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RESEARCH PAPER

Regression of aortic valve stenosis by ApoA-I mimetic peptide infusions in rabbits

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Background and purpose: Aortic valve stenosis (AVS) is the most common valvular heart disease, and standard curative therapy remains open heart surgical valve replacement. The aim of our experimental study was to determine if apolipoprotein A-I (ApoA-I) mimetic peptide infusions could induce regression of AVS.
Experimental approach: Fifteen New Zealand White male rabbits received a cholesterol-enriched diet and vitamin D_2 until significant AVS was detected by echocardiography. The enriched diet was then stopped to mimic cholesterol-lowering therapy and animals were allocated randomly to receive saline (control group, $n=8$) or an ApoA-I mimetic peptide (treated group, $n=7$), three times per week for 2 weeks. Serial echocardiograms and *post mortem* valve histology were performed.
Key results: Aortic valve area increased significantly by 25% in the treated group after 14 days of treatment ($P=0.012$). Likewise, aortic valve thickness decreased by 21% in the treated group, whereas it was unchanged in controls ($P=0.0006$). Histological analysis revealed that the extent of lesions at the base of valve leaflets and sinuses of Valsalva was smaller in the treated group compared with controls ($P=0.032$). The treatment also reduced calcification, as revealed by the loss of the positive relationship observed in the control group ($r=0.87$, $P=0.004$) between calcification area and aortic valve thickness.
Conclusions and implications: Infusions of ApoA-I mimetic peptide lead to regression of experimental AVS. These positive results justify the further testing of high-density lipoprotein (HDL)-based therapies in patients with valvular aortic stenosis. Regression of aortic stenosis, if achieved safely, could transform the clinical treatment of this disease.
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Keywords: valves; stenosis; apolipoproteins

Abbreviations: ApoA-I, apolipoprotein A-I; AVS, aortic valve stenosis; CSA, cross-sectional area; HDL, high-density lipoprotein; LVOT, left ventricular outflow tract; ROA, region of analysis; V_1/V_2, ratio of velocities in the LVOT vs across the aortic valve; VTI, velocity time integral

Introduction

Aortic valve stenosis (AVS) is the most common valvular heart disease in developed countries (Freeman and Otto, 2005). As severe symptomatic AVS usually leads to considerable morbidity and death in less than 5 years if left untreated, open heart surgical valve replacement remains the primary management (Carabello, 2002). Histopathological, experimental and clinical data suggest that calcific AVS is an active disease process with lipoprotein deposition, inflammation and active leaflet calcification (Freeman and Otto, 2005). Although there are some similarities between AVS and atherosclerosis, their pathophysiology and treatments differ significantly. In terms of pathophysiology, a bicuspid aortic valve is present and contributes to the disease in a significant number of patients with AVS, whereas atherosclerosis is not due to a structural congenital abnormality. Rheumatic heart disease can also lead to AVS, whereas it is usually not associated with atherosclerosis. Calcific AVS of the elderly, the most frequent type in the western countries, is often treated by isolated aortic valve replacement without the need for associated coronary bypass surgery. In terms of medical treatments, statins have been shown to be protective in patients with coronary disease and to halt progression or induce regression of atherosclerosis (Nissen *et al.*, 2006). However, in a recent randomized clinical trial, the progression of AVS was not prevented by intensive statin therapy (Cowell *et al.*, 2005). Similarly, angiotensin-converting enzyme inhibitors have been shown to be cardioprotective in several large-scale clinical trials but have failed to slow progression of AVS.

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There is an inverse relationship between plasma levels of high-density lipoprotein (HDL) cholesterol and coronary artery disease. Studies in animals with experimental atherosclerosis have demonstrated that apolipoprotein A-I (ApoA-I) Milano/phospholipid complexes rapidly mobilize cholesterol and thereby reduce atherosclerotic plaque burden (Ameli *et al*., 1994; Shah *et al*., 2001). In addition, two clinical studies have suggested that infusions of reconstituted HDL could induce rapid improvement of coronary atherosclerosis (Nissen *et al*., 2003; Tardif *et al*., 2007). The total to HDL cholesterol ratio has been shown to correlate significantly with the rate of progression of AVS, with more rapid progression occurring in patients with a higher ratio (Yilmaz *et al*., 2004). Because HDL has anti-inflammatory properties and promotes reverse cholesterol transport, we hypothesized that an HDL-based therapy may also induce regression of AVS. ApoA-I is a structural component of HDL that mediates many of its beneficial effects, including enhanced reverse cholesterol transport (Meyers and Kashyap, 2005). The ApoA-I mimetic peptide used in our study is capable of forming an amphipathic α-helix in the presence of lipids, as found in ApoA-I (Khan *et al*., 2003; Bodary *et al*., 2004). Hence, we tested the ability of this ApoA-I mimetic peptide complexed with phospholipids, mimicking nascent HDL, to induce regression of calcific AVS in a previously described rabbit model (Drolet *et al*., 2003). If this could be achieved safely, medical treatment of AVS and its regression may transform our clinical approach to this frequent disease.

