Regression of Atherosclerosis Induced by Liver-Directed Gene Transfer of Apolipoprotein A-I in Mice

Rajendra K. Tangirala, PhD; Kazuhisa Tsukamoto, MD; Sam H. Chun, BA; David Usher, PhD; Ellen Pure’, PhD; Daniel J. Rader, MD

Background—The ability of apolipoprotein (apo)A-I to induce regression of preexisting atherosclerotic lesions has not been determined, and a mouse model of atherosclerosis regression has not yet been reported.

Methods and Results—LDL receptor–deficient mice were fed a western-type diet for 5 weeks to induce atherosclerotic lesions. A second-generation recombinant adenovirus encoding human apoA-I or a control adenovirus were injected intravenously in order to express apoA-I in the liver. Three days after injection, total apoA-I levels in mice injected with the apoA-I–expressing adenovirus were $216 \pm 16.0$ mg/dL, compared with $68.0 \pm 3.0$ mg/dL in control virus–injected mice ($P<0.001$). HDL cholesterol levels in mice injected with the AdhapoA-I vector 7 days after injection were $189 \pm 21.0$ mg/dL, compared with $123 \pm 8.0$ mg/dL in control virus-injected mice ($P<0.02$). Total and non-HDL cholesterol levels did not differ between the 2 groups. Atherosclerotic lesion area was quantified by en face analysis of the aorta and cross-sectional analysis of the aortic root. Compared with baseline mice, atherosclerosis progressed in mice injected with the control adenovirus. In contrast, in mice expressing apoA-I compared with baseline mice, total en face aortic lesion area was reduced by 70% and aortic root lesion was reduced by 46%. Expression of apoA-I was associated with a significant reduction in the fraction of lesions occupied by macrophages and macrophage-derived foam cells.

Conclusions—Liver-directed gene transfer of human apoA-I resulted in significant regression of preexisting atherosclerotic lesions in LDL receptor–deficient mice as assessed by 2 independent methods. (Circulation. 1999;100:1816-1822.)

Key Words: atherosclerosis • liver • genes • apolipoproteins

Epidemiological data indicate a strong inverse association between HDL cholesterol levels and coronary heart disease (CHD).1–3 Low levels of HDL cholesterol are associated with significantly increased risk of CHD.4 Genetic syndromes of high HDL are associated with longevity and decreased incidence of CHD.5,6 Thus, the concept of intervention targeted toward HDL as a method of preventing or treating atherosclerotic cardiovascular disease is attractive.7,8

See p 1762

HDLs are macromolecular complexes consisting of lipids and a variety of apolipoproteins, enzymes, and transfer proteins.9 Apolipoprotein A-I (apoA-I) is the major protein component of HDL. Animal studies have provided substantial support for the concept that injection or expression of apoA-I inhibits the initiation and progression of atherosclerosis. Weekly intravenous injection of purified rabbit apoA-I reduced the progression of atherosclerosis in cholesterol-fed rabbits (despite no change in HDL cholesterol levels).10 Transgenic overexpression of human apoA-I in hyperlipidemic Watanabe heritable hyperlipidemic rabbits delayed the development of atherosclerosis.11 Overexpression of human apoA-I in transgenic mice reduced atherogenesis in C57BL/6 mice fed a high-fat diet,12 in apoE-deficient mice,13,14 and in human apo(a)–transgenic mice.15 Somatic gene transfer of human apoA-I reduced neointima formation after endothelial denudation in apoE-deficient mice16 and reduced progression of atherosclerosis in human apoA-I transgenic/apoE-deficient mice.17 These studies indicate that apoA-I is effective in delaying the progression of atherosclerosis in animals. However, there are substantially fewer data regarding the ability of HDL or apoA-I to induce regression of preexisting atherosclerotic lesions, a question more relevant to the potential therapeutic application of this approach in patients with established atherosclerotic disease. In their pioneering studies, Badimon and colleagues18 demonstrated that weekly intravenous injection of rabbit HDL induced regression of atherosclerotic lesions in cholesterol-fed rabbits. However, in addition to apoA-I, HDL contains a variety of other proteins that have been demonstrated to have antiatherogenic properties, including apoE,19,20 apoA-IV,21,22 lecithin–cholesterol acyltransferase (LCAT),23 cholesteryl ester transfer protein...
Therefore, we used somatic gene transfer in an established mouse model of atherosclerosis to test the hypothesis that expression of apoA-I alone would induce regression of preexisting atherosclerotic lesions. We found that hepatic expression of human apoA-I induced significant regression of established atherosclerotic lesions in LDL receptor (LDLR)–deficient mice after 4 weeks.

