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Combined Effects of Cholesterol Reduction and

Apolipoprotein A-I Expression on Atherosclerosis in LDLR Deficient Mice

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Short Title: Combined Effects of Cholesterol reduction and ApoA-I expression on Atherosclerosis

This manuscript contains 5 figures and 2 tables.

Abstract

Reduction of total and LDL cholesterol reduces atherosclerosis and clinical cardiovascular events. High density lipoprotein (HDL) cholesterol levels have a strong inverse association with atherosclerosis, and overexpression of apolipoprotein A-I (apoA-I), the major protein component of HDL, reduces atherosclerosis in hypercholesterolemic animals. However, little is known about the potential for additive or synergistic effects between cholesterol reduction and apoA-I overexpression on atherosclerosis. In the current study, we tested the hypothesis that significant reduction of plasma cholesterol combined with overexpression of apoA-I would reduce atherosclerosis to a greater extent than either one alone. We used somatic gene transfer of the LDL receptor (to induce cholesterol reduction) and apoA-I in LDLR deficient mice fed a Western type diet and compared the combination to expression of each gene alone and to controls. Although the reduction of cholesterol was transient, expression of the LDLR alone significantly reduced atherosclerosis by 45% compared with controls. Overexpression of human apoA-I alone reduced atherosclerosis by 42% compared with controls and was not different than expression of the LDLR alone. Co-expression of the LDLR with apoA-I resulted in significantly higher levels of apoA-I and a greater effect on plasma HDL cholesterol levels than expression of apoA-I alone. Although co-expression of the LDLR and apoA-I reduced atherosclerosis by 37% compared to controls, the reduction in atherosclerosis was no greater than that seen with expression of the LDLR alone or apoA-I alone. In summary, in this relatively short-term murine model, reduction of cholesterol enhanced the lipoprotein effects of apoA-I expression but did not result in greater reduction in atherosclerosis compared to either one alone.

Keywords: apolipoprotein A-I, low density lipoprotein receptor, atherosclerosis, high density lipoprotein cholesterol, gene transfer

Introduction

Long-term reduction of total and low density lipoprotein (LDL) cholesterol reduces atherosclerosis in animals (1) and clinical cardiovascular events in humans (2). However, the molecular mechanisms by which cholesterol reduction impacts on atherosclerotic lesions to reduce clinical events and the kinetics of these effects are not yet fully understood. Importantly, reduction in plasma cholesterol does not eliminate the risk of cardiovascular event (3). Therefore, there remains continued interest in other interventions that may be additive to cholesterol reduction in reducing atherosclerosis and cardiovascular risk.

Epidemiological data indicates a strong inverse association between high density lipoprotein (HDL) cholesterol levels and atherosclerotic cardiovascular disease (ASCVD) in humans (4,5). Genetic syndromes of high HDL cholesterol are associated with longevity and a decreased incidence of ASCVD (6), and low levels of HDL cholesterol are associated with significantly increased risk of ASCVD (7). Apolipoprotein A-I (apoA-I) is a 27kDa protein synthesized in liver and intestine, and the major protein component of HDL. Studies in hypercholesterolemic animal models have provided substantial support for the concept that apoA-I is protective against atherosclerosis. Regular injection of human HDL (8) or rabbit apoA-I (9) into cholesterol-fed rabbits reduced atherosclerosis. Overexpression of apoA-I in hyperlipidemic Watanabe heritative hyperlipidemic rabbits decreased the development of atherosclerosis (10). Transgenic overexpression of human apoA-I raised HDL cholesterol levels and inhibited atherosclerosis in C57BL/6 mice on high-fat diet (11), in apolipoprotein E deficient mice (12,13), and in human apo(a)-transgenic mice (14). Adenoviral overexpression of human apoA-I increased HDL cholesterol level and inhibited the neointima formation after carotid endothelial denudation in apoE deficient mice (15). Somatic adenoviral overexpression of human apoA-I inhibited the progression of atherosclerosis in apoA-I transgenic/apoE deficient mice (16). Finally, liver directed gene transfer using a second generation adenovirus increased HDL cholesterol levels and induced regression of pre-existing atherosclerosis in LDLR deficient mice (17). Therefore, both types of intervention--cholesterol reduction as well as increasing of apoA-I levels--are effective in reducing atherosclerosis in hypercholesterolemic animals. However, the effects

of apoA-I overexpression when combined with intervention to reduce cholesterol levels have never been investigated. Nothing therefore is known about the potential for additive or synergistic effects between cholesterol reduction and apoA-I overexpression on atherosclerosis.