Methods

Animals and experiments

Animal care and procedures complied with the Canadian Council on Animal Care guidelines and were approved by the institutional ethics committee for animal research.

We used an animal model adapted from that described by Drolet *et al*. (2003). Fifteen male New Zealand White rabbits (2.7–3.0 kg, aged 12–13 weeks) were fed with a 0.5% cholesterol-enriched diet plus vitamin D_2 (50 000 IU day^{-1}) in the drinking water until significant AVS, as defined by a $\geqslant$10% decrease of aortic valve area or of the transvalvular velocities ratio (ratio of velocities in the left ventricular outflow tract (LVOT) vs across the aortic valve; V_1/V_2), could be detected by echocardiography (12.9 ± 2.4 weeks).

The animals then returned to a standard diet (without vitamin D_2) to mimic cholesterol-lowering therapy and were randomly assigned to receive either saline (control group, $n=8$) or the ApoA-I mimetic peptide (treated group, $n=7$). Rabbits were given injections through the marginal ear vein of saline or of the ApoA-I mimetic peptide (25 mg kg^{-1}) complexed with phospholipids, three times per week for 2 weeks. Echocardiograms were performed serially (see below), including every 3–4 days throughout the randomized treatment period. At 2 days after their last infusion, the animals underwent a final echocardiogram and were killed, and the aortic valves were removed for histological analyses. Blood samples were obtained through the ear artery at baseline, prior to treatment and before death. Total cholesterol, HDL cholesterol, triglycerides and calcium levels were measured with an automated filter photometer system (Dimension RxL Max; Dade Behring, Deerfield, IL, USA).

ApoA-I mimetic peptide

The ApoA-I mimetic peptide purity as assessed by HPLC and mass spectral analysis was greater than 98%. The peptide was complexed with egg sphingomyelin and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Khan *et al*., 2003) in a 1:1:1 weight ratio by mixing the components in saline and performing multiple heating and cooling cycles until the solution appeared perfectly clear. The solution containing the complexes was lyophilized in aliquots for long-term storage. Fresh solution was reconstituted every week under sterile conditions and kept at 4 °C. Biological activity of the complex was tested in rats for its ability to mobilize cholesterol and to raise HDL cholesterol in blood transiently following an intravenous injection of the peptide; circulating total cholesterol level more than doubled 45 min after a single injection in rats (data not shown).

Echocardiography

Transthoracic echocardiographic studies were performed at baseline, on a weekly basis starting at 8 weeks of hypercholesterolaemic diet until significant AVS developed, and then after 4, 7, 10 and 14 days of ApoA-I mimetic peptide or saline control treatments. Studies were carried out with an S12 probe using a standard echocardiographic system (Sonos 5500; Philips Medical Systems, Bothell, WA, USA). Intramuscular injections of ketamine (45 mg kg^{-1}) and midazolam (0.75 mg kg^{-1}) were used for sedation.

Parasternal long- and short-axis views of the aortic valve were recorded to assess leaflet morphology. LVOT diameter was measured in a zoomed parasternal long-axis view, and LVOT cross-sectional area (CSA_{LVOT}) was calculated according to $CSA_{LVOT} = \pi$ (LVOT diameter/2)^2. LVOT velocity (V_1) and velocity time integral (VTI_{LVOT}) were obtained with a pulsed-wave Doppler placed proximal to the aortic valve in the apical five-chamber view. Continuous wave Doppler interrogation across the aortic valve was used to obtain transvalvular maximal velocity (V_2) and VTI (VTI_{AO}) in the same view. V_1/V_2 ratio was calculated in the pretreatment period to determine AVS development. Aortic valve area was obtained at each time point by the continuity equation and was equal to $CSA_{LVOT}(VTI_{LVOT}/VTI_{AO})$. Aortic valve thickness in its middle portion was measured at end diastole in a zoomed parasternal long-axis view at baseline, before randomized treatment and at the final echocardiographic measurement.

The average of three consecutive cardiac cycles was used for each measurement. Special care was taken to obtain similar imaging planes on serial examinations by reviewing previous recordings before follow-up study. All echocardiographic imaging and measurements were performed throughout the protocol by the same experienced investigator blinded to the randomized treatment assignment.