Methods

Construction of Recombinant Adenoviruses

A second-generation recombinant adenovirus encoding the human apoA-I cDNA was constructed as previously described. Briefly, pAdCMVapoAI was linearized with NheI and cotransfected into 293 cells along with adenoviral DNA isolated from H5.110CMVlacZ digested with Clal, and cells were overlaid with agar and incubated at 32°C for 15 days. Plaques positive for the apoA-I cDNA were subjected to a second round of plaque purification, and the recombinant adenovirus (designated AdhapoA-I) was expanded in 293 cells at 32°C. The null adenovirus (designated Adnull) was constructed and expanded in an identical manner. The purified viruses were stored in 10% glycerol/PBS at −80°C.

Animal Studies

Eight-week-old female LDLR-deficient mice (backcrossed 6 times with C57BL/6 mice) were obtained from Jackson Laboratory (Bar Harbor, Me) and were fed a western diet27 (normal chow supplemented with 0.15% cholesterol and 20% butterfat). Five weeks after initiation of the diet, mice were randomly assigned to 3 groups; 1 group of mice (n=15) was killed for baseline quantification and examination of atherosclerotic lesions, and the remaining mice were injected intravenously with AdhapoA-I (n=12) or control Adnull virus (n=11) at a dose of 6.0×10^9 particles (2.0×10^8 pfu)/g body wt. Blood was obtained from the retro-orbital plexus after a 4-hour fast 1 day before injection, 3 days after injection, and weekly over the course of the next 4 weeks. Blood samples collected into a tube containing EDTA, NaN3, gentamicin, PMSF, and benzamidine (final concentrations, 2 mmol/L, 0.2%, 0.77%, 1 mmol/L, and 1 mmol/L, respectively) were immediately centrifuged to obtain plasma, which was stored either at −20°C for plasma lipid analyses or at 4°C for fast protein liquid chromatography (FPLC). Mice were killed 4 weeks after injection for quantification and morphological characterization of atherosclerotic lesions.

Plasma ApoA-I, HDL, and Lipid Levels in LDLR-Deficient Mice Injected With Control and Human ApoA-I–Expressing Second-Generation Adenovirus

<table>
<thead>
<tr>
<th>Day</th>
<th>TG (mg/dL)</th>
<th>Cholesterol</th>
<th>ApoA-I</th>
<th>A-I/HDL Ratio</th>
<th>TG (mg/dL)</th>
<th>Cholesterol</th>
<th>ApoA-I</th>
<th>A-I/HDL Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>500</td>
<td>1183</td>
<td>1032</td>
<td>151</td>
<td>64</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(35)</td>
<td>(107)</td>
<td>(77)</td>
<td>(15)</td>
<td>(8)</td>
<td>(0.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>491</td>
<td>1347</td>
<td>1182</td>
<td>165</td>
<td>68</td>
<td>0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(45)</td>
<td>(82)</td>
<td>(76)</td>
<td>(13)</td>
<td>(3)</td>
<td>(0.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>312</td>
<td>1566</td>
<td>1443</td>
<td>123</td>
<td>66</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(26)</td>
<td>(76)</td>
<td>(71)</td>
<td>(8)</td>
<td>(3)</td>
<td>(0.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>347</td>
<td>1515</td>
<td>1372</td>
<td>143</td>
<td>64</td>
<td>0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(42)</td>
<td>(96)</td>
<td>(87)</td>
<td>(12)</td>
<td>(4)</td>
<td>(0.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>217</td>
<td>1176</td>
<td>1057</td>
<td>119</td>
<td>54</td>
<td>0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(35)</td>
<td>(65)</td>
<td>(55)</td>
<td>(12)</td>
<td>(2)</td>
<td>(0.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>321</td>
<td>1298</td>
<td>1137</td>
<td>161</td>
<td>57</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(35)</td>
<td>(53)</td>
<td>(40)</td>
<td>(7)</td>
<td>(4)</td>
<td>(0.04)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TG indicates triglyceride. Data represent mean expressed as mg/dL±SEM (in parentheses). Day 0 plasma values were before virus injection and day 3 through 28 values were after injection.

