Human Apolipoprotein A-II Protects Against Diet-Induced Atherosclerosis in Transgenic Rabbits

Yao Wang,* Manabu Niimi,* Kazutoshi Nishijima, Ahmed Bilal Waqar, Ying Yu, Tomonari Koike, Shuji Kitajima, Enqi Liu, Tomohiro Inoue, Masayuki Kohashi, Yuka Keyamura, Tomohiro Yoshikawa, Jifeng Zhang, Loretta Ma, Xiaohui Zha, Teruo Watanabe, Yujiro Asada, Eugene Y. Chen, Jianglin Fan

Objective—Apolipoprotein (apo) A-II is the second major apo of high-density lipoproteins, yet its pathophysiological roles in the development of atherosclerosis remain unknown. We aimed to examine whether apo A-II plays any role in atherogenesis and, if so, to elucidate the mechanism involved.

Methods and Results—We compared the susceptibility of human apo A-II transgenic (Tg) rabbits to cholesterol diet-induced atherosclerosis with non-Tg littermate rabbits. Tg rabbits developed significantly less aortic and coronary atherosclerosis than their non-Tg littermates, whereas total plasma cholesterol levels were similar. Atherosclerotic lesions of Tg rabbits were characterized by reduced macrophages and smooth muscle cells, and apo A-II immunoreactive proteins were frequently detected in the lesions. Tg rabbits exhibited low levels of plasma C-reactive protein and blood leukocytes compared with non-Tg rabbits, and high-density lipoproteins of Tg rabbit plasma exerted stronger cholesterol efflux activity and inhibitory effects on the inflammatory cytokine expression by macrophages in vitro than high-density lipoproteins isolated from non-Tg rabbits. In addition, β-very-low-density lipoproteins of Tg rabbits were less sensitive to copper-induced oxidation than β-very-low-density lipoproteins of non-Tg rabbits.

Conclusion—These results suggest that enrichment of apo A-II in high-density lipoprotein particles has atheroprotective effects and apo A-II may become a target for the treatment of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: apolipoproteins  atherosclerosis  lipoproteins  metabolism  transgenic rabbits

Plasma levels of high-density lipoproteins (HDLs) are closely associated with the risk of atherosclerotic coronary artery disease.1 HDLs are macromolecules and contain equal amounts of lipids and proteins.2 Apolipoprotein (apo) A-I and apo A-II constitute the major protein components of HDLs, comprising 70% and 20% of the total, respectively. Although apo A-I atheroprotective functions (such as reverse cholesterol transport, anti-inflammation, and antioxidation) have been extensively investigated and well established,3,4 relatively little is known about the physiological significance of apo A-II in lipid metabolism and atherosclerosis.5 Synthesized predominantly in the liver and small amounts made in the intestine, apo A-II is a 77-aa protein and exists as a disulfide-linked homodimer (MW, 17.4 kDa) in plasma.

In humans, apo A-II levels do not correlate with plasma HDL-cholesterol (HDL-C) levels, yet clinical and epidemiological studies have yielded conflicting results about the relationship between plasma apo A-II levels and coronary heart disease. Some studies showed that plasma apo A-II levels are inversely associated with coronary artery heart disease, as are HDL-C and apo A-I levels,6 and low plasma apo A-II is associated with increased risk of myocardial infarction.7,8 In addition, there was found to be a strong inverse relationship between plasma apo A-II levels and risk of future coronary artery disease in an apparently healthy population.9 Nevertheless, patients who are deficient in apo A-II gene did not show any increased susceptibility to coronary artery disease,10 and the −265C polymorphism in the apo A-II promoter region was shown to be associated with decreased plasma apo A-II concentration and reduced risk of coronary artery disease.11

Animal studies along with in vitro studies also generated controversial results. Most of these studies indicate that apo A-II is poorly antiatherogenic or even proatherogenic because replacement of apo A-I with apo A-II impairs HDL antiatherogenic functions.12-14 This notion is supported by the finding that the expression of murine apo A-II gene increases aortic

Received on: September 10, 2012; final version accepted on: November 11, 2012.

From the Department of Molecular Pathology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo-City, Yamanashi, Japan (Y.W., M.N., T.K., T.I., A.B.W., J.F.); Analytical Research Centre for Experimental Sciences, Saga University, Saga, Japan (K.N., S.K., T.W.); Research Institute of Atherosclerotic Disease, Xi’an Jiaotong University School of Medicine, Xi’an, China (E.L.); Free Radical Research Project, Otsuka Pharmaceutical Co Ltd, Tokushima, Japan (M.N., M.K., Y.K., T.Y.); Cardiovascular Center, Department of Internal Medicine, University of Michigan, Ann Arbor, MI (J.Z., Y.E.C.); Chronic Disease Program, Ottawa Hospital Research Institute, Ottawa, ON, Canada (L.M., X.Z.); and Department of Pathology, University of Miyazaki School of Medicine, Miyazaki, Japan (Y.A.).

