Decreased Susceptibility to Diet-Induced Atherosclerosis in Human Apolipoprotein A-II Transgenic Mice

Anne Tailleux, Muriel Bouly, Gérard Luc, Graciela Castro, Jean-Michel Caillaud, Nathalie Hennuyer, Philippe Poulain, Jean-Charles Fruchart, Nicolas Duverger, Catherine Fiévet

Abstract—Studies performed in vivo have been controversial regarding the implication of human apolipoprotein (apo)A-II in the atherogenic process. Expression of human apoA-II in transgenic mice fed a chow diet leads to (1) a bimodal distribution of high density lipoprotein (HDL) size as in humans, (2) a reduction in total cholesterol concentration that is mainly due to a reduction in non–HDL cholesterol level, and (3) a dramatic reduction in mouse endogenous apoA-I and apoA-II. After 20 weeks on an atherogenic diet, transgenic mice had reduced total cholesterol concentrations because of a reduction in cholesterol associated with all lipoprotein classes. Endogenous apoA-I and apoA-II were also dramatically decreased in transgenic mice. The mean area of atherosclerotic lesions was drastically decreased in transgenic mice (−44%, \( P=0.0027 \)) compared with control mice. The amount of aortic surface covered by lesions was positively correlated with very low density lipoprotein cholesterol (\( P<0.01 \)) and intermediate density lipoprotein cholesterol levels (\( P<0.05 \)). Transgenic mice were protected against the development of atherosclerosis despite a marked decrease in HDL cholesterol and apoA-I concentrations. This protection may be related to the marked reduction in circulating low density lipoprotein (very low density and intermediate density lipoprotein) levels in transgenic mice.

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Key Words: atherosclerosis ■ transgenic mice ■ lipoproteins

In most populations, plasma HDL levels correlate inversely with the incidence of atherosclerotic cardiovascular disease.\(^1\) Of the 2 major HDL-associated apolipoproteins, apoA-I and apoA-II, the protective effect of apoA-I is established, whereas relatively little is known about the role of apoA-II in HDL metabolism and how this might relate to the antiatherogenic properties of HDL. In humans, apoA-II levels do not correlate with HDL cholesterol (HDL-C) levels.\(^2\) However, in some epidemiological studies, apoA-II levels are inversely correlated with coronary heart disease susceptibility, as are HDL-C and apoA-I.\(^1\) One patient with total apoA-II deficiency did not show any lipoprotein alteration.\(^3\) In contrast, clinical observations and tissue culture studies suggest that increased levels of apoA-II may be proatherogenic by increasing the concentration of HDL containing both apoA-I and apoA-II, a particle with properties thought to be less favorable than HDL containing only apoA-I.\(^4\)

The availability of genetically engineered mice and the assessment of their susceptibility to atherogenesis provide new substrates to study the effect of gene expression on lipoprotein profile and atherosclerosis development. Transgenic mice expressing mouse apoA-II\(^5\) support a role for murine apoA-II in determining HDL structure and levels. These mice have a marked increase in HDL-C and non–HDL-C levels, and they develop severe atherosclerosis even on a chow diet. HDLs from murine apoA-II transgenic mice are unable to prevent LDL oxidation and exhibit proinflammatory properties.\(^6\) Some studies performed in apoA-II–deficient mice showed that murine apoA-II displays some antiatherogenic properties, such as the maintenance of the plasma HDL pool, whereas others showed proatherogenic properties, such as decreasing the clearance of lipoprotein remnants and promotion of insulin resistance.\(^7\) Mice overexpressing human apoA-II\(^8\) do not have elevated HDL-C levels or marked differences in lipid and apolipoprotein profiles, but they do have a modified HDL structure. These mice show a tendency to be protected against atherogenesis.\(^8\) In contrast, human apoA-II coexpression with human apoA-I in mice moderates the antiatherogenic effect of apoA-I.\(^8\) Expression of human apoA-II in mice, described by Marzal-Casacuberta et al,\(^9\) leads to a reduction in HDL-C levels in addition to reduced activity of endogenous lecithin-cholesterol acyltransferase (LCAT). In these mice, human apoA-II is a proatherogenic molecule when it is expressed in mice at a level similar to that in humans but only when the animals are fed a high-fat high-cholesterol diet.\(^10\) Finally, it has been reported that mice expressing human apoA-II present the potentially proatherogenic association of postprandial hypertriglyceridemia and low plasma HDL.\(^11\) Taken together, these results indicate that

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Correspondence to Anne Tailleux, PhD, Département d’athérosclérose et INSERM U. 325, Institut Pasteur, 1, rue du Professeur Calmette, 59019 Lille Cedex, France. E-mail Anne.Tailleux@pasteur-lille.fr

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2453
the role of human apoA-II in atherosclerosis remains poorly defined and controversial.

