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Biochemical and Biophysical Research Communications 346 (2006) 14–18
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Overexpression of apolipoprotein AV in the liver reduces plasma triglyceride and cholesterol but not HDL in ApoE deficient mice

Wei Huang [a], Nan Bi [b], Xiaohong Zhang [a], Yanan Wang [a], Baosheng Chen [b], George Liu [a,*]

[a] *Institute of Cardiovascular Sciences and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Peking University, 38 Xue Yuan Road, Beijing 100083, PR China*

[b] *Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100005, PR China*

Received 27 April 2006
Available online 22 May 2006

Abstract

It has been shown that adenovirus-mediated overexpression of human ApoAV (hApoAV) in C57BL/6 mice results in decreased plasma triglyceride (TG) and total cholesterol (TC) levels with a major reduction occurring in the HDL fraction. In order to study the effect of ApoAV on hypercholesterolemic mice, an adenoviral vector expressing hApoAV was constructed and injected into ApoE deficient mice. High levels of hApoAV mRNA in the liver and ApoAV proteins in the liver and plasma were detected. The treatment reduced plasma TG levels by 50% and 75%, and TC levels by 45% and 58% at day 3 and 7, respectively, after treatment as compared with a control group treated with Ad-hAP (human alkaline phosphatase). Plasma HDL-C levels remained unaltered, which were different from normolipidemic mice. These findings suggest that ApoAV might serve as a therapeutic agent for hyperlipidemic disorder.

Keywords: Adenoviral vector; Apolipoprotein AV; Apolipoprotein E; Triglyceride; Cholesterol; Hypercholesterolemia; High density lipoprotein

Apolipoprotein AV (ApoAV) is a newly discovered gene among apolipoprotein gene families which was identified by comparative sequence analysis of the mammalian APOA1/C3/A4 gene cluster [1]. ApoAV gene has been associated with different plasma triglyceride (TG) levels, familial combined hyperlipidemia, and increased risk of cardiovascular disease [2–5]. Considering the independent risk effect of hypertriglyceridemia on coronary artery disease, it is conceivable that ApoAV might be used as a potential therapeutic agent.

Functional studies in mice indicated that ApoAV plays an important role in controlling plasma TG levels. Overexpression of human ApoAV (hApoAV) and deletion of ApoAV were found to have significantly decreased and increased plasma TG levels, respectively, [1,6]. Both decreased hepatic secretion of VLDL [7,8] and/or increased catabolism of TG-rich lipoproteins via interaction with lipoprotein lipase (LPL) might be involved in reduction of plasma TG by ApoAV [8–10]. Grosskopf et al. recently reported that the production rate of VLDL-TG was not altered in ApoAV deficient (ApoAV−/−) mice, but affinity of the LDL receptor for VLDL was reduced [11].

It has been shown that overexpression of Ad-ApoAV (20-fold higher than control) in C57BL/6 mice resulted in 40% reduction of plasma total cholesterol (TC) with a major decrease in HDL fraction [6,8]; whereas in ApoAV−/− mice, plasma HDL-C increased 30% [11]. HDL-C is well known for its inverse relationship to atherosclerosis and its anti-atherosclerosis effects have been widely studied. Therefore, the reduction of HDL-C by high expression of ApoAV could limit its potential clinical application.

It was noted that the above studies were all carried out in a mouse model of common C57BL/6 genetic background. Therefore, we wondered whether in certain hyperlipidemic conditions ApoAV may exert its hypolipidemic effect with minimal effect on HDL. In this study, we

* Corresponding author. Fax: +8610 82802769.
E-mail address: vangeorgeliu@gmail.com (G. Liu).

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doi:10.1016/j.bbrc.2006.05.072

therefore choose the well-established hypercholesterolemic mouse model, the ApoE deficient (ApoE−/−) mouse [12], for exploration of the potential therapeutic application of the ApoAV gene.