*Significantly different from Adnull values, P<0.01.
En Face Quantification of Atherosclerotic Lesions in the Aorta

Mice were killed, and after the aorta had been perfused with ice-cold PBS for 10 minutes via the left ventricle, the heart was exposed, and the lower half of the heart was cut off at the base in a plane perpendicular to the aortic root. The upper half of the heart with aortic root was embedded in OCT and frozen at $-80^\circ C$. The remainder of the aorta was removed by cutting off minor branching arteries and fixed in formal-sucrose (4% paraformaldehyde, 5% sucrose, 20 $\mu$m EDTA, pH 7.4). After the adventitial and adipose tissue had been removed, aortas were cut open longitudinally, stained with Sudan IV, and pinned out as previously described.28 The extent of atherosclerosis in the aortas was quantified by use of images of aortas captured with a Dage-MTI 3CCD 3-chip color video camera (Dage-MTI Inc) connected to a Leica MZ 12 dissection microscope. The captured 24-bit digitized color images were analyzed, and the lesion areas covering the aortas were determined by use of Image Pro Plus image analysis software (Media Cybernetics). The acquisition of aortic images and the analysis of lesion areas were both performed in a blinded fashion.

Immunohistochemistry and Quantification of Atherosclerotic Lesions in the Aortic Root

The fresh-frozen OCT-embedded hearts were used for immunohistochemistry of lesions in the aortic root. Serial sections (8 $\mu$m) of the aortic root were mounted on masked slides. Sections were fixed in acetone, air-dried, and rehydrated in PBS containing 0.02% NaN$_3$, and blocked with 1% BSA in PBS/NaN$_3$. For detection of macrophages, sections were reacted with monoclonal rat anti-murine MAC-1 antibody,29 monoclonal hamster anti-CD18,30 and monoclonal hamster anti-CD11c,30 followed by incubation with mouse anti-rat IgG or goat anti-hamster IgG in the presence of 200 $\mu$g/mL normal mouse IgG. Antibody reactivity was detected with horseradish peroxidase–conjugated biotin-streptavidin complexes and developed with diaminobenzidine tetrahydrochloride (DAB) as substrate. Immunostaining was photographed on a Leica microscope. Images were captured digitally with a video camera connected to a Leica microscope. The digitized color images were analyzed and lesion and macrophage areas determined with Image Pro Plus image analysis software (Media Cybernetics). Total lesion area was quantified by manual tracing of entire intimal lesions in 4 equally spaced aortic root sections per mouse. Macrophage area was quantified in the same sections by determination of the area within lesions stained by the macrophage markers noted above. The acquisition of images and analysis of lesions were performed in a blinded fashion.

Analytical Methods

The plasma total cholesterol, HDL cholesterol, and triglyceride levels were measured enzymatically on a Cobas Fara II (Roche Diagnostic Systems Inc) with Sigma reagents (Sigma Chemical Co) as described.26 Total plasma apoA-I levels were quantified by use of an immunoturbidimetric assay (Sigma) as previously described.26 Human apoA-I concentrations were quantified with an ELISA. Maxisorb 96-well, flat-bottom microtiter plates (NUNC) were coated overnight at 4°C with 100 $\mu$L per well of 5 $\mu$g/mL monoclonal
ApoA-I Expression and Effects on Plasma Lipids

Injection of AdhapoA-I in LDLR-deficient mice resulted in expression of human apoA-I, which was sustained through the 4 weeks of the experiment (Figure 1). Total plasma apoA-I levels were also increased as a result of the human apoA-I expression (Table). Expression of human apoA-I resulted in increased HDL cholesterol levels (Table). This increase was seen after FPLC gel filtration of lipoproteins from plasma 7 days after injection (Figure 2). The ratio of total apoA-I to HDL cholesterol was significantly higher in the AdhapoA-I–injected mice compared with the Adnull-injected mice at all time points (Table), consistent with the presence of relatively lipid-poor apoA-I. Total and non-HDL cholesterol levels were not different between the 2 groups (Table).