In the present study, using transgenic mice expressing human apoA-II, we have provided in vivo evidence of the protective role of apoA-II in the development of atherosclerotic lesions. This protective process seems to be independent of HDL pathway.

Methods

Animals and Diets
Female mice expressing the human apoA-II gene controlled by the antithrombin III gene promoter in the C57BL/6J genetic background were compared with their littermates. Mice were housed in stainless-steel cages in groups of 5 on a 12-hour light-dark cycle and maintained on water and food ad libitum.

Control and transgenic mice were assigned for 10 weeks to a regular chow diet and then were switched for 20 weeks to the atherogenic diet. Mouse chow diet contained (wt/wt) <0.03% cholesterol and 4.5% animal fat but no casein or sodium cholate. The high-fat high-cholesterol diet contained 1.25% cholesterol, 15% fat, 7.5% casein, and 0.5% sodium cholate.

Blood was taken from the retro-orbital plexus after the animals had been fasted overnight and were placed under chloroform anesthesia. Serum was separated by low-speed centrifugation and kept at 4°C until analysis (<1 week).

Lipid, Lipoprotein, and Apolipoprotein Analyses
Total cholesterol (TC), free cholesterol (FC), triglycerides (TGs), and phospholipids were measured by using commercially available kits (Boehringer-Mannheim). Cholesterol esters (CEs) were calculated as 1.68×(TC–FC).

Human apoA-II was quantified by rocket immunodiffusion with the use of ready-to-use plates (Sebia). Serum levels of mouse apoA-I and apoA-II were measured by immunonephelometric assays with the use of specific polyclonal antibodies.

Lipoprotein cholesterol profiles were obtained by fast protein liquid chromatography (FPLC) as described.13 This system allows for separation of the main lipoprotein classes: VLDL, IDL (when present at a sufficient level), LDL, and HDL. Cholesterol was dosed continuously and directly at the column exit, whereas human apoA-II concentrations were determined in the pooled eluted fractions. Accumulated cholesterol data were analyzed by the Millenium 20/0 program (Waters). Non–HDL-C was calculated as the sum of VLDL-C, IDL-C, and LDL-C, respectively. VLDL-C, IDL-C, and LDL-C were measured in postheparin plasma incubated with a gum arabic–stabilized TG emulsion containing glycerol tri[1-14C] as previously described.18

Lipolytic Activity
The activities of lipoprotein lipase and hepatic lipase (HL) were measured in postheparin plasma incubated with a gum arabic–stabilized TG emulsion containing glycerol tri[1-14C] as previously described.18

Quantitative Atherosclerosis Measurement
One hundred sections (10 μm thick) were prepared from the top of the left ventricle, where the aortic valves were first visible, up to a position in the aorta at which the valves were just disappearing from the field. The sections were stained with oil red O. Ten of the 100 sections, each separated by 90 μm, were used for morphometric evaluation with a computerized Biocom morphometric system. Data are expressed as the mean lesion size of these 10 sections.

Statistical Analysis
Values were reported as mean±SEM. Statistical differences were determined by the Mann-Whitney U test. Spearman correlation coefficients were used to determine the significance of linear relationships between sets of measurements. A value of P<0.05 was accepted as statistically significant.

Results

Characterization of the Human ApoA-II Transgenic Mouse Model Fed a Chow Diet

**Lipid Concentration**
Table 1 shows lipid and apolipoprotein concentrations. A reduction in TC was observed in the transgenic group compared with the control group because of a reduction in non–HDL-C, whereas HDL-C only exhibited a tendency to decrease. However, it is noteworthy that the HDL-C/TC and non–HDL-C/TC ratios were significantly increased and reduced, respectively. The FC level was significantly decreased in the transgenic mouse group compared with control group,