In the current study, we tested the hypothesis that significant reduction of plasma cholesterol combined with overexpression of apoA-I would reduce atherosclerosis to a greater extent than either one alone. We used LDL receptor deficient mice fed a Western type diet as our animal model and somatic gene transfer of the LDL receptor (LDLR)(to induce cholesterol reduction) and apoA-I as our interventions. LDLR deficient mice fed a high cholesterol diet have high plasma cholesterol levels and develop substantial atherosclerosis (18). Adenovirally-mediated gene transfer of the LDLR to the livers of chow-fed LDLR deficient mice markedly but transiently reduced plasma cholesterol levels (19). In this study, we compared the combination of transient cholesterol reduction and apoA-I overexpression to each intervention alone and to controls in order to determine whether cholesterol reduction and apoA-I overexpression have additive effects on atherosclerosis in this murine model of atherosclerosis.

Methods

Mouse Studies

We utilized a recombinant first-generation adenovirus, kindly provided by Dr. Karen Kozarsky, for gene transfer of the human LDLR (20). For gene transfer of human apoA-I, we utilized a second-generation adenovirus (17,21). As control adenoviruses, we used first and second generation vectors encoding beta galactosidase (AdlacZ and tsAdlacZ, respectively). A total of 37 male LDLR deficient mice (back-crossed at least 10 times to C57BL/6 mice), 28 weeks old, obtained from Jackson Labs were fed with a Western type diet (normal chow supplemented with 0.15% cholesterol and 20% butter fat) for 10 weeks before gene transfer. At 38 weeks of age, mice were divided into 4 groups based on their plasma cholesterol levels so that the baseline cholesterol levels were not different among the four groups. Mice were injected intravenously with the four different combinations of first- and second-generation adenoviruses (1.0×10^{11} particles of each virus, 2.0×10^{11} particles total dose): 1) AdlacZ+tsAdlacZ, 2) AdLDLR+tsAdlacZ, 3) AdlacZ+tsAdapoA-I, and 4) AdLDLR+tsAdapoA-I). All mice received the same total dose of virus. Each group contained 9-10 mice. Blood was obtained from the retroorbital plexus after 4h fast prior to injection and on days 3, 7, 14, 21, 28, and 42 after adenovirus injection for analysis of transgene expression and lipids. Aliquots of plasma were stored at -20 °C until quantification. All mice were killed 6 weeks after virus injection to quantify the extent of atherosclerotic lesions. This experiment included mice injected with apoE adenovirus and LDLR + apoE adenoviruses, the results of which are the focus of a separate manuscript (Kawashiri et., manuscript enclosed). The mice injected with control vector and LDLR vectors were used as comparators for both papers.

En Face Quantification of Atherosclerotic Lesions in the Aorta

The extent of atherosclerosis in the whole aorta was analyzed as previously described (17). Mice were anesthetized with 3mg of xylozine and 3mg of ketamine injection to peritoneum. After the aorta was gently perfused with ice-cold PBS via the left ventricle, the heart was cut and the rest of aorta, from ascending aorta to common iliac artery, was removed and fixed in 10%

formalin. After the adventitial and adipose tissue was removed, the aorta were cut open longitudinally, then stained with Sudan-IV solution (0.5% SudanIV, 35% ethanol, 50% acetone) for 8 minutes and destained with 80% ethanol for 5 minutes to eliminate background staining. The image was captured with the use of a Leica MZ12 microscope and digitized, and Sudan IV stained lesion area was quantified using Image Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD). All data capture and quantification was performed in a blinded fashion.

Analytical Methods

The plasma total cholesterol and triglyceride levels were measured on Cobas Fara II (Roche Diagnostic System Inc) with Sigma reagents (Sigma Chemical Co) as described (21). Human specific apoA-I concentrations were quantified with an ELISA using commercially available kit (AlerCHEK, Portland, ME). Plasma samples from each group were pooled at each time point. Pooled plasma obtained on days 0, 3, 7, and 14 from each group were subjected to fast protein liquid chromatography (FPLC) gel filtration (Pharmacia LKB Biotechnology) on two Superose 6 columns as described (21,22). Cholesterol concentrations in the fractions were determined with an enzymatic assay (Wako Pure Chemical Industries, Ltd). The amount of cholesterol in each class was calculated using FPLC fractions #3-#10 for VLDL, #11-#28 for IDL/LDL, and #29-#40 for HDL. After separating the pooled plasma samples on FPLC, 6 µl samples from two adjacent fractions were pooled and subjected to 10% SDS-PAGE. Human specific apoA-I was detected using a mouse anti-human apoA-I monoclonal antibody, a kind gifted of Dr. David Usher. Peroxidase-labeled goat anti-mouse antibody (Jackson Immuno Research) was used for detection.