Quantitative Effects of ApoA-I Expression on Preexisting Atherosclerotic Lesions

Representative examples of en face Sudan IV–stained aortas from mice at baseline, injected with Adnull, and injected with AdhapoA-I are shown in Figure 3. Compared with baseline mice, lesions in the Adnull-injected mice progressed over 4 weeks, whereas those in AdhapoA-I–injected mice regressed. These data are illustrated quantitatively in Figure 4A. Total aortic lesion area was 161 210±37 210 μm² in baseline mice, 202 680±43 930 μm² in Adnull-injected mice, and 48 150±10 700 μm² in AdhapoA-I–injected mice (P<0.02 AdhapoA-I versus baseline and P<0.001 AdhapoA-I versus Adnull). Expression of human apoA-I induced a 70% regression in lesion area compared with baseline mice and a 76% reduction compared with control mice. Lesion area was significantly reduced in AdhapoA-I mice both in the aortic arch and in the remainder of the aorta.

Total lesion area was also quantified in the aortic root (Figure 4B). Mice injected with Adnull demonstrated a modest 28% progression over 4 weeks compared with base-
line mice. In contrast, mice injected with AdhapoA-I demonstrated a significant 46% regression of aortic root lesions compared with baseline mice and a 58% reduction compared with control mice. Thus, expression of human apoA-I induced regression of atherosclerosis over 4 weeks as assessed by 2 independent methods of quantification at 2 different sites within the aorta.

To examine the relationship between liver-derived apoA-I expression and the extent of atherosclerotic lesions, the correlation between total apoA-I exposure and atherosclerotic lesion area by en face analysis was evaluated. The analysis of the 2 parameters showed a negative correlation between the apoA-I exposure and the extent of atherosclerotic lesions in the aorta ($r = -0.63$, $P < 0.002$) (Figure 5).

**Effects of ApoA-I Expression on Lesion Morphology**

To determine the morphological effects of apoA-I expression on atherosclerotic lesions, we performed immunohistochemistry on fresh-frozen sections in the aortic root. Representative sections are shown in Figure 6. Aortic root lesions in AdhapoA-I–injected mice appeared less rich in foam cells and more fibrotic than lesions in Adnull-injected mice. The percent of total lesion area occupied by macrophages and macrophage-derived foam cells was significantly reduced in apoA-I–expressing mice compared with baseline and control injected mice (Figure 7).

**Discussion**

In this study, we demonstrate that somatic gene transfer and expression of human apoA-I in the liver induced regression of preexisting atherosclerotic lesions in mice after 4 weeks. Previous studies demonstrated that transgenic apoA-I overexpression inhibited the progression of atherosclerotic lesions in rabbits$^{10,11}$ and mice.$^{12-15}$ However, regression of preexisting atherosclerotic lesions potentially involves physiological mechanisms other than inhibition of progression and is more relevant to the development of a therapeutic approach for established atherosclerotic cardiovascular disease. Badimon and colleagues$^{18}$ demonstrated that weekly intravenous injection of rabbit HDL induced regression of atherosclerotic lesions in cholesterol-fed rabbits. However, HDL contains a variety of proteins that have antiatherogenic properties. Therefore, we used somatic gene transfer to test the hypothesis that specific expression of apoA-I alone would induce regression of preexisting atherosclerotic lesions in a mouse model of atherosclerosis.

Although HDL contains apoA-I, and plasma levels of HDL cholesterol and apoA-I are highly correlated, it cannot be assumed that the antiatherogenic properties of HDL are entirely due to the presence of apoA-I. HDL has been demonstrated in vitro to decrease neutrophil degranulation and superoxide production,$^{32}$ inhibit LDL oxidation,$^{33}$ protect endothelial cells against the effects of oxidized LDL,$^{34}$ and prevent the cytokine-induced upregulation of adhesion molecules on endothelial cells,$^{35}$ effects that have not been demonstrated to be specifically due to the apoA-I component of the HDL particle. HDL contains a variety of proteins that have antiatherogenic properties in addition to apoA-I that have been shown to be antiatherogenic in vivo, including apoE,$^{19,20}$ apoA-IV,$^{21,22}$ LCAT,$^{23}$ CETP,$^{24}$ and paraoxonase.$^{25}$ The ability of apoA-I to induce cholesterol efflux from cells$^{16}$ and therefore promote the process of reverse cholesterol transport$^6$ is thought to be 1 major mechanism of its ability to inhibit atherogenesis. It is believed that lipid-poor apoA-I, rather than HDL-associated apoA-I, is more likely to gain access to the arterial intima and serve as the initial acceptor of cholesterol that has desorbed
from cells. However, even apoA-I-deficient plasma has the ability to induce substantial cholesterol efflux from cells, at least partly because of the presence of apoE and apoA-IV. Therefore, considerable additional experimentation will be required to dissect the antiatherogenic effects of apoA-I itself, independent of other components and properties of HDL that may also contribute to its antiatherogenic effects.

