Recombinant Apolipoprotein A-I Milano Reduces Intimal Thickening After Balloon Injury in Hypercholesterolemic Rabbits

Sean Ameli, MD; Anna Hultgards-Nilsson, PhD; Bojan Cercek, MD; Prediman K. Shah, MD; James S. Forrester, MD; Hans Ageland, PhD; Jan Nilsson, MD, PhD

**Background** Several epidemiological studies have shown an inverse relation between high-density lipoprotein (HDL) cholesterol levels and coronary heart disease. Recently, observational studies have suggested a similar inverse relation between HDL and restenosis after coronary balloon angioplasty. Despite these observations, it is unclear whether this inverse relation reflects a direct vascular protective effect of HDL or atherogenic content. We investigated the effect of recombinant apo A-I Milano (apo A-I M), a mutant of human apo A-I with Arg-173 to Cys substitution, on intimal thickening after balloon injury in cholesterol-fed rabbits.

**Methods and Results** Cholesterol feeding was initiated 18 days before injury and continued until the time of death. Eight rabbits received intravenous injections of 40 mg of apo A-I M linked to a phospholipid carrier on alternate days, beginning 5 days before and continuing for 5 days after balloon injury of femoral and iliac arteries. Eight rabbits received the carrier alone, and four received neither apo A-I M nor the carrier. Three weeks after balloon injury, apo A-I M–treated rabbits had significantly reduced intimal thickness compared with the two control groups (mean±SD): 0.49±0.29 versus 1.14±0.38 mm² and 1.69±0.43 mm², P<.002 by ANOVA). The intima-media ratio was also significantly reduced by apo A-I M (0.7±0.2 versus 1.5±0.5 and 2.1±0.1, P<.002 by ANOVA) compared with the two controls. The fraction of intimal lesion covered by macrophages, as identified by immunohistochemistry using macrophage-specific monoclonal antibody, was significantly less in apo A-I M–treated rabbits compared with carrier-treated animals (25.3±17% versus 59.4±12.3%, P<.005). Aortic cholesterol content, measured in an additional 10 rabbits, did not differ significantly between apo A-I M–treated animals (n=5) and carrier-treated controls (n=5).

**Conclusions** Apo A-I M significantly reduced intimal thickening and macrophage content after balloon injury in cholesterol-fed rabbits without a change in arterial total cholesterol content. Although the precise mechanism of action remains to be defined, these findings are consistent with a direct vascular effect of apo A-I, which could have potential therapeutic implications. (Circulation. 1994;90:1935-1941.)

**Key Words** • restenosis • apolipoproteins • angioplasty • atherosclerosis

Several epidemiological studies have shown that the plasma concentration of high-density lipoprotein (HDL) is inversely correlated with the incidence of coronary artery disease. A similar inverse relation has been demonstrated for the plasma level of apolipoprotein A-I (apo A-I), the major protein component of HDL. Recently, a similar inverse relation has been described between the plasma level of HDL and restenosis rate after coronary balloon angioplasty.

The HDL molecule contains two major apoproteins: apo A-I and apo A-II. Immunochromatography has revealed the existence of two HDL subclasses: one containing apo A-I and the other containing both apo A-I and apo A-II. It is only the apo A-I–containing HDL subclass that is reduced in patients with premature coronary artery disease. Whether HDL or apo A-I is a marker for other risk factors for atherosclerosis or has a direct vascular protective effect is not entirely clear. Nevertheless, studies of transgenic animals that overexpress human apo A-I or apo A-II have suggested that elevated levels of apo A-I are antiatherogenic, whereas elevated levels of apo A-II are proatherogenic. Similarly, a reduction in the magnitude of intimal lesions and regression of preexisting lesions in rabbits after administration of homologous HDL suggests a direct vascular protective effect of HDL.

In 1980, a familial lipoprotein disorder characterized by a remarkable absence of atherosclerotic vascular disease despite markedly reduced plasma levels of HDL cholesterol was described in a family from Limone sul Garda in Northern Italy. Subsequent studies have shown that this disorder is associated with the presence of a mutant form of apo A-I with a single amino acid substitution, Arg-173 to Cys, which favors the formation of dimers. This mutant form of human apo A-I, known as apo A-I Milano (apo A-I M), constitutes 75% to 84% of the apolipoprotein content in affected subjects. All individuals with this mutation are heterozygous for the
mutant allele and have very low levels of HDL, virtually no HDL–2, reduced apo A-I levels, and a high incidence of elevated triglyceride levels without an increased incidence of vascular disease. The apo A-I M has a shortened residence time with more rapid catabolism, and its presence also accelerates the catabolism of apo A-I, accounting for its reduced blood level in subjects with the mutation. The substitution of cysteine for arginine appears to substantially alter the amphipathic nature of the α-helical fragment of apo A-I, increasing exposure of its hydrophobic residues. This structural modification is associated with a higher kinetic affinity of apo A-I M for lipids and an easier dissociation from lipid-protein complexes, which could contribute to its accelerated catabolism and increased efficiency for uptake of tissue lipids. Although the exact mechanism by which the apo A-I M mutation confers relative protection against vascular disease remains to be fully defined, its enhanced efficacy in removing cholesterol from tissues could be one such mechanism. The gene for apo A-I M has been cloned, and a genetically engineered version of the mutant protein is available for investigation.