Statistical Analysis

Atherosclerotic lesion area data and lipids data on each day were subjected to ANOVA with the Kruskal-Wallis test, followed by the Dunn's multiple comparison test. Statistical significance for all comparisons was assigned at $p<0.05$. Graphs represent mean ± SEM values.

Results

Transgene expression and effects on plasma cholesterol levels

Male LDLR deficient mice on a Western diet were injected with adenoviral vectors encoding the LDLR alone, apoA-I alone, LDLR plus apoA-I, or control LacZ. Mean plasma levels of human apoA-I in mice co-expressing the LDLR (91 mg/dl) were 55% higher than in the mice injected with the apoA-I vector alone (59 mg/dl) on day 7 (Figure 1). Human apoA-I could be detected in both groups of mice injected with apoA-I vector but remained significantly higher in mice co-expressing the LDLR even after 4 weeks (apoA-I alone; 12 mg/dl, LDLR plus apoA-I; 22 mg/dl,).

The effects on plasma total cholesterol levels are shown in Table 1. Mice injected with control vector (AdlacZ) experienced transiently decreased levels of plasma cholesterol, which returned to baseline by day 42. As expected, mice injected with the LDLR vector had a significant 75% decrease in cholesterol that was transient, similar to that previously reported in chow-fed mice (19) and cholesterol levels returned to the level of the control mice by day 14. Expression of apoA-I alone did not change total cholesterol levels throughout experimental period compared with control virus injected mice. Co-expression of human apoA-I and LDLR reduced plasma cholesterol levels at the same degree as that of LDLR alone. The effects on plasma triglyceride levels are shown in Table 2. The plasma triglyceride levels of the mice injected with control vector decreased transiently compared with baseline, which returned to baseline 14 days after vector injection. The expression of LDLR alone significantly decreased plasma triglyceride levels by 72% on day 7. On the other hand, mice expressing apoA-I alone had a modest, transient increase in plasma triglyceride levels that were not significantly different than controls. The plasma triglyceride levels of the mice co-expressing the LDLR and apoA-I were 87 % higher than those expressing the LDLR alone and were not different than those expressing apoA-I alone on day 7.

Effects on Lipoprotein Distribution

In order to determine the effects of LDLR and apoA-I expression on the cholesterol

distribution in lipoprotein classes, pooled plasma samples were subjected to FPLC gel filtration. On day 0, there were no significant differences between the 4 groups (Figure 2a). On day 3, expression of LDLR alone markedly reduced apoB-containing lipoprotein cholesterol (Figure 2b). Human apoA-I expression alone did not change lipoprotein cholesterol distribution (Figure 2b). Co-expression of apoA-I with LDLR did not change apoB-containing lipoprotein cholesterol compared with expression of LDLR alone, but resulted in a substantial increase in the HDL cholesterol peak. On day 7, the mice expressing the LDLR alone continued to have markedly reduced VLDL and LDL (Figure 2c). Expression of apoA-I alone did not affect the HDL cholesterol peak (Figure 2c). However, co-expression of apoA-I with the LDLR increased cholesterol in both VLDL and IDL/LDL fractions compared with expression of the LDLR alone, and shifted the HDL cholesterol peak to larger particle size (Figure 2c).

To determine the distribution of human apoA-I in lipoproteins, Western blotting for human apoA-I was performed using FPLC fractions on day 3 (Figure 3). When apoA-I was expressed alone, apoA-I accumulated mainly in fraction #33-42, which was corresponded to the HDL cholesterol peak. When the LDLR was co-expressed with apoA-I, more apoA-I was seen in larger particles of HDL compared with apoA-I expression alone.

