due to decreased catabolism of larger HDL particles, suggesting that the size of HDL may modulate the selective HDL-C uptake by the liver (10). In human-LCAT transgenic mice, the liver uptake of HDL was reduced by 41%, resulting in a substantial increase of large HDL particles that might be atherogenic (11) due to the fact that mice lack cholesteryl ester transfer protein (CETP) and that continued increase of cholesteryl ester in HDL by high levels of LCAT changes both the size and lipid composition of HDL. When CETP was coexpressed in LCAT transgenic mice, HDL size and composition changed and the animals were protected from atherosclerosis (12). These data suggest that under normal conditions in which CETP is present as in humans, increased LCAT activity is likely to increase HDL cholesterol and size and might reduce the risk for atherosclerosis. Our previous data suggest that the two largest, spherical, cholesteryl ester-rich HDL particles, α-1 and α-2, are good substrates for SR-BI in a human hepatoma cell line (13).

Our aim was to gain insight into the role that LCAT plays in HDL metabolism as well as to better understand LCAT deficiency states. We have examined apoA-I-, -A-II-, -A-IV-, -C-I-, -C-III-, and -E-containing HDL subpopulation profiles in LCAT-deficient homozygotes and heterozygotes and in control subjects. The data we present indicate that LCAT plays a very significant role in HDL particle metabolism, composition, and remodeling.

MATERIALS AND METHODS

Subjects

We examined plasma obtained from 11 homozygous LCAT-deficient subjects of Italian (n = 7), Japanese (n = 3), and US (n = 1) origin, as well as from 11 heterozygous LCAT-deficient subjects from Italy. Plasma obtained from gender-matched control subjects from the US (n = 15), Italy (n = 4), and Japan (n = 3) was used in this comparison. Homozygous and heterozygous subjects from Italy have been described previously (14). All homozygous subjects had primary hypoalphalipoproteinemia as defined by a plasma HDL-C level below the 5th percentile for the age- and gender-matched general populations of the specific countries. One homozygous subject from Japan had FED; however, none of the measured parameters of this subject were different by more than 1 SD from those of the other 10 homozygotes.

Sample handling and measurements

Blood was collected from all subjects after an overnight fast and immediately placed on ice. Plasma was separated by low-speed centrifugation at 4°C and was stored at −°80 C until use. Samples were sent to the Lipid Metabolism Laboratory at Tufts University on dry ice and were thawed in a 37°C water bath for 1–2 min and then placed on ice just before use. Plasma total cholesterol, HDL-C, and triglyceride (TG) levels were determined using standard enzymatic techniques. Plasma concentrations of apoA-I, -A-II, and -B were determined by immunoturbidimetry. Plasma concentrations of apoA-IV, -C-I, -C-III, and -E were estimated by dot-blot analyses and expressed as arbitrary units. LCAT gene analyses, activity, and mass measurements were performed as described previously (14). HDL subpopulations were determined by nondenaturing two-dimensional PAGE, immunoblotting, and image analysis as described previously (15). Four microliters of plasma was applied and electrophoresed on a vertical-slab agarose gel (0.7%) in the first dimension at 250 V until the α-mobility front moved 3.5 cm from the origin. The agarose gel was sliced, and the strips were applied onto 3–35% nondenaturing concave gradient polyacrylamide gels. In the second dimension, gels were electrophoresed to completion at 250 V for 24 h at 10°C, followed by electrotransfer to nitrocellulose membranes at 30 V for 24 h at 10°C. The specific apolipoproteins were immuno-localized on the membrane with mono-specific goat anti-human primary and ^{125}I-labeled secondary antibodies [immunopurified rabbit F(ab')$_2$ fraction against goat IgG]. The bound ^{125}I-labeled secondary antibody was quantified in a FluorImager (Molecular Dynamics). Each membrane was first probed for the apolipoprotein of primary interest and than reprobed for apoA-I for reference.

