Rapid Communication

Overexpression of Human Apolipoprotein A-II in Transgenic Mice Does Not Impair Macrophage-Specific Reverse Cholesterol Transport In Vivo

Noemí Rotllan, Vicent Ribas, Laura Calpe-Berdiel, Jesús M. Martín-Campos, Francisco Blanco-Vaca, Joan Carles Escola-Gil

Background—Overexpression of human apolipoprotein (apo) A-II in transgenic mice induces high-density lipoprotein (HDL) deficiency, and increased atherosclerosis susceptibility only when fed an atherogenic diet. This may, in part, be caused by impairment in reverse cholesterol transport (RCT).

Methods and Results—[3H]cholesterol-labeled macrophages were injected intraperitoneally into mice maintained on a chow diet or an atherogenic diet. Plasma [3H]cholesterol did not differ from human apoA-II transgenic and control mice at 24 or 48 hours after the label injection. On the chow diet, human apoA-II transgenic mice presented increased [3H]cholesterol in liver (1.3-fold) and feces (6-fold) compared with control mice (P<0.05). The magnitude of macrophage-specific RCT did not differ between transgenic and control mice fed the atherogenic diet.

Conclusions—Human apoA-II maintains effective RCT from macrophages to feces in vivo despite an HDL deficiency. These findings suggest that the increased atherosclerotic lesions observed in apoA-II transgenic mice fed an atherogenic diet are not caused by impairment in macrophage-specific RCT. (Arterioscler Thromb Vasc Biol. 2005;25:e128-e132.)

Key Words: apolipoprotein A-II ■ atherosclerosis ■ high-density lipoprotein ■ macrophages ■ reverse cholesterol transport

High-density lipoprotein (HDL) is thought to protect against atherosclerosis by extracting cholesterol from macrophages and delivering it to liver, from where it will be, in part, eliminated via bile and feces in a process usually known as reverse cholesterol transport (RCT). Several studies have assessed overall RCT by measuring peripheral cholesterol synthesis, which in the steady state approximates centripetal cholesterol efflux to the liver. These studies have shown that overexpression or underexpression of apolipoprotein (apo) A-I does not perturb the RCT rate. However, they do not provide information on specific RCT from macrophages, a most important cholesterol-loaded cell in atherosclerotic lesions.

HDL are classified according to their content of the major apolipoproteins apoA-I and apoA-II. ApoA-I has a protective role against atherosclerosis, as demonstrated by the analysis of apoA-I transgenic mice. Recently, tracing of reverse [3H]cholesterol transport from macrophages to feces in apoA-I transgenic mice unambiguously demonstrated increased RCT in this atherogenic-resistant animal. In contrast, the role of apoA-II is less understood. The overexpression of mouse apoA-II has been found to be pro-atherogenic. Human apoA-II transgenic mice have also generally been found to display increased atherosclerosis susceptibility, but exclusively when fed an atherogenic diet or when cross-bred with apoE-deficient mice and fed a regular chow diet. Mechanisms by which apoA-II expression may promote atherogenesis include impairing inhibition of the oxidative modification of apoB-containing lipoproteins and increasing inflammatory response of vascular cells. A further major pro-atherogenic mechanism of apoA-II expression may involve reduced RCT. Although the role of apoA-II on individual steps in the RCT pathway has been studied and reviewed in detail, little is known as to how apoA-II regulates the entire RCT rate in vivo.

The present study tested the ability of human apoA-II to induce the macrophage-specific RCT pathway in vivo. Cholesterol-loaded P388D1 macrophage-like cells were used as...
the cell model because they express most of the cellular determinants of intracellular recycling of lipoprotein cholesterol and exhibit apoptotic characteristics of vascular cells of atherosclerotic plaque.