Materials and methods

Construction of adenoviral vector. Total RNA was first extracted by Trizol (Invitrogen) in human liver tissue. An RT-PCR fragment containing the entire coding region of hApoAV cDNA was cloned in pMD18-T (Invitrogen). Then, *Xba*I–*Kpn*I fragments were subcloned in pShuttle 2. Ligation in vitro with the adenoviral backbone vector pAdeno-X (Adeno-X™ Expression System 1, Bioscience Clontech, BD, USA) after PI-Sce I and I-Ceu I double digestions yielded recombinant pAd-hApoAV. After transforming, amplification, and purification, recombinant pAd-hApoAV was transfected in the adenovirus packaging cell line HEK 293 (Invitrogen). It was then finally propagated, purified, and titered as described in the application manual for the AdEasy vector system (Qbiogene, USA).

Animal procedures. Female ApoE−/− mice, 2 months of age, were supplied by Peking University Animal Center. Animals were housed and allowed free access to tap water and standard laboratory chow. The 'Principles of Laboratory Animal Care' (NIH publication No. 85-23, revised 1996) were followed and the experimental protocol was approved by the Animal Care Committee, Peking University Health Science Center.

Ad-hApoAV or control virus Ad-hAP [13] at 2×10^9 ifu in 0.2 ml PBS was injected in the tail vein of the mice ($n = 6$ for each group). Mice were fasted for 4 h and blood samples were taken from the retro-orbital plexus before, 3 and 7 days after virus infection. Seven days after the treatment, blood samples were taken before and 30 min after heparin injection (1 IU/g i.p.) for lipid analysis, Western blot, and LPL activity measurement. The liver was then harvested, snap-frozen in liquid nitrogen, and stored at −80 °C for real-time PCR and Western blot analysis.

RNA isolation and quantitative real-time RT-PCR. Total RNA from the liver was extracted using Tri reagent (Molecular Research Center, USA) and first-strand cDNA was generated by using a RT kit (Invitrogen, USA). Quantitative real-time PCR was performed using following primer sets: Human ApoAV (5′-CCGCGACCCTGAAAGACA-3′, 5′-CAAAGCC CAAGCCTCGTC-3′); Mouse ApoAV (5′-CTGGCACAGGAGAACC TGA-3′, 5′-TTTGACTCGGTCGGTATGGT-3′); ACAT (5′-TGAAGG AAGTCTACATGGG-3′, 5′-CTGTTCCTGCCGTGAGATA-3′); HMG-CoAR (5′-CTGTGGCTGGAATTATGAG-3′, 5′-CACAAGGCATT CCACAAGA-3′); LDL-R (5′-TGAAGAATGTGGTGGCTC-3′, 5′-CT GCCTGGGACTGAATCT-3′); LRP-1 (5′-TGCCAATGAGACCGTA TG-3′, 5′-TCCTGTCGTCAATGTCGT-3′); SREBP-1 (5′-AGGATA GCCAGGTCAAAG-3′, 5′-ATCTCTGCTCTCTGCCTC-3′); Mouse β-actin (5′-TCA GAA GGA CTC CTA TAG TGG-3′, 5′-TCT CTT TGA TGT CAC GCA CG-3′).

Amplifications were performed in 35 cycles using an opticon continuous fluorescence detection system (MJ Research) with SYBR green fluorescence (Molecular Probes, Eugene, USA). Each cycle consisted of heating denaturation for 45 s at 94 °C, annealing for 45 s at 55 °C, and extension for 60 s at 72 °C. All samples were quantitated by using the comparative CT method for relative quantitation of gene expression, normalized to β-actin [14].

Western blot analysis. Mouse plasma (0.8 μl) or liver homogenate (80 μg protein extracted by RIPA solution) was subjected to electrophoresis on 12% SDS–PAGE and transferred to nitrocellulose membranes (Sigma, MO, USA). After blocking with 5% bovine serum albumin for 1 h, the membrane was probed with 1:700 goat anti-hApoAV polyclonal antibody (provided by Prof. D. Liu, Peking Union Medical College), followed by horseradish peroxidase-conjugated secondary antibody (rabbit anti-goat, 1:2000). The reaction was detected by chemiluminescence and exposed to Kodak X-Omat film (Kodak, Rochester, USA). ApoAV protein level was normalized to that of GAPDH by using a mouse anti-human monoclonal antibody.

