Inhibition of rupture of established atherosclerotic plaques by treatment with apolipoprotein A-I

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Aims
Plasma concentrations of high-density lipoprotein (HDL)-cholesterol correlate inversely with the incidence of myocardial infarction in humans. We investigated the effect of treatment with human apolipoprotein A-I (apoA-I), the principal protein of HDL, on plaque disruption in an animal model.

Methods and results
Seventy apolipoprotein E knockout mice were induced to develop atherosclerotic lesions in the brachiocephalic artery by feeding a high-fat diet for 9 weeks. Mice then received twice-weekly treatment with human apoA-I (8 mg/kg) or vehicle, for 2 weeks. The incidence of acute plaque disruption was reduced by 65% in mice receiving apoA-I (P < 0.01). Plaques in treated mice had a more stable phenotype, with an increase in smooth muscle cell (SMC): macrophage ratio (P = 0.05), principally the consequence of an increase in the number of SMC in plaques. In the fibrous cap, there were reductions in matrix metalloproteinase-13 (−69%, P < 0.0001) and S100A4, a marker of SMC de-differentiation (−60%, P < 0.0001). These results indicate that 2 weeks of treatment with small amounts of human apoA-I produces more stable plaques in a mouse model.

Conclusion
Treatment with apoA-I has the potential to stabilize plaques and prevent plaque rupture in humans.

Keywords
Apolipoprotein A-I • Plaque rupture • Atherosclerosis • Smooth muscle cells

1. Introduction
Atherosclerosis is a chronic disease characterized by the accumulation of lipids and inflammatory cells within the walls of arteries. These deposits form plaques which remain clinically silent for much of the course of the disease. In its later stages, the disease may be complicated by life-threatening events such as acute plaque rupture or erosion followed by coronary thrombosis, particularly when the necrotic core is exposed.¹,² This can lead to vascular occlusion and its potentially devastating clinical sequelae.

Plaque rupture occurs when there is a breach in the integrity of the extracellular matrix of the plaque, usually in the shoulder region or in the collagen and elastin-rich layer (fibrous cap) that overlies the necrotic core.¹ Chronic arterial inflammation is associated with an increased turnover of the extracellular matrix.³,⁴ Smooth muscle cells (SMCs) are the major secretors of matrix, while enzymes such as matrix metalloproteinases (MMPs) and cathepsins⁵ remodel the extracellular matrix and may weaken the plaque.³ Thus, interventions that increase SMC numbers and matrix deposition or reduce the secretion of matrix-degrading enzymes have the potential to stabilize plaques.

The observation that apolipoprotein E (apoE) knockout mice develop unstable lesions in the brachiocephalic artery⁶–¹⁰ has provided a model in which to investigate how specific factors may impact on plaque disruption. Williams et al.⁶ have defined plaque disruption in this model as breaches of the fibrous cap combined with the presence of red blood cells within the body of the plaque. The requirement for intraplaque haemorrhage, which can only occur during life, eliminates the possibility of a post-mortem tissue artefact being labelled erroneously as a rupture.

Human epidemiological studies suggest that high-density lipoproteins (HDLs) protect against atherosclerosis.¹¹ Possible cardioprotective effects of HDL include their ability to promote efflux of cholesterol from macrophages and their ability to inhibit oxidation and inflammation in the artery wall.¹² Previous animal studies have shown that high concentrations (>140 mg/dL) of apolipoprotein A-I (apoA-I), the principal structural protein of HDL, can retard the development of atherosclerosis in apoE knockout mice,¹³–¹⁵ and in
C57BL/6 mice fed an atherogenic diet. In addition, apoA-I slowed the progression of atherosclerosis after arterial transplantation and caused plaque regression in LDL-receptor-deficient mice after liver-directed gene transfer of apoA-I. However, these studies did not directly address the issue of changes in actual plaque stability, which would be desirable for any potential clinical intervention. We have therefore investigated the effects of short-term treatment with low doses of human apoA-I on the incidence of plaque rupture in apoE knockout mice.