| TABLE 1. Concentrations of Lipids and Apolipoproteins in Serum From Control and Human ApoA-II Transgenic Mice Fed a Regular Chow Diet |
|-------------------------------------------------|------------------|------------------|
| Concentration, mg/dL                          | Control          | Transgenic       |
| TC                                             | (n=20)           | (n=14)           |
| 71±2                                           | 64±2*            |
| VLDL-C                                         | 0.60±0.19        | 0.80±0.37        |
| LDL-C                                         | 14±1             | 10±1             |
| HDL-C                                         | 57±2             | 53±1             |
| HDL-C/TC                                       | 0.81±0.01        | 0.84±0.01†        |
| Non–HDL-C/TC                                   | 0.19±0.01        | 0.16±0.01†        |
| FC/CE                                         | 0.27±0.02        | 0.21±0.01*        |
| TG                                            | 52±1             | 59±2†            |
| Human apoA-II                                  | ····             | 43±7             |
| Murine apoA-I                                  | 112±5            | 64±3‡            |
| Murine apoA-II                                 | 54±4             | 31±1§            |

Values are mean±SEM.
*P<0.01, †P<0.05, and ‡P<0.001 for transgenic vs control mice.
Atherogenic diet, %

Chow diet, %

and Transgenic Mice Fed a Chow Diet or an Atherogenic Diet

Equilibrium Ultracentrifugation From Pooled Sera From Control

TABLE 3. Mass Composition of HDL Particles Isolated by

Ultracentrifugation From Pooled Sera From Control

and Transgenic Mice Fed a Chow Diet or an Atherogenic Diet

HDL preparation isolated from a pooled sample of 3 mice in each group.

Table 2: Kinetic Parameters of Murine ApoA-I and ApoA-II in Control and Transgenic Mice

ApoA-I ApoA-II

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=3)</th>
<th>Transgenic (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCR, pool/h</td>
<td>0.161±0.03</td>
<td>0.125±0.03*</td>
</tr>
<tr>
<td>PR, mg·d⁻¹·kg⁻¹</td>
<td>173.13±37.69</td>
<td>126±24.30*</td>
</tr>
<tr>
<td></td>
<td>0.219±0.02</td>
<td>0.183±0.05*</td>
</tr>
<tr>
<td></td>
<td>72.89±9.03</td>
<td>51.79±13.47*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. FCR indicates fractional catabolic rate; PR, production rate.

*P<0.05 for transgenic vs control mice.

whereas CE concentrations were not different, thus leading to an FC/CE ratio significantly lower in transgenic animals. TG concentrations were significantly higher in the transgenic group.

Apolipoprotein Analysis

The mean level of human apoA-II expression in the transgenic mice was 43±7 mg/dL, a concentration spanning the range in humans. The expression of human apoA-II was associated with a dramatic reduction (~40%) in murine apoA-I and apoA-II (Table 1). No human apoA-II was detectable in the VLDL and LDL fractions separated by FPLC, whereas the totality of this apolipoprotein was associated with the HDL peak fraction (data not shown).

To understand the metabolic mechanism underlying the decreased endogenous apolipoprotein levels, turnover studies were performed in control and transgenic mice with murine apoA-I and apoA-II. Results in Table 2 showed a decreased production rate in the transgenic group for murine apoA-I and apoA-II (~27% and ~28%, respectively). Moreover, the catabolism of both apolipoproteins was reduced in transgenic mice (~22% for apoA-I and ~16% for apoA-II).

HDL Composition and Size

HDL from transgenic mice was shown to contain dimeric human apoA-II in addition to murine apoA-I and monomeric apoA-II (data not shown) as previously described. HDL isolated from control mice consists of 1 population with a mean Stokes diameter of 9 nm and contained apoA-I and apoA-II. In the transgenic mice, the HDL population displayed a bimodal size distribution. A first HDL population with a mean diameter of 9 nm was shown to contain human apoA-II in addition to murine apoA-I and apoA-II, showing the existence of chimeric particles containing human and murine apolipoproteins. Conversely, a second HDL population with a mean Stokes diameter of 8 nm was free of murine apolipoproteins and contained exclusively human apoA-II. The lipid and protein composition of ultracentrifugally isolated total HDL showed no differences between the 2 groups of chow-fed animals (Table 3).

Cholesterol Efflux

To examine the properties of serum-promoting cholesterol efflux in vitro, we measured the efflux of labeled cholesterol from the Fu5AH hepatoma cells after a 4-hour incubation (Figure 1). All serum samples promoted cholesterol efflux. However, mean cholesterol efflux promoted by the incubation of samples from the transgenic group was slightly but significantly lower than that from the control group (~23% of control value, P<0.05).

Enzymatic Activities

The LCAT activity showed a tendency to increase in serum from transgenic animals compared with serum from control animals (15.96±1.41 versus 13.92±0.87 nmol/mL per hour, mean±SEM), but the difference was not significant.