Plasma lipid analysis. Plasma TC and TG were determined by using enzymatic methods (Sigma kits, MO, USA). Plasma HDL-C was measured after precipitation of ApoB-containing lipoproteins with an equal volume of a 20% polyethylene glycol solution, as described previously [15].

Plasma LPL activity analysis. Post-heparin plasma was obtained as described above and LPL activity was determined by incubation of 10 μl plasma with ^{3}H-triolein emulsion substrate prepared as described [16] at 37 °C for 60 min. Enzyme activity was expressed as mU/ml (1 mU corresponds to 1 nmol free fatty acid generated per minute).

Statistical analysis. All data are presented as means ± SEM. Statistical comparison between the two groups was performed using Student's t test. A value of $p \leqslant 0.05$ was considered statistically significant.

Results

Adenoviral overexpression of hApoAV in mice

The expression of Ad-hApoAV was evaluated by real-time PCR measurement at mRNA levels in liver, and also

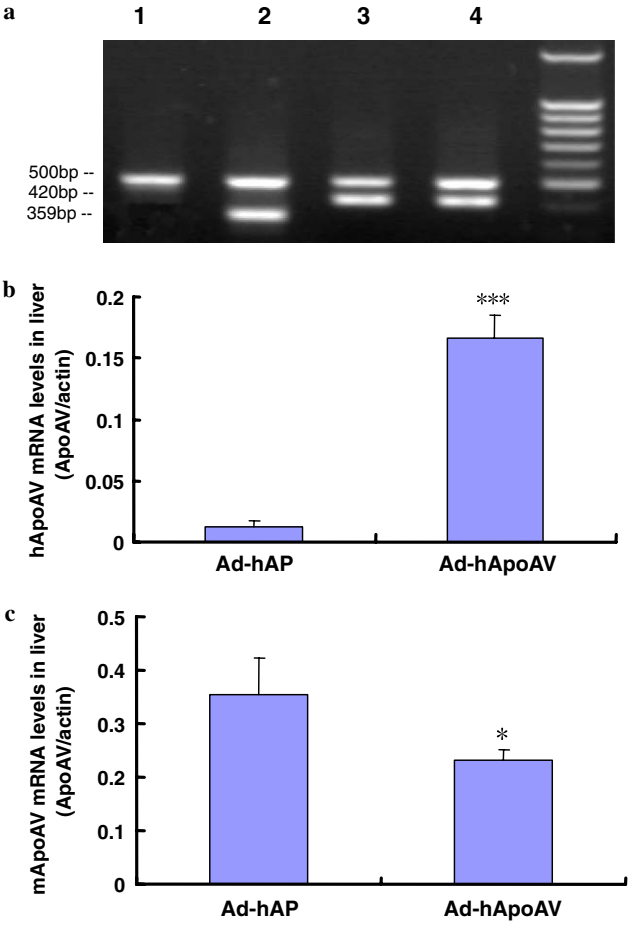

Fig. 1. Analysis of human ApoAV and mouse ApoAV mRNA expression in liver by RT-PCR (a) and real-time quantification (b,c). (a) Lanes 1 and 2, samples from Ad-hAP and Ad-hApoAV groups, respectively, using the human ApoAV gene special primers; lanes 3 and 4, samples from Ad-hAP and Ad-hApoAV groups, respectively, using mouse ApoAV gene special primers. The 359 bp band corresponds to human ApoAV, the 420 bp band corresponds to mouse ApoAV, and the 500 bp band corresponds to mouse β-actin. In (b,c), human and mouse ApoAV mRNA expression in liver was measured by relative quantitative real-time RT-PCR. Results were normalized to mouse β-actin. *$p < 0.05$; ***$p < 0.001$ for Ad-ApoAV groups compared with the Ad-hAP control group ($n = 6$ for each group).

by Western blot to determine protein levels in liver and plasma.