*These authors contributed equally to this work.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.112.300445/-/DC1. Correspondence to Jianglin Fan, Department of Molecular Pathology, University of Yamanashi, 1110 Shimokato, Yamanashi 409-3898, Japan. E-mail jianglin@yamanashi.ac.jp

© 2012 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.112.300445
atherosclerosis in transgenic (Tg) mice fed even on a chow diet. On the other hand, a study using apo A-II knock-out mice showed that murine apo A-II may have somewhat antiatherogenic properties, such as in the maintenance of the plasma HDL pool. Furthermore, expression of human apo A-II gene in Tg mice led to either increased or decreased susceptibility to atherosclerosis dependent on high-fat diets. Therefore, it is still poorly understood whether human apo A-II is involved in the development of atherosclerosis or whether apo A-II–rich HDLs are beneficial for cardiovascular protection. To investigate the pathophysiological roles of human apo A-II in lipoprotein metabolism and atherosclerosis, our laboratory generated human apo A-II Tg rabbits, a species that normally does not have an endogenous apo A-II gene. On a chow diet, apo A-II Tg rabbits exhibited mild hyperlipidemia (mean plasma total cholesterol in males, 74 mg/dL in Tg versus 65 mg/dL in non-Tg; females, 129 mg/dL in Tg versus 68 mg/dL in non-Tg) and 50% reduction of HDL-C compared with non-Tg littermates, but did not develop spontaneous atherosclerosis (Koike et al, unpublished data). To examine whether human apo A-II plays any role in the development of atherosclerosis, we fed Tg and non-Tg rabbits with a cholesterol diet and compared their susceptibility to atherosclerosis. Our studies showed that expression of human apo A-II protects against cholesterol diet-induced atherosclerosis in Tg rabbits through enhancement of cholesterol efflux activity and anti-inflammatory functions.

**Methods**

**Animals**

Tg Japanese white rabbits expressing the human apo A-II genomic DNA were generated in our laboratory as described previously. Human apo A-II was mainly expressed in the liver. Plasma levels of human apo A-II of Tg rabbits were 29.38±6.3 mg/dL in males and 35.06±10.45 mg/dL in females, which are similar to those of healthy humans (31–35 mg/dL). Tg rabbits along with sex- and age-matched non-Tg littermates (4–5 months old) were used for the current study. All rabbits were fed with a diet containing ≥ 0.3% cholesterol and 3% soybean oil for 16 weeks. A-II animal experiments were performed with the approval of the Animal Care Committee of the University of Yamashani and Saga and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Plasma lipids (total cholesterol, triglycerides, and HDL-C), lipoproteins, apo A-I and apo A-II, CETP activity, whole blood cells, and C-reactive protein levels along with serum paraoxonase/arylesterase 1 activity and hepatic expression were measured. Lipoproteins and HDL size were also analyzed by high-performance liquid chromatography. See the details in the online-only Data Supplement.

**Analysis of Aortic and Coronary Atherosclerosis**

The aortic and coronary atherosclerosis lesions were quantified using the method as described previously. To detect whether apo A-II was present in lesions, rabbit aortic arch along with human carotid arteries and aortas were immunohistochemically stained with goat polyclonal Ab against human apo A-II (See the online-only Data Supplement for all Abs). For negative control staining, the sections were immunostained with nonspecific goat IgG as the primary Ab or without adding the primary Ab. In addition, fresh aortic specimens of rabbits and human autopsy cases were homogenized in ice-cold suspension buffer (0.02 mol/L Tris–HCl, pH 7.5). From each sample, 45 μg of the crude protein was fractionated by 4%–20% gradient SDS-PAGE under the nonreducing conditions and probed with human apo A-II Ab.

**Cholesterol Efflux Assay**

To analyze whether apo A-II affects cholesterol efflux capacity of HDLs, we compared Tg-HDLs with non-Tg HDLs for their cholesterol efflux capacity in vitro. To investigate whether ATP-binding cassette transporter A-1 (ABC-A1) was involved in the cholesterol efflux mediated by apo A-II, we performed the cholesterol efflux assay using baby hamster kidney cells stably transfected with a mifepristone-inducible vector containing an insert encoding ABC-A1 or without insert (MOCK). See the details in the online-only Data Supplement.

**Analysis of Anti-inflammatory Effects of HDLs**

To examine whether apo A-II affects the anti-inflammatory functions of HDLs, we compared the Tg-HDLs with non-Tg HDLs in terms of their inhibitory effects on tumor necrosis factor-α, interleukin-6, and monocyte chemoattractant protein-1 expression in vitro. U937 monocyte-derived macrophages were incubated with 20 μg/mL of lipopoly saccharide (L4391, Sigma-Aldrich, St. Louis, MO) alone (as a control) or lipopolysaccharide with different concentrations of HDL, isolated from either Tg or non-Tg rabbits for 24 hours. Then, cells were lysed and total RNA was extracted for determination of the gene expression of tumor necrosis factor-α, monocyte chemoattractant protein-1, and interleukin-6 by real-time reverse transcriptase-polymerase chain reaction. The primers and protocol used for reverse transcriptase-polymerase chain reaction are shown in the online-only Data Supplement.