Data analysis

Means and standard deviations were calculated for all study groups. Data obtained from homozygotes and heterozygotes were compared with data from controls using ANOVA analyses. A two-tailed $P < 0.05$ was considered as significant.

RESULTS

Table 1 shows data on LCAT mass and activity as well as on lipids and apolipoproteins in controls (n = 22), heterozygotes (n = 11), and homozygotes (n = 11) for LCAT deficiency. Heterozygotes had 39% of the LCAT activity and 87% of the LCAT mass of controls. They had lower apoA-I (−22%), apoA-II (−19%), HDL-C (−15%), and

TABLE 1. Characteristics of study participants

	Controls (n = 22)	Heterozygotes (n = 11)	Homozygotes (n = 11)
Male/female	17/5	7/4	10/1
LCAT mass (μg/ml)	4.60 ± 1.01	4.02 ± 1.07	1.04 ± 0.96*
LCAT activity (nmol/ml/h)	33.0 ± 18.1	20.21 ± 1.6*	0.44 ± 0.66*
Total cholesterol (mg/dl)	200 ± 38	171 ± 37*	112 ± 63*
LDL-C (mg/dl)	126 ± 33	99 ± 33*	65 ± 54*
HDL-C (mg/dl)	54 ± 13	46 ± 12*	9 ± 5*
TG (mg/dl)	137 ± 88	127 ± 45	203 ± 146*
apoA-I (mg/dl)	140 ± 25	109 ± 17*	34 ± 11*
apoA-II (mg/dl)	38 ± 4	31 ± 5	11 ± 6*
apoB (mg/dl)	96 ± 16	97 ± 25	31 ± 17*

ApoA-I, apolipoprotein A-I; HDL-C, HDL cholesterol; TG, triglyceride. Data are mean ± SD.
* Significantly different ($P < 0.05$) from controls.

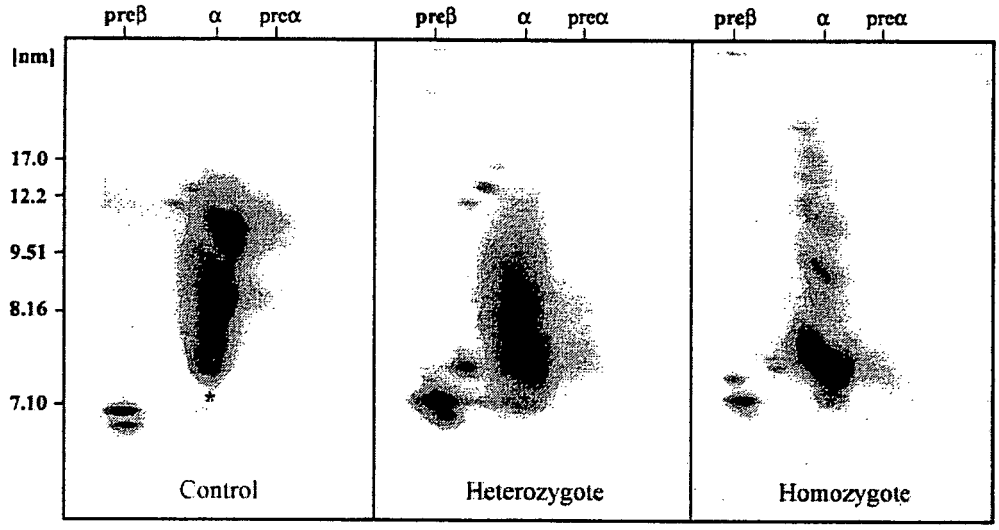

Fig. 1. Apolipoprotein A-I (apoA-I)-containing HDL subpopulations of representative control, heterozygous, and homozygous LCAT-deficient subjects separated by two-dimensional, nondenaturing agarose-PAGE. The asterisk represents the endogenous human serum albumin marking the α-mobility front.