2. Methods

2.1 Reagents

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

2.2 Animals and diet

A total of 70 male apoE knockout mice on a C57BL/6 background were fed a high-fat diet containing 21% fat supplemented with 0.15% (wt/wt) cholesterol (Specialty Feeds, Glen Forrest, WA, Australia) from 6 weeks of age onwards. Mice were housed in the Biological Facility at the Heart Research Institute, University of Sydney. The investigation complies with the Australian code of practice for the care and use of animals for scientific purposes (Approval #2008/024A) and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996): PHS Approved Animal Welfare Assurance University of Sydney A5029-01.

2.3 Isolation and reconstitution of apolipoprotein A-I for infusion

HDLs (density 1.07–1.21 g/mL) were isolated from pooled human plasma (Gribbles Pathology, Adelaide, SA, Australia) by sequential ultracentrifugation at 4°C. ApoA-I was isolated from HDL as previously described. Immediately prior to use, apoA-I was extensively dialysed against endotoxin-free PBS. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA).

2.4 Administration of apolipoprotein A-I to mice

After 15 weeks of age and 9 weeks of a high-fat diet, mice were randomized to receive either human apoA-I (8 mg/kg of bodyweight) in PBS or PBS alone (controls) by tail vein injection twice weekly for 2 weeks.

2.5 Preparation of apoA-I-containing discoidal HDL

Discoidal HDL, reconstituted with 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC, Avanti Polar Lipids, Alabaster, AL, USA), was prepared by the cholate dialysis method. For every 2 mg of apoA-I protein to be reconstituted, 87 µL of PLPC [100 mg/mL PLPC in chloroform/methanol 2:1 (v/v)] was used. Discs were dialysed into endotoxin-free PBS and used within 1 week of preparation.

2.6 Removal of the brachiocephalic artery

Four days after the final injection of either human apoA-I or PBS, mice were anaesthetized with inhaled methoxyflurane, 1 mL of blood was collected by cardiac puncture and mice sacrificed by cervical dislocation. After perfusion (PBS followed by 4% paraformaldehyde), the brachiocephalic artery was removed with part of the aortic arch and right subclavian artery retained to aid with orientation. Specimens were further fixed in 4% paraformaldehyde overnight.

2.7 Histology

Brachiocephalic arteries were embedded in paraffin. Beginning at the origin of the brachiocephalic artery, sections were cut every 3 µm for 150 µm into the artery and mounted onto silane-coated slides.

2.8 Identification of plaque disruption

The origin of the brachiocephalic artery, and then every 10th section thereafter, for a total of 6 sections, were stained using Verhoeff’s iron haematoxylin method and counterstained with Van Giesen’s stain (VVG). Sections were examined microscopically for evidence of a breach in collagen and elastin-rich layer overlying the necrotic core.

2.9 Morphometry

Images were captured digitally with a Zeiss Axio (Imaging Solutions, North Ryde, NSW, Australia) and used for computerized morphometric analysis (Axiovision rel 4.5). The section at which the plaque was of the greatest cross-sectional area was analysed. The following parameters were characterized as previously described, cross-sectional areas of the artery, the intima, the lumen, and the media; maximum intimal thickness; maximum medial thickness. Buried fibrous layers were identified and counted.

2.10 Analysis of plaque composition

Images of VVG-stained delipidated paraffin sections were digitally processed to monochrome causing delipidated areas to appear as white. The white area was then expressed as a percentage of the total plaque area; a method previously validated against oil red O staining. One section from each animal was stained with picrosirius red (0.1%) and imaged to quantify plaque collagen content.