In the present study, we tested the hypothesis that administration of recombinant apo A-I M would reduce intimal thickening after balloon injury in cholesterol-fed rabbits.

Methods

Twenty male New Zealand White rabbits weighing 3 to 4 kg were housed individually at an animal facility accredited by the American Association of Accreditation of Laboratory Animal Care under conditions of 12-hour light/dark periods. The protocol was approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center. The rabbits were fed standard chow supplemented with 1% cholesterol and 3% peanut oil during the entire study period. Eighteen days after initiation of the cholesterol-rich diet, eight rabbits received an intravenous injection of 40 mg of apo A-I M contained in a carrier of phosphatidylycholine-choline complex (dissolved in 10 mL of saline) in the left marginal ear vein every other day during a 10-day period. Eight other rabbits received the carrier phosphatidylycholine-choline complex without apo A-I M, and four received no treatment. Recombinant apo A-I M used in this study was supplied by Pharmacia Therapeutics, where it was produced using an inducible Escherichia coli expression system in which the product was secreted into the culture medium, mostly in its dimeric form. After fermentation, the filtered medium was purified by a number of chromatographic techniques using matrices such as Q-Sepharose FF, phenyl Sepharose FF, and Superdex 200 to separate dimeric and monomeric forms of the protein. Concentration and buffer exchanges were performed using ultrafiltration technique. The purified material was analyzed with respect to endotoxin level, identity, purity, and concentration. Final formulation of the protein as a phosphatidylcholine-protein complex was performed by dissolving phosphatidylcholine in cholate and then adding the protein solution. Cholate was then removed by dialysis.

Balloon injury was performed 4 days after the start of injections of apo A-I M or its carrier. Before balloon injury, all animals were anesthetized with ketamine (35 mg/kg body wt) and xylazine (5 mg/kg body wt). With standard sterile technique, a right carotid artery cutdown was performed, an intravenous injection of 1000 U heparin was given, and a 2.5-mm angioplasty catheter was introduced with a guidewire (high torque-floppy 0.014). Under fluoroscopic guidance, the catheter was advanced through the aorta to the femoral or iliac artery. The balloon was then inflated to a constant pressure of 8 atm for 1 minute. This step was repeated with a 1-minute interval, at the end of which the inflated balloon was retracted to the bifurcation of common iliac arteries and deflated. The catheter and wire were removed, the right carotid artery was ligated, and the overlying skin was sutured. In the animals receiving apo A-I M or its carrier, the balloon injury was performed after the second injection. All animals were returned to their housing fully awake, and routine postoperative care and follow-up were instituted. Cholesterol feeding was continued until death. Three weeks after the injury, the animals were anesthetized as described and exanguinated while the arteries were perfusion-fixed with 0.9% normal saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) (pH 7.2) under a constant pressure of 150 cm H2O for 20 minutes. The iliac and femoral arteries were removed and immersed in 4% paraformaldehyde for 3 hours and then transferred to a solution of 15% sucrose in PBS and stored overnight at 4°C. Three 0.5-mm sections of the iliac and femoral arteries were mounted on OCT medium for cryosectioning.

To determine the effect of apo A-I M on tissue cholesterol content, 10 additional rabbits were fed a cholesteral-rich diet for 4 weeks. During the last 10 days of cholesterol feeding, five rabbits received alternate-day injections of 40 mg of apo A-I M in phosphatidylycholine-choline complex, whereas the remaining five rabbits received only the carrier without apo A-I M. Aortas from these additional rabbits were removed, dried, weighed, and kept frozen at −70°C until processing for tissue cholesterol measurements.

