Inhibition of Arterial Thrombus Formation by ApoA1 Milano

Dayuan Li, Sharon Weng, Baichun Yang, Dani S. Zander, Tom Saldeen, Wilmer W. Nichols, Saeed Khan, Jawahar L. Mehta

Abstract—The mutant form of human apoA1, known as apoA1 Milano, is formed as a result of arginine 173 to cysteine substitution and inhibits experimental atherosclerosis in cholesterol-fed animals. This study was designed to determine if apoA1 Milano would modify arterial thrombogenesis. Sprague Dawley rats were intravenously administered the carrier alone (n=8) or apoA1 Milano (20 mg·kg⁻¹·d⁻¹ for 4 to 10 days, n=17). The abdominal cavity was opened, and the abdominal aorta was isolated. Whatman paper impregnated with 35% FeCl₃ was wrapped around the surface of the aorta, and aortic flow was recorded continuously. In carrier-treated rats, an occlusive platelet-fibrin-rich thrombus was formed in 21.2±4.1 (mean±SD) minutes. Treatment of rats with apoA1 Milano markedly delayed time to thrombus formation (38.8±11.9 versus 21.2±4.1 minutes, P<0.01), inhibited platelet aggregation (25±7% versus 50±11%, P<0.01), and reduced weight of the thrombus (18.5±1.8 versus 23.7±2.3 mg/cm, P<0.01). Total cholesterol and HDL levels remained similar in both groups of rats, but plasma apoA1 Milano levels were elevated in apoA1 Milano–treated rats. In in vitro studies, incubation of platelets with apoA1 Milano reduced ADP-induced platelet aggregation by about 50%, but apoA1 Milano had no direct effect on vasoreactivity. This study provides further evidence for critical role of platelets in thrombosis. Use of apoA1 Milano offers a novel approach to inhibit arterial thrombosis. (Arterioscler Thromb Vasc Biol. 1999;19:378-383.)

Key Words: apoA1 ◆ apolipoproteins ◆ lipoproteins, HDL ◆ thrombosis

Thrombus formation in the atherosclerotic coronary artery is the immediate cause of acute myocardial ischemia. Coronary artery thrombosis often is initiated by abrupt disruption of the atherosclerotic plaque and deposition and activation of platelets on the subendothelial layers in the disrupted plaque.¹,² Experimental and clinical studies have shown reduction in atherosclerosis and cardiac events with aggressive lowering of serum total and LDL cholesterol levels.³–⁵ Epidemiological studies also show an inverse relationship between the plasma levels of HDL cholesterol and atherosclerosis.⁶–⁷ Plasma levels of apoA1, the major protein component of HDL cholesterol, are reduced in patients with premature coronary artery disease.⁸ Whether increased HDL cholesterol or apoA1 is a marker for protection from athero-sclerosis or has a direct vasoprotective effect is not known; however, studies in transgenic animal models that overexpress apoA1 suggest that elevated levels of apoA1 are antiatherogenic.⁹

In 1980, Franceschini et al¹⁰ described a family from Limone sur Garda in Northern Italy with a lipoprotein disorder characterized by a striking absence of atherosclerosis despite very low plasma levels of HDL cholesterol. Subsequent studies by this group¹¹ showed that the absence of atherosclerosis in this family was associated with the presence of a mutant form of apoA1 with a single amino acid substitution, arginine 173 to cysteine, which favors the formation of dimers. This mutant form of apoA1, thereafter termed apoA1 Milano, constitutes a large component of the apolipoprotein content in the affected family. All subjects in this family were found to be heterozygous for the mutant allele and had very low levels of HDL cholesterol, high triglyceride levels, and yet no atherosclerosis. The apoA1 Milano has a shortened residence time and causes rapid catabolism of apoA1 in these subjects.¹² The substitution of cysteine for arginine appears to alter the amphipathic nature of the α-helical fragment of apoA1, increasing exposure of its hydrophobic residues.¹³ This structural modification is associated with high affinity of apoA1 Milano for lipids in the lipid-protein complexes and their easy removal. The gene for apoA1 Milano has been cloned by Pharmacia, and the genetically engineered version of the mutant protein has been used in experimental studies. In a study by Ameli et al,¹⁴ administration of the genetically engineered apoA1 Milano caused a marked reduction in the magnitude of intimal lesions and regression of preexisting lesions in cholesterol-fed rabbits.