TG (−7%) values compared with controls. Homozygotes had about 1% of the LCAT activity and about 23% of the LCAT mass of controls. They had significantly lower HDL-C (−83%), apoA-I (−76%), apoA-II (−71%), apoB (−68%), and LDL-C (−49%), and 48% higher TG values than controls.

Figure 1 and **Table 2** summarize data on apoA-I-containing HDL subpopulations in controls and in heterozygous and homozygous LCAT-deficient subjects. Heterozygotes had one extra particle in the preβ-1 region (preβ-1×); however, all of their other apoA-I-containing HDL subpopulations were comparable to controls in electrophoretic mobility and size. ApoA-I distribution in heterozygotes was shifted toward the smaller HDL particles: there was a 2-fold increase in preβ-1 level, a 23% increase in α-4, and a 45% increase in preα-4 levels compared with controls. There were significant decreases in the concentrations of all the other HDL particles, whereas the mean concentration of α-3, an intermediate-sized particle, was similar to that of controls. In homozygotes, the majority of apoA-I was detected in small, lipid-poor, disc-shaped HDL particles (preβ-1 and α-4). Despite the low plasma concentrations of apoA-I in homozygous subjects, the apoA-I concentrations of these particles were comparable to those of controls. We have also observed larger (~8 nm–20 nm) apoA-I-containing HDL particles with α-mobility in many of the homozygotes. **Figure 2** represents the distribution of apoA-II-containing particles—superimposed on apoA-I-containing particles—in representative control, heterozygous, and homozygous LCAT-deficient subjects. In control subjects, α-2 and α-3 HDL contain apoA-I and apoA-II. In heterozygotes, some apoA-II was detected in the preβ-1 region but the majority of apoA-II was distributed in the α-2 and α-3 subpopulations, with a slight shift toward the smaller α-3 particles, compared with controls. In contrast to controls, homozygotes had a very low level of apoA-II, which was detected in a small, lipid-poor particle, comigrating with the regular LpA-I preβ-1 HDL particles. Total or partial LCAT deficiency had no significant effect on the concentration of apoA-IV or the distribution of apoA-IV-containing HDL particles (**Fig. 3**). There were no significant differences between heterozygotes and controls in apoC-I concentration and distribution (**Fig. 4**). In contrast, homozygotes had significantly lower apoC-I levels, and their apoC-I was found on the top of the gel with β-mobility, indicating that apoC-I was present solely in VLDL particles, not in α-mobility HDL particles, as in controls and heterozygotes. The concentrations and distribution of apoC-III were significantly different between LCAT-deficient subjects and controls (**Fig. 5**). In controls, the majority of apoC-III comigrated with apoA-I in α-1 and α-2 HDL, and some was also found

TABLE 2. Concentrations of HDL subpopulations as determined by apoA-I content

	Controls (n = 22)	Heterozygotes (n = 11)	Homozygotes (n = 11)
Preβ-1_x	Not detectable	0.7 ± 0.9*	1.6 ± 1.0*
Preβ-1_a	8.2 ± 3.2	14.6 ± 5.4*	7.9 ± 4.0
Preβ-1_b	4.1 ± 1.6	10.3 ± 8.1*	1.8 ± 1.1*
Preβ-2_a	0.7 ± 0.4	0.3 ± 0.2*	Not detectable
Preβ-2_b	1.0 ± 0.5	0.5 ± 0.3*	Not detectable
Preβ-2_c	0.5 ± 0.3	0.2 ± 0.2*	Not detectable
α-1	16.7 ± 8.9	11.0 ± 8.6*	
α-2	39.1 ± 9.6	25.3 ± 6.7*	11.6 ± 2.4
α-3	24.3 ± 5.6	23.2 ± 5.5	
α-4	13.4 ± 3.6	16.5 ± 3.5*	12.1 ± 7.0
Preα-1	5.2 ± 3.3	0.9 ± 0.9*	
Preα-2	6.2 ± 2.4	1.9 ± 1.1*	0.6 ± 0.4
Preα-3	3.4 ± 1.4	2.0 ± 1.0*	
Preα-4	1.1 ± 0.4	1.6 ± 0.8*	0.6 ± 1.0

Data are mean (mg/dl) ± SD.
* Significantly different ($P < 0.05$) from control.