**Methods**

**Mice and Diets**

All animal procedures were in accordance with published recommendations. Human apoA-II transgenic mice (line 11.1) were developed as previously described (Department of Medicine and Molecular and Cell Biology, Baylor College of Medicine, Houston, Tex.). Two-month-old C57BL/6 (control) and human apoA-II transgenic mice were maintained on a regular chow diet (Rodent Toxicology Diet; B&K Universal, UK) containing 0.02% cholesterol or an atherogenic diet (TD 88051; Harlan Teklad, Madison, Wis) containing 1.25% cholesterol, 15% fat, and 0.5% sodium cholate for 3 months. Male and female mice were used in equal proportions. All animal manipulations began at 12:00 PM with the mice fed ad "n ad libitum.

**Lipid, Apolipoprotein, and Fecal Analyses**

The methods used for plasma analyses have been described in detail elsewhere. Lipids were extracted from 1 gram of feces with isopropl alcohol-hexane and the ratio of [14C] to [3H] in collected over the 3 following days. Lipids were extracted from stools with isopropl alcohol-hexane and the ratio of [14C] to [3H] in each sample determined. Percent cholesterol absorption was calculated from these data as described.

**Cell Culture and [3H]Cholesterol Loading**

Low-density lipoprotein (LDL) (50 mg of apoB) obtained by sequential ultracentrifugation was radiolabeled by 18-hour incubation at 37°C with a sonicated suspension of phosphatidyl-choline and acetylated by sequential addition of acetic anhydride. The modified and labeled LDL was extensively dialyzed against saline.

The methods used for plasma analyses have been described in detail elsewhere. Lipids were extracted from 1 gram of feces with isopropl alcohol-hexane and the ratio of [14C] to [3H] in each sample determined. Percent cholesterol absorption was calculated from these data as described.

**Plasma Lipid and Fecal Parameters in Control and Human ApoA-II Transgenic (ApoAIITg) Mice of Line 11.1 on Either a Chow Diet or an Atherogenic Diet**

<table>
<thead>
<tr>
<th></th>
<th>Chow Diet</th>
<th>Atherogenic Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>ApoAIITg (n=9)</td>
<td>Control (n=9)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>2.0±0.1</td>
<td>1.4±0.1*</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.5±0.1</td>
<td>0.8±0.1*</td>
</tr>
<tr>
<td>Non-HDL cholesterol, mmol/L</td>
<td>0.5±0.1</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>Human apo A-II, mg/dl</td>
<td>0±0</td>
<td>85.7±6.0</td>
</tr>
<tr>
<td>Fecal cholesterol output, µmol/day</td>
<td>4.6±0.4</td>
<td>3.7±0.3</td>
</tr>
<tr>
<td>Fecal bile acid output, µmol/day</td>
<td>0.3±0.1</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Intestinal cholesterol absorption, %†</td>
<td>89.2±1.0</td>
<td>89.4±0.8</td>
</tr>
</tbody>
</table>

HDL and non-HDL cholesterol were measured after phosphotungstic-MgCl<sub>2</sub> precipitation of plasma. Fecal bile acids was determined as previously described. Cellular uptake of [3H]cholesterol labeled LDL resulted in the accumulation of large quantities of cholesteryl esters visible as free droplets in the cytoplasm of Oil red O-stained P388D1 mouse macrophages. These cells were washed, equilibrated, detached with cell scrapers, and resuspended in saline. The distribution of [3H] cholesterol between free cholesterol and cholesteryl ester was determined by thin-layer chromatography.