A 359 bp band, specific for the hApoAV gene, was detected by RT-PCR only in the Ad-hApoAV group, but not in the Ad-hAP control group while the endogenous mouse ApoAV gene specific 420 bp band and a 500 bp band corresponding to mouse β-actin were present in both groups (Fig. 1a). Real-time quantitation of hApoAV mRNA expression was significantly higher in Ad-hApoAV mice than in Ad-hAP control mice (Fig. 1b, $p < 0.001$). The mouse endogenous ApoAV gene expression was decreased in Ad-hApoAV treated mice as compared with the controls (Fig. 1c, $p < 0.05$).

Western blot probed by anti-ApoAV antibody revealed a band of 39 kDa corresponding to the size of ApoAV protein in both groups in the liver and plasma. The intensity of ApoAV band was 3- and 10-fold higher in liver (after correction against an internal standard GAPDH) and in plasma, respectively, in the Ad-hApoAV group than in the control group (Fig. 2a and b, $p < 0.001$).

Biological effects of hApoAV overexpression

Triglyceride levels in mice receiving Ad-hApoAV were decreased 50% and 75% at day 3 and 7 after gene transfer compared with the control group (Fig. 3, $p < 0.01$ and $p < 0.001$). Furthermore, in these ApoE−/− mice, plasma TC levels were also decreased 45% at day 3 (Fig. 4, from 490 ± 94 to 270 ± 160 mg/dl, $p < 0.02$), and 58% at 7 days (Fig. 4, from 618 ± 90 to 260 ± 110 mg/dl, $p < 0.001$) after gene transfer. However, plasma HDL-C levels remained unchanged (26 ± 1.0 vs. 23.9 ± 3.5 mg/dl). Post-heparin plasma LPL activity was also unaltered in the Ad-hApoAV treated group than in the control group (463.6 ± 40.0 vs. 440.0 ± 57.7 mU/ml).

Overexpression of hApoAV in ApoE−/− mice did not alter hepatic mRNA levels of several genes influencing plasma cholesterol levels (ACAT, HMG-CoAR, LDL-R, LRP-1, and SREBP-1) as compared with the control group (Table 1).