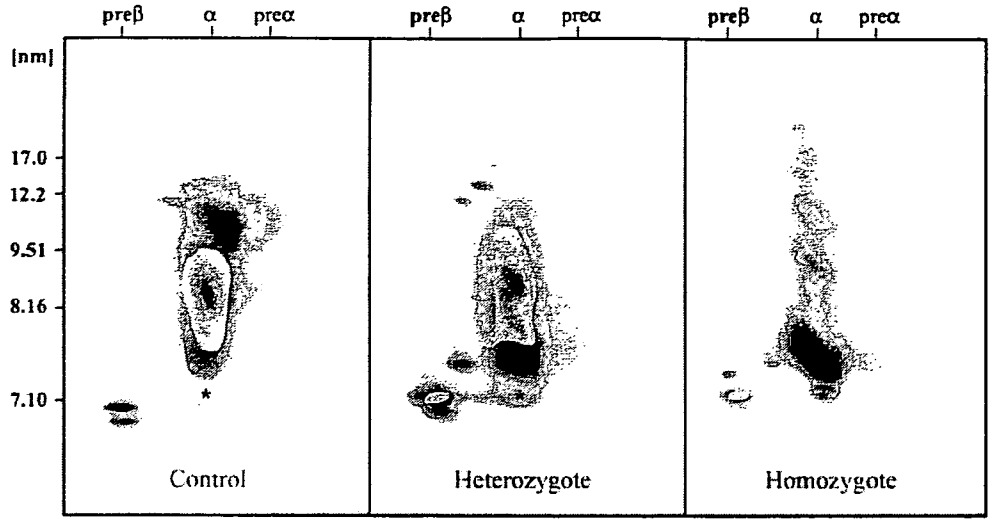

Fig. 2. ApoA-II-containing HDL subpopulations of representative control, heterozygous, and homozygous LCAT-deficient subjects superimposed on the image of apoA-I-containing subpopulations. LCAT-deficient subjects have apoA-II in small, preβ-migrating HDL particles. The asterisk represents the endogenous human serum albumin marking the α-mobility front.

in the α-3 and α-4 size range, with no comigration with apoA-I. In contrast, practically all of the apoC-III was detected in small, lipid-poor HDL particles in homozygotes and heterozygotes. ApoE-containing particles migrated with β-preβ-mobility in the size range between 12 nm and VLDL size, with a median diameter of 16.5 nm in controls (**Fig. 6**) and no overlap with apoA-I-containing HDL particles. In heterozygotes, apoE was also found in large β-preβ-mobility particles, with no comigration with apoA-I. Interestingly, the size of apoE-containing particles was somewhat increased in heterozygotes compared with controls. Homozygotes had much less apoE than controls. ApoE concentrations in the larger particles decreased, and smaller apoE-containing particles appeared in the plasma of homozygotes.

DISCUSSION

The purpose of this study was to gain insight into the role that LCAT plays in HDL metabolism as well as to better understand LCAT deficiency states. Characteriz-

