HDL antiinflammatory properties were assessed as previously described.\textsuperscript{11} Briefly, 100 \(\mu\)g of LDL, or 50 \(\mu\)g of HDL+100 \(\mu\)g of control human LDL were added to a confluent monolayer of human aortic endothelial cells. Supernatants were collected and tested for monocyte chemotactic activity using a Neuroprobe chamber (Neuro Probe) with a polycarbonate filter of 5 \(\mu\)m pore size separating the upper and lower wells. Supernatants were added to the bottom chamber and freshly isolated monocytes to the top chamber. Membranes were fixed with 1% glutaraldehyde and stained with 0.1% Crystal Violet and the number of migrated monocytes was determined microscopically and expressed as mean±SD.
Lesion Analysis
Three weeks after injection, aortas were analyzed for atheromatous lesions as previously described. Briefly, the heart including the proximal aorta was isolated and embedded in OCT compound. Serial 10-μm thick cryosections of aorta were stained with Oil Red O and hematoxylin. The mean area of lipid staining per section from 10 sections was determined for each mouse. Image-Pro Plus software was used for quantification (Media Cybernetics).

Statistical Analysis
Statistical significance was determined by student t test. A value of P<0.05 was considered statistically significant.

Results
Recombinant adenoviral vectors capable of expressing green fluorescent protein (AdGFP) or active human PON3 protein together with GFP (AdPON3) were generated as previously described (supplemental Figure IA and IB, available online at http://atvb.ahajournals.org). To determine the utility of these first generation adenoviruses to initiate transgene expression in our animal model, a time course and tissue distribution study was performed by injecting 26 week-old apoE−/− mice with AdGFP. Liver targeting by adenoviruses after systemic injection is rapid and indeed, adenovirus mediated transgene expression of GFP was detected in the livers of mice at 1, 7, 14, and 21 days after injection by RT-PCR analysis (supplemental Figure II).

After intravenous administration of AdPON3, human PON3 message was detected in all livers tested (Figure 1A). As previously reported, transgene expression was also detected in numerous tissues including the lungs, kidneys, spleen, as well as the aorta (Figure 1A). Quantitative real time PCR analysis on mRNA isolated from individual mouse livers showed that transgene expression peaked at day 7 but was undoubtably present throughout the course of the study (supplemental Figure III). Three weeks after adenovirus administration, PON3 lactonase activity, using lovatide levels, or in total, HDL, and VLDL/LDL cholesterol were unable to detect human PON3 in either serum or HDL fractions of mice administered AdPON3 (data not shown). Further evaluation of endogenous mouse PON3 revealed that although it is expressed in the liver, in contrast to human PON3, mouse PON3 does not associate with HDL in the circulation.

To study the effects of PON3 overexpression on atheroma progression in vivo, 3×10^11 viral particles of AdPON3 or control AdGFP were injected into 26 week-old apoE−/− mice. There was no significant difference in body weight, triglyceride levels, or in total, HDL, and VLDL/LDL cholesterol levels between AdGFP and AdPON3 treated mice (Table). However, mice treated with AdPON3 exhibited a significant suppression in atheroma formation relative to their AdGFP-treated control counterparts (Figure 3). With an average lesion area of 3.44×10^5±0.36 μm^2, 26 week-old apoE−/− mice on normal chow clearly have established atheromatous lesions. Three weeks after adenovirus administration, the lesion area of AdGFP-treated mice progressed to 3.90×10^5±0.25 μm^2. In contrast, AdPON3-treated mice exhibited a capacity to suppress the progression of atheroma formation, with lesions averaging 3.27×10^5±0.26 μm^2 (P<0.05 versus AdGFP). Moreover, apoE-null mice given PBS alone did not differ significantly in lesion development when compared with AdGFP-treated group (Figure 3).

Serum Antiatherogenic Capacity
To determine the potential mechanism(s) by which elevated levels of PON3 inhibited the progression of atheroma formation, the antiatherogenic capacity, or more specifically, the antioxidant capacity and cholesterol efflux potential of serum was assessed. Three weeks after adenovirus administration, serum from AdPON3-treated mice exhibited an increase in antioxidant capacity, containing significantly lower levels of lipid hydroperoxides compared with mice treated with...
AdGFP (3592.8±1028.3 versus 5640.8±1602.1 ng LOOH, \( P<0.05 \); Figure 4A). In addition, LDL isolated from AdPON3-treated mice also contained significantly lower levels of lipid hydroperoxides relative to controls (669.1±301 versus 438.6±101 ng LOOH, \( P<0.05 \); Figure 4B). Furthermore, serum from AdPON3-treated mice demonstrated an enhanced ability to efflux cholesterol from cholesterol-loaded macrophages relative to their AdGFP treated counterparts (12.83±0.64 versus 10.19±0.75% cholesterol efflux, \( P<0.05 \); Figure 4C). These improvements in serum antioxidant capacity and efflux potential were independent of PON1 activity, apoAI protein levels, and LCAT activity (supplemental Figure IVA through IVC).