The amount of cholesterol in each lipoprotein fraction was calculated using the FPLC data. After control virus injection, both VLDL (Figure 4a) and IDL/LDL cholesterol (Figure 4b) decreased but HDL cholesterol (Figure 4c) did not change. Expression of the LDLR alone markedly decreased both the VLDL (90%) and IDL/LDL cholesterol (60%) levels, but did not change the HDL cholesterol level. Expression of apoA-I alone had little effect on the lipoprotein classes compared with controls. Interestingly, co-expression of apoA-I with LDLR blunted the effect of the LDLR expression on VLDL, especially by day 14. Co-expression of the LDLR with apoA-I had a more significant effect of increasing HDL cholesterol compared with either one alone.

Effects of LDLR and apoA-I expression on atherosclerosis

Atherosclerosis was quantified by *en face* analysis of the lipid-stained aortas (Figure 5).

Although expression of the LDLR alone decreased cholesterol levels for only up to 14 days, it resulted in a significant 45% ($p<0.05$ vs control) reduction in atherosclerotic lesion area. Overexpression of apoA-I alone also resulted in a significant 42% ($p<0.05$ vs control) reduction in lesion area. Co-expression of apoA-I and the LDLR reduced atherosclerosis significantly by 37% ($p<0.05$ vs. control) compared with controls. However, the combined expression of apoA-I and LDLR did not have a greater effect on atherosclerosis than either one alone.

Discussion

In these studies, we tested the hypothesis that the combination of cholesterol reduction and apoA-I overexpression would reduce atherosclerosis to a greater degree than either one alone. We used LDLR deficient mice fed a Western diet, an established murine atherosclerosis model. Previous studies in LDLR deficient mice had confirmed that expression of the LDLR using gene transfer effectively reduced plasma cholesterol levels in mice fed chow (19) and that overexpression of apoA-I reduced atherosclerosis in mice fed a Western diet (17). We hypothesized that the cholesterol reduction induced by LDLR expression combined with overexpression of apoA-I would result in a more substantial reduction in atherosclerosis than either intervention alone.

As expected, expression of the LDLR alone had a marked effect in reducing plasma cholesterol levels in this mouse model, although the effects using this first generation adenoviral vector were transient, lasting only up to 14 days. Interestingly, this rapid and transient reduction of plasma cholesterol resulted in a significant reduction in atherosclerosis compared with control mice. We reported previously that more than 7 months of cholesterol reduction induced by adeno-associated virus mediated gene transfer of the VLDL receptor reduced the development of atherosclerosis in the same mouse model (23). The current report suggests that even short-term reduction in plasma cholesterol can have a significant impact on atherosclerosis progression.

As expected, expression of apoA-I alone reduced atherosclerosis compared with the control group. Interestingly, in this experiment apoA-I expression alone did not increase HDL cholesterol. This experimental design differed in a few key ways from our previous report (17). First, the dose of the second generation tsAdhapoA-I was 33% lower than our previous reports (17,21). Second, the mice that received tsAdhapoA-I also received an equivalent dose of a control first generation AdLacZ adenovirus, which could have influenced apoA-I expression. Finally, the mice were male (not female as in previous reports), were 30 weeks older, were fed the western diet for 5 weeks longer, and had approximately 40% higher cholesterol levels. Although expression of apoA-I alone did not increase HDL cholesterol levels, it significantly

decreased atherosclerosis compared with controls. This suggests that apoA-I can exert anti-atherogenic effects independent of its effects on HDL cholesterol levels.

Interestingly, co-expression of the LDLR with apoA-I resulted in higher apoA-I levels compared with mice expressing apoA-I alone. Furthermore, in mice expressing both the LDLR and apoA-I, HDL cholesterol levels were significantly increased to a greater extent compared with expression of either transgene alone. Frénais et al. reported that both catabolism and production rate of HDL-apoA-I increased in heterozygous FH patients (24). The increased levels of plasma human apoA-I in the mice co-expressed with apoA-I plus LDLR might be explained, at least partially, by a decreased catabolic rate of human apoA-I. Our finding here suggests that interventions to raise apoA-I and HDL cholesterol levels may be more effective when coupled with reduction in total and LDL cholesterol levels.