In the present study, injection of a recombinant second-generation adeno virus resulted in sustained expression of human apoA-I in LDLR-deficient mice. Although plasma levels of human apoA-I decreased between 7 and 28 days, at 28 days they were still in the same range as in human apoA-I transgenic/apoE-deficient mice in which human apoA-I expression reduced the progression of atherosclerosis. HDL cholesterol levels were increased only modestly and were not sustained, but the total apoA-I/HDL cholesterol ratios were significantly increased throughout the 4 weeks, suggesting that much of the apoA-I was contained in particles that were smaller and less lipid-rich. Despite its modest effect on HDL cholesterol levels, apoA-I expression resulted in significant regression of atherosclerotic lesions. These results suggest that expression of apoA-I alone has a significant impact on atherosclerosis and that increases in HDL cholesterol per se are not required for the antiatherogenic effect of apoA-I.

ApoA-I expression also induced morphological changes in lesions consistent with greater reduction in macrophages and macrophage-derived foam cells relative to nonmacrophage content. Loss of macrophage-derived foam cell mass could be consistent with an effect of apoA-I in promoting cholesterol efflux from foam cells. However, it is possible that apoA-I directly induced the death of existing foam cells by necrosis or apoptosis, and more experiments will be necessary to address these hypotheses. This study suggests that somatic gene transfer will be a useful tool to examine the in vivo cellular and molecular mechanisms by which apoA-I and other antiatherogenic proteins modulate the size and composition of preexisting atherosclerotic lesions.

Despite the clinical efficacy of cholesterol reduction in reducing cardiovascular events, novel therapeutic approaches to atherosclerosis are still needed, and apoA-I is an attractive target for therapeutic intervention. Purified human apoA-I has been safely infused into humans with modest effects on plasma apoA-I and HDL cholesterol concentrations. Repeated infusions of recombinant apoA-I protein represents a possible approach to treatment of atherosclerosis. The results presented here support the concept that somatic liver-directed gene transfer and systemic expression of apoA-I could be another potential strategy for treating established atherosclerotic disease. Within only 4 weeks of apoA-I gene transfer, lesions had regressed in size and changed in morphology. Further experiments will be necessary to determine the natural history of these regressed lesions once the plasma human apoA-I has been reduced to very low levels. It is possible that the effects of inducing substantial lesion regression could persist considerably longer than the transgene expression itself. If this were the case, short-term high-level expression of apoA-I could modify existing atherosclerotic lesions and potentially have longer-term benefit with regard to atherosclerotic disease and its sequelae.

In summary, gene transfer and expression of human apoA-I in the livers of LDLR-deficient mice for 4 weeks induced significant regression of preexisting atherosclerosis as well as morphological changes in lesions. Somatic gene transfer will be a useful tool to examine the in vivo cellular and molecular mechanisms by which apoA-I and other antiatherogenic proteins modulate the size and composition of preexisting atherosclerotic lesions and could potentially be developed as a novel clinical approach to the treatment of atherosclerotic cardiovascular disease.

Acknowledgments

This work was supported in part by grant HL-55323-01 from the National Heart, Lung, and Blood Institute; a Grant-in-Aid from the American Heart Association; and a grant from the W.W. Smith Charitable Trust. Dr Tsukamoto was supported in part by the Mochida Memorial Foundation for Medical and Pharmaceutical Research. We are indebted to Pearle Smith for excellent technical assistance and to Dr Jane Glick for helpful discussions.

References


Regression of Atherosclerosis Induced by Liver-Directed Gene Transfer of Apolipoprotein A-I in Mice

Rajendra K. Tangirala, Kazuhisa Tsukamoto, Sam H. Chun, David Usher, Ellen Puré and Daniel J. Rader

_Circulation_. 1999;100:1816-1822
doi: 10.1161/01.CIR.100.17.1816

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/100/17/1816

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/