Somatic Gene Transfer of Human ApoA-I Inhibits Atherosclerosis Progression in Mouse Models

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Background—Apolipoprotein (apo) A-I is the major component of HDL, and it displays antiatherogenic properties.

Methods and Results—The human apoA-I gene has been transferred into different mouse models by use of a recombinant adenovirus under the control of an RSV-LTR promoter (AV RSV apoA-I). Administration of AV RSV apoA-I to C57BL/6 mice resulted in moderate expression of human apoA-I for 3 weeks, leading to a transient elevation (40% at day 11 after injection) of HDL cholesterol concentration. In contrast, administration of AV RSV apoA-I to human apoA-I–transgenic mice induced a large increase of human apoA-I and HDL cholesterol concentrations (300% and 360%, respectively, at day 14 after injection) for 10 weeks, indicating that an immune response to the transgene was one major hurdle for long-term duration of expression. Recombinant adenovirus expressing human apolipoprotein A-I (AV RSV apoA-I) was also injected into human apoA-I–transgenic/apoE-deficient mice, which are prone to develop atherosclerosis. Over a 6-week period, overexpression of human apoA-I inhibited fatty streak lesion formation by 56% in comparison with control.

Conclusions—Somatic gene transfer of human apoA-I prevents the development of atherosclerosis in the mouse model. (Circulation. 1999;99:105-110.)

Key Words: genes ■ apolipoproteins ■ atherosclerosis

More than half of patients with angiographically confirmed coronary heart disease (CHD) before the age of 60 years have a familial lipoprotein disorder. Reduced HDL cholesterol is the most common lipoprotein abnormality (39% of cases of CHD). Epidemiological studies have consistently demonstrated a strong inverse correlation between plasma levels of HDL cholesterol and the incidence of CHD. HDL and its major apolipoprotein (apo), apoA-I, are thought to directly limit the development of atherosclerosis. In humans, apoA-I is synthesized by both liver and intestine. ApoA-I represents 70% of the protein component of HDL, and its concentration is directly correlated with HDL cholesterol levels. ApoA-I plays a predominant role in the molecular architecture of HDL. ApoA-I–containing lipoproteins are heterogeneous in hydrated densities and sizes. In addition to the epidemiological data, several animal studies have shown that apoA-I prevents atherosclerosis development. Infusion of apoA-I–containing lipoproteins in rabbits inhibits lesion formation. Overexpression of human apoA-I in specific inbred strains of animals, natural cholesterol–fed C57BL/6 mice and New Zealand White rabbits, or in genetically engineered strains of animals, apoE-deficient mice and human apo(a)–transgenic mice, protects against atherosclerosis. ApoA-I–containing lipoproteins appear to exert their antiatherogenic effect by (1) facilitating reverse cholesterol transport, during which cholesterol excess is transported away from cells of extrahepatic tissues and carried back to the liver, where it can be eliminated or reused; (2) inhibiting lipoprotein oxidation; and (3) directly protecting the vessel wall from damages.

Recently, gene therapy approaches for atherosclerosis using adenovirus-mediated transfer of human apoA-I gene in normal mice demonstrated a transient expression of the transgene associated with elevations of HDL concentration. In these studies, human apoA-I expression was under the control of cytomegalovirus (CMV) promoter. CMV promoter has been used extensively to achieve high expression of genes. It displayed a strong transient activity when expressed in an adenoviral vector. However, its activity is greatly reduced after a short period of time; human apoA-I expression lasted only 2 weeks. Moreover, expression controlled by CMV promoter is rapidly downregulated or repressed in the liver and is not an attractive candidate promoter for hepatic long-term expression. Another viral...
promoter, the Rous sarcoma virus long-terminal repeat (RSV-LTR) promoter, which allowed high and long-duration (>1 month) expression in the liver, has also been described.\textsuperscript{22} Therefore, to increase the duration of expression and to generate conclusive data on the role of apoA-I in HDL metabolism and its effect on atherogenesis progression in animal models, we constructed an adenoviral vector encoding human apoA-I under the control of the RSV-LTR promoter. In addition, because the transgene is of human origin and thus can generate an immune response in mice,\textsuperscript{23} human apoA-I-transgenic mice were used to abolish this immune reaction and then improve the duration of the transgene expression. In this article, we demonstrate that somatic adenovirus-mediated apoA-I gene transfer inhibits the development of atherosclerosis in the mouse model.