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Because HDL cholesterol fraction per se decreases platelet aggregation and negates the stimulatory effect of oxidized LDL cholesterol on platelet aggregation, and apoA1 Milano in particular facilitates removal of LDL cholesterol, we hypothesized that administration of recombinant apoA1 Milano would inhibit platelet-dependent thrombus formation. This hypothesis was tested in a rat model of arterial thrombosis.

Materials and Methods

Study Protocol

Twenty five Sprague Dawley (Harlan, Indianapolis, IN) rats weighing 250 to 350 g were injected with standard rat chow. Seventeen rats received an intravenous injection of 20 mg/kg of apoA1 Milano in a carrier (or vehicle) of phosphatidylcholine-choline complex (dissolved in saline) in the tail vein every day for 8 to 10 days. Eight rats received an intravenous injection of the carrier of phosphatidylcholine-choline complex. Recombinant apoA1 Milano used in this study was supplied by Pharmacia and Upjohn.

An arterial thrombus model described by Kurz et al was used in this study. All animals were anesthetized with pentobarbital (30 mg/kg). Abdominal cavity was opened and approximately 1.2 cm length abdominal aorta was isolated. The aortic blood flow was recorded continuously by an ultrasonic Doppler flow probe (Crystal Biotech). The signal from the Doppler flow probe was calibrated against an electromagnetic flow probe, and the flow was expressed in mL/min as described earlier. Whatman paper soaked in 35% FeCl3 was wrapped around the external surface of the aorta. When thrombus was formed, thrombus along with the exposed aorta was taken out and weighed.

About 3 mL of blood was collected for platelet aggregation, measurement of plasma lipids, and apoA1 Milano levels. A segment of the thrombus along with aorta was saved in 2.5% glutaraldehyde, and another segment was saved in 10% neutral buffered formalin. ApoA1 Milano–treated rats were studied in parallel with carrier-treated rats.

Platelet Preparation and Aggregation

Blood was mixed thoroughly with 3.8% sodium citrate (9:1). Blood was centrifuged at 1200 rpm for 10 minutes at room temperature to obtain platelet-rich plasma (PRP) and centrifuged again at 3000 rpm for 15 minutes to obtain platelet-poor plasma (PPP). Platelet count in PRP was counted and kept at about 2 × 10^10 to 3 × 10^11 cells/mL. ADP (final concentration 20 μmol/L) was used as stimulus for platelet aggregation as described previously. All platelet aggregations were performed in a 4-channel Chronolog aggregometer. Every time platelet aggregation was performed in an apoA1 Milano–treated rat, platelet aggregation was also done in a vehicle-treated rat. In other in vitro studies, PRP was incubated with A1 Milano (0.6 mg/mL) or the vehicle for 60 minutes at 37°C and platelet aggregation in response to ADP determined.

Plasma HDL and Total Cholesterol Measurement

The blood 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 apoB-containing lipoproteins with phosphotungstic acid.

ApoA1 Milano Levels

Plasma levels of apoA1 Milano were determined by an ELISA by Dr P.K. Shah of the University of California, Cedar-Sinai Medical Center, Los Angeles, CA. The ELISA uses 2 different mouse monoclonal antibodies (MAbs) developed against recombinant apoA1 Milano. The first MAb was used to coat the microtiter wells. Standard recombinant apoA1 Milano and appropriate dilution of the serum samples were incubated. Bound apoA1 Milano was detected with the other MAb, which was biotinylated. The plate was developed using alkaline phosphatase, and the absorbance was read at 405 nm.