To validate our measurements, we subjected six rabbits to five repeated echocardiographic studies every 3–4 days apart,

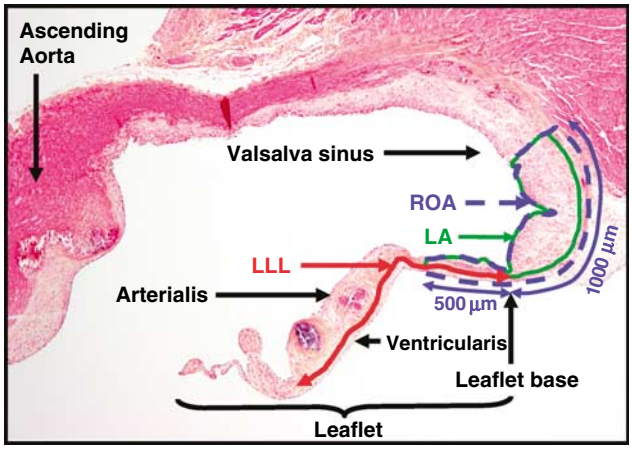

Figure 1 Histomorphological measurements on aortic valve sections. The ROA was composed of 1000 µm of the Valsalva sinus from the leaflet base and 500 µm of the leaflet from the leaflet base. ROA, region of analysis; LA, lesion area; LLL, leaflet lesion length.

with three consecutive cardiac cycles analysed for each recording. The coefficient of variation (s.d./mean × 100%) between three consecutive cardiac cycles was 2.72 ± 1.40% for the measurement of aortic valve area from the 30 echocardiograms. The coefficient of variation between the five repeated echocardiographic studies was 2.19 ± 0.95% for the measurement of aortic valve area from the six rabbits.

Histomorphometry

The ascending aorta and aortic valve were opened longitudinally and the three valvular cusps were separated. Two cusps were immediately frozen in an embedding medium and stored at −80 °C. The third cusp was immersion-fixed in 10% buffered formalin at 4 °C for 24 h and embedded in paraffin. Stained or immunohistochemically labelled tissue sections obtained from the central third of each cusp were analysed with a computer-based digitizing image system (Image Pro Plus, version 5.1) using a light microscope (BX41, Olympus, Tokyo, Japan) connected to a digital video camera (Q-Color3; Olympus). The region of analysis (ROA) (Figure 1) was composed of 1000 µm of the Valsalva sinus from the leaflet base and 500 µm of the leaflet from the leaflet base. Lesion area and leaflet lesion length were also measured.

Histochemistry

Haematoxylin-phloxin-safran, von Kossa and Sirius red-stained sections were prepared for routine examination, tissue calcification and collagen studies, respectively. Collagen fibre types I and III were quantified as previously described (Busseuil *et al*., 2004), on Sirius red picric acid-stained sections under polarized light. For immunohistochemistry evaluation, all sections were preincubated with either mouse immunoglobulin G2a monoclonal antibody against rabbit macrophage (RAM11) (1:100 dilution) or rabbit smooth muscle cell α-actin (Clone 1A4) (1:200 dilution). Species-appropriate biotinylated secondary antibodies were applied, followed by streptavidin-horseradish peroxidase complex, visualized with amino-9-ethylcarbazol and counterstained by Mayer's haematoxylin. Smooth muscle cells, macrophages and calcification areas were quantified in the ROA on digital images acquired at × 40 magnification. Images from each section were digitally captured with the same illumination settings, and automatic computer-based analysis was performed with the same colour threshold for all specimens. Data are expressed as % labelled area in the ROA.

For assessment of tissue-free cholesterol, 5-µm cryosections fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) were stained with filipin. Sections were incubated for 1 h at room temperature in filipin complex dissolved in dimethyl sulphoxide and diluted in phosphate-buffered saline, mounted in Vectashield and viewed by fluorescence microscopy using a Zeiss Axiovert 200 M microscope with the 4′-6-diamidino-2-phenylindole filter set. Images were acquired with an AxioCam MRm digital camera mounted with a × 0.63 C-mount adapter. Filipin data are expressed as arbitrary units of fluorescence intensity.

Statistical analyses

Data are presented as mean ± s.d. For the 'pretreatment' period, repeated measures analysis of variance models were used to study the echocardiographic and serum measurements across time and between groups (treated vs control groups). Models with time, group and group × time interaction as independent variables were used and comparisons between groups at a given time point were undertaken only in the presence of a significant group × time interaction. Otherwise, global conclusions were drawn based on the main group effects of the model. For the randomized treatment period, repeated measures analysis of covariance models were used to study the echocardiographic and serum measurements across time and between groups (treated vs control groups), adjusted for the baseline value of the response variable. The group × time interaction was also included in the analysis of covariance models and comparisons between groups at a given time point were undertaken only in the presence of a significant group × time interaction. Otherwise, global conclusions were drawn based on the main group effects of the model. Histological variables were compared between treated and control groups using Student's *t*-test. Relationships between histomorphometry and echocardiographic variables were evaluated using Pearson correlation coefficient. All analyses were performed with SAS release 8.2 (SAS Institute Inc., Cary, NC, USA) and conducted at the 0.05 significance level.