Methods

Recombinant Adenovirus Preparation

Recombinant vectors were constructed on the basis of pAV RSV–β-galactosidase (βGal) by standard techniques as described previously.\textsuperscript{24} The coding region from apoA-I cDNA,\textsuperscript{25} generated by reverse transcription–polymerase chain reaction from HepG2 cells, is cloned under the control of the RSV-LTR promoter from pAV RSV-βGal and the bGH polyadenylation site from the pRc/CMV plasmid (Invitrogen). The recombinant adenovirus was constructed by in vivo homologous recombination in 293 cells between shuttle plasmids and AV RSV-βGal and plaque purified. Recombinant adenosivirus encoding no transgene (AV Empty) was constructed by homologous recombination in Escherichia coli as described.\textsuperscript{26} AV Empty contains the CMV promoter in place of E1 adenovirus sequences but encodes no transgene. High-titer stocks, 10^11 pfu/mL determined by plaque assays on 293 cells, were produced in 293 cells and purified by CsCl gradients.

Animal Experiments

The study protocol was approved by the Animal Use Committee of Rhône-Poulenc Rorer. C57BL/6 mice, human apoA-I–transgenic mice,\textsuperscript{27} and apoE-deficient mice expressing human apoA-I\textsuperscript{10} have been described previously. All mice had a C57BL/6 background. Mice 8 to 10 weeks old were treated by tail-vein injection of purified recombinant adenosivirus stocks. Blood was taken from the retro-orbital plexus of mice fasted for 3 hours. Plasma was separated by centrifugation at 2800g for 20 minutes at 4°C. At the end of the experiment, animals were killed for histological analysis. Injections of 1×10^6, 3×10^6, and 1×10^9 pfu of adenovirus in mice resulted in expression of the transgene. Very high inflammation in the liver and some difficulties in recovering were observed in mice receiving the adenovirus-induced acute-phase response. Therefore, 3×10^9 pfu of the virus was chosen as the optimal dose for mice.

Protein and Lipoprotein Analysis

Cholesterol was measured colorimetrically with a commercially available kit (Boehringer Mannheim). Plasma lipoprotein distribution was assayed by analytical gel filtration chromatography, with a Superose 6 HR 10/30 column (Pharmacia). Plasma levels of human apoA-I were determined by rocket immunoelectrophoresis (Sebia). There was no cross-reactivity between human and mouse apoA-I in this assay. The size of plasma human apoA-I–containing lipoproteins was determined by nondenaturing gradient polyacrylamide gel electrophoresis\textsuperscript{28} followed by a Western blot analysis using specific anti–human apoA-I antibodies.\textsuperscript{29} Blots were analyzed by quantitative scanning densitometry (Hoefer GS-300).

Results

Adenovirus-Mediated Human ApoA-I Gene Transfer in Normal Mice

In vivo expression of human apoA-I was assessed by injection of increasing doses of AV RSV apoA-I in normal C57BL/6 mice (data not shown). At the optimal dose (3×10^9 pfu per animal), plasma levels of human apoA-I peaked at day 14 (130±10 mg/dL) and decreased to the detection levels (n=5) 31 days after injection (Figure 1). Analysis of lipoprotein profiles 14 days after vector administration indicated the presence of a large amount of HDL, and a significant increase (+40%) of HDL cholesterol was measured (Figure 1). No
effect on lipoprotein profile was observed in AV RSV βGal–treated mice (n=5).

Adenovirus-Mediated Human ApoA-I Gene Transfer in Human ApoA-I–Transgenic Mice
To avoid the effect of the immune reaction against the human protein, transgenic mice for human apoA-I were used. Basal levels of human apoA-I were 143±5 mg/dL, whereas endogenous mouse apoA-I was undetectable. Transgenic mice were injected with 3×10^9 pfu of the AV RSV apoA-I (n=10) or with PBS (n=5). Plasma levels of human apoA-I and HDL cholesterol increased at day 7 and remained significantly elevated during the 6 weeks of the experiment (P<0.001 and P<0.001 for human apoA-I and HDL cholesterol levels, respectively). Human apoA-I–transgenic mice injected with PBS showed no variation in the human apoA-I levels.

