Recombinant Apolipoprotein A-I\textsubscript{Milano} Infusion Into Rabbit Carotid Artery Rapidly Removes Lipid From Fatty Streaks

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Abstract—Apolipoprotein A-I\textsubscript{Milano} (AIM), a natural variant of human apolipoprotein A-I, confers to carriers a significant protection against vascular disease. In previous studies, administration of recombinant AIM-phospholipid (AIM-PL) complexes to hypercholesterolemic rabbits markedly inhibited neointimal formation after arterial injury; moreover, repeated injections of AIM-PL in apoE-deficient mice significantly reduced atherosclerosis progression. The objective of the present study was to determine if a single localized infusion of AIM-PL complexes administered directly to atheromatous lesions could promote plaque regression. Lipid-rich, atheromatous plaques were generated at both carotid arteries of 25 rabbits by applying a perivascular electric injury, followed by 1.5% cholesterol diet for 90 days. Rabbits were infused with either saline, phospholipid vesicles, or 3 different AIM-PL doses (250, 500, or 1000 mg of protein) delivered through an intravascular ultrasound (IVUS) catheter positioned at the origin of the right carotid. The lesions at the left carotid artery were therefore exposed to the agents systemically. Infusion of AIM-PL at the 2 highest doses caused reduction of right carotid artery plaque area by the end of 90-minute infusion as assessed by IVUS analysis. Plaque area regression was confirmed by histology in carotid arteries receiving direct (500 and 1000 mg doses) and systemic (500 mg dose) delivery, 72 hours after the start of the treatment. Plaque lipid content was associated with significant and similar decreases in Oil Red O staining in both arteries. These results suggest AIM-PL complexes enhanced lipid removal from arteries is the mechanism responsible for the observed plaque changes. (Circ Res. 2002; 90:974-980.)

Key Words: rabbit ■ lipid-rich plaques ■ apolipoprotein A-I\textsubscript{Milano} ■ regression

The protective role of high-density lipoproteins (HDLs) against atheromatous vascular disease is widely accepted and is generally attributed to the HDL function in reverse cholesterol transport, the process by which excess cholesterol in peripheral tissues is conveyed to the liver for excretion. A number of in vitro studies have clearly established the capacity of HDL to extract cholesterol from a variety of cell types, including cholesterol-loaded macrophages, the typical cellular components of human unstable plaques. In agreement with these results in vitro, the administration of homologous HDLs to cholesterol-fed rabbits with established atherosclerotic lesions was able to reduce significantly aortic lipid deposition. A direct demonstration that HDLs remove lipids from the arterial wall came from studies in intact rat arteries, where HDL proved effective in depleting the intimal layer of lipids that accumulate after perfusion with atherogenic lipoproteins. This process may also be effective in humans. The infusion of synthetic HDL made of apolipoprotein A-I (apoA-I) and phosphatidylcholine into healthy volunteers led to an increased cholesterol efflux with production of small HDLs. Administration of recombinant pro–apoA-I complexed with phospholipids to hypercholesterolemic patients enhanced bile acid and neutral sterol excretion, indicative of a stimulated reverse cholesterol transport.

The possibility that a variant form of apoA-I, ie, apolipoprotein A-I\textsubscript{Milano} (AIM), originally described in a population with low cardiovascular risk, might be linked to improved arterial protection has received considerable attention. A recombinant form of the AIM mutant has been produced in E. coli and used in a variety of in vitro and in vivo experiments to assess its therapeutic potential against vascular disease. Preclinical studies in laboratory animals have shown that repeated injections of AIM-phospholipid (AIM-PL) complexes prevent neointimal formation after different arterial injuries (eg, balloon angioplasty or perivascular manipulation). These same complexes were shown to inhibit the progression or possibly induce the regression of atheromatous lesions in apoE-deficient mice by reducing the lipid and...
localized infusion of AIM-PL complexes administered directly to lipid-rich atheromatous plaques via an accessible site, ie, the rabbit carotid artery, could directly and acutely reduce the atheromatous burden. In view of the limited knowledge of potential effects of such a treatment, a short-term evaluation (90 minutes at the end of infusion) was performed by intravascular ultrasound (IVUS) and histomorphometric analysis of lesion area and lipid content was evaluated subacutely (72 hours after infusion). These studies address the potential use of infusible synthetic HDL as a therapeutic option for the removal of lipid from arterial plaques.

