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

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Abstract—Apolipoprotein A-I_{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 common 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 a 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_{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 in vitro findings, 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_{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 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. 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: (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 recombiant 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 obtained from animals 90 minutes after treatment with cholesterol-fed rabbits (n=7) were similar among the treatment groups (II). Posttreatment profiles were obtained from animals 90 minutes after treatment with saline (III), DPPC (IV), 250 mg AIM (V), 500 mg AIM (VI), or 1000 mg AIM (VII). Profiles represent a single (saline) or the average of 2 profiles (all other groups). B, Plasma AIM concentrations in rabbits infused with 3 different doses of AIM-PL complexes: 250 mg (triangles), 500 mg (circles), and 1000 mg (squares) of protein. The infusion started at time 0 and lasted 90 minutes; the animals were euthanized at 72 hours. Values are expressed as mean±SD (n=5).

Intravascular ultrasound (IVUS) and histological analyses were totally blinded with respect to the treatment.

Figure 1. A, Change in lipoprotein unesterified cholesterol after treatments. Serum lipoproteins from rabbits before and 90 minutes after treatments were separated by high-performance gel-filtration chromatography for online analysis of lipoprotein unesterified cholesterol. Reference serum from a chow-fed rabbit was obtained after selective precipitation with dextran sulfate-MgCl₂. 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.

macrophage accumulation in arterial plaques. These studies demonstrate a benefit of subchronic administration of AIM-PL complexes to affect a variety of processes involved in the formation and progression of vascular lesions. The present study was conducted to determine whether a single...
Results

Features of Arterial Lesions

Electrical injury at the common carotid arteries followed by cholesterol feeding generates vascular lesions poorly echoreflective by IVUS, enriched of extracellular lipids and macrophages, and relatively devoid of smooth muscle cells by histology.\(^1\) 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)\(^2\)\(^9\) 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

**Figure 2.** Plasma HDL cholesterol levels, measured at euthanasia (72 hours after the start of infusion), in rabbits treated with saline, DPPC liposomes, or 3 different doses of AIM-PL complexes. Values are expressed as mean±SD (n=5). \(^*P<0.01\) and \(†P<0.0001\) vs saline; \(‡P<0.05\) and \(§P<0.0005\) vs DPPC.

**Figure 3.** Example of IVUS analysis performed at the point of maximal stenosis before (A) and after (B) 1000 mg AIM-PL infusion. Plaque area, delimited by the yellow tracing, was reduced by 43% after treatment.

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.
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 (i.e., 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 systemically, 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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<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>
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<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>
<td></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>
<td></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>
<td></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>
<td></td>
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</tbody>
</table>

Data are normalized vs baseline values and are expressed as mean±SD. *$P<0.05$, †$P<0.01$ vs 0 minutes.

**Figure 4.** Histological measurement of plaque area at maximal stenosis in the right (A) and in the left (C) carotids of rabbits treated with saline, DPPC liposomes, or 3 different doses of AIM-PL complexes; (B) correlation between plaque area measurements by IVUS and histology of right carotid artery at maximal stenosis. A and C, Values are expressed as mean±SD (n=5). *$P<0.005$, †$P<0.05$, and ‡$P<0.05$ vs saline; §$P<0.01$, //$P<0.05$, and ¶$P<0.05$ vs DPPC. B, Each dot represents plaque CSA at maximal stenosis of individual animals infused with either saline (open diamond), DPPC (open squares) or 250 mg (filled triangles), 500 mg (filled circles), and 1000 mg (filled squares) of protein as AIM-PL complexes.
apoA-I immunoreactivity was found in sections from saline- and DPPC-treated animals, but large amounts of immunoreactive AIM were detected in the plaques of both carotids of all animals receiving AIM-PL infusions (Figure 5).

**Discussion**

A number of studies in the past few years suggest that repeated infusions of HDL, or apoA-I or AIM complexed with phospholipid, may have a direct antiatherosclerotic effect in various animal models of human atherosclerosis. The present investigation clearly indicates that a single intracarotid infusion of AIM-PL complexes leads to a significant, rapid reduction of a localized carotid lesion, induced by perivascular damage followed by a cholesterol-rich diet.

Similar to observations in humans administered synthetic HDL containing recombinant pro-apoA-I,7 the infusion of AIM-PL, even for a limited length of time (90 minutes), resulted in a remarkable rise of HDL-cholesterol levels persisting up to 72 hours after the administration. AIM levels in plasma were still detectable at euthanasia of the rabbits, 72 hours after the start of infusion. The AIM half-life of 40 hours evaluated in the present study is twice that of homologous rabbit or human apoA-I observed in kinetic studies performed in rabbits.2,6 Similarly, kinetic studies of dimeric AIM in humans clearly indicated an increased residence time in the circulation compared with monomeric AIM or wild-type apoA-I. This finding was recently confirmed in a clinical investigation comparing AIM to the chemically similar apoA-I variant. The delayed turnover of the AIM dimer cannot be attributed with certainty to any metabolic characteristic of the product, and it is consistent with the described structural stability of AIM dimer complexed with phospholipids and of naturally occurring AIM containing particles in the carriers. This may certainly contribute to the apparently improved capacity for cell cholesterol removal, as shown in a variety of in vitro and ex vivo experiments.

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.

**Figure 5.** Immunohistological analysis of the right carotid of rabbits infused with saline (A) or AIM-PL complexes (1000 mg of protein) (B), using a specific antibody that recognizes AIM but not rabbit apoA-I (magnification ×80).

<table>
<thead>
<tr>
<th>Table 2. Effect of AIM-PL Infusion on Plaque Lipid Content</th>
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<tr>
<td></td>
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<tr>
<td>Saline</td>
</tr>
<tr>
<td>DPPC</td>
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<tr>
<td>AIM 250 mg</td>
</tr>
<tr>
<td>AIM 500 mg</td>
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<tr>
<td>AIM 1000 mg</td>
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</table>

Data are expressed as percent of Oil Red O–positive staining ±SD.

*P*<0.01, †P*<0.05, ‡P*<0.001, §P*<0.0005 vs saline; ||P*<0.05, ¶P*<0.0005, #P*<0.05 vs DPPC.
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 HDL on regression and/or stabilization of complicated, fibrofatty human atheromass.

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

This work was supported by the Istituto Superiore di Sanità, Rome, Italy (ISS project “Gene Therapy”) and by Esperion Therapeutics (Ann Arbor, Mich). We are very grateful to Dr Silvia Fabbri, Dr Ivano Eberini, and Rose Ackermann for their technical assistance and advice.

References


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Circ Res. 2002;90:974-980; originally published online April 18, 2002;
doi: 10.1161/01.RES.000018422.31717.EE
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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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.