**Measurement of In Vivo Transport of Cholesterol From Macrophages to Feces**

Two preparations of [3H]cholesterol-labeled macrophages were used. First, 19 chow-fed mice (10 controls and 9 transgenics) were injected intraperitoneally with [3H]cholesterol-labeled P388D1 mouse macrophages (11×10<sup>6</sup> cells containing 2.5×10<sup>6</sup> dpm in 0.5 mL of saline in each mouse). Second, 17 mice fed the atherogenic diet (9 controls and 8 transgenics) were injected intraperitoneally with [3H]cholesterol-labeled macrophages (13.5×10<sup>6</sup> cells containing 3.0×10<sup>6</sup> dpm). In both experiments, 55% of the labeled cholesterol content of macrophages was in esterified form. Mice were then individually housed in metabolic cages and stools were collected over the next 2 days. Plasma dpm were determined at 24 and 48 hours by scintillation counting. HDL-associated [3H]cholesterol was measured after precipitation with phosphotungstic acid and magnesium ions (Roche Diagnostics GmbH, Germany) and non-HDL-associated [3H]cholesterol calculated by the difference between [3H]cholesterol in plasma and HDL. At 48 hours, mice were euthanized and livers removed. Lipids were extracted with isopropl alcohol-hexane and the distribution of [3H] cholesterol between free cholesterol and cholesteryl ester was determined. The [3H] tracer detected in the fecal bile acids was determined as previously described. The amount of [3H] tracer was also expressed as a fraction of the injected dose.

**Statistical Methods**

Unpaired Student t test or Mann–Whitney U test were used to compare data obtained from control and human apoA-II transgenic mice on both types of diet. GraphPad Prism 4.0 software (GraphPad, San Diego, Calif) was used to perform all statistical analyses. P<0.05 was considered statistically significant.

**Results**

ApoA-II transgenic mice showed several previously reported phenotypes, such as decreased cholesterol in HDL and
increased apoB-containing lipoproteins after being fed the atherogenic diet (Table). The 11.1 transgenic mice fed the atherogenic diet had increased cholesterol levels in very-low-density lipoprotein and LDL (7.4 and 3.2 mmol/L, respectively) compared with those of the control group (2.2 and 0.8 mmol/L, respectively). Fecal cholesterol and bile acid output and intestinal cholesterol absorption did not differ between human apoA-II mice and control mice (Table). No gender differences were found in any of the parameters studied.

**Macrophage-Specific RCT in Chow-Fed Human ApoA-II Transgenic Mice**

After intraperitoneal injection of [3H]cholesterol-labeled macrophages, plasma [3H]cholesterol levels from human apoA-II transgenic mice, both at 24 and 48 hours, were not significantly different from those of control mice (Figure 1A). Approximately 70% of radiolabeled [3H]cholesterol was bound to HDL, both in control and transgenic mice at 24 hours, decreasing significantly in transgenic mice at 48 hours (35% versus 55% in control mice; Figure 1A). The major part of plasma labeled cholesterol was esterified in both groups of mice at 24 hours (66% and 67% in control and transgenic mice, respectively). At 48 hours after injection, the tracer corresponding to plasma esterified cholesterol was reduced in transgenic mice (51%) compared with control mice (65%). The percentage of liver labeled esterified cholesterol (compared with total cholesterol) was 51% and 42% in control mice and transgenic mice, respectively. The [3H]tracer detected in liver, fecal cholesterol, and bile acids of transgenic mice did not differ significantly from that of control mice (Figure 2B).

We repeated this experiment with transgenic mice of line 25.3 that expressed low concentrations of human apoA-II (14 mg/dL) after 6 weeks on the atherogenic diet. Again, the [3H]cholesterol detected in liver showed no differences between transgenic and control mice (339 and 339 dpm, respectively). No difference was found in [3H] tracer of feces (3146±339 and 2557±297 dpm, respectively; n=5 in each group).

**Discussion**

Previous studies by our group indicated that the proatherogenic susceptibility to atherosclerosis in our human apoA-II transgenic mice fed an atherogenic diet might be caused by impaired RCT secondary to HDL deficiency. The latter was caused in part by decreased apoA-I levels, which, as a major cofactor of lecithin:cholesterol acyltransferase, induced a functional lecithin:cholesterol acyltransferase deficiency in these mice. In contrast, the main conclusion of the present study is that the overexpression of human apoA-II effectively maintains RCT from macrophages to liver and feces.