Unexpectedly, there were not additive effects of cholesterol reduction and apoA-I expression on atherosclerosis compared with either intervention alone in this model. One possible interpretation of this result is that cholesterol reduction and apoA-I overexpression reduce atherosclerosis through similar mechanisms that are partially redundant. For example, cholesterol reduction may result in reduced influx of cholesterol into the vessel wall and therefore, if absolute efflux rates remain constant, net efflux of cholesterol from vessel wall may increase. ApoA-I expression alone may promote net efflux as well, and the combination of the two interventions may not be able to promote a greater net efflux than either one alone. However, a number of important caveats to the interpretation of these results should be mentioned. Expression of apoA-I increased plasma triglyceride levels in mice expressing the LDLR and this could partially offset the benefit of apoA-I expression in this setting. The LDLR deficient mouse fed a western diet has a relatively high level of HDL cholesterol and apoA-I at baseline and an animal model that has low HDL cholesterol and apoA-I may be more likely to demonstrate additive effects. Although the expression of human apoA-I was robust, higher levels of apoA-I expression may have a greater additive effect. Most importantly, these experiments were in a short-term six weeks model, and therefore may not apply to longer-term studies. Additional studies in longer-term animal models are required. However, the data presented here suggest

that it can't be assumed that these two different interventions are additive and that future studies should be designed carefully to address this issue.

In summary, apoA-I and LDLR were expressed alone or in combination in the livers of LDLR deficient mice fed a Western type diet to test the additive effects on the development of atherosclerosis. (1) Overexpression of the LDLR alone reduced cholesterol levels markedly but transiently, (2) rapid and transient cholesterol reduction derived from LDLR expression significantly reduced atherosclerosis; (3) overexpression of apoA-I alone did not change plasma cholesterol levels but significantly reduced atherosclerosis; (4) co-expression of the LDLR with apoA-I increased apoA-I and HDL cholesterol levels compared with apoA-I expression alone, and (5) co-expression of the LDLR plus apoA-I did not reduce atherosclerosis to a greater extent than expression of either gene alone.

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Figure legends

Figure 1

Changes in plasma human specific apoA-I. Plasma human specific apoA-I were measured using commercially available ELISA kit (AlerCHEK Portland , ME) as described in Methods. (squares) Mice injected AdlacZ/tsAdlacZ ; (diamonds) does not appear in figure because the same value as squares, mice injected AdLDLR; (open circles) mice injected tsAdapoA-I; (filled circles) mice injected tsAdapoA-I with AdLDLR. Data are mean ± SE. *There were significant difference ($p<0.05$) between apoA-I alone and apoA-I plus LDLR by ANOVA with the Kruskal-Wallis test.

Figure 2

FPLC cholesterol profile. Pooled plasma samples were subjected to gel filtration using Superose 6 columns, and cholesterol level in each fraction was measured by using an enzymatic kit. (a) data from samples collected before injection of vectors, (b) data from samples collected 3 days after injection of vectors, and (c) data from samples collected 7 days after injection of vectors. (squares) Mice injected AdlacZ/tsAdlacZ ; (diamonds) mice injected AdLDLR; (open circles) mice injected tsAdapoA-I; (filled circles) mice injected tsAdapoA-I with AdLDLR.

Figure 3

Distribution of human specific apolipoprotein A-I. 6 µl samples from two adjacent FPLC fractions were pooled and subjected to SDS-PAGE, followed by Western blotting analysis with the antibody against human apoA-I. The last lane on the right is a positive control of human apoA-I transgenic mouse.

Figure 4

Cholesterol changes in lipoprotein subclasses. Pooled plasma samples were subjected to gel filtration using 6 columns, followed by cholesterol measurement in each fraction. After calculating the percentage of cholesterol recovered in the (a) VLDL, (b) IDL/LDL, and (c) HDL fractions the

actual VLDL-C, IDL/LDL-C, and HDL-C levels in plasma were calculated by multiplying each by the plasma cholesterol levels. (squares) Mice injected AdlacZ/tsAdlacZ; (diamonds) mice injected AdLDLR; (empty circles) mice injected tsAdapoA-I ; (filled circles) mice injected tsAdapoA-I with AdLDLR

Figure 5

Extent of atherosclerosis in LDLR deficient mice fed a Western diet 10 weeks and an additional 6 weeks after injection with (Cont) AdlacZ/tsAdlacZ; (LDLR) AdLDLR; (apoA-I) tsAdapoA-I ; (apoA-I+LDLR) tsAdapoA-I with AdLDLR Aortic surfaces covered by lesions measured by en face were expressed as a percent of total aortic area. There was significant difference ($p<0.05$) between 4 groups by ANOVA with the Kruskal-Wallis test, * significant different from Control. ($p<0.05$)