Lipoprotein lipase activity was comparable in the transgenic and control groups (194±26 versus 206±30 nmol/mL per minute, mean±SEM). HL activity was significantly higher in the transgenic group compared with the control group (135±7 versus 67±3 nmol/mL per minute, mean±SEM; P<0.001).

Lipoprotein Profile of Transgenic Mice Fed a High-Fat High-Cholesterol Diet

Administration of an atherogenic diet for 20 weeks did not change body mass (20.6±1.5 versus 20.4±1.5 g) or liver weight (2.5±0.3 versus 2.3±0.4 g) in the transgenic and control groups, respectively.

Figure 1. Cellular cholesterol efflux with serum from mice fed a chow diet (A) or an atherogenic diet (B). Serum from transgenic (black bar) and control (white bar) mice was incubated with cholesterol-enriched Fu5AH cells, and cholesterol efflux by the cells was measured after 4-hour incubation at 37°C. Values are expressed as percentages of initial cholesterol present in the cells and still found in the medium after incubation. Results are presented as mean±SEM. *P<0.05 for transgenic (n=10) vs control (n=10) mice.
TABLE 4. Concentrations of Lipids and Apolipoproteins in Serum From Control and Human ApoA-II Transgenic Mice After 20 Weeks of an Atherogenic Diet

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Concentration, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>Control (n=20)</td>
</tr>
<tr>
<td></td>
<td>Transgenic (n=14)</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>259±5</td>
</tr>
<tr>
<td>IDL-C</td>
<td>78±3</td>
</tr>
<tr>
<td>LDL-C</td>
<td>54±1</td>
</tr>
<tr>
<td>LDL-C/TC</td>
<td>34±1</td>
</tr>
<tr>
<td>HDL-C</td>
<td>92±3</td>
</tr>
<tr>
<td>HDL-C/TC</td>
<td>0.35±0.01</td>
</tr>
<tr>
<td>Non–HDL-C/TC</td>
<td>0.64±0.01</td>
</tr>
<tr>
<td>FC/CE</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td>TG</td>
<td>43±2</td>
</tr>
<tr>
<td>Human apoA-II</td>
<td>...</td>
</tr>
<tr>
<td>Murine apoA-I</td>
<td>121±2</td>
</tr>
<tr>
<td>Murine apoA-II</td>
<td>56±2</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.001, †P<0.05, and ‡P<0.01 for transgenic vs control mice.

Lipid Concentration
As expected, animals fed the atherogenic diet developed high levels of cholesterol, especially IDL fractions, which became highly detectable by FPLC (Table 4). A marked lower level of TC concentration was observed in the transgenic group compared with the control group because of the lower cholesterol concentration associated with all lipoprotein fractions (VLDL, IDL, LDL, and HDL). It is important to note that the HDL-C/TC and non–HDL-C/TC ratios were comparable in the 2 mouse groups. The FC/CE ratio showed no difference between the transgenic and the control mice. As on the chow diet, murine apoA-I and apoA-II concentrations associated with the atherogenic diet were lower in the transgenic group, but the decrease was lesser than in mice fed the chow diet. Mass composition of HDL showed no major differences between control and transgenic mice, except for a slight increase in protein content (Table 3).

Cholesterol Efflux
Figure 1 shows that the mean cholesterol efflux promoted by the incubation of samples from the transgenic group was slightly but significantly lower than that from the control group (–22% of control value, P<0.05).

Enzymatic Activities
Significantly higher LCAT activity was observed in transgenic mice compared with control mice (36.62±1.97 versus 29.78±2.37 nmol/mL per minute, mean±SEM; P<0.005).

For lipoprotein lipase activity, a decreasing trend was observed in the transgenic group compared with the control group (976±78 versus 1177±188 nmol/mL per minute, respectively; mean±SEM). By contrast, HL activity appears slightly higher in the transgenic group compared with the control group (293±24 versus 213±27 nmol/mL per minute, respectively; mean±SEM). The extent of the changes was not statistically significant.