Materials and Methods

Experimental Protocol

Male New Zealand White rabbits (Charles River, Calco, Italy) were used for the study. Procedures involving animals and their care were conducted in accordance with institutional guidelines that are in compliance with national and international laws and policies. All lipid-rich plaque formation was induced at both common carotid arteries of rabbits by perivascular injury followed by a dietary treatment for 90 days as described. At 90 days after surgery, 5 groups of rabbits, 5 animals each, received one of the following treatments administered through an IVUS catheter inserted into the right carotid artery: (1) saline; (2) dipalmitoylphosphatidylcholine (DPPC) liposomes (2500 mg); (3) AIM-PL complexes (250 mg protein+625 mg DPPC); (4) AIM-PL complexes (500 mg protein+1250 mg DPPC); and (5) AIM-PL complexes (1000 mg protein+2500 mg DPPC).

The recombinant AIM-PL preparation used in this study has been described previously and contains AIM in dimeric form. All rabbits received the same volume, ie, 50 mL, at a constant rate of 0.55 mL/min, over a fixed period of 90 minutes. IVUS evaluations of the infused carotid artery were performed before and then at 30 minutes, 60 minutes, and 90 minutes after the start of the infusion. Blood samples for biochemical analyses were collected at time 0 (before the infusion), 45 minutes, 90 minutes (end of infusion), 6 hours, 24 hours, 48 hours, and 72 hours, ie, before euthanasia. Histological analyses were performed on the right and left carotid arteries to evaluate the local and systemic effects of the treatments. Operators responsible for animal care, treatment administration, IVUS, and histological analyses were totally blinded with respect to the treatment.

IVUS Imaging

IVUS evaluations were performed using a mechanical IVUS system (ClearView, Boston Scientific). The carotid plaque at maximum stenosis was specifically evaluated, as described, before, during, and at the end of the infusion by 2 independent observers using computerized planimetry (TapeMeasure, Indec Systems). X-ray analysis allowed positioning of the ultrasound probe at the site of maximal stenosis for the following histology. Media thickness cannot be measured accurately by IVUS; therefore, plaque cross sectional area (CSA) was measured as external elastic membrane (vessel) CSA minus the lumen CSA.

Biochemical Evaluations

Plasma total cholesterol and triglycerides were determined with standard enzymatic techniques. HDL cholesterol levels were measured after selective precipitation with dextran sulfate-MgCl₂. Lipoprotein profiles for unesterified cholesterol were measured before and at the end of infusion (90 minutes), were performed by high-performance gel-filtration chromatography as previously described. Plasma levels of AIM were measured by an immunoturbidimetric assay using a polyclonal antibody against human apoA-I (Roche Diagnostics, Basel, Switzerland) that recognizes both human apoA-I and AIM, but not rabbit apoA-I.
Histological Evaluation and Quantitation of Lesions

Seventy-two hours after the infusion, rabbits were euthanized and carotids were included in O.C.T. compound (BDH Laboratory Supplies); sections were stained as described. AIM detection was performed by using the same anti-human apoA-I antibody used for AIM plasma quantification.

Plaque area was measured for each carotid on 5 sections with maximal plaque accumulation as described previously. Adjacent sections were taken at 150-μm intervals, within the area corresponding to the maximal plaque accumulation visualized by IVUS. For comparison with the IVUS findings, the atherosclerotic plaque was measured as intima plus media CSA and calculated as external elastic membrane CSA minus the lumen CSA. Quantification of the percentage of plaque area covered by lipids (ie, with positive staining for Oil Red O) was performed by 2 independent observers for each rabbit by dividing the CSAs calculated at each time point by the baseline (time 0) measurement. No significant differences in the baseline plaque and vessel CSAs were found between the groups.

Calculations and Statistical Analyses

Data are expressed as mean±standard deviation (SD). Analysis of variance (ANOVA) was used to compare interobserver variability measurements obtained by histology or IVUS. Group differences were tested for statistical significance by using 2-way ANOVA for repeated measurements, followed by Bonferroni post hoc test; a value of P<0.05 was considered statistically significant. Pearson correlation coefficient was calculated for statistical comparison of plaque measurements obtained by IVUS and histological analysis.

Results

Features of Arterial Lesions

Electrical injury at the common carotid arteries followed by cholesterol feeding generates vascular lesions poorly echorefractive by IVUS, enriched of extracellular lipids and macrophages, and relatively devoid of smooth muscle cells by histology. This is also the case of animals undergoing infusion of AIM-PL complexes, as assessed by histological examination of 3 animals euthanized without treatment (data not shown).

Plasma Lipid/Lipoprotein Changes

Total plasma cholesterol did not change significantly throughout the entire experiment. A rise of plasma triglycerides, with a peak at 24 hours after the start of the infusion, was observed in all animals treated with DPPC liposomes or AIM-DPPC complexes; a smaller increase of plasma triglycerides was found in saline-infused animals (data not shown).