**Evaluation of ApoB-Containing Lipoprotein Oxidizability**

In Tg rabbit plasma, a small amount of apo A-II was also detected in apoB-containing lipoprotein particles of Tg rabbits; thus, we investigated whether apo A-II affects oxidation of these particles. For this experiment, we isolated 4 apoB-containing lipoprotein fractions (d<1.006 g/mL, B-very-low-density lipoprotein [VLDL]), d=1.006–1.02 g/mL [intermediate density lipoprotein], d=1.02–1.04 g/mL [intermediate density lipoprotein and large LDL], and d=1.04–1.06 [LDL]) from cholesterol-fed Tg and non-Tg rabbits. Each lipoprotein fraction (50 μg/mL protein) was dissolved in phosphate buffer (10 mmol/L, pH 7.4) with 0.16 mol/L NaCl. The kinetics of copper-induced lipoprotein oxidation was determined by monitoring the change of the conjugate-diene absorbance at 234 nm at 37°C with a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA) as described previously. Absorbance of lag-time, maximal oxidation speed (V max), and maximal diene concentrations were calculated for the estimation of oxidation sensitivity.

**Statistical Analysis**

All data are expressed as mean±SD. Statistical significance was determined by the Mann-Whitney U test for nonparametric analysis of the lesions of coronary arteries. Student t test was used to compare the results of other assays. In all cases, statistical significance was set at P<0.05.

**Results**

**Plasma Lipids and Lipoproteins**

Tg and non-Tg developed similar hypercholesterolemia (Figure IA in the online-only Data Supplement, top). Compared with those of non-Tg rabbits, plasma average triglycerides levels of Tg rabbits were high, but there was no statistical significance after 10 weeks (males) or 4 weeks (females) after cholesterol diet feeding (Figure IA in the online-only Data Supplement, middle). HDL-C levels of Tg rabbits were significantly lower than those of non-Tg rabbits on a chow diet as reported previously but did not change with a cholesterol diet. In contrast, HDL-C levels of non-Tg rabbits were decreased after cholesterol diet feeding and became lower than those of Tg rabbits at the end of the experiment (Figure IA in the online-only Data Supplement,
Furthermore, cholesterol diet feeding led to the reduction of plasma apo A-I levels in both non-Tg and Tg rabbits, but apo A-II levels were not affected in Tg rabbits (Figure IB in the online-only Data Supplement). Hepatic expression of apo A-I was low in Tg rabbits, but not significantly different at 16 weeks. There was no difference in CETP activity between the 2 groups (Figure IB in the online-only Data Supplement).

Agarose gel electrophoresis analysis of lipoprotein profiles revealed that Tg rabbits had less α-migrating HDLs than non-Tg rabbits, whereas pre-β-migrating HDLs became prominent (Figure IC in the online-only Data Supplement). Analysis of the density fractions of lipoproteins further confirmed that almost all HDL1–3 in Tg rabbits migrated to the pre-β density fraction of lipoproteins (Figure IC in the online-only Data Supplement). Hepatic expression of apo A-II was low in Tg rabbits, but not significantly different at 16 weeks. There was no difference in CETP activity between the 2 groups (Figure IB in the online-only Data Supplement). ApoB and apoE contents in apoB-containing particles and HDL particles were similar in both the groups.

**Aortic and Coronary Atherosclerosis**

Analysis of en face aortic sudanophilic area revealed that Tg rabbits had significantly smaller atherosclerotic lesions of the whole aorta than non-Tg rabbits (Figure 1A). The whole lesion

**Figure 1.** Analysis of atherosclerotic lesions of aorta and coronary arteries. A, Representative photographs of pinned-out aortic trees stained with Sudan IV from non-transgenic (Tg) and Tg rabbits (Figure IE in the online-only Data Supplement). B, Representative micrographs of the aortic lesions from male non-Tg and Tg rabbits. Serial paraffin sections were stained with hematoxylin-eosin (HE) and elastica van Gieson (EVG) or immunohistochemically stained with monoclonal antibodies (mAbs) against either macrophages (Mφ) or α-smooth muscle actin for smooth muscle cells (SMC; left). Intimal lesions on EVG-stained sections and positively immunostained areas of macrophages and SMC were quantified with an image analysis system (right). Each dot represents the lesion area of an individual animal. B, Representative micrographs of the aortic lesions from male non-Tg and Tg rabbits. Serial paraffin sections were stained with heparinase/β-glucuronidase (HE) from non-transgenic (Tg) and Tg rabbits (Figure IE in the online-only Data Supplement). C, The heart was cut into 7 blocks, and blocks I and II containing left and right coronary trunks were sectioned in 5-μm intervals (3 sections from each block) and stained with EVG. Representative micrographs of coronary atherosclerosis of the left main trunks stained by EVG (left). Coronary stenosis=lesion area/total lumen area×100(%) was measured and is expressed as percentage (right). LCA indicates left coronary artery trunk; and RCA, right coronary artery trunk.
surface area was significantly reduced by 67% in male Tg rabbits (40%↓ in aortic arch, 87.6%↓ in thoracic, and 81.1%↓ in abdominal aorta versus non-Tg) and 45% in female Tg rabbits (26.8%↓ in aortic arch, 70.5%↓ in thoracic, and 46.5%↓ in abdominal aorta versus non-Tg). Histological examinations showed that the aortic lesions were mainly composed of infiltrating macrophages and smooth muscle cells intermingled with extracellular matrix (Figure 1B, left). Morphometric analysis revealed that the microscopic atherosclerotic lesion area was significantly reduced in all parts of the aorta in Tg rabbits: 85%↓ in aortic arch, 90%↓ in thoracic aorta, and 73.9%↓ in abdominal aorta in Tg males and 69.6%↓ in aortic arch, 83%↓ in thoracic aorta, and 40%↓ in abdominal aorta in Tg females compared with those in each counterpart in non-Tg rabbits.