2.11 Immunohistochemistry

Sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval. Sections stained for α-smooth muscle actin (α-SMA) were blocked using the M.O.M. kit (Vector Laboratories, Burlingame, CA, CA, USA). All other sections were blocked with 10% serum, from the species in which the secondary antibody was raised, in tris-buffered saline. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 20 min. Sections were stained for SMC (mouse monoclonal anti-α-SMA, 1:100, Sigma-Aldrich, Castle Hill, NSW, Australia), macrophages (rat anti-mouse mac-3, 1:50, Pharmingen, North Ryde, NSW, Australia), S100A4 (rabbit polyclonal anti-mouse S100A4, 1:500, Abcam, Cambridge, UK), matrix-degrading proteinase (MMP)-13 (sheep polyclonal anti-mouse MMP-13, 1:100, Abcam, Cambridge, UK) intercellular adhesion molecule-1 (goat polyclonal anti-mouse ICAM-1, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), vascular cell adhesion molecule-1 (rabbit polyclonal anti-mouse VCAM-1, 1:100, Santa Cruz Biochemistry). The primary antibody was visualized with an appropriate secondary, avidin–biotin complex (ABC, Vector Laboratories Burlingame, CA, USA) and diaminobenzidine. The area of diaminobenzidine positive staining was measured with image analysis software (ImageJ, National Institutes of Health, Bethesda, Maryland, USA).

2.12 Assessment of apoptosis

A DNA fragmentation detection kit (TdT-FragEL, Calbiochem, Kilsyth, VIC, Australia) was used according to the manufacturer’s protocol. A rabbit monoclonal antibody to cleaved PARP (Abcam, Cambridge, UK) was used at 1:350 dilution with a goat anti-rabbit biotin secondary (1:2000, Vector Laboratories Burlingame, CA, USA).
2.13 Analysis of plasma lipids
Human apoA-I was assayed using double-layer enzyme-linked immunosorbent assay (ELISA) using goat anti-human apoA-I and horse radish peroxidase-conjugated goat anti-human apoA-I, (both from Biodesign, Saco, ME, USA). The anti-human antibody did not react with endogenous murine apoA-I. Mouse apoA-I was assayed by triple-layer ELISA (goat anti-mouse apoA-I, rabbit anti-mouse apoA-I, and horse radish peroxidase-conjugated goat anti-rabbit IgG, all from Biodesign). Goat anti-mouse apoA-I used does not react with human apoA-I. Total cholesterol, triglycerides, and HDL cholesterol concentrations were measured by enzymatic colorimetric assay (Roche, Castle Hill, NSW, Australia).

2.14 Statistical analyses
Values are expressed as means ± SEM. Statistical comparisons of plaque disruption were made with Fisher’s exact test. The number of buried fibrous layers was analysed using the Mann–Whitney U test. In all other cases comparisons were made by Student’s-t test with Welch’s correction where appropriate. The software used for statistical analyses was Prism 4 for Windows (GraphPad Software, La Jolla, CA, USA). A value of P < 0.05 was considered significant.

3. Results

3.1 Plasma lipids
There were no significant differences in the plasma concentrations of total cholesterol, triglyceride, or HDL-cholesterol between animals that were infused with human apoA-I and those that received PBS alone (Table 1). Although 4 days had passed since the last injections were given, human apoA-I was still detectable in plasma collected at sacrifice (12 ± 8 μg/dL). Human apoA-I was not detectable in the plasma of mice that received PBS alone, indicating no cross-reactivity with endogenous murine apoA-I. There was no difference in murine apoA-I concentration between the groups (Table 1).

3.2 Effects of apoA-I on acute plaque disruption
Brachiocephalic arteries were step sectioned and every 10th section examined for evidence of plaque disruption and intraplaque haemorrhage (Figure 1A). In the group of mice that received human apoA-I, combined intraplaque haemorrhage and plaque disruption was found in 6 of 35 animals (17%) while in the control group these were present in 17 of 35 animals (49%) (P < 0.05) (Figure 1B). No more than a single disruption was ever detected in plaques, and it was always associated with intraplaque haemorrhage.

Buried fibrous layers are SMC-rich, elastin-rich regions within the body of the plaque, usually located beneath a layer of foam cells. It has been suggested that they might represent previous ruptures that have healed. There was no difference in the average number of buried fibrous layers per plaque (control, 1.9 ± 0.2; apoA-I, 1.7 ± 0.2, P = 0.67).