Materials

The cholesterol-enriched diet was from Harlan (Indianapolis, IN, USA); vitamin D_2 from Sigma (Markham, ON, Canada). The ApoA-I mimetic peptide (H-Pro-Val-Leu-Asp-Leu-Phe-Arg-Glu-Leu-Leu-Asn-Glu-Leu-Leu-Glu-Ala-Leu-Lys-Gln-Lys-Leu-Lys-OH) (Khan *et al*., 2003; Bodary *et al*., 2004) were synthesized by Polypeptide Laboratories (Torrance, CA, USA); egg sphingomyelin and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine were from Avanti Polar Lipids (Alabaster, AL, USA); embedding medium, OCT Tissue-Tek (Sakura, McGaw

Park, IL, USA); haematoxylin-phloxin-safran, von Kossa and Sirius red (F3B), BDH (UK). Mouse immunoglobulin G2a monoclonal antibodies, RAM11 and Clone 1A4 were from Dako (Mississauga, Ontario, Canada); filipin complex, Sigma (Canada); Vectashield, Vector Laboratories (Burlingame, CA, USA).

Results

Serum lipids and calcaemia

There was no significant difference between the groups during the pretreatment period (the hypercholesterolaemic diet period) for serum levels of total cholesterol ($P=0.942$), HDL cholesterol ($P=0.787$), triglycerides ($P=0.906$) and calcaemia ($P=0.727$). Comparing the results before and after the 2-week treatment period, total cholesterol levels were also statistically similar in both groups ($P=0.470$): values were 20.46 ± 3.52 and 20.13 ± 5.18 mM at the time of random group allocation for treatment and 13.78 ± 6.24 and 17.57 ± 10.32 mM before death in the control and treated groups, respectively. There was no statistically significant difference between groups for HDL cholesterol levels during the treatment period ($P=0.374$). HDL cholesterol was 0.50 ± 0.20 and 0.50 ± 0.15 mM at the time of random group allocation and 0.39 ± 0.17 and 0.45 ± 0.17 mM before death. During this period, triglyceride levels were also similar ($P=0.544$). There was no significant difference between groups for calcaemia during the treatment period ($P=0.832$), with values of 3.31 ± 0.29 and 3.15 ± 0.37 mM before random group allocation and 3.22 ± 0.11 and 3.22 ± 0.12 mM before death in both groups.

Development of AVS during the period of hypercholesterolaemic diet and vitamin D_2 supplementation

The induction time for AVS in animals administered the cholesterol plus vitamin D_2 diet was similar for control and treated groups (12.8 ± 2.2 vs 13.0 ± 2.9 weeks; $P=0.852$). There was a significant difference between aortic valve area at baseline and at the end of the hypercholesterolaemic diet period ($P<0.0001$). The aortic valve area decreased similarly in both groups by an amount almost identical for control and treated rabbits (from $24.2 \pm 4.1\,mm^2$ at baseline to $19.0 \pm 2.7\,mm^2$ in controls and from 24.7 ± 3.9 to $19.1 \pm 2.6\,mm^2$ in the treated group) during the period of AVS development ($P=0.852$). Therefore, the aortic valve area decreased by 20.5 ± 4.2 and $21.6 \pm 3.7\%$ before random allocation of treatment in the control and treated groups, respectively.

At the end of the period of hypercholesterolaemic diet, the V_1/V_2 ratio had decreased significantly from baseline ($P<0.0001$), with no significant differences between groups before random allocation ($P=0.914$).

The progression of AVS with treatment: echocardiography

As illustrated in Figure 2a, during the treatment period (from AVS detection to after 2 weeks of treatment), a significant group × time interaction was observed for aortic valve area ($P=0.013$). Using repeated measures analysis of covariance models, echocardiographic measurements revealed significant increases in the aortic valve area in the treated group compared with controls after 7 days (relative increases of 14.2 ± 3.5 vs $3.9 \pm 3.4\%$), 10 days (relative increases of 19.8 ± 3.5 vs $7.6 \pm 4.2\%$) and 14 days of treatment (relative increases of 24.6 ± 2.0 vs $12.9 \pm 3.5\%$) (Figure 2a).

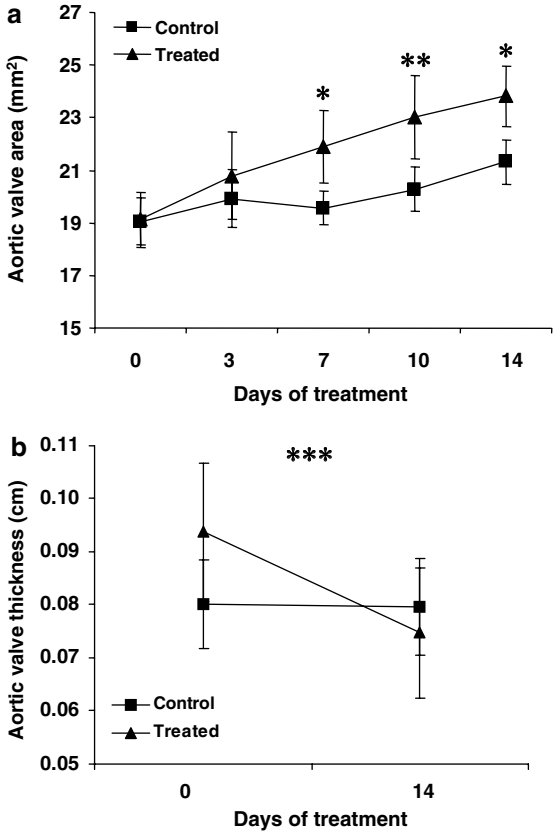

Figure 2 Echocardiographic measurements obtained during the ApoA-I mimetic peptide treatment period. Day '0' corresponds to the end of cholesterol plus vitamin D_2 diet and the beginning of ApoA-I mimetic peptide treatment period. (**a**) Aortic valve area values, $^*P \leqslant 0.05$; $^{**}P \leqslant 0.01$. (**b**) Aortic valve thickness values, $^{***}P \leqslant 0.001$. ApoA-I, apolipoprotein A-I.