**LDL Induced Monocyte Chemotaxis and HDL Antiinflammatory Properties**

The modification of LDL in the subendothelial space of the artery wall is believed to trigger a number of proinflammatory events including the recruitment of monocytes into the intima. HDL has been shown to inhibit this process.\(^7\) To determine whether PON3 protects against atherosclerosis by modulating the properties of circulating lipoproteins, LDL-induced monocyte chemotactic activity and the capacity of HDL to protect against LDL-induced monocyte chemotaxis was determined. LDL isolated from AdPON3-treated mice was less susceptible to oxidation, inducing significantly less monocyte chemotactic activity compared with LDL from AdGFP-treated mice (5.86±2.28 versus 11.18±3.97 monocytes, \( P<0.05 \); Figure 5A). Moreover, HDL isolated from AdPON3-treated mice exhibited enhanced antiinflammatory properties; significantly inhibiting LDL-induced monocyte chemotactic activity relative to mice treated with AdGFP (2.67±0.83 versus 6.66±1.65 monocytes, \( P<0.05 \); Figure 5B).

**Discussion**

Epidemiological, genetic, and biochemical studies support an antiatherogenic role for PON1. PON3 exhibits high sequence homology with PON1, with \( \approx 61\% \) similarity at the protein level and \( \approx 68\% \) at the nucleotide level. In humans, PON3 like PON1 has been reported to associate with HDL in the circulation.\(^6,7\) Together, these observations suggest that PON3, similar to PON1, may possess antiatherogenic properties, and in vitro studies have supported a protective role for PON3 in atherogenesis.\(^7\) The aim of this study was to determine whether an increase in PON3 protein could protect against the progression of atherosclerosis in vivo. We chose to use an adenoviral vector to overexpress PON3 in apoE\(^{-/-}\) mice to determine the effects of a brief elevation in PON3 protein in a setting of established atherosclerosis. Our results demonstrate that elevated levels of PON3 suppress the progression of atheromatous lesion formation in 26 week-old apoE\(^{-/-}\) mice independent of significant changes in serum lipid levels.

Although the precise mechanism by which PON3 suppresses lesion formation is unknown, the reduction in atheroma is, in part, mediated by the ability of PON3 to enhance the antiatherogenic properties of lipoproteins. Oxidized lip-
Lipoproteins have been shown to induce a number of proinflammatory events that lead to the formation of fatty streak lesions. In previous studies, lipoproteins incubated with supernatants from cells overexpressing PON3 were less proinflammatory and contained fewer lipid hydroperoxides. In this study, serum and LDL from AdPON3 treated mice exhibited lower levels of oxidized lipids, containing significantly less lipid hydroperoxides than their control counterparts. Moreover, LDL isolated from AdPON3 treated mice was significantly less susceptible to oxidative modification, whereas HDL from these same mice demonstrated enhanced antiinflammatory properties.

In contrast to PON1, very little PON3 is associated with HDL in humans. Interestingly, PON3 protein is virtually undetectable in mouse HDL. In the present study, we were unable to detect either human PON3 or endogenous mouse PON3 in the serum of AdPON3-treated mice by Western blotting. Incidentally, because of nonspecific hydrolysis of lovastatin in mouse plasma, PON3 specific activity could not be determined. However, adenovirus-mediated transgene expression was clearly detected in a number of tissues/organs, including the aorta, kidney, liver, lung, and spleen (Figure 1A). These results suggest that the mechanism, by which PON3 modulates lipoprotein properties, is likely occurring at the site(s) of lipoprotein synthesis. Indeed, AdPON3 administration led to a ∼2-fold increase in PON3 lactonase (LV hydrolase) activity in the livers of mice 3 weeks after injection (Figure 1B). Previous studies using purified recombinant PON proteins have shown that among the PON family, LV hydrolase is specific to PON3.

Generation of VLDL and subsequently LDL particles, and HDL particles begin with the secretion of apolipoproteins and lipids and end in the liver with the reuptake or exchange of lipoprotein particles and lipids, respectively. Yang and colleagues recently reported that overexpression of the intracellular antioxidant enzymes catalase or catalase/Cu/Zn-superoxide dismutase (SOD) led to the retardation of atheroma development in apoE−/− mice. In addition, mice overexpressing catalase or catalase/Cu/Zn-SOD exhibited significantly lower levels of F2-isoprostanes in the plasma and aorta relative to control apoE−/− mice and mice overexpressing only Cu/Zn-SOD. The results of this study suggest that (1) the activity and expression levels of cell-associated antioxidant enzymes can modulate the oxidative status of plasma components, and (2) hydrogen peroxide plays a role in the lipid peroxidation process that is implicated in atherogenesis. Indeed, PON1 has been reported to exhibit peroxidase-like activities, and PON3 may be directly reducing lipid hydroperoxides through a similar activity.