Plasma and Aortic Tissue Cholesterol Measurement

Blood samples were taken from the peripheral ear artery of each animal before initiation of cholesterol supplementation as well as on the days of injury and death. The samples were centrifuged at 1400g for 10 minutes, and the supernatant was collected. Total serum cholesterol was determined by enzymatic technique, and serum HDL cholesterol was measured after precipitation of apo B-containing lipoproteins with phosphotungstic acid. Aortic tissue samples were homogenized in 50 mL of ice-cold methanol using a tissue homogenizer. Tissue cholesterol content was then extracted in chloroform as described by Folch et al. After evaporation, the material was dissolved in 4 mL of isopropanol, and the cholesterol content was determined as described by Zlatkis et al.

Apo A-I M Levels and Antibodies to Apo A-I M

The circulating level of apo A-I M was determined by an ELISA at Pharmacia Therapeutics. The ELISA uses two different mouse monoclonal antibodies (MAbs) developed against recombinant apo A-I M. The first MAb was used to coat the microtiter wells. Standard recombinant apo A-I M and appropriate dilutions of the serum samples were incubated. Bound apo A-I M was detected with the other MAb, which was biotinylated. The plate was developed using alkaline phosphatase, and the absorbance was read at 405 nm.

The presence of antibodies to apo A-I M was determined by a radioimmunoassay at Pharmacia Therapeutics. Serial dilutions of serum samples were incubated with 125I-labeled plasma apo A-I M. The antibody-antigen complex was precipitated with a commercial anti-rabbit cellulose suspension (SAC-CEL, IDS Ltd) and measured in a gamma counter.

Immunohistochemistry

The semithin (10- μm) sections were mounted on Superfrost glass slides, rinsed in PBS, and incubated with blocking serum for 1 hour. The sections were washed three times in PBS, permeabilized by transfer to PBS/0.2% Triton X-100 for 5 minutes, rinsed in PBS, and incubated with PBS/1.5% blocking serum for 30 minutes. Excess blocking serum was blotted from
the coverslips, and the sections were incubated with antibodies against macrophages (RAM 11) (Dako Corp) or smooth muscle–specific α-actin (HHF 35) (Dako Corp) for 18 hours at room temperature in a humidifying chamber. Controls included an irrelevant primary antibody as well as omission of the primary antibody. After being washed three times in PBS, the sections were incubated with biotinylated anti-mouse IgG for 60 minutes, washed three times in PBS, and stained with avidin/biotinylated alkaline phosphatase for 60 minutes. The sections were finally incubated with a solution of the alkaline phosphatase substrate for 30 minutes in the dark and counterstained with hematoxylin.

Morphometric Analysis

Ten-millimeter-thick sections of iliac and femoral arteries, taken at predetermined levels (proximal, mid, and distal), were cut at least 1 mm apart and stained with hematoxylin or by immunohistochemistry as described above and examined under microscopy. Total intimal area (area within the internal elastic lamina minus the luminal area) and the medial area (area between the internal elastic lamina and the external elastic lamina) were determined using the OPTILAB 2.03 image-analysis program (Grafix) on a Macintosh Ili computer. The percent of intimal surface area occupied by macrophages (as defined by immunostaining) was also measured by a blinded observer. The interobserver and intraobserver variabilities of these measurements in our laboratory were 2.8% and 2.7%, respectively.

Statistical Analysis

Values are given as mean±SD. Between-group comparisons were made with an unpaired t test. One-way ANOVA was used to compare three experimental groups followed by unpaired t test with Bonferroni’s correction. A corrected value of P<.05 was considered significant.

Results

Blood and Aortic Tissue Cholesterol Levels

The three groups of animals showed no significant changes in body weight during the course of the study and did not differ significantly in their plasma levels of total and HDL cholesterol (Table). The total aortic cholesterol content in apo A-I M–treated animals was similar to that of animals receiving the carrier alone.

Circulating Apo A-I M Level and Antibodies to Apo A-I M

At the time of injury, 4 days after the first injection, plasma apo A-I M level was 122±58 mg/dL in the apo A-I M–treated animals but undetectable in the two control groups (Table). Apo A-I M was no longer detected at the time of death, ie, 17 days after the last injection in the apo A-I M–treated animals. Conversely, antibodies against apo A-I M were detected in apo A-I M–treated animals at the time of death but not at the time of injury.

Intimal Thickness and Intima-to-Media Ratio

The intimal thickness of uninjured arteries averaged 0.02±0.02 mm², and balloon injury, without any treatment, resulted in a significant increase in the intimal thickness to 1.69±0.43 mm². Compared with no treatment, the carrier alone reduced the mean intimal thickness by 32% to 1.14±0.43 mm² (P=NS), whereas treatment with apo A-I M resulted in a 71% reduction in intimal thickness to 0.49±0.29 mm² (P<.002) (Figs 1 and 2). Compared with the carrier alone, apo A-I M reduced intimal thickness by 57% (P<.002).