Aortic valve thickness was assessed by echocardiography and measured before and after 14 days of treatment. A significant group × time interaction was also observed for aortic valve thickness ($P=0.005$). No significant difference was found between groups at the randomization time point but a significant decrease of aortic valve thickness was observed in the treated group as compared with the control group after 14 days (Figure 2b).

Histology

All the animals presented aortic valve lesions. Lesions consisted of a cap of new tissue composed of multiple layers of foam cells giving way gradually to fibrotic material with fewer foam cells from about half-way down into the lesion. Lesions generally developed from the sinotubular area, covering the whole of the sinus of Valsalva and extending

to the cusp base and up to one-half to two-thirds of the proximal leaflet arterialis. In contrast, lesions on the leaflet ventricularis were not common and were less severe.

Histomorphometry

Figure 3a illustrates the type of lesion produced in this model. The lesion area as a percentage of ROA decreased significantly in the ApoA-I mimetic peptide-treated group compared with the control group (Figure 3b). Interestingly, when the values for lesion area (as % ROA), determined by histomorphometry, were compared with the aortic valve areas, determined by echocardiography, by pooling values from both groups, a negative correlation was found between them (Figure 3c). Similarly, analysis of pooled data from both groups revealed a negative correlation between aortic valve area and the percentage of total leaflet length occupied by the lesion (or leaflet lesion length/total leaflet length × 100) ($r = -0.70$, $P = 0.004$). However, there was no significant difference in the sizes of these lesions (as % total leaflet length) between the treated and control groups (55.7 ± 24.3 and 72.3 ± 11.7%; $P = 0.109$).