Figure 2. Effects of adenovirus-mediated gene transfer on the transgene expression in human apoA-I–transgenic mice. Mice were injected with 3×10^9 pfu of AV RSV apoA-I (●) or with PBS (■). Plasma levels of human apoA-I and HDL cholesterol increased at day 7 and remained significantly elevated during the 6 weeks of the experiment (P<0.001 and P<0.001 for human apoA-I and HDL cholesterol levels, respectively). Human apoA-I–transgenic mice injected with PBS showed no variation in the human apoA-I levels.

mg/dL) was observed after human gene transfer for all time points. Altogether, these data demonstrated that human apoA-I gene delivery in mice that displayed mouse- or human-type HDL was distinct in term of levels and duration of transgene expression.

We investigated modifications of HDL structure after human apoA-I gene transfer. Before and after infection by the adenovirus, apoA-I–containing lipoproteins of human apoA-I–transgenic mice had a bimodal distribution, with major peak sizes of 9.1±0.4 and 12.1±0.3 nm (Stokes diameters) corresponding to HDL3 and HDL2 fractions. These data were in agreement with those previously reported. These data indicated that no alteration of HDL structure occurred after human apoA-I gene delivery in human apoA-I–transgenic mice. Analysis of the distribution of human apoA-I by analytical gel filtration chromatography indicated that all human apoA-I was associated with HDL (data not shown).

Adenovirus-Mediated Human ApoA-I Gene Transfer in ApoE-Deficient Mice Expressing Human ApoA-I
ApoE-knockout/human apoA-I–transgenic mice were created by crossing human apoA-I–transgenic mice with apoE-deficient mice. Female mice (9 weeks old) were infused with 3×10^9 pfu of the AV RSV apoA-I (●), AV Empty (■), or PBS (◇). Plasma levels of human apoA-I and HDL cholesterol increased at day 14 and remained significantly elevated during the 6 weeks of the experiment (P<0.001 and P<0.001 for human apoA-I and HDL cholesterol levels, respectively). Human apoA-I–transgenic mice injected with PBS or AV Empty showed no variation in human apoA-I and HDL cholesterol levels.
Expression of human transgene increased HDL cholesterol level (Figure 3) from 60±4 to 174±10 mg/dL 14 days after injection. HDL cholesterol concentration remained elevated in AV RSV apoA-I–infused mice during the experiment. HDL cholesterol levels were correlated with human apoA-I concentrations (P<0.005). Non-HDL cholesterol concentrations in apoE-deficient mice expressing human apoA-I were 469±12 mg/dL (basal level). There was no difference in non-HDL cholesterol levels between groups of mice treated with AV RSV apoA-I, AV Empty, or PBS during the experiment.

Atherosclerosis progression in the proximal aorta was determined. Analysis of fatty-streak lesions (Figure 4) indicated that mean lesion areas were significantly higher in the PBS (n=14) and AV Empty (n=8) groups of mice than in the AV RSV apoA-I–treated mice (n=21) (P<0.01 and P<0.01, respectively). Mean lesion area for the PBS group was 16 872±1897 μm². Lesions in mice treated with AV Empty and AV RSV apoA-I were 94.31±5.44% and 56.4±1.52% of control (PBS group), respectively. Mean lesion areas (logarithmically calculated) were highly correlated with plasma concentrations of human apoA-I and HDL cholesterol (P<0.01 for both). Initial mean lesion areas (day 0) were 2038±403 μm², ie, 12.08±0.64% of control (PBS group). These data indicated that overexpression of human apoA-I retarded atherosclerosis development in apoE-deficient mice expressing human apoA-I.

To gain insight into the molecular mechanism of the protective effect mediated by human apoA-I, the promotion of cholesterol efflux from cells was investigated. Cholesterol efflux (Figure 5) promoted from cholesterol-preloaded Fu5AH cells was 1.6-fold greater (P<0.001) with the incubation of sera (14 days after injection) from the AV RSV apoA-I–treated mice than from PBS-treated mice. There was no difference between cholesterol efflux induced by the incubation of sera from PBS- and AV Empty–treated mice.