Morphologic Analysis of Thrombus

The method for scanning electron microscopy was similar to the method described previously by. Essentially, aortic segments were fixed in 2.5% glutaraldehyde and then placed in 1% osmium tetroxide in 1% cacodylate buffer (pH 7.2). After several washes in cacodylate buffer, aortic segments were dehydrated in graded alcohols and 1% acetone and then refrigerated overnight in amyl acetate. Specimens were dried to the critical point and coated with silver in a Hummer 5 sputter coating system (Anatech Ltd). Under a dissecting microscope, tissues were cut with a razor blade for full exposure of the intimal surface. Specimens were examined with a Hitachi S450 scanning electron microscope.

For light microscopic examination, tissues from carrier and apoA1 Milano–treated rats were fixed in 10% neutral buffered formalin for 3 days, processed routinely through alcohols and xylene, and then embedded in paraffin. Five micron thick sections were cut at 2 levels in the paraffin block and stained with standard hematoxylin-eosin stain. Additional sections were stained with a Prussian blue (iron) stain. Other sections were stained to illustrate fibrin and platelets using Carstairs’ method; in brief, 5 μm thick paraffin sections were hydrated to water, placed in 5% ferric alun for 5 minutes, rinsed in running tap water, stained in Mayer’s hematoxylin for 5 minutes, and then rinsed again in running tap water. Slides were placed for 1 hour in picric acid-orange G solution (composition: 20 mL saturated aqueous picric acid, 80 mL saturated picric acid in isopropanol, and 0.2 g orange G) and then rinsed in distilled water. For 5 minutes, slides were placed in ponceau-fuchsin solution (composition: 0.5 g acid fuchsin, 0.5 g ponceau 2R, 1 mL acetic acid, and distilled water; final volume 100 mL) and then rinsed in distilled water. Slides were treated with 1% phosphotungstic acid until the muscle appeared red and the background pale pink and then rinsed in distilled water. Slides were stained with anilin blue solution (1 g anilin blue in 100 mL 1% acetic acid) for 30 minutes, rinsed in several changes of distilled water, dehydrated, cleared, and then mounted. With a fixation time >48 hours, Carstairs’ method for fibrin and platelets produces differential staining of fibrin (bright red), platelets (gray-blue to navy), collagen (bright blue), muscle (red), and red blood cells (yellow).

ApoA1 Milano and Vascular Reactivity

To determine the direct effect of apoA1 Milano on vascular reactivity, rat aortic rings (4 to 5 mm) were obtained from 6 different rats. Care was taken to avoid any unnecessary manipulation of vessels. The rings were then mounted onto wire stirrups connected to force transducers (Kistler Morse) and placed in custom-designed tissue-organ baths filled with oxygen-saturated (95% O2, 5% CO2) Krebs-Ringer buffer (composition in mmol/L: NaCl 118, KCl 4.7, CaCl2 1.3, KH2PO4 1.2, MgCl2 1.2, NaHCO3 12.5, Na-EDTA 0.01, and glucose 11.1, pH 7.4). The rings were then stretched to and maintained at a preload tone of 2 g for approximately 60 minutes. During the period of equilibration, rings were incubated with apoA1 Milano (0.6 mg/mL) or carrier for 1 hour at 37°C. Buffer was changed every 30 minutes and continuously bubbled with 95% O2, 5% CO2. After equilibration, rings were exposed to cumulative concentrations of norepinephrine (NE, 10^−7 to 10^−5 M) to determine the vasococontractor response. Other rings were contracted with NE (10^−2 to 10^−6 M) to obtain 60% to 70% of maximal contraction, then exposed to the endothelium-dependent receptor-mediated vasorelaxant acetylcholine (Acetylcholine (ACH), 10^−5 to 10^−6 M) to determine endothelium-dependent vasorelaxation.

Statistical Analysis

All data are given as mean ± SD. ANOVA was used to compare the 2 experimental groups followed by unpaired t-test with Bonferroni’s correction. A P value of ≤0.05 was considered significant.

Results

Blood Total Cholesterol, LDL, and ApoA1 Milano Levels

Both group of rats showed no significant change in body weight during the course of the study and did not differ...
significantly in their plasma levels of total cholesterol. The HDL cholesterol levels were slightly lower (P, NS) in the apoA1 Milano–treated rats. Plasma apoA1 Milano levels were identified only in the apoA1 Milano–treated rats (Table).