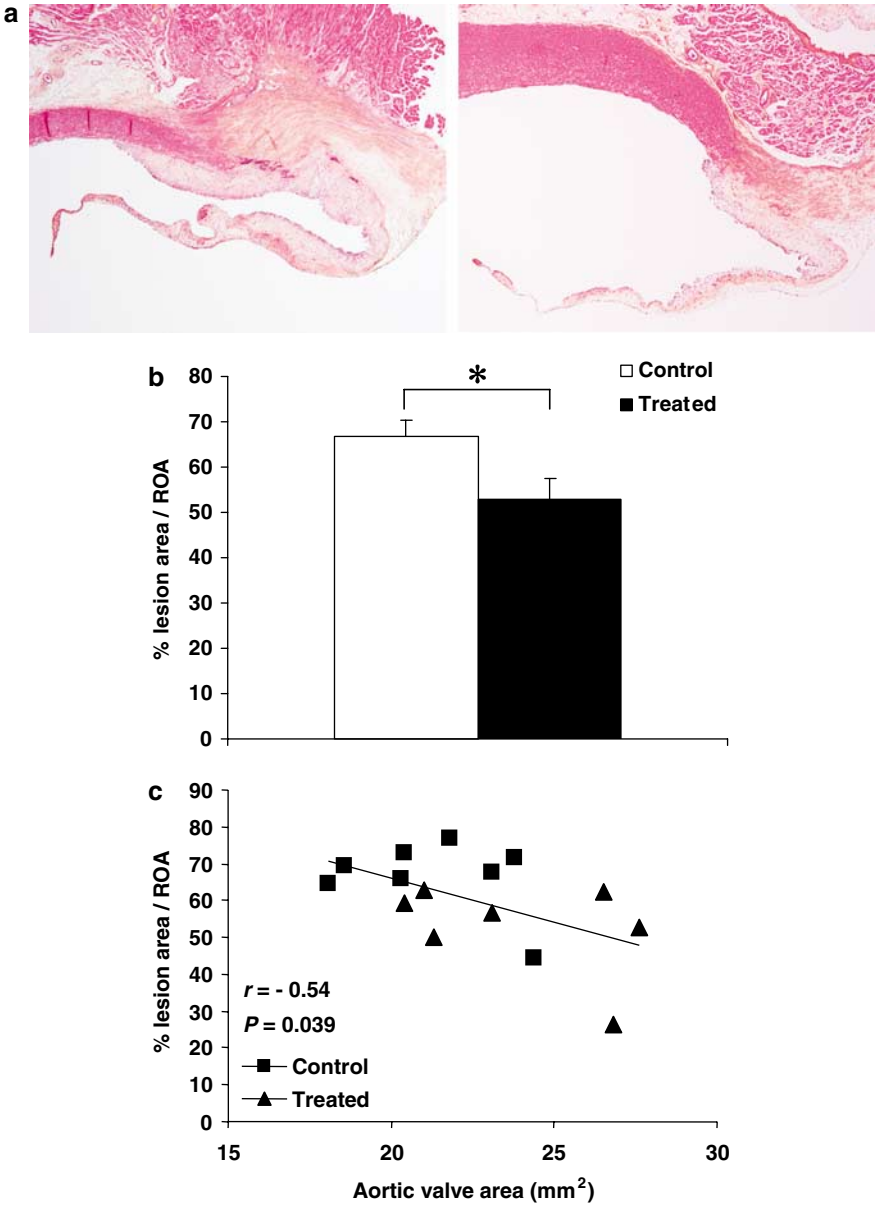

Figure 3 (**a**) Haematoxylin-phloxin-safran (HPS)-stained aortic valve sections from control (left) and treated (right) animals. Lesion area was less extensive in the sinus of Valsalva and on the aortic valve leaflet of treated (LA/ROA = 45.1%) compared with control (LA/ROA = 74.2%) animals. (**b**) Comparison of the LA, expressed as a percentage of the ROA, in aortic valves from control and treated groups, *$P = 0.032$. (**c**) Correlation between AVA and LA, expressed as a percentage of the ROA, in aortic valves from both groups. AVA, aortic valve area; LA, lesion area; ROA, region of analysis.