The mean intima-to-media ratio in animals receiving no treatment was 2.1±0.1. Compared with no treatment, the carrier alone resulted in a 28% reduction in this ratio to 1.5±0.5 (P=NS), whereas treatment with apo A-I M resulted in a 67% reduction to 0.7±0.2 (P<.002). Compared with the carrier alone, apo A-I M reduced this ratio by 53% (P<.002).

Intimal Macrophage Content

Microscopic examination revealed the presence of macrophages in the media as well as the neointima (Fig 2). In the media, the macrophages were present in areas of cellular degeneration. The macrophage content of the media did not appear to differ between the groups of animals. However, compared with the carrier alone, apo A-I M reduced the fraction of intima covered by macrophages by 57% (59.4±12.3% versus 25.3±17%, P<.005).

Discussion

Main Findings

The major finding in this study was that recombinant human apo A-I M produced a substantial reduction (57% to 71%) in intimal thickening after balloon injury in the hypercholesterolemic rabbit. The reduction in intimal thickening was associated with a >50% reduction in intimal macrophage content without a significant change in circulating or aortic cholesterol content.

Potential Mechanisms of Reduced Intimal Thickening by Apo A-I M

The precise mechanisms by which recombinant apo A-I M reduced intimal thickening remain unclear. It has
been suggested that the potential mechanism by which the apo A-I M mutation confers relative protection against vascular disease may be related to its enhanced efficacy in removing cholesterol from tissues.\textsuperscript{15} The substitution of cysteine for arginine in apo A-I M appears to substantially alter the amphipathic nature of the $\alpha$-helical fragment of apo A-I, increasing exposure of its hydrophobic residues.\textsuperscript{15} This structural modification is associated with a higher kinetic affinity of apo A-I M for lipids and an easier dissociation from lipoprotein complexes, which could contribute to its accelerated catabolism and increased efficiency for uptake of tissue lipids.\textsuperscript{14} The protective effect of HDL against atherosclerosis has been attributed, in large part, to the ability of HDL to facilitate reverse cholesterol transport from peripheral tissues to the liver for removal or reutilization.\textsuperscript{18} In this process of reverse cholesterol transport, apo A-I serves as a ligand for binding of HDL to its putative receptor on cell membranes. The findings of Badimon et al\textsuperscript{10,11} in the hypercholesterolemic rabbit are consistent with this hypothesis. In a study of cholesterol-fed rabbits, administration of intravenous homologous HDL--very high-density lipoprotein (VHDL) fraction for 8 weeks significantly reduced the extent of aortic fatty streak formation.\textsuperscript{10} In an extension of this study, Badimon et al also reported apparent regression of intimal lesions produced by 90 days of cholesterol feeding when homologous HDL-VHDL was administered over 4 weeks.\textsuperscript{11} In both of these studies,\textsuperscript{10,11} HDL-VHDL--treated animals had a >50% reduction in the aortic total and esterified cholesterol content without a significant difference in circulating lipid levels compared with control animals. A reduction in intimal thickening has also been reported in balloon-injured arteries of transgenic rats overexpressing human apo A-I.\textsuperscript{19} In the present study, we did not observe a significant reduction in either the blood or the aortic tissue total cholesterol content with the administration of apo A-I M. These seemingly discrepant results may have occurred because of a longer duration (30 to 60 days) of treatment in the study of Badimon et al compared with a treatment period of only 10 days for apo A-I M in the present study. Furthermore, the difference in results may be attributed to the fact that Badimon et al used homologous HDL-VHDL lipoprotein fraction derived from rabbits by plasmapheresis, whereas in the present study, a recombinant form of a mutant of human apo A-I was used.