Atherosclerotic Lesions
After 20 weeks of the cholesterol-rich diet, animals were euthanized, and the extent of aortic lesions was measured.
of apoA-I. Although the function of these particles remains unknown, it has been reported that apoA-II–HDLs (in the absence of apoA-I or apoA-I/apoA-II–HDL) become efficient acceptors and/or donors of apoC peptides and apoE and may serve as surrogate acceptors of FC from peripheral cells. As already described, a dramatic decrease in murine apoA-I and apoA-II was observed in the transgenic mice. Steady-state apolipoprotein levels are dependent on 2 pathways, endogenous synthesis and degradation or tissue clearance, which are quantifiable as the mean of the production rate and the fractional catabolic rate, respectively. In the present study, the dramatic reduction in endogenous murine apolipoproteins in the transgenic group was the result of a decrease in production and catabolism. The decrease in the production rates of both apolipoproteins was predominant, leading to a reduction in circulating murine apoA-I and apoA-II.

Cholesterol efflux from peripheral cells is the first step of reverse cholesterol transport, the series of metabolic steps by which excess of cholesterol is returned from peripheral cells to the liver either for excretion from the body or for recycling. In this way, cholesterol efflux may participate in preventing atherosclerosis. Transgenic mouse serum showed a slightly decreased capacity compared with that of control mice. This could be related to the significant decrease in apoA-I concentration reported in transgenic mice.

Because of its unique function as the key enzyme involved in the esterification of intravascular FC, LCAT may not only modulate HDL concentrations in plasma but may also have an important role in the processes of reverse cholesterol transport and atherosclerosis. In the present study, the transgenic group exhibits a tendency toward an increase in LCAT activity. In addition, hepatic lipase plays an important role in the metabolism of HDL by contributing in the exchanges of lipids and apolipoproteins between IDL and HDL and, consequently, in the remodeling of HDL₂ to HDL₃. HL activity was significantly higher in transgenic than in control mice. Finally, the dramatic decrease in apoA-I concentrations and the slight decrease in cellular cholesterol efflux in transgenic mice fed a chow diet may suggest an atherogenic role for apoA-II. Conversely, when some data are taken into consideration (decrease in non–HDL-C level, the tendency of LCAT activity to increase, and higher HL activity), the animals’ phenotypes tended toward protection against atherosclerosis.

After 20 weeks on an atherogenic diet, human apoA-II transgenic mice developed less extensive aortic atherosclerosis than did control mice. The mechanism by which transgenic mice are protected may be activated by different parameters, including atherogenic or antiatherogenic lipoprotein levels and/or composition, LCAT activity, lipase activities, or else interaction between several of these. First of all, it was determined that both groups of mice had the same mean body weight at the time of euthanasia to demonstrate that protection against atherosclerosis development was independent of their feeding. A higher LCAT activity along with a tendency to increased HL in the transgenic group could have a role in the antiatherogenic process by activating reverse cholesterol transport. Most striking was the consistent decrease in the TC level in the transgenic group, which was due to a drop in cholesterol in each lipoprotein class. This affected equally the atherogenic lipoproteins (VLDL, IDL, and LDL) and the nonatherogenic ones (HDL), inasmuch as the HDL-C/TC and non–HDL-C/TC ratios were no different between the 2 genotypes. The same can be said of the mass composition of ultracentrifugally isolated HDL particles (Table 2). VLDL, IDL, and LDL particles (data not shown). This suggests that the reduction in cholesterol was due to a decrease in the number of circulating lipoproteins in each class.

Inasmuch as previous studies have clearly demonstrated the protective effect of HDL and apoA-I against atherosclerosis, the drop in HDL-C and apoA-I levels in our transgenic animals came as a contradiction and may appear as a paradoxical result because the transgenic mice developed fewer lesions than the control mice. This is reinforced by the slight decrease in cholesterol efflux measured in vitro. However, because no correlation was observed between the extent of atherosclerotic lesions and HDL-C or apoA-I concentrations, it could be supposed that neither absolute apoA-I nor HDL-C levels have a significant role in determining antiatherogenicity in these animals. It has been generally accepted for some years that lipids and lipoproteins are not exclusive parameters implicated in the atherogenic process. Oxidation, inflammation, and even vascular wall remodeling are actors in atherosclerotic lesion development.

In humans, considerable evidence from clinical and epidemiological studies suggests that TG-rich lipoprotein remnants play a role in the pathogenesis of atherosclerosis. In the present study, VLDL-C and IDL-C levels were both positively correlated (P<0.01 and P<0.05, respectively) with the area of atherosclerotic lesions, whereas HDL-C levels were not. Therefore, we can question whether the protection against atherosclerosis in human apoA-II transgenic mice reported in the present study is related to the decrease in remnant lipoproteins rather than to changes occurring in the HDL fraction. However, further studies are needed to elucidate the mechanism underlying the role of apoA-II in the remnant metabolism.

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