3.3 Effects of ApoA-I on plaque morphometry
There was no difference between groups in the average cross-sectional areas of the following: brachiocephalic artery, arterial lumen, intima, or media (Table 2). Nor was there any difference between the groups in terms of maximum intimal thickness, maximum medial thickness, or overall thickness of the collagen and elastin-rich layer overlying the necrotic core (fibrous cap), measured at the thinnest point (Table 2).

3.4 Effects of ApoA-I on the cellular composition of plaques
The average number of nucleated cells in the plaques was unchanged after human apoA-I treatment (control, 130 ± 10; apoA-I, 130 ± 6, P = 1.0). Macrophages were mainly found in the shoulder regions of the plaque and occasionally in the media underlying the plaque. The number of macrophages, expressed as a percentage of nucleated cells in the plaque, did not differ between treatment groups (control, 51 ± 5%; apoA-I treated, 49 ± 4%, P = 0.8) (Figure 2A). In both groups of animals the media in the uninvolved arterial wall was rich in cells which stained positively for α-SMA, while the medial layer immediately beneath the lipid core of plaques was largely devoid of such cells (Figure 3A and B). The number of α-SMA positive cells within the plaque however was significantly higher in apoA-I-treated mice (Figure 2B; control, 9.4 ± 1.2%; apoA-I treated, 25.7 ± 2.3%, P < 0.001).

The ratio of α-SMA positive cells to macrophages is an indicator of plaque stability. Treatment with apoA-I significantly increased this ratio (P < 0.05), demonstrating that human apoA-I promoted a more stable plaque phenotype.

The percentage area of plaque occupied by apoptotic cells, as determined by TUNEL staining, was not different between groups (control, 1.7 ± 0.3%; apoA-I treated, 1.8 ± 0.2%, P = 0.88). Since there was also no difference in the total number of cells in plaques from apoA-I-treated or control mice or in the number of macrophages in the plaques, this indicates that the α-SMA positive cells in the apoA-I-treated mice appeared at the expense of some other cell type.

Table 1 Plasma lipid analyses

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total cholesterol (mmol/L)</th>
<th>HDL-cholesterol (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
<th>Mouse apoA-I (mg/dL)</th>
<th>Human apoA-I (μg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35.9 ± 1</td>
<td>0.7 ± 0.7</td>
<td>1.9 ± 0.7</td>
<td>108 ± 4</td>
<td>ND</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>36.4 ± 1</td>
<td>0.6 ± 0.2</td>
<td>2.0 ± 0.7</td>
<td>102 ± 5</td>
<td>12 ± 8</td>
</tr>
</tbody>
</table>

Plasma lipid concentrations were determined at the time of sacrifice in apoE knockout mice that received infusions of human apoA-I, 8 mg/kg of bodyweight, in PBS (n = 35) or an equivalent volume of PBS alone (control) (n = 35), twice weekly for 2 weeks. Plasma was obtained at the time of sacrifice. Values are expressed as mean ± SEM; ND, not detected.
3.5 Effects of ApoA-I on the collagen and lipid content of plaques

The total lipid content of the plaques was similar in the apoA-I treated and control mice (control, 11 ± 2%; apoA-I treated, 15 ± 3%; \( P = 0.30 \)). The plaque collagen content, as determined by picrosirius red staining under polarized light, was similar in the apoA-I treated and control mice, and occupied just over 20% of the plaque area (control, 22.8 ± 2.8%; apoA-I treated, 20.8 ± 2.8%, \( P = 0.62 \)).