Lipoprotein-free cholesterol profiles of rabbits infused with saline or DPPC liposomes did not show at the end of infusion considerable variations compared with pretreatment, whereas AIM-PL infusion caused a dose-dependent increase, mostly within the HDL fraction (Figure 1A).

The infusion of AIM-PL complexes caused a progressive, dose-dependent increase of plasma AIM concentration up to the end of the infusion, followed by a slow decline till euthanasia (Figure 1B). The AIM areas under the curve (AUC) increased proportionally with rising doses (3995, 8113, 15 702 mg/dL per hour, with AIM infusion of 250, 500, and 1000 mg, respectively). Detectable amounts of circulating AIM were still found at the end of the experiment, ie, 72 hours after the start of the infusion. The AIM half-life was estimated at about 40 hours. Sustained changes were observed for plasma HDL cholesterol levels. HDL cholesterol was 2- to 4-fold higher in the animals infused with AIM-PL complexes compared with saline-infused rabbits (Figure 2). The HDL cholesterol elevation in the groups infused with 500 and 1000 mg of AIM-PL complexes was statistically significant versus both saline and DPPC treatments (Figure 2). Higher HDL-cholesterol levels were found in animals infused with DPPC liposomes, compared with saline-infused rabbits, but this difference did not achieve statistical significance (P=0.719).

Treatment Effect by IVUS Evaluation

Plaque areas (calculated as vessel area—lumen area) at the site of maximum stenosis of the right (infused) carotid arteries were measured by IVUS at different time points during the course of the infusion (Figure 3). No significant differences in the baseline plaque and vessel CSAs were found in the 5 groups. Plaque CSAs values were normalized for each rabbit by dividing the CSAs calculated at each time point by the baseline (time 0) measurement. No significant changes in the average plaque CSA were found during the...
infusion of either saline, DPPC liposomes, or low-dose AIM-PL complexes (Table 1). The infusion of the intermediate dose of AIM-PL complexes caused a rapid reduction of plaque CSA, with no further changes at later time points. Average plaque CSA decreased progressively by 30% at the end of infusion, in animals treated with the highest dose of AIM-PL complexes (Table 1). No variation in vessel size (ie, CSAs) was detected during AIM-PL infusion, indicating that vasodilator effects are not likely the cause of the plaque reduction observed by IVUS.

Treatment Effect by Histology
Seventy-two hours after the start of infusion, the animals were euthanized and 5 sections of each carotid artery at the site of maximal stenosis were analyzed to calculate average plaque CSA. Consistent with the results of the IVUS evaluation performed at the end of the infusion, no significant changes were found in the right carotid artery of rabbits infused with either saline, DPPC, or low-dose (250 mg) AIM-PL complexes. On the contrary, in rabbits infused with 500 and 1000 mg of AIM-PL, plaque CSAs were 42% and 32% lower, respectively, compared with saline-infused rabbits, and 32% and 20% lower, respectively, when compared with DPPC-treated animals (Figure 4A). A significant correlation was indeed found between plaque-area measurements detected by IVUS at the end of infusion and by histology at euthanasia \((r=0.82; \ P<0.001)\) (Figure 4B). Histological analysis of left carotid arteries, which underwent the same perivascular manipulation, exposed to treatment systematically, displayed a trend similar to that observed for right carotids, with plaque reductions of 29% (500 mg) and 13% (1000 mg) compared with saline treatment, and 25% (500 mg) and 11% (1000 mg) compared with DPPC treatment (Figure 4C).

The lipid content of arterial plaques in the right and left carotids was evaluated by Oil Red O staining and calculated as percentage of Oil Red O-positive area over the total plaque area. A significant, dose-dependent reduction of plaque lipid content was found in both carotids of AIM-infused rabbits, whereas no changes were detected in animals receiving DPPC liposomes (Table 2).