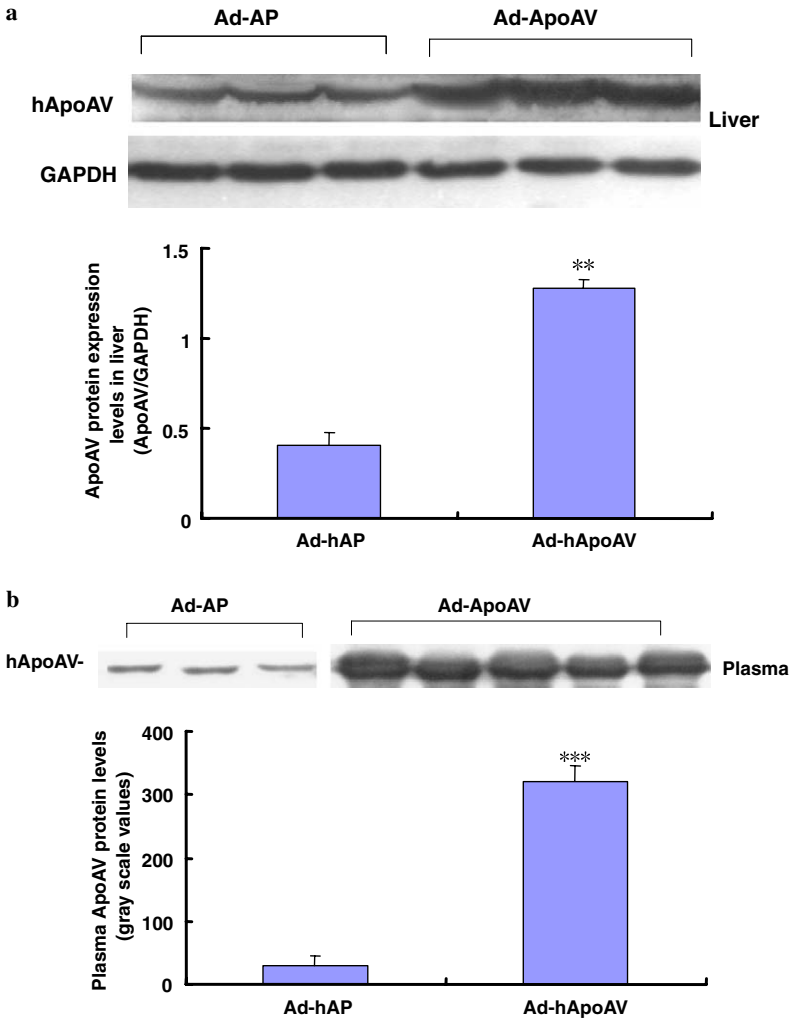

Fig. 2. Western blot analysis and quantitative representation of ApoAV protein levels in liver (a) and in plasma (b). (a) A band of 39 kDa corresponds to hApoAV protein using a goat anti-human polyantibody, a band of 36 kDa corresponds to GAPDH protein in liver. Results of quantification were normalized to mouse GAPDH. (b) A band of 39 kDa corresponds to hApoAV protein in 0.8 μl plasma. $^{**}p < 0.002$; $^{***}p < 0.001$ for Ad-ApoAV groups compared with the Ad-hAP control group. $n = 6$ for each group. Ad-ApoAV, representing samples from the Ad-hAPOAV treated group; Ad-AP, representing samples from the Ad-hAP treated group.

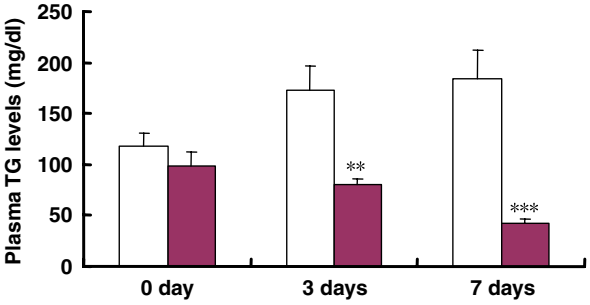

Fig. 3. Plasma triglyceride (TG) levels at day 0, 3, and 7 after Ad-hAPOAV gene transfer in ApoE−/− mice. □, Ad-hAP treated group; ■, Ad-hAPOAV treated group. $^{**}p < 0.01$; $^{***}p < 0.001$ for Ad-ApoAV groups compared with Ad-hAP control group ($n = 6$ for each group).

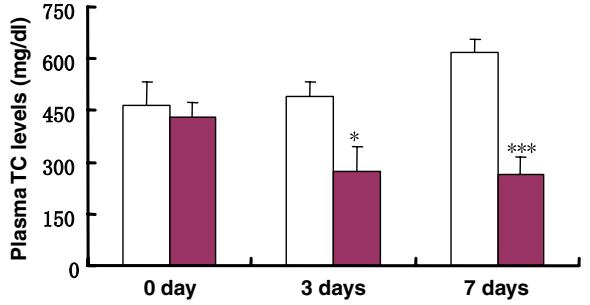

Fig. 4. Plasma total cholesterol (TC) levels at day 0, 3, and 7 after Ad-hAPOAV gene transfer in ApoE−/− mice. □, Ad-hAP treated group; ■, Ad-hAPOAV treated group. $^{*}p < 0.05$; $^{***}p < 0.001$ for Ad-ApoAV groups compared with the Ad-hAP control group ($n = 6$ for each group).