Immunohistochemical staining showed that decreased aortic lesion areas in Tg rabbits were caused by marked reduction of macrophages and smooth muscle cells (Figure 1B, right).

In addition to aorta, male Tg rabbits had significantly smaller lesions in both left and right coronary arteries, whereas in female Tg rabbits, the left coronary artery stenosis was significantly less severe than in non-Tg rabbits (Figure 1C).

Detection of Apo A-II Immunoreactive Proteins in the Lesions
Although apo A-II was not found in the nonlesional area of Tg rabbits, apo A-II was detected beneath endothelial cells adjacent to the lesions of aorta (Figure 2A, top). Apo A-II was often

![Figure 2.](http://atvb.ahajournals.org/)

Figure 2. Demonstration of apolipoprotein (apo) A-II immunoreactive proteins in lesions of transgenic (Tg) rabbit and human atherosclerosis. Representative micrographs of normal intima and early-stage lesions (A), and fatty streak (foam cell-rich lesions; B, top) of Tg rabbits. Serial paraffin sections were stained with hematoxylin-eosin (HE) or Abs against human apo A-II alone or double-stained with apoA-II (stained as red) and macrophage (stained as blue) Abs (labeled as apo A-II/Mϕ). Normal intima and atherosclerotic intima (grossly) of Tg rabbits were analyzed by 4% to 20% SDS-PAGE under nonreducing conditions and followed by immunoblotting using human apo A-II polyclonal Ab (B, bottom). C, Representative micrographs of advanced lesion from human carotid artery (top). Serial paraffin sections of the lesions were stained with HE, polyclonal Ab against human apo A-II, and double-staining with Abs against apo A-II (stained as red) and human macrophage (stained as blue). Human aortic atherosclerotic intima of 3 autopsy cases was analyzed by 4% to 20% SDS-PAGE under nonreducing conditions and followed by immunoblotting using human apo A-II polyclonal Ab (bottom). Proteins isolated from U937-derived macrophages were used as the negative control and 1 μL of human plasma was used as the positive control. GAPDH proteins are shown at the bottom to indicate the relative amount of proteins loaded in each lane.
observed in the fatty streak where apo A-II was located around macrophages as shown by double immunostaining with both apo A-II and macrophage Abs (Figure 2A, middle and bottom). The specificity of apo A-II immunostaining was confirmed by the observation that (1) apo A-II was not stained in non-Tg aortic lesions and (2) apo A-II was not stained when the primary Ab was omitted or replaced with a nonspecific goat IgG in the lesions of Tg rabbits (Figure 2B, top). The presence of apo A-II dimer (17 kDa) in the lesions of Tg rabbits was further confirmed by nonreducing SDS-PAGE followed by Western blotting in the aortic intima of Tg rabbits but not in non-Tg rabbits (Figure 2B, bottom). We also examined the lesions of human aortic atherosclerosis obtained from autopsy. Similar to the lesions of Tg rabbits, apo A-II-immunoreactive proteins were mainly located around macrophage-derived foam cells (Figure 2C). To investigate the interactions between apo A-II and foam cells, we examined foam-cell–rich lesions and found that apo A-II–immunoreactive proteins were mainly located around macrophage-derived foam cells. Proteins (HDLs) on cytokine expression in macrophages. U937 macrophages were treated with lipopolysaccharide (LPS) or in the presence of HDL3 isolated from either non-transgenic (Tg) or Tg rabbit plasma for 24 hours and mRNA expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) was measured by real-time reverse transcriptase-polymerase chain reaction. HDLs were fractionated by SDS-PAGE and shown in the middle insert. Representative data from 3 separate experiments are shown. Data are expressed as mean±SD. *P<0.05, **P<0.01 vs non-Tg.

### Anti-Inflammatory Effects of Apo A-II–Rich HDLs

The finding that apo A-II expression in Tg rabbits led to the reduction of atherosclerosis prompted us to examine the possible mechanisms involved. We first investigated whether apoA-II affects inflammatory state. We measured plasma C-reactive protein levels (a robust inflammatory marker) and serum paraoxonase/arylesterase 1 activity, an enzyme present in HDLs that is important for their anti-inflammatory and antioxidation functions. Tg rabbits exhibited significantly lower levels of plasma C-reactive protein but higher paraoxonase/arylesterase 1 activity than non-Tg littersmates after cholesterol diet feeding at 16 weeks (Figure 3 top). We also measured white blood cell numbers. Although there was no difference between Tg and non-Tg rabbits on a chow diet, Tg rabbits had significantly fewer total white blood cells with 42% neutrophils (P=0.08) and 44% monocytes (P=0.25), but no changes in lymphocytes than those in non-Tg rabbits on a cholesterol diet at 16 weeks (Figure 3, bottom). There was no difference in platelet and red blood cell counts between the 2 groups (data not shown).

Addition of either Tg-HDLs or non-Tg HDLs at doses of 10–40 μg/mL significantly inhibited the expression of tumor necrosis factor-α and interleukin-6 in lipopolysaccharide-stimulated macrophages (Figure 4). When a comparison was made between Tg and non-Tg HDLs with regard to their suppressive capacity, Tg-HDLs had significantly stronger effects on the tumor necrosis factor-α and interleukin-6 expression than did non-Tg HDLs. Furthermore, Tg-HDLs showed inhibitory effects on monocyte chemoattractant protein-1 expression, which was not obvious in non-Tg HDLs.