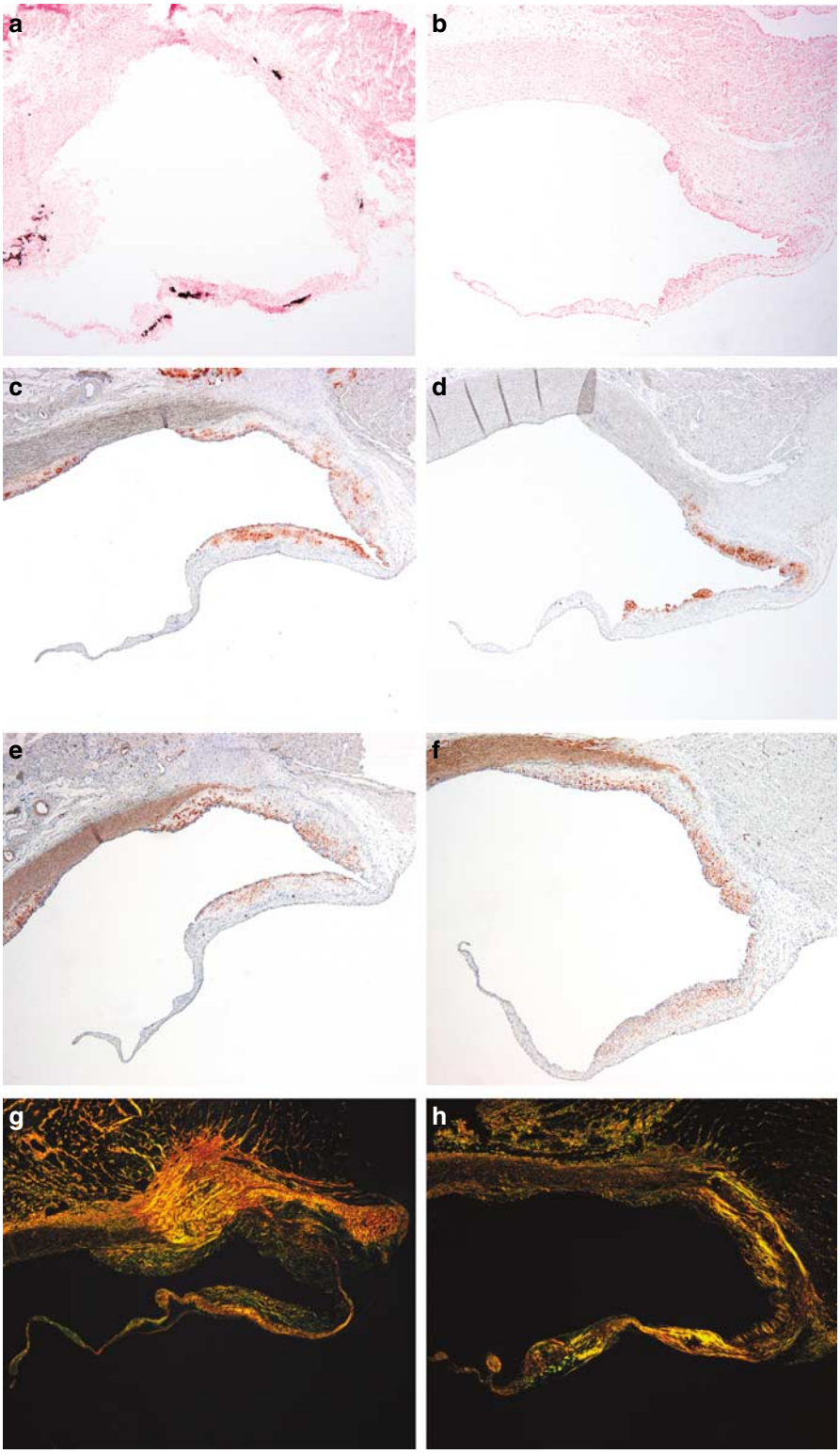

Figure 4 Aortic valve sections (× 4 magnification). Left column, control group; right column, treated group. The orientation from the ventricle to ascending aorta is from right to left. Labelling of: (**a**, **b**) calcifications (von Kossa staining); (**c**, **d**) macrophages (RAM11 antibody); (**e**, **f**), smooth muscle cells (α-smooth actin antibody); (**g**, **h**) collagen fibres (red-yellow (type I) and green (type III) with picrosirius red polarization analysis).

Histochemistry

Foci of calcifications were observed in the majority of animals (Figures 4a and b). The number of rabbits (%) with calcium deposits in the lesion core in the sinuses of Valsalva was 57% (4/7) in the treated group and 88% (7/8) in controls. Quantification of the area of the sinus of Valsalva occupied

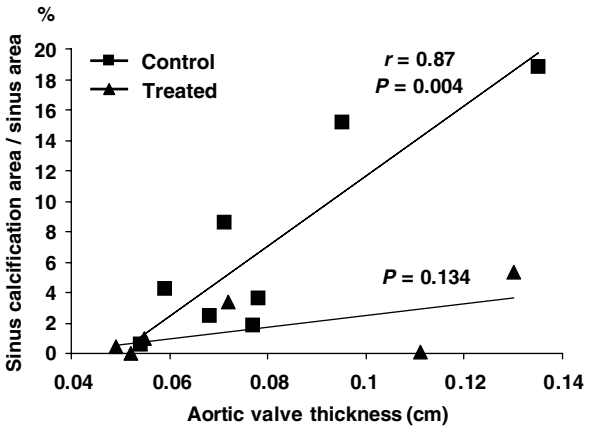

Figure 5 Correlation between aortic valve thickness, determined by echocardiography, and the calcification foci expressed as a percentage of the sinus of Valsalva area, in animals from each group. Note that a value (triangle) from one treated animal is hidden behind a square at the bottom left of the figure.

by calcifications, revealed by von Kossa staining, showed that the treated group tended to have less (77% decrease) calcification than controls (1.6 ± 2.0 vs $6.9 \pm 6.7\%$; $P = 0.063$). Further, when the relationship between the percentage of calcification foci area within the sinus area and aortic valve thickness (determined by echocardiography) were plotted for the control and treated groups (Figure 5), the loss of a positive correlation between these two variables was revealed after treatment, indicating that the treatment reduced calcification.

Free cholesterol, as detected by filipin staining, was present throughout the whole lesion area of the aortic valve in almost all control animals, whereas the fluorescent signal was numerically lower at the luminal edge of lesions in most animals treated with the ApoA-I mimetic peptide. Assessment of the fluorescent signal within the first 10 μm at the luminal edge of lesions showed a 41% decrease in free cholesterol in treated animals compared with controls but this difference did not reach statistical significance (221 ± 54 vs 377 ± 229 arbitrary units of fluorescence intensity; $P = 0.231$). The area occupied by macrophages (34.5%) was about twice as large as smooth muscle α-actin-positive areas (17.1%), with no significant differences between groups (Figures 4c–f). The percentage of collagen type III fibres (light green) was higher than collagen type I fibres (red-yellow) in the ROA ($19.7 \pm 5.5\%$ for type III vs $6.6 \pm 4.1\%$ for type I in controls, $P = 0.00009$; $19.1 \pm 11.3\%$ for type III vs $5.5 \pm 5.6\%$ for type I in treated rabbits, $P = 0.015$; Figures 4g–h). However, the percentages of collagen fibres (types I and III) in the ROA were similar in both groups ($P = 0.671$ and $P = 0.883$, respectively).

Discussion and conclusions

This is the first study to demonstrate that infusions of an ApoA-I mimetic peptide lead to significant regression of experimental AVS. Compared with the control group, ApoA-I mimetic peptide infusions induced greater improvement of aortic valve area and a significant reduction in aortic valve thickness. These favourable changes in AVS severity detected by echocardiography were accompanied by a significant decrease in lesion extent in the leaflet base region (shown in histological sections) as well as decreased calcifications, revealed by the loss of positive relationship between calcification area and aortic valve thickness.