The significant reduction in intimal thickening produced by apo A-I M without a reduction in aortic tissue cholesterol content suggests that it may work through mechanisms other than or in addition to reverse cholesterol transport. Recent studies have demonstrated that oxidative modification of low-density lipoprotein (LDL), the main source of cholesterol ester in the foam cells of the early atherosclerotic lesion, is a key event that renders LDL highly atherogenic.\textsuperscript{20} The highly atherogenic properties of oxidized LDL have been attributed to its ability to stimulate monocyte attachment, migration, and retention in the arterial wall; monocyte transformation into macrophages and foam cells through the scavenger receptor pathway; T-cell activation; and endothelial dysfunction.\textsuperscript{20-24} Recent studies demonstrating that HDL impedes the oxidative modification of LDL suggest yet another mechanism by which HDL may produce its antiatherogenic effects.\textsuperscript{25-27} In the in vitro study of Parthasarathy et al,\textsuperscript{25} the macrophage-induced degradation of LDL that had been incubated with copper or iron was significantly inhibited by HDL. Furthermore, Mackness et al\textsuperscript{26} showed that HDL substantially decreased lipid peroxide formation from LDL in a concentration-dependent fashion without affecting the lag phase for conjugated diene formation. In contrast to the effects of HDL, antioxidant vitamins were shown to prolong the lag phase for conjugated diene formation without a significant effect on the formation of lipid peroxides.\textsuperscript{26} These findings suggest that antioxidant effects of HDL are mediated by
mechanisms that are different than those of antioxidant vitamins. HDL also inhibits the induction and production of monocyte chemotactic protein (MCP-1), reduces the cytotoxicity of oxidized LDL to endothelial and smooth muscle cells, scavenges lipid peroxides from peripheral tissues, and modifies the inflammatory response by inhibiting complement polymerization and promotes fibrinolysis. The precise mechanism(s) by which HDL inhibits oxidative modification of LDL are not clearly defined. Recent data suggest that the antioxidant effects of HDL may be in large part related to enzymes such as paraoxonase and platelet-activating factor acetylhydrolase associated with HDL. It is well known that monocyte recruitment into the arterial wall and monocyte-derived foam cell formation are promoted by oxidized LDL. These inflammatory cells have the ability to modulate the production of growth factors and cytokines that influence arterial smooth muscle cell migration, proliferation, and matrix secretion. In our study, apo A-I M substantially reduced the intimal inflammatory response, raising the possibility that apo A-I M may also inhibit LDL oxidation and/or the consequences of LDL oxidation. This hypothesis is consistent with the known ability of oxidized LDL to stimulate the growth of vascular smooth muscle cells and the inhibition of neointima formation by antioxidants in balloon-injured hypercholesterolemic rabbits.

**Experimental Protocol**

Our protocol used two sequential 1-minute balloon inflations of 8 atm each followed by pullback of the inflated catheter to the iliac bifurcation. This approach was shown to result consistently in intimal disruption in our pilot experiments. Since cellular processes responsible for the vascular response to balloon injury are triggered immediately after injury, we chose to begin administration of apo A-I 5 days before balloon injury to maximize any potential effects on the response to injury. The duration of administration and the time of death were chosen to minimize confounding effects of a possible immune response to human recombinant apo A-I M. Antibodies to apo A-I M were detected at the time of death but not at the time of balloon injury. The circulating lipid levels were not significantly altered by apo A-I M, except for a transient decrease in native...
HDL cholesterol levels noted when the animals were receiving apo A-I M. These findings are consistent with kinetic studies that have shown that apo A-I M undergoes accelerated catabolism and increases the catabolic rate of native or wild-type apo A-I as well, accounting for reduced circulating levels and a shortened residence time.\textsuperscript{14} It is of interest to note that the phospholipid carrier also produced a reduction in intimal thickening; however, the magnitude was small in comparison to the effect observed with apo A-I M, and it did not achieve statistical significance. The reasons for the modest effects observed with the carrier remain to be determined.

**Potential Significance and Clinical Relevance of the Study**

The results of the present study lend further support to the concept that apo A-I and HDL have a direct inhibitory effect against the formation of intimal lesions. Taken together with clinical and epidemiological studies showing an inverse relation between HDL or apo A-I and coronary heart disease\textsuperscript{1-4} and restenosis,\textsuperscript{5,6} these findings offer a potentially novel therapeutic approach against atherosclerosis as well as restenosis.

**Study Limitations**

Several potential limitations of our study should be pointed out. First, although a reduction in intimal inflammatory response observed with apo A-I M is consistent with the possibility that apo A-I M inhibits LDL oxidation or its consequences, no data to support such a hypothesis are provided. Second, apo A-I M treatment did not reduce aortic cholesterol content, but we cannot exclude the possibility that cholesterol or cholesterol ester content was reduced in the injured arteries. Measurement of tissue cholesterol content requires a relatively large amount of tissue, which precluded measurement in the injured arteries, thus necessitating the use of a separate series of animals for this purpose. Third, although recombinant apo A-I M was shown to reduce intimal thickness, its comparative effect to that of wild-type apo A-I needs to be defined. Fourth, the results produced by apo A-I M in rabbits may not necessarily extend to other species. Finally, the optimum dose and duration of administration necessary to achieve maximal effects of apo A-I M and any potential side effects need to be defined. Experiments are under way in our laboratory to address these issues.

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