Table 1
Hepatic mRNA levels of several genes influencing plasma cholesterol levels by real-time PCR

	Ad-ApoAV	Ad-AP	p values
ACAT	0.112 ± 0.023	0.164 ± 0.057	0.183
HMG	1.477 ± 0.733	1.534 ± 0.226	0.472
LDL-R	1.120 ± 0.618	0.613 ± 0.150	0.227
LRP	0.110 ± 0.041	0.086 ± 0.019	0.306
SREBP1	0.325 ± 0.096	0.449 ± 0.062	0.152

Values are expressed as means ± SEM. No differences were observed between the Ad-ApoAV treated group and the Ad-AP control groups ($n = 5$).

Discussion

In the present study, overexpression of hApoAV via adenoviral gene transfer in hypercholesterolemic ApoE deficient mice reduced both plasma TG and TC levels to an extent similar to that reported in normolipidemic wild-type C57BL/6 mice [6,8]. It was of interest to note that a significant reduction of TC only occurred in non-HDL fractions since plasma HDL-C remained unaltered.

The mechanism by which Ad-ApoAV overexpression reduces plasma HDL in wild-type mice is not well understood. At high concentrations (20-fold higher in plasma as compared with the control), ApoAV might competitively inhibit ApoAI or HDL-mediated efflux of TC from cells, thus lowering plasma HDL-C levels [6]. It has been suggested that HDL-C reduction may relate simply to the LPL stimulation effect of ApoAV in Ad-ApoAV treated mice. HDL is the major TC containing fraction in the rodent models, and overexpression of LPL has been shown to result in reduction of plasma HDL-C [8].

In our study, plasma LPL activity was unchanged after Ad-ApoAV gene transfer. The same results have been found in the ApoAV transgenic and knockout mice by Merkel et al. [17]. In another in vitro study by co-incubation of purified ApoAV with bovine LPL in reaction mixtures, Lookene et al. did not observe direct effect of ApoAV on LPL activity [10]. Both of them further showed that ApoAV might act by guiding VLDL and chylomicrons to heparan sulfate proteoglycans (HSPG) bound LPL for lipolysis [10,17].

The most important function of ApoE is mediating uptake of remnants of CM/VLDL by the liver. Thereby, in ApoE−/− mice, 80% of TC consists of remnants of CM and VLDL, whereas LDL constitutes 20% and only 5% is HDL [12]. HDL-C levels did not change in our study. Therefore, remnants of CM/VLDL were the major decreased fractions in the 58% reduction of plasma TC in Ad-hApoAV treated ApoE−/− mice.

Recent studies have shown a close association between ApoAV and HSPG. ApoAV could promote the binding of HSPG and CM in vitro [10]. Reduction of plasma TC levels by Ad-hApoAV in ApoE−/− mice might be caused by increase of hepatic uptake of the CM/VLDL remnants mediated by HSPG independent of ApoE. It is therefore possible that in the absence of ApoE, ApoAV could act as ligand between HSPG and CM/VLDL remnants to partially compensate the effect of ApoE in lipoprotein remnant clearance.

In search for potential molecules interacting with ApoAV, we also evaluated hepatic mRNA levels of several genes known to influence plasma cholesterol metabolism (i.e. ACAT, HMG-CoAR, LDL-R, LRP-1, and SREBP-1). They did not alter in our study. These results were agreed with Schaap et al. [8]. The exact mechanism(s) by which Ad-ApoAV decreases plasma TC levels in wild-type and ApoE−/− mice requires further investigation.

In conclusion, adenoviral overexpression of hApoAV reduced significantly both circulating TG and TC levels with minimal effect on HDL-C in ApoE deficient mice. These findings suggest that such plasma lipid lowering effects of ApoAV could be used as a potential therapeutic agent in certain forms of hypercholesterolemia, such as ApoE mutated type III hyperlipoproteinemia, and further investigation is thus warranted.

Acknowledgments

This project was supported in part by Major National Basic Research Program of the People's Republic of China (No. G20000056902), Program for Changjiang Scholars Innovative Research Team in University (PCSIPT) and a grant from the National Natural Science Foundation of

the People's Republic of China (No. 30470811) awarded to W.H. We are grateful to Dr. Michael Blackstein for critical reading of our manuscript.

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