**Discussion**

This study described the effects of administration of recombinant adenovirus encoding human apoA-I in different mouse models. The use of adenoviral vector permitted a high level of human apoA-I expression, which was associated with increased HDL cholesterol concentrations. The use of the RSV-LTR promoter to control the transgene expression as well as of transgenic mice as recipient mice allowed a long-term duration of human apoA-I expression. These modifications of HDL metabolism led to an inhibition of atherosclerosis development in the apoE-deficient mouse model.

Adenovirus-mediated gene delivery of human apoA-I demonstrated that it is possible to increase the levels of apoA-I and HDL cholesterol in the plasma of human apoA-I–transgenic mice by nearly 300%, whereas only moderate increases of these parameters were observed in normal mice. The dramatic difference in the level of transgene expression between transgenic and nontransgenic mice reflected the lack of a reaction against the protein of foreign origin. The levels of human apoA-I expression obtained in transgenic animals were higher than in the nontransgenic models, and the duration of expression was also increased from weeks to months. Nevertheless, an immune reaction against viral proteins may still be present with this first-generation adenoviral vector and may contribute to the extinction of transgene expression by elimination of virus-transduced cells. Recently, several groups have reported solutions to this problem, such as transient immunosuppression, neonatal administration of the vector, or modification of the viral vector. We showed here that immune response against the foreign protein is one major hurdle to obtaining stable expression for several months and to producing data in the study of lipoprotein metabolism and atherogenesis. In addition, the use of RSV-LTR promoter, which may appear less strong in direct transgene expression at the peak level in comparison with that of a CMV promoter, allowed a constant expression for a longer period of time without side effects such as the hypertriglyceridemia that was observed.

To assess the beneficial effect of apoA-I on atherosclerosis, we evaluated human apoA-I gene transfer in apoE-deficient mice expressing basal levels of human apoA-I. ApoE-knockout mice develop atherosclerotic lesions resembling those observed in humans. We demonstrated here that...
overexpression of human apoA-I after adenovirus-mediated transfer of the human apoA-I gene was associated with a 2-fold increase of HDL cholesterol and a 2-fold decrease in the development of atherosclerotic lesions over a 6-week period. HDL- and apoA-I–containing lipoproteins have been proposed to protect against atherogenesis by removing excess cholesterol in peripheral cells and transporting it back to the liver. Although other mechanisms have been proposed for the antiatherogenic role of HDL, the present in vitro cellular cholesterol efflux study supports this hypothesis. In addition, of course, direct vascular protective effects of apoA-I may also occur. Gene transfer of apoe, LDL receptor, or VLDL receptor in apoE- and LDL receptor–deficient animals (reviewed in Reference 37) has been used successfully and resulted in a modification of plasma lipid concentrations to normal levels, ie, a correction of the lipoprotein phenotype. We showed here that overexpression of apoA-I, which did not normalize the lipoprotein profile, can reduce atherosclerotic lesion formation.

The vast majority of hypoalphalipoproteinemic patients having some residual apoA-I levels in the circulation develop premature CHD. Data emerging from large epidemiological surveys have suggested that each 1% increase in HDL cholesterol plasma concentration results in a 3% to 4% decrease in CHD risk. In addition, overproduction of human apoA-I has been found in some subjects and was associated with high HDL cholesterol levels, an absence of CHD, and longevity. Therefore, overexpression of apoA-I to higher-than-normal concentrations can be considered as potential therapy to increase HDL concentration and induce inhibition or regression of atherosclerotic lesions in a large population. We have recently reported another potential therapy for hypoalphalipoproteinemic patients with adenosine-mediated transfer of the human lecithin-cholesterol acyltransferase (LCAT) gene. Human LCAT gene transfer in human apoA-I–transgenic mice led to an increase of HDL cholesterol and human apoA-I plasma levels, probably due to a delay in apoA-I–containing lipoprotein catabolism. Similar constructs and animal models will be necessary to compare or combine these 2 approaches.

The present study demonstrates the potential of gene transfer strategy in the appropriate animal model to gain insight into the role of apoA-I in lipoprotein metabolism as well as in the atherosclerotic process.

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References


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