**Platelet Aggregation in ApoA1 Milano–Treated Rats**

Treatment with apoA1 Milano markedly inhibited platelet aggregation in each of the treated rats (mean 25 ± 7% versus 50 ± 11% in vehicle-treated rats, n = 17 and n = 8, respectively; P < 0.01). Representative examples of platelet aggregation patterns in the 2 groups of rats are shown in Figure 1. There was no difference in platelet counts in the 2 groups of rats.

**Time to Thrombosis and Weight of Thrombus**

Application of FeCl₃ in vehicle-treated rats resulted in oscillations in aortic blood flow for about 15 minutes. This was followed by rapid decrease in blood flow and eventually total cessation, indicating occlusive thrombus formation. Once the flow had totally ceased, there was no spontaneous return of flow over 1 hour of observation in all vehicle-treated animals. A typical pattern of thrombus formation in a vehicle-treated rat is shown in Figure 1.

Treatment of rats with apoA1 Milano markedly delayed time to thrombus formation, and the mean value increased 86% to 38.8 ± 11.9 minutes (compared with 21.2 ± 4.1 minutes in the vehicle-treated group) (Figure 2). Following cessation of blood flow, the thrombus was often unstable as evident from return of flow transiently in all apoA1 Milano–treated rats. A representative example of markedly delayed time to thrombus formation and unstable thrombus in an apoA1 Milano–treated rat is shown in Figure 1. The weight of the thrombus in all apoA1 Milano–treated rats was much less than in the vehicle-treated rats (Figure 2).

**Morphology of the Thrombus**

Scanning electron microscopic examination of the aortic region with thrombus revealed endothelial disruption, deposition of platelets on the intimal surface, fibrin strands, and red blood cells, especially at the base of the thrombus (Figure 3A). Cross-sectional view of the aortas of vehicle-treated animals showed extensive platelet-fibrin deposition along the entire intimal surface. The main body of the thrombus consisted of large number of red blood cells tethered with fibrin, but few platelets (Figure 3B). There was no discernible difference in the morphology of the thrombus in the 2 groups of rats.

Light microscopic examination of the thrombi (Figure 4) revealed irregular masses of platelets with focal connections to the intimal surfaces, and variable numbers of red blood cells, accounting for the majority of the thrombus. Fibrin occupied spaces between the platelet aggregates and the red blood cells, particularly adjacent to the arterial wall. Subintimal deposits of crystalline material were present and stained blue with the Prussian blue stain, consistent with transvascular penetration of FeCl₃ (Figure 4). Again, there were no discernible differences in the morphology of the thrombi in the carrier-treated and apoA1 Milano–treated groups.

**Direct Effect of ApoA1 Milano on Platelet Aggregation**

Incubation of PRP with apoA1 Milano markedly decreased ADP-induced platelet aggregation in each of the 6 rats (mean platelet aggregation at 5 minutes: 22.8 ± 10.7% versus 54.5 ± 20.9%; P < 0.01).

**ApoA1 Milano and Vasoreactivity**

Incubation of aortic rings with apoA1 Milano had no effect on vasoconstrictor response to NE (EC₅₀: 5.5 ± 0.4 × 10⁻⁷ M versus 5.6 ± 0.4 × 10⁻⁷ M; P, NS). Likewise, incubation of aortic rings with apoA1 Milano had no effect on relaxant response to Ach (IC₅₀: 5.1 ± 0.4 × 10⁻⁸ M versus 5.0 ± 0.2 × 10⁻⁸ M; P, NS).