We used a rabbit model of AVS developed by Drolet *et al*. (2003), in which aortic valve calcification occurs significantly and reproducibly, similar to the clinical condition. After the animals had received a cholesterol-enriched diet and vitamin D_2 supplementation for approximately 13 weeks, echocardiographic measurements revealed a 21% decrease in aortic valve area. Two-dimensional imaging showed increased valve thickness and echogenicity compatible with leaflet sclerosis and areas of calcification. Histological examination confirmed leaflet thickening and calcium deposition both in the sinuses of Valsalva and at the leaflet base. Hence, this study provides echocardiographic and histological evidence for the beneficial effects of an ApoA-I mimetic peptide on experimental calcific AVS. The increase in aortic valve area was observed as early as 7 days after the initiation of active treatment, and was improved by 24% at 14 days. In fact, in rabbits given ApoA-I mimetic peptide infusions, the aortic valve area almost returned to the normal value present before the start of the hypercholesterolaemic diet. In contrast, discontinuation of the cholesterol-enriched diet with vitamin D_2 supplementation in the control group (to mimic lipid lowering) only led to a mild increase in aortic valve area, which confirms the additional beneficial effects of the peptide. Aortic valve thickness was also significantly reduced after only 14 days of treatment with the peptide, as shown by echocardiography. Interestingly, aortic valve area determined by echocardiography correlated inversely with indices of lesion extent, assessed histologically. ApoA-I mimetic peptide infusions also led to a significant reduction in the percentage of lesion area in the region of histological analysis around the valvular leaflet base. Furthermore, the large reduction in the extent of valvular calcifications that almost reached statistical significance may be of clinical importance, given that calcific AVS of the elderly is the most frequent form of stenosis encountered in developed countries. This may mean that the presence of aortic valve calcifications not only does not preclude obtaining favourable results with the peptide but also that this approach may even regress the valvular calcifications themselves. Possible mechanisms of action of HDL-based therapy leading to regression of valvular calcifications include improved endothelial function and integrity, antioxidant and/or anti-inflammatory effects, and better leaflet repair by increased incorporation of bone marrow-derived progenitor cells (Mohler, 2004; Feng *et al*., 2008). It is difficult to predict whether this treatment would also be efficacious in severe calcified aortic stenosis. Only a randomized clinical trial will allow us to answer this important question. Whether this finding may also apply to mitral valve and/or annular calcifications is unknown but it may also be of clinical significance.

An ApoA-I mimetic peptide complexed with phospholipids may stimulate reverse cholesterol transport in a manner similar to native ApoA-I (Navab *et al*., 2005). This ApoA-I

mimetic peptide has been shown to mobilize cholesterol in pre-clinical studies and also to elevate transiently circulating HDL cholesterol levels in patients (Khan *et al*., 2003; Bodary *et al*., 2004). In our preliminary experiment using the ApoA-I mimetic peptide in rats, circulating total cholesterol levels more than doubled 45 min after a single injection. In the present study, the blood samples taken at the time of death probably missed in part the large but transient increase in circulating cholesterol levels. This explains why the higher circulating cholesterol levels observed after the end of the treatment period did not reach statistical significance (although the mean total cholesterol level was 27% higher in the peptide group than in controls at that time point). Automated quantification methods used to measure HDL cholesterol are not sensitive enough to quantify the ApoA-I mimetic peptide-based particles, and for that reason total cholesterol is a better measurement in this setting. The higher circulating levels of total cholesterol in rabbits treated with the peptide compared with controls (observed after 2 weeks of treatment), therefore, probably indicate enhanced mobilization of tissue cholesterol. The qualitative difference in fluorescence intensity at the luminal edge of valvular lesions, shown using filipin staining, may indicate that this peptide induces a greater cholesterol efflux to circulating lipoproteins, but this important matter will require additional studies. Potential anti-inflammatory and antioxidant effects of the peptide (Navab *et al*., 2004) were not assessed in the present study. However, as the plasma cholesterol values were very high for a long period in the animals prior to random allocation to peptide or saline infusions, it remains possible that this ApoA-I mimetic may have intrinsic anti-inflammatory properties despite the lack of significant difference between study groups in the area occupied by macrophages. In this context, more prolonged therapy would probably have been needed to reveal any decrease in macrophage area. Nevertheless, the similar cell and collagen content between groups confirms, along with echocardiographic and other data, that the pretreatment diet resulted in similar lesion type and severity prior to random assignment to therapy. This makes the echocardiographic and histological results obtained later, after peptide infusions, even more convincing.

Limitations

This proof-of-concept study has limitations. First, the duration of observation was relatively short after initiation of treatment; however, aortic valve area after 2 weeks of ApoA-I mimetic peptide infusions was already almost back to normal, that is similar to that observed before the hypercholesterolaemic diet. Second, although treatment with the ApoA-I mimetic peptide significantly reduced aortic valve thickness, the difference between groups at the end of the study was relatively small. Nevertheless, the observed reduction in valve thickness is consistent with the favourable effects on the aortic valve area, determined by echocardiography, and the aortic valve lesion, measured by histology.

In conclusion, infusions of an ApoA-I mimetic peptide lead to regression of experimental calcific AVS. HDL-based therapies should be tested in patients with valvular aortic stenosis. Regression of aortic stenosis, if achieved safely, could transform the clinical approach to this disease.

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Conflict of interest

A provisional patent application was submitted concerning the results contained in this article by the Montreal Heart Institute and J-CT is mentioned as an author.

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