We have previously shown that PON2, another member of the PON gene family, is a ubiquitously expressed cell-
associated protein with antioxidant properties capable of preventing cell-mediated oxidative modification of low density lipoprotein. Most recently, we have reported that mice deficient in PON2 develop significantly larger (2.7-fold) atherosclerotic lesions compared with their wild-type counterparts. Interestingly, enhanced inflammatory properties of LDL, attenuated antiatherogenic capacity of HDL, and a heightened state of oxidative stress coupled with an exacerbated inflammatory response from PON2-deficient macrophages were determined to be the main mechanisms behind the larger atherosclerotic lesions in PON2-deficient mice. Thus, it is plausible that PON3 is also playing an antiatherogenic role through mechanisms similar to those used by PON2.

The ability of HDL to extract cholesterol from peripheral tissues including the artery wall is believed to protect against atherogenesis. A number of agents that enhance the reverse cholesterol transport pathway have been shown to decrease the formation of atheroma. In addition to HDL, a number of other factors in the serum have been shown to modulate cholesterol efflux, including various free apolipoproteins, enzymes, and lipids. Because we did not detect either the endogenous mouse PON3 or human PON3 on HDL from AdPON3-injected apoE−/− mice, we tested whole serum in place of isolated lipoprotein fractions to include all the potential factors that influence cholesterol efflux. We found that serum from mice treated with AdPON3 exhibited enhanced abilities to efflux cholesterol from cholesterol-loaded macrophages compared with their control counterparts (Figure 4C).

The exact mechanism by which PON3 expression influences serum cholesterol efflux potential is not known. However, a plausible mechanism for the improved cholesterol efflux in AdPON3 mice may be the improved antioxidant function of HDL. Indeed, prooxidant HDL has been reported to be less effective in effluxing cholesterol from cholesterol-loaded macrophages relative to control HDL. Our data suggest that HDL from AdPON3-treated mice was significantly more protective against LDL oxidation in a monocyte chemotaxis assay, when compared with HDL from AdGFP treated mice (Figure 5) suggesting that PON3 overexpression improved HDL function in this study.

PON1, ApoAI, and LCAT are HDL associated proteins that play a significant role in the reverse cholesterol pathway. Previous reports have demonstrated that oxidative stress can influence the function of these proteins in the cholesterol transport pathway. However, there was no significant difference in serum apoAI protein levels or in serum LCAT activity between AdPON3-treated mice and their control counterparts. Thus, the improvement in serum cholesterol efflux seen in AdPON3-treated mice can be attributable to enhanced HDL function and/or serum components other than PON1, ApoAI, and LCAT.

Taken together, the data presented in this study demonstrates for the first time that in a setting of established atherosclerosis, elevated levels of PON3 can enhance the cholesterol efflux potential of serum, decrease LDL oxidation, increase the antioxidant properties of HDL, and ultimately protect against the progression of atherosomatic lesion formation in vivo.

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Disclosures

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Supplemental Figure I. Adenovirus mediated gene expression and activity in HEK 293 cells. A. Protein extracts from HEK 293 cells were collected after transducing with AdPON3 for the indicated amount of time. 10 µg of protein extracts were subjected to western analysis for PON3. B. Protein extracts from HEK 293 cells were collected after transducing with AdPON3 or AdGFP for the indicated amount of time and 2 µg of protein extracts were subjected to PON3 lactonase activity assay as described in Methods.
**Supplemental Figure II.** Adenovirus-mediated GFP expression. 3x1011 viral particles of AdGFP were injected into 26 week old apoE-/- mice. Livers were collected on day 0, day 1, day 7, day 14, and day 21 post-injection, RNA extracted, and GFP expression analyzed by RT-PCR.

**Supplemental Figure III.** Adenovirus-mediated human PON3 expression. ApoE-/- mice were intravenously administered AdPON3 and sacrificed at day 0, 1, 7, 14, and 21 post-injection. Total RNA was isolated from livers (n=4) and real-time RT-PCR was then performed. Data presented as ratio of human PON3 transgene to mouse GAPDH expression (*: p<0.05).
Supplemental Figure IV. Effect of adenoviral treatment on PON1, ApoAI, and LCAT. Three weeks after treatment with either AdGFP (n=7) or AdPON3 (n=9), (A) serum PON1 activity towards paraoxon was measured, (B) mouse apoAI protein levels were assessed by western blot analysis, and (C) LCAT activity (described under methods) was determined by taking the ratio of emission intensities between 470nm (which represents the intact substrate) and 390nm (which represents the LCAT hydrolyzed monomer). Values are expressed as mean±SD.
Supplemental Figure IV

C.

LCAT activity
(470/390nm)

AdGFP  AdPON3

0.00  0.01  0.02  0.03  0.04  0.05  0.06