**Discussion**

**Cholesterol and Thrombosis**

It is generally presumed that high levels of LDL cholesterol lead to enhanced thrombus formation, probably a result of reduced vasomotion and enhanced platelet aggregability. This has been attributed in part to the loss of NO release and its activity in cholesterol-rich platelets and vascular tissues.
Most episodes of acute myocardial infarction are a result of formation of occlusive platelet-rich thrombus in the atherosclerotic narrowed coronary arteries. There is also evidence that myocardial injury is greater in hypercholesterolemic animals subjected to coronary artery occlusion; this is related to diminished vasomotion of the coronary artery and enhanced platelet aggregation in the coronary arterioles. Sawa et al described a 2-fold increase in plasma levels of plasminogen activator inhibitor-1 (PAI-1) as well as PAI-1 mRNA in hypercholesterolemic rabbits with indwelling catheters. Abela et al showed that hypercholesterolemia causes markedly large thrombi in rabbits subjected to balloon injury. However, there are no data on the influence of alteration in lipid components on in situ platelet-fibrin rich thrombus formation that is akin to human arterial thrombus. Also, there are no data on the influence of modification of HDL cholesterol on thrombogenicity.

Observations in the Current Study
The major finding in this study was that administration of recombinant apoA1 Milano produces a substantial inhibition of platelet aggregation and delays formation of arterial thrombus in rats. In vitro incubation of platelets with apoA1 Milano for a short period also decreased ADP-induced platelet aggregation. However, there was no significant effect of apoA1 Milano on vasoconstriction or endothelium-dependent relaxation.

Figure 2. Prolongation of time to thrombus and reduction in the weight of the thrombus in apoA1 Milano–treated rats compared with vehicle-treated rats. Number of rats in each group is 17 and 8, respectively. Data in mean±SD.

Figure 3. Morphology of the thrombus in a carrier-treated rat as determined by scanning electron microscopy. Beginning of the thrombus is characterized by endothelial disruption, large number of platelets adherent to the intimal surface, and red blood cells tethered with fibrin bands (A). Cross-section of the aorta through the body of the thrombus shows very large number of platelet-fibrin aggregates adherent to the intimal surface of the entire vessel wall. The core of the thrombus consists primarily of red blood cells with inter-twined fibrin strands (B).

Figure 4. Light microscopic morphology of the aortic thrombus in a carrier-treated rat. A and B, Carstairs’ method for fibrin and platelets. Irregular aggregates of platelets (P) demonstrate connections to the intimal surface of the aorta. The center of the thrombus consists primarily of red blood cells (yellow). Fibrin (red) is interspersed between the platelet aggregates and red blood cells. Original magnification ×40 (A) and ×200 (B). C, Prussian blue (iron) stain. Subendocardial FeCl₃ deposits (dark blue). Original magnification ×40.
Model of Arterial Thrombosis

The model of arterial thrombosis used in these studies has been used to study the effect of a variety of thrombolytic effect of a variety of agents. The in situ thrombus induced in the rat aorta by external application of FeCl₃, is akin to human intracoronary thrombus in its cellular composition and fibrin content. Light microscopy showed penetration of FeCl₃ across the vessel wall (Prussian blue stain), adherence of large numbers of platelets to the subendothelial layers at several points along the circumference of the vessel wall, and the central mass of the thrombus consisting predominantly of red blood cells with interspersed fibrin. These are also characteristics of arterial thrombi in human coronary arteries. The rapidity with which the thrombus is formed in this model depends on the concentration of FeCl₃ applied on the external surface of the blood vessel. The thrombus, once formed, is stable and not subject to spontaneous dissolution. We chose the 35% FeCl₃ concentration because this concentration causes occlusive thrombus formation in 18 to 24 minutes in all rats weighing about 300 g. Because of the relatively low cost of animals and the ability to study formation of occlusive thrombus, vascular reactivity, and platelet aggregation, this model may be considered useful in evaluation of different pharmacologic agents that modulate platelet function, vascular reactivity, and lipid profile.

Mechanism of Antithrombotic Effect of ApoA1 Milano

It has been suggested that apoA1 Milano provides relative protection against vascular disease related to its ability to remove cholesterol from tissues. The apoA1 mutant appears to substantially alter the amphipathic nature of the a-helical fragment of apoA1, thus increasing the exposure of its hydrophobic residues. This structural modification is associated with a higher kinetic affinity of apoA1 Milano 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.

The protective effect of HDL against atherosclerosis has accordingly been attributed in large part to the ability of HDL to facilitate reverse cholesterol transport from peripheral tissues to the liver for removal or revitalization. Badimon et al have shown that HDL cholesterol can reverse atherosclerotic lesions in cholesterol-fed rabbits. Burkey et al showed that elevated apoA1 fractions reduce aortic smooth muscle cell proliferation, a key early feature of atherosclerosis.

Recent studies from our laboratory have indicated that it is the oxidized LDL cholesterol fraction that enhances platelet aggregation and downregulates NO synthase expression. Studies from other laboratories have shown that HDL inhibits oxidation of LDL cholesterol. We have also shown that HDL cholesterol blocks the platelet stimulatory affect of LDL cholesterol, and this effect of HDL cholesterol appears in large part mediated by reversal of the suppressive effect of LDL cholesterol on NO synthase in platelets. Stimulation of NO synthesis in the endothelial cells and platelets has a powerful antithrombotic effect. This phenomenon has been adequately demonstrated in animal models of thrombosis, wherein NO donors were used. The current studies showed that administration of apoA1 Milano or in vitro incubation of PRP with apoA1 Milano markedly reduced platelet aggregation. Whether platelet NO production or its activity is enhanced by apoA1 Milano cannot be discerned from this study. However, vascular endothelium-dependent relaxation was not altered when aortic rings were incubated ex vivo with apoA1 Milano. It is, nonetheless, reasonable to postulate that platelet inhibition and/or modulation of vascular reactivity are operative in delaying thrombus formation in apoA1 Milano–treated animals.

Activation of endogenous fibrinolytic pathways by apoA1 fraction of HDL cholesterol has also been well characterized. A similar activation of fibrinolytic pathway in apoA1 Milano–treated rats may relate to unstable thrombus and its tendency to spontaneously dissolve.

The light and scanning electron microscopy findings showed in a semiquantitative fashion that the composition of the thrombus in carrier- and apoA1 Milano–treated rats was similar, which indicates that platelets are able to aggregate and form an occlusive thrombus in the presence of extensive arterial endothelial injury despite treatment with apoA1 Milano. This suggests that the inhibitory effects of these agents are quantitative rather than qualitative.

Limitations of the Study

There are some important limitations of the study. First, it would have been ideal to conduct similar studies with wild-type apoA1 to determine if prolongation of time to thrombosis and inhibition of platelet aggregation are unique to the administration of apoA1 Milano. It is possible that wild-type apoA1 can decrease platelet aggregation and hence prolong time to thrombosis. Second, the study lacked information on apoA1 levels in rats treated with carrier or apoA1 Milano. Because apoA1 Milano has been characterized to induce catabolism of apoA1 as well as HDL cholesterol, it is possible that the apoA1 levels were low in apoA1 Milano–treated rats. This concept gains support from the observation of lower HDL cholesterol levels in apoA1 Milano–treated rats (41.8 vs. 49.4 mg/dL in carrier-treated rats). Similar observation was made by Ameli et al in rabbits fed high cholesterol diet and administered apoA1 Milano. Third, the apoA1 Milano levels were somewhat lower in this study compared with another study wherein a similar dose was given to rabbits. The differences may relate to differences in species (rat versus rabbit), diet (regular diet versus high cholesterol diet), and study end-point (thrombosis versus atherosclerosis). In the original report on apoA1 mutant, a wide range of apoA1 levels (35 to 116 mg/dL) were identified in the index family. In the transgenic mice, saturated fat diet increased apoA1 levels markedly from 38 to 58 mg/dL. Our studies were conducted in fasting rats fed regular chow. In further studies, all these issues will need to carefully evaluated.

Conclusion

This study confirms a critical role of platelet activation in arterial thrombosis. The use of apoA1 Milano—or possibly use of modalities that may increase apoA1 only—offers a novel approach directed to the inhibition of platelet-mediated thrombosis. However, the precise dose and duration of administration of apoA1 mutant needs to be determined and the consistency of these findings across species assessed.
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