AIM Immunodetection in the Carotids
To understand whether the infused AIM-PL complexes were able to penetrate into the vascular lesion, arterial plaque sections were immunostained with a polyclonal antibody against human apoA-I, which cross-reacts with AIM. No

**TABLE 1. Plaque Area Changes After AIM-PL Infusion**

<table>
<thead>
<tr>
<th>Time, min</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1</td>
<td>1.058±0.095</td>
<td>0.985±0.070</td>
<td>0.997±0.040</td>
</tr>
<tr>
<td>DPPC</td>
<td>1</td>
<td>0.906±0.104</td>
<td>0.928±0.045</td>
<td>0.915±0.199</td>
</tr>
<tr>
<td>AIM 250 mg</td>
<td>1</td>
<td>0.955±0.156</td>
<td>0.956±0.138</td>
<td>0.968±0.169</td>
</tr>
<tr>
<td>AIM 500 mg</td>
<td>1</td>
<td>0.856±0.046</td>
<td>0.894±0.020*</td>
<td>0.882±0.007†</td>
</tr>
<tr>
<td>AIM 1000 mg</td>
<td>1</td>
<td>0.828±0.115</td>
<td>0.757±0.088*</td>
<td>0.711±0.143†</td>
</tr>
</tbody>
</table>

Data are normalized vs baseline values and are expressed as mean±SD.

*P<0.05, †P<0.01 vs 0 minutes.
Several antiatherosclerotic properties have been attributed to HDL and apoA-I. Very recently, Spieker et al. reported clinical findings indicative of a restored endothelial function in hypercholesterolemic patients after infusion with reconstituted HDL. No significant increase of forearm blood flow during HDL infusion was observed, supporting our findings of no direct vasodilatory effect by synthetic HDL. The rapid plaque reduction observed by IVUS monitoring during AIM-PL infusion suggests direct cholesterol removal from the lipid-rich plaques. Extracellular lipids present in the carotid lesions, may be removed by AIM-PL complexes, leading to plaque remodeling and reduced plaque area. Indeed, AIM was able to penetrate into rabbit plaques, as assessed by immunohistochemical analysis, and a significant dose-dependent reduction of plaque lipid content was detected by Oil Red O staining (see online Figure, which can be found in the online data supplement available at http://www.circresaha.org). Recently, Shah et al. documented similar results in apoE-null mice, 48 hours after infusion of a single high dose (400 mg/kg) of AIM phospholipid complexes.

Historological analysis of the contralateral (left) carotid plaques was performed to assess the systemic effect of the AIM-PL infusion on lipid-rich arterial lesions. Plaque area quantification of the left carotid artery showed a tendency for reduced plaque area in all groups receiving AIM-PL complexes; however, this reduction was significant only in rabbits receiving the mid dose of AIM-PL (500 mg). The AIM-PL complexes significantly reduced plaque lipid content at the mid and high doses to a similar degree for both carotid arteries. Altogether, these results suggest that systemic administration of AIM-PL could treat plaques in vascular beds distant from the administration site. An obvious benefit of this type of therapy is that systemic administration may afford global benefit to the entire vascular tree, including those sites that are not accessible for local treatments or surgical interventions.

Direct evaluation of regression and/or stabilization of lipid-rich, rupture-prone, atherosclerotic plaques in the clinic, ie, by direct monitoring of the plaque by technologies such as IVUS, has generally indicated a relatively weak effect with current pharmaceutical interventions. For example, studies in statin-treated individuals showed that unstable lipid-rich plaque volume was only reduced by a minimal extent at the 3-year interval. Interestingly, Linker et al. showed that a pharmacological treatment of hyperlipidemic patients, with a drug combination significantly raising HDL levels, could markedly reduce coronary plaque volume compared with no significant effect (only a stabilization) of LDL cholesterol lowering induced by a statin. The remarkable effects of AIM-PL on plaque size and lipid content in our animal model of lipid-rich, macrophage-rich arterial lesion argues for the use of synthetic HDL as an effective innovative therapeutic.
intervention. Our model, as well as any other rabbit model of atherosclerosis, does not reproduce all the features of “complicated” or “advanced” human lesions because of the almost complete lack of fibrotic components. However, our results, together with those by Shah et al in an animal model in which arterial plaques more closely resemble human atherosclerotic lesions, suggest a potential effect of synthetic lipid on regression and/or stabilization of complicated, fibrofatty human atheromas.

A limitation of the present study is that human apoA-I was not utilized for direct comparisons. Difficulty in obtaining large amounts of recombinant apolipoprotein formulations is, in fact, still the limit for any studies of direct vascular intervention.

The present study provides direct evidence for the potential therapeutic value of a recombinant apolipoprotein, complexed to phospholipids, for directly reducing the lipid content of atheromatous plaques in a well-established animal model. Although it is not known whether the acute beneficial effect of the treatment will persist, the simplicity of the procedure allows for the long-term evaluation of this therapy with and without additional pharmaceutical interventions. The actual therapeutic potential will be evaluated in the clinic.

Acknowledgments

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Example of histochemical revelation of lipid content in rabbit soft plaques after infusion with saline (control) and maximal dose of recombinant AIM.