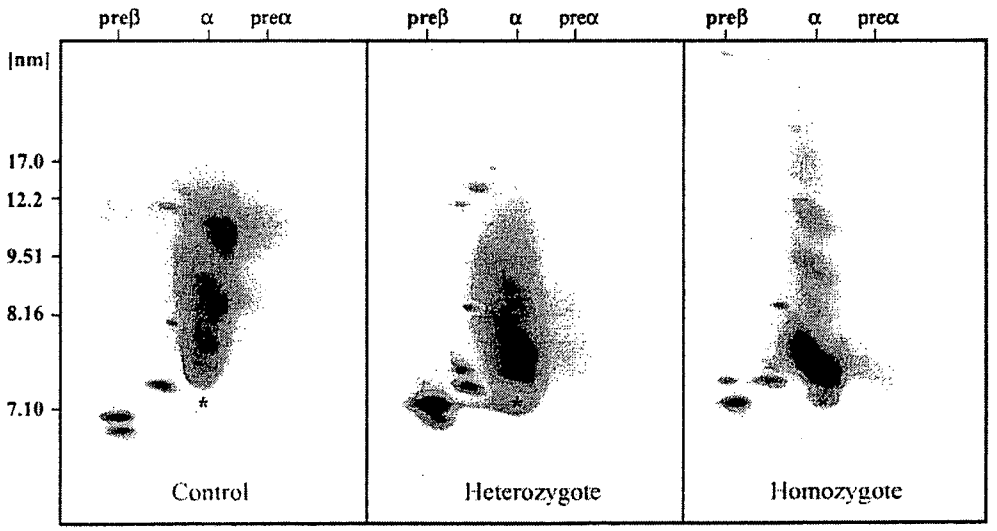

Fig. 3. ApoA-IV-containing HDL subpopulations of representative control, heterozygous, and homozygous LCAT-deficient subjects superimposed on the image of apoA-I-containing subpopulations. Total or partial LCAT deficiency has no significant effect on the distribution of apoA-IV-containing HDL particles. The asterisk represents the endogenous human serum albumin marking the α-mobility front.

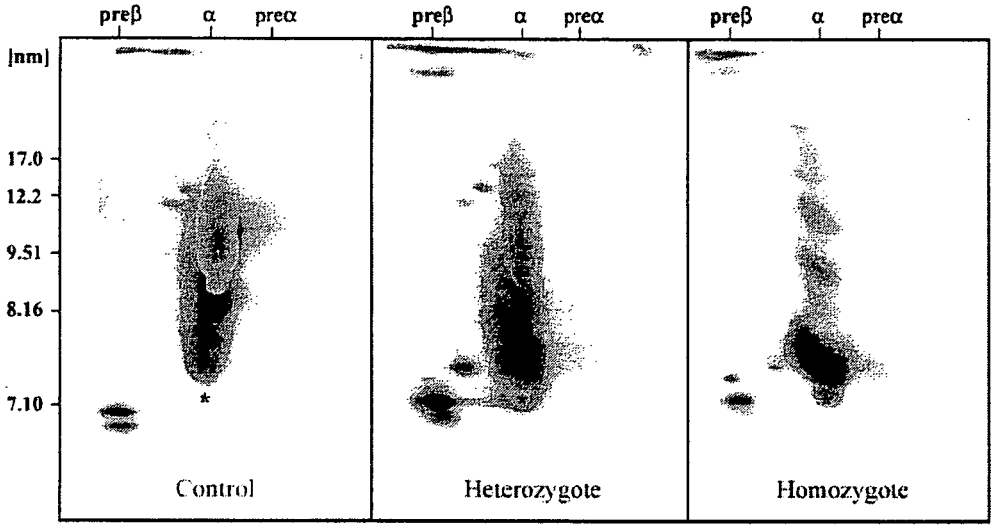

Fig. 4. ApoC-I-containing HDL subpopulations of representative control, heterozygous, and homozygous LCAT-deficient subjects superimposed on the image of apoA-I-containing subpopulations. In homozygotes, apoC-I has only been detected on the top of the gel with β-mobility (VLDL) in contrast to controls and heterozygotes. The asterisk represents the endogenous human serum albumin marking the α-mobility front.

ing HDL particles in patients with rare inborn errors of HDL metabolism has been helpful in better understanding HDL particle metabolism and reverse cholesterol transport. We have documented that Tangier disease patients had: *1*) apoA-I only in the preβ-1 HDL particles, *2*) no apoA-II-containing HDL, and *3*) decreased size of apoE HDL. ApoA-IV was not significantly influenced by the lack of ABCA1-mediated cellular cholesterol efflux