It is noteworthy, however, that the human apoA-II overexpression in chow-fed mice enhanced RCT from macrophages to liver and feces, whereas the same RCT was
unaltered in human apoA-II transgenic mice fed the atherogenic diet. Absolute radioactivity was similar in plasma of control and transgenic mice, demonstrating that the dietary and genotypic variations that strongly affected the concentration of different lipoprotein subclasses in the different mice groups were not critical, at least at the time points studied (24 and 48 hours). Given that in transgenic mice HDL concentrations are decreased, these results also indicate an increased relative radioactive label in HDL. We detected the presence of “lipid-free” human apoA-II in our transgenic mice by native gradient polyacrylamide gel electrophoresis (data not shown). This would be consistent with an efficient role of apoA-II–containing small HDL in inducing cholesterol efflux through ABCA1.11 However, apoA-II transgenic mice present increased labeled lipid uptake from HDL by the liver when fed a chow diet.25 Thus, increasing cholesterol efflux from macrophages and increasing delivery of cholesterol to the liver by HDL may explain our observations. However, several in vitro studies including our own experiments indicate that apoA-II exerts a negative effect on scavenger receptor type B-1 (SR-BI)–specific cholesteryl ester uptake.18,25 Although a recent study suggested that SR-BI is responsible for the bulk of liver HDL-selective cholesteryl ester uptake,26 a minor SR-BI–independent mechanism for cholesterol-poor HDL has also been observed in mice.26 Thus, it is possible that SR-BI–independent pathways27–29 contribute to the enhanced uptake of HDL-derived cholesteryl ond found in the liver of human apoA-II transgenic mice.

Importantly, RCT from macrophages to feces is unaltered in human apoA-II transgenic mice fed the atherogenic diet despite an increased HDL-cholesteryl ester selective uptake by the liver (Ribas et al, unpublished data) and an increased atherosclerosis susceptibility.11 A marked increase in [3H]cholesterol bound to non-HDL particles was observed in the experiment performed with mice fed an atherogenic diet (Figure 2). Considering that mice do not show cholesteryl ester transfer protein activity, the increase in [3H]cholesterol bound to non-HDL particles may, in part, be caused by the action of plasma phospholipid transfer protein that exchanges bound to non-HDL particles may, in part, be caused by the action of plasma phospholipid transfer protein that exchanges lipoprotein-poor HDL has also been observed in mice.26 Thus, it is possible that SR-BI–independent pathways27–29 contribute to the enhanced uptake of HDL-derived cholesteryl

may be a possible explanation for these observations as well as for the comparatively reduced excretion of [3H]cholesterol in feces of mice fed the atherogenic diet with respect to their measured liver labeled cholesterol.

As a potential limitation of the study, it should be borne in mind that it remains to be established whether the P388D1 cholesterol-loaded cell behaves exactly as do endogenous murine macrophages with respect to RCT.

In conclusion, our studies show, for the first time to our knowledge, that human apoA-II effectively maintains RCT from macrophages to feces in vivo even in a situation of HDL deficiency. They may also indicate, together with the previous work of other groups, that apoA-II exerts at least part of its pro-atherogenic effect by counteracting antioxidant properties of HDL.15,16 rather than by impairing macrophage-specific RCT.

Acknowledgments

We are grateful to Christine O’Hara for editorial assistance. This work was funded by FIS grants 03/1058, G03–181 and C03–08. J.C.E.-G. is a Ramón y Cajal researcher, funded by the Ministerio de Educación y Ciencia.

References


2. Jolley CD, Woollett LA, Turley SD, Dietschy JM. Centripetal cholesterol flux to the liver is dictated by events in the peripheral organs and not by the plasma high density lipoprotein or apolipoprotein A-1 concentration. J Lipid Res. 1998;39:2143–2149.


Overexpression of Human Apolipoprotein A-II in Transgenic Mice Does Not Impair Macrophage-Specific Reverse Cholesterol Transport In Vivo
Noemí Rotllan, Vicent Ribas, Laura Calpe-Berdiel, Jesús M. Martín-Campos, Francisco Blanco-Vaca and Joan Carles Escolà-Gil

Arterioscler Thromb Vasc Biol. 2005;25:E128-E132; originally published online June 30, 2005;
doi: 10.1161/01.ATV.0000175760.28378.80
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/9/E128