Figure 3. Analysis of plasma C-reactive protein (CRP), paraoxonase/arylesterase 1 (PON1) activity (top), and white blood cells (bottom). Plasma levels of CRP of non-transgenic (Tg; white dot) and Tg rabbits (gray dots) were measured by ELISA. Serum PON1 activity was measured using 3 different substrates. Data are expressed as mean±SD. *P<0.05, **P<0.01 vs non-Tg. n=6–13 from each group containing both males and females.

Figure 4. Inhibitory effects of high-density lipoproteins (HDLs) on cytokine expression in macrophages. U937 macrophages were treated with lipopolysaccharide (LPS) or in the presence of HDLs isolated from either non-transgenic (Tg) or Tg rabbit plasma for 24 hours and mRNA expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) was measured by real-time reverse transcriptase-polymerase chain reaction. HDLs were fractionated by SDS-PAGE and shown in the middle insert. Representative data from 3 separate experiments are shown. Data are expressed as mean±SD. *P<0.05, **P<0.01 vs non-Tg.
Cholesterol Efflux Capacity

Next, we examined whether there was any difference between Tg-HDLs (containing both apo A-I and apo A-II) and non-Tg HDLs (containing only apo A-I particles) in terms of their cholesterol efflux ability. Clearly, Tg-HDLs (both HDL2 and HDL3) were more efficient in removing cellular cholesterol from THP-1 macrophages than non-Tg HDLs at all doses (10, 30, and 100 μg/mL; Figure 5A). We further compared the purified apo A-I and apo A-II without lipids and showed that, among 3 batches of apo A-I and 2 batches of apo A-II, their cholesterol efflux capacities were almost identical (Figure III in the online-only Data Supplement). In addition to THP-1 macrophages, similar results were also obtained when mouse RAW264.7 macrophages (either unloaded or loaded with cholesterol) were used (data not shown). Using ABCA-1–transfected baby hamster kidney cells, we further demonstrated that functional ABCA-1 was essential for both apo A-I– and apo A-II–mediated cholesterol efflux.

Both mock and cells expressing an ABCA-1–defective mutant (A937V) failed to efflux cholesterol under identical conditions (Figure 5B). In addition, apoA-I and apoA-II did not seem to interfere with each other because apoA-I/apoA-II combination removed cholesterol as efficiently as apoA-I or apoA-II alone (Figure 5B).

ApoB-Containing Lipoprotein Oxidizability

Because a small amount of apo A-II is present in apoB-containing particles (Figure IV in the online-only Data Supplement), we next examined the possible role of apo A-II in apoB-containing lipoprotein oxidation. β-VLDL, the major atherogenic lipoprotein in cholesterol-fed rabbits, was the most sensitive to oxidation among the 4 apoB-containing lipoprotein fractions. Compared with that of non-Tg rabbits, oxidizability of β-VLDL but not intermediate density lipoprotein and LDL fractions of Tg rabbits was significantly diminished (Figure 6).

Discussion

In this study, we have demonstrated that human apo A-II, the second major apo of HDLs, protected against cholesterol-diet–induced atherosclerosis in Tg rabbits. Expression of human apo A-II at the physiological levels of healthy humans significantly suppressed the development of aortic atherosclerosis by 67% in male and 45% in female Tg rabbits compared with that in non-Tg rabbits. Interestingly, coronary atherosclerosis was also significantly decreased in Tg rabbits. Atherosclerotic lesions of Tg rabbits were characterized by reduced macrophages and smooth muscle cells. This finding was initially surprising and unexpected because apo A-II expression in Tg rabbits led to mild elevation of plasma lipids and low HDL-C attributable to inhibition of lipoprotein lipase activity. In spite of this, under similar atherogenic hypercholesterolemia, overall effects of human apo A-II are atheroprotective as shown in the current study. Several mechanisms may be operative underlying the antiatherogenic functions of apo A-II in Tg rabbits. The first possible mechanism for apo A-II antiatherogenicity may be attributed to its potent anti-inflammatory activity. This contention is supported by the fact that Tg rabbits exhibited lower levels of inflammatory marker, C-reactive protein, and blood leukocytes (both neutrophils and monocytes) along with high paraoxonase/arylesterase 1 activity compared with non-Tg rabbits. Furthermore, Tg HDLs showed stronger suppressive activity on inflammatory cytokine expression of macrophages in vitro than did non-Tg HDLs. This finding is in contrast to a report that mouse apo A-II–rich HDLs of Tg mice could potentially be proinflammatory. We speculate that such a difference may be caused by different apo A-IIs expressed in different animals (ie, human dimer apo A-II in Tg rabbits versus murine monomer apo A-II in Tg mice). In support of our observations, Yamashita et al recently reported that plasma apo A-II is a potent anti-inflammatory bioactive protein because the administration of apo A-II led to the reduction of leukocyte infiltration and production of T cell–related cytokines in Con A–induced hepatitis in mice. Because Tg-HDLs are rich in phospholipids, it is necessary to investigate whether apo A-II also affects lipopolysaccharide–binding activity of HDL in future.

Figure 5. A. Analysis of cholesterol efflux capacity of high-density lipoproteins (HDLs). Apolipoprotein (Apo) A-I and apo A-II contents were analyzed by SDS-PAGE and stained with Coomassie brilliant blue (top). Each lane represents 1 sample from 1 rabbit. [H] acetylated–low-density lipoprotein (LDL)–loaded human THP-1 macrophages were incubated with different doses of HDLs for 24 hours and data are expressed as percent cholesterol effluxed (bottom). n=3 for each group. **P<0.01, ***P<0.001 vs non-transgenic (Tg). B, ATP-binding cassette transporter A-1 (ABCA-1) is required for apo A-I– and apo A-II–mediated cholesterol efflux. Cholesterol efflux assay was performed using baby hamster kidney (BHK) cells transfected with either mock or ABCA-1 vectors as described. ABCA-1 is essential for both apo A-I– and apo A-II–mediated cholesterol efflux (left). In the presence of ABCA-1 inhibitors cyclosporin A (CsA), EGTA, and tacrolimus (FK506), both apo A-I– and apo A-II–mediated cholesterol efflux activity in ABCA-1–transfected BHK cells was inhibited (right). Representative data of 3 independent experiments are shown.
Secondly, we found that HDLs isolated from Tg rabbits show increased cholesterol efflux capacity from macrophages compared with HDLs from non-Tg rabbits in vitro, suggesting that enrichment of apo A-II in HDL particles favors cholesterol efflux and thus inhibits foam cell formation in Tg rabbits. We also compared human lipid-free apo A-II with apo A-I with regard to cholesterol efflux activity and found that apo A-II is actually as efficient as apo A-I. Furthermore, functional ABCA-1 is required for lipid-free apo A-II-mediated cholesterol efflux, identical to apo A-I. Taken together, these data indicate that lipid-free apo A-II functions similarly to apo A-I in terms of cholesterol efflux via an ABCA-1–mediated mechanism. Moreover, there is a synergistic effect in HDL particles for cholesterol efflux when both apo A-I and apo A-II are present as shown in Tg HDLs. This finding supports the results showing that the plasma of human apo A-II Tg mice exhibited greater cholesterol efflux from J774 macrophages. It has been controversial whether enrichment or replacement of apo A-I with apo A-II impairs HDL-C efflux functions. Huang et al purified α-migrating HDLs containing both apo A-I and apo A-II (LpA-I/A-II) from human plasma by immunosubtracting 2-dimensional-PAGE and showed that LpA-I/A-II particles are less efficient for cholesterol efflux than LpA-I particles in fibroblasts, whereas other studies failed to demonstrate differences in the ability of LpA-I and LpA-I/A-II to remove cholesterol from hepatoma cells, fibroblasts, smooth muscle cells, and bovine endothelial cells. In addition, enrichment of apo A-II in Tg mouse HDLs did not affect the ability for cholesterol efflux from human macrophages compared with that in HDLs of wild-type mice. It has been reported that SR-B1 removes cholesterol esters from LpA-I/A-II particles more effectively than from LpA-I particles.

Because a small amount of apo A-II was also contained in apo-B–containing particles of Tg rabbits, it is likely that apo A-II affects their susceptibility to oxidation. We found that β-VLDL (the major atherogenic lipoprotein in cholesterol-fed animals) isolated from Tg rabbits showed less susceptibility to copper-induced oxidation than β-VLDL of non-Tg rabbits, which serves as another molecular mechanism for the atheroprotection shown in human apo A-II Tg rabbits. Of note, a particularly interesting finding of the current study is the first demonstration of apo A-II immunoreactive proteins in the lesions of both Tg rabbit and human atherosclerosis. In the lesions, apo A-II is mainly present extracellularly in intimate association with macrophages. It has been reported that apo A-II exerts an important role in HDL binding and selective lipid uptake through SR-B1 and CD36 receptors. Nevertheless, how apo A-II enters the arterial intima and what pathophysiological roles apo A-II plays in the lesions deserve further investigation. In the future study, it is necessary to clarify whether apoA-II exerts antiatherogenic effects in hepatic lipase Tg rabbits because normal rabbits are considered having low hepatic lipase activity. In conclusion, our studies provide evidence that human apo A-II, the second major apo of HDLs, has a potent antiatherogenic function through several mechanisms. Enrichment of apo A-II in HDLs promotes cholesterol efflux from the cells, and inhibits inflammation and oxidation. It remains to be verified, however, whether apo A-II may become a novel therapeutic target for the treatment of atherosclerosis like apo A-I mimetic peptides.

### Sources of Funding
This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (71790514, 19390099, 21659078, and 22390068 to J. Fan), National Institutes of Health (HL088391, HL068878, HL105114 to Y.E. Chen), a research grant for cardiovascular disease from the Ministry of Health, Labor and Welfare of Japan, and a research grant from AstraZeneca (J. Fan). Y.E. Chen is an established investigator of the American Heart Association (0840025 N).


Human Apolipoprotein A-II Protects Against Diet-Induced Atherosclerosis in Transgenic Rabbits

Yao Wang, Manabu Niimi, Kazutoshi Nishijima, Ahmed Bilal Waqar, Ying Yu, Tomonari Koike, Shuji Kitajima, Enqi Liu, Tomohiro Inoue, Masayuki Kohashi, Yuka Keyamura, Tomohiro Yoshikawa, Jifeng Zhang, Loretta Ma, Xiaohui Zha, Teruo Watanabe, Yujiro Asada, Eugene Y. Chen and Jianglin Fan

Arterioscler Thromb Vasc Biol. published online December 13, 2012; Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231 Copyright © 2012 American Heart Association, Inc. All rights reserved. Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2012/12/13/ATVBAHA.112.300445

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/12/13/ATVBAHA.112.300445.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Material

Supplemental Methods

Analysis of lipoproteins using high-performance liquid chromatography (HPLC)
Rabbit plasma isolated at 15 weeks were analyzed using HPLC according to the method by Okazaki\textsuperscript{1} at Skylight Biotech Inc.(Akita, Japan). In brief, 4 μl whole plasma was separated by gel filtration chromatography and the eluent was continuously monitored at 550 or 600 nm after an online enzymatic reaction. Total cholesterol, free cholesterol and phospholipids in each fraction were measured. Cholesteryl ester was determined by subtracting the value of free cholesterol from total cholesterol. In addition, HDL size was measured.

Analysis of plasma apo A-I and apo A-II, and CETP activity
Rabbit apo A-I and human apo A-II were analyzed by Western blotting and immunonephelometry (Apo A-I auto and Apo A-II auto, Daiichi, Sekisui Medical Co, Tokyo, Japan) as described previously\textsuperscript{2}. Because anti-human apo A-I antibody can only partially react with rabbit apo A-I, the values obtained by this method are lower than actual values so only used for comparison between non-Tg and Tg rabbits and expressed as arbitrary units (a.u.). Liver expression of apo A-I was analyzed by real-time RT-PCR and primers were listed in Supplemental Table I. CETP activity was measured using the method described previously\textsuperscript{2}.

Analysis of serum paraoxonase 1 (PON1) activity and hepatic mRNA expression
PON1 is an enzyme mainly present in HDL particles that are involved in anti-oxidation of LDLs. For the measurement of PON1 activity in serum, paraoxon and phenyl acetate were used as substrates for evaluating paraoxonase and arylesterase activity (Relassay, Gaziantep, Turkey)\textsuperscript{3} whereas gamma-thiobutyrolactone was used for measuring homocysteine-thiolactonase (HTLase) activity (Alfresa Pharma Corporation, Osaka, Japan)\textsuperscript{4}. Hepatic expression of PON1 was analyzed using real-time RT-PCR method and primers were listed in Supplemental Table I.
Cholesterol efflux assay

To analyze whether apo A-II affects cholesterol efflux capacity of HDLs, we compared Tg-HDLs with non-Tg HDLs for their cholesterol efflux capacity *in vitro* using a method reported previously. We isolated HDL$_2$ (d=1.08~1.10 g/ml) and HDL$_3$ (d=1.10~1.121 g/ml) from cholesterol-fed Tg and non-Tg rabbits by density gradient ultracentrifugation. HDLs were dialyzed against 0.15 M NaCl and 0.01% EDTA overnight and sterilized using a Millipore filter (0.22 μl). At the same time, we used apo A-II and apo A-I purified from human plasma using delipidation and a gel permeation chromatographic technique (Sigma-Aldrich, St. Louis, MO). Human monocytic cells (THP-1) were obtained from ATCC and maintained in 1640 RPMI medium supplemented with 10% fetal bovine serum. These cells were induced to differentiate into macrophages using 50 ng/ml phorbol 12-myristate 13-acetate for 3 days. Then, cells were incubated with [$^3$H]cholesterol-containing acetylated-LDL for 24 h to load the macrophages with cholesterol. Equilibrated cholesterol-enriched cells were washed with PBS twice and incubated with 0.3 ml of 1640 RPMI with 0.5% bovine serum albumin containing different concentrations of HDLs, apo A-I, or apo A-II. After 24 h, the medium was removed and were counted. Data are expressed as percent cholesterol effluxed. To investigate whether ATP-binding cassette transporter A-1 (ABCA-1) was involved in the cholesterol efflux mediated by apo A-II, we performed the cholesterol efflux assay using baby hamster kidney (BHK) cells stably transfected with a mifepristone-inducible vector containing an insert encoding ABCA-1 or without insert (mock as a control).
Supplemental Tables

Supplemental Table I. Primers used for Real-Time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td></td>
</tr>
<tr>
<td>PON1</td>
<td>Forward: ATTTGGGATTAGCGTGCTATTTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTGTCACTTTGAGGCTCGTGGATAA</td>
</tr>
<tr>
<td>apoA-I</td>
<td>Forward: CTTCTGGACGAATTCCAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTCAGCTTCCTCGAGGTCCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: ATCACTGCCACCCAGAAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTGAGTTTCCCGTTCAGCTC</td>
</tr>
<tr>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>apoA-II</td>
<td>Forward: CACAGACACCAAGGACAGAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCACACATGGCTCCTTTG</td>
</tr>
<tr>
<td>TNF-a</td>
<td>Forward: ACAACATGACACTGAAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGAAGGAGCTAGAAGAAAGC</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward: AGCCGAGCTATGAACTCCTTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCGGCTACATCTTGGGAATC</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward: AGCAAGTGTCGGAAGAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAGTTGCGGTTGCTGTCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: CAATGACCCCTCTAGGACCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCATCGCCCACTTGGATT</td>
</tr>
</tbody>
</table>

Supplemental Table II. Antibodies used for the current study

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Working dilution</th>
<th>Species</th>
<th>Manufactures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Immunohistochemical staining</td>
</tr>
<tr>
<td>RAM11</td>
<td>x400</td>
<td>Mouse</td>
<td>DakoCytomation, Carpinteria, CA</td>
</tr>
<tr>
<td>HHF35</td>
<td>x300</td>
<td>Mouse</td>
<td>Dako Corporation, Carpinteria, CA</td>
</tr>
<tr>
<td>KP1</td>
<td>x100</td>
<td>Mouse</td>
<td>DakoCytomation Denmark A/S, Glostrup, Denmark</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>x1200</td>
<td>Goat</td>
<td>Rockland Inc., Gilbertsville, PA</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>x100</td>
<td>Sheep</td>
<td>AbD Serotec, Kidlington, UK</td>
</tr>
<tr>
<td>ApoB</td>
<td>x1000</td>
<td>Goat</td>
<td>Rockland Inc., Gilbertsville, PA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Western Blotting</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>x4000</td>
<td>Goat</td>
<td>Rockland Inc., Gilbertsville, PA</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>x2500</td>
<td>Sheep</td>
<td>AbD Serotec, Kidlington, UK</td>
</tr>
<tr>
<td>GAPDH</td>
<td>x3000</td>
<td>Mouse</td>
<td>Imgenex Corporation, San Diego, CA</td>
</tr>
</tbody>
</table>

KP1, human CD68 macrophage; RAM11, rabbit monocyte/macrophage; HHF35, smooth muscle α-actin
Supplemental Figures

Supplemental Figure I. Plasma lipids and lipoproteins

Supplemental Figure I-A. Plasma TC, TG, and HDL-C levels of male (left) and female (right) rabbits are shown. Data are expressed as mean ± SD. N= 7 and 13 for male Tg and non-Tg, and 8 and 10 for female Tg and non-Tg. *P<0.05, **P<0.01 vs. non-Tg.
Supplemental Figure I-B. Plasma apo A-I and apo A-II were analyzed by Western blotting (top) and quantified by turbidimetric immunoassay (middle). Apo A-I concentrations are expressed as arbitrary units (a.u.) because anti-human apo A-I Ab can only partially cross-react with rabbit apo A-I. Data are expressed as mean ± SD.*P<0.05, vs. non-Tg. N= 6 ~14 from each group containing both males and females. Hepatic expression of apo A-I mRNA was analyzed by real-time RT-PCR (bottom left). N=4 for each group. CETP activity was measured by CETP activity assay kit (BioVision) (bottom right) and data are expressed as mean ± SD. N=8 for each group.
Supplemental Figure I-C. Agarose gel electrophoresis of whole plasma lipoproteins. Whole plasma (2 μl) from representative non-Tg and Tg rabbits was resolved by 1% agarose gel electrophoresis and stained for neutral lipids with Fat Red 7B. The electrophoretic mobilities of lipoprotein classes are indicated by arrows. Decreased α-migrating lipoproteins and increased pre-β-migrating lipoproteins were seen in Tg rabbit plasma.

Supplemental Figure I-D. Analysis of lipoprotein density fractions. Plasma lipoproteins from a non-Tg and a Tg rabbit were separated by sequential density ultracentrifugation using the density ranges shown above the gels. Equal volumes of each fraction (8 μL) were resolved by electrophoresis in a 1% agarose gel. Lipoproteins were visualized using Fat Red 7B staining (left). The quantification of TC and TG contents in lipoproteins is shown on the right. N= 6 and 8 for male Tg and non-Tg, and 4 and 9 for female Tg and non-Tg.
Supplemental Figure I-E. HPLC analysis of lipoprotein CE, FC and PL. Chylomicrons (CM), VLDLs, LDLs and HDLs were divided into subclasses according to their diameter size range. Data are expressed as mean ± SD. *P<0.05 or **P<0.01, vs. non-Tg. N= 4 from each group.
Supplemental Figure I-F. Analysis of apolipoproteins in lipoprotein density fractions. Equal amounts of each fraction (5 μL) were resolved by electrophoresis by 4-20% SDS-PAGE. Apolipoproteins were visualized using Coomassie brilliant blue staining. A large mass above apoE in HDL₃ is albumin.
Supplemental Figure II. Negative control immunohistochemical staining of apo A-II in atherosclerotic lesions.

Supplemental Figure II. Aortic lesions of Tg were immunohistochemically stained with the primary Ab goat anti-human apo A-II, or non-specific goat IgG or without adding the primary Ab. Aortic lesions of non-Tg were immunohistochemically stained with the primary Ab goat anti-human apo A-II.
Supplemental Figure III. Analysis of cholesterol efflux capacity of lipid-free apo A-I and apo A-II

Supplemental Figure III. Purified human apo A-I (three different preparations) and apo A-II (two different preparations) were used for determination of cholesterol efflux capacity as for HDLs. Experiments were performed using [$^3$H] acetylated-LDL-loaded human THP-1 macrophages.

Supplemental Figure IV. Demonstration of apo A-II in Tg β-VLDL by Western blotting.

Supplemental Figure IV. Human plasma was used as a positive control.
References