3.6 Effects of ApoA-I on inflammatory markers associated with atherosclerotic plaques

Treatment with apoA-I did not affect any of the markers of inflammation examined. Staining for ICAM-1 was confined to the endothelium and there was no change in ICAM-1 staining when expressed as a percentage of total endothelium (control, 0.4 ± 0.1%; apoA-I treated, 0.5 ± 0.1%, \( P = 0.3 \)). Nor were there any changes in the fraction of the plaque area which stained for VCAM-1 (control, 0.5 ± 0.1%; apoA-I treated, 0.4 ± 0.1%, \( P = 0.50 \)), MCP-1 (control, 0.2 ± 0.1%; apoA-I treated, 0.2 ± 0.1%, \( P = 0.99 \)), or iNOS (control, 41.5 ± 10.2%; apoA-I treated, 41.02 ± 7.8%, \( P = 0.51 \)). The interaction between the immune mediator CD40 and its ligand CD40L (CD154) can trigger a cascade of inflammatory events at different stages of atherosclerotic plaque development and increase the expression of the adhesion molecules VCAM, ICAM and the release of cytokines such as MCP-1.\(^7\) The extent of positive staining in plaques did not differ between treated and untreated groups for CD40 (control, 27.9 ± 5.79%; apoA-I treated, 33.9 ± 5.94%, \( P = 0.48 \)) or CD40L (control, 26.3 ± 8.4%; apoA-I treated, 24.1 ± 5.5%, \( P = 0.37 \)). Together these data suggest that in this model, apoA-I does not have significant anti-inflammatory effects.

3.7 Effects of ApoA-I on fibrous cap composition

Since the fibrous cap is an important determinant of plaque stability,\(^26\) we compared the composition of the fibrous cap in plaques from treated and control mice. Very few macrophages were present in the fibrous caps in either group but we found a significant increase in the fibrous cap area occupied by \(\alpha\)-SMA positive cells in the apoA-I-treated mice (Figure 4A; control, 8.6 ± 2.4%; apoA-I treated, 19.2 ± 3.6%, \( P < 0.02 \)). The proportion of fibrous cap area positive for TUNEL, a marker of apoptosis, was not changed by treatment.
with human apoA-I (control, 2.8 ± 0.6%; apoA-I treated, 2.8 ± 0.6%; P = 1.0), indicating that the increase in α-SMA in the apoA-I treated group was not brought about by a decrease in apoptosis. Staining for cleaved PARP in the fibrous cap, a primary target for active caspase 3, did not differ between groups (control, 13.3 ± 1.9%; apoA-I treated, 13.4 ± 0.8%; P = 0.96) providing further evidence that apoA-I did not exert an anti-apoptotic effect in this model.

The calcium-binding protein S100A4 is associated with proliferation and migration in certain cell types. It has been reported that SMC can de-differentiate into a synthetic phenotype, which expresses S100A4. This phenotype is also associated with a reduction in α-SMA expression. The fibrous caps of plaques in mice that received human apoA-I had significantly less immunostaining for S100A4 than those of controls (control, 1.5 ± 0.2%; apoA-I treated, 0.6 ± 0.1%; P < 0.001) (Figures 3C and D, 4B). Migration of SMC requires collagenolytic activity; this is associated with synthetic and not contractile SMC. Expression of the collagenase MMP-13 is associated with unstable plaques in humans and was found to be decreased in the fibrous caps of human apoA-I-treated mice (control, 32.3 ± 4.5%; apoA-I treated, 10.0 ± 2.9%, P < 0.0003) (Figures 3E and F, 4C). In summary, the fibrous caps from the apoA-I-treated mice contained more α-SMA positive cells and fewer cells expressing the synthetic SMC phenotype, characterized by S100A4 expression and MMP-13 production than those from the control mice, together these changes would contribute to a more stable plaque.

4. Discussion

These studies show that treatment with exogenous human apoA-I can prevent the acute rupture of established atherosclerotic plaques in an apoE knockout mouse model. ApoA-I treatment was introduced after 9 weeks of fat feeding, when plaques in the proximal brachiocephalic artery of the apoE knockout mouse were already undergoing a high rate of spontaneous disruption. Four injections of apoA-I over a 2-week period markedly and significantly reduced the incidence of acute plaque disruption and intraplaque haemorrhage. The incidence of acute plaque disruption in the control group was comparable with that previously reported.

Unstable lesions are likely to possess increased numbers of macrophages and decreased numbers of SMC, and the ratio of SMC to macrophages has accordingly been used previously as an indirect indicator of plaque stability. We report here that treatment with apoA-I substantially increased the ratio of SMC to macrophages in plaques, which is consistent with the decrease in the incidence of spontaneous plaque disruption in the treated animals, and supports the use of this indirect phenotypic marker.

The effect of human apoA-I on atherosclerosis in apoE knockout mice has been investigated previously. In a study by Plump et al., apoE knockout mice were crossed with transgenic mice expressing human apoA-I. In these chow-fed mice, early atherosclerosis was profoundly inhibited in the group of mice with average plasma levels of 275 mg/dL human apoA-I; no decrease in lesion area however was observed in mice with lower plasma apoA-I levels (153 mg/dL). In the present study, injections of human apoA-I (8 mg/kg), which produces peak plasma levels of 15 mg/dL of human apoA-I, significantly altered plaque composition and stability without any change in plaque size or lipid content. Since human apoA-I has a half-life of 20 h in apoE knockout mice, and injections were given twice weekly, significant changes in plaque composition were achieved with average plasma levels of 2.5 mg/dL of human apoA-I.

In the study of Rong et al., atherosclerotic lesions from fat-fed apoE knockout donor mice were transplanted into chow-fed apoE knockout mice that had been genetically engineered to express human apoA-I. The plasma concentration of human apoA-I in these animals was 38 mg/dL, so was more than twice peak plasma apoA-I levels in the present study. Animals expressing the human apoA-I transgene still had significant further progression of lesions, though this was slower than in controls. Lesions exposed to exogenous apoA-I however contained fewer macrophages and more SMCs. Similarly, when apoA-I levels were raised in high-fat fed LDL receptor-deficient mice or apoE knockout mice by adenoviral gene transfer, the lesions that developed contained significantly more α-SMA-positive cells and a more collagen-rich extracellular matrix.
Pedersen et al. also reported a significant increase in SMC (α-SMA-positive cells) in the aortic root of uremic apoE knockout mice after receiving twice weekly injections of apoA-I (100 mg/kg) for 7 weeks. In this study, as in the present study, there was no evidence of changes in markers of inflammation or in the cross-sectional area of the lesions following apoA-I injection.

In the present study, we show that a short course of a low dose of apoA-I significantly inhibited the occurrence of acute plaque rupture in a mouse model. These results must be interpreted with the reservation that it remains to be conclusively proved that plaque disruptions in mice share the same aetiology as human plaque ruptures. It was clear however that treatment with low doses of apoA-I produced many changes in plaque composition consistent with a more stable plaque phenotype. The number of α-SMA-positive cells, particularly in the fibrous cap of the plaque was significantly increased. Furthermore, there was a decrease in the MMP-13 and in SMCs that were positive for the de-differentiation marker protein S100A4. In human atherosclerotic lesions, MMP-13 mRNA is found in both macrophage foam cells and SMC of the fibrous caps of unstable plaques, indicating an association between plaque instability and MMP-13 expression.

Indeed, there appears to be an inverse relationship between MMP-13 and plaque collagen content: in apoE/MMP-13 double knockout mice there is an increase in the absolute amount of collagen in aortic root plaques compared with apoE single-knockout controls. Treatment with apoA-I also markedly reduced the number of cells that stained positively for S100A4. As SMC de-differentiate, α-SMA expression decreases and the expression of other proteins increases at different stages of the differentiation process. Both S100A4 and MMP-13 are associated with ‘synthetic’ or de-differentiated SMC and not with ‘contractile’ SMC. Caps rich in contractile SMC are likely to be more biomechanically resilient and thus better able to withstand shear stresses than caps containing few contractile cells. In addition, synthesis of MMP-13 by de-differentiated SMC could weaken the extracellular matrix of the fibrous cap, rendering it more prone to rupture. The reductions in MMP-13 and S100A4 caused by ApoA-I suggest that the phenotype of SMC in the fibrous cap is altered.
cap was altered in a beneficial way, possibly accounting for the decrease in the incidence of plaque rupture in the treated animals.

This study is thus the first to show that exogenously administered apoA-I, using a clinically feasible regime, produces profound changes in plaque composition that translate into a reduction in the incidence of actual plaque disruption. These effects are exerted despite the intervention being given at a time when the incidence of spontaneous plaque disruption was at its highest. These findings have potentially major implications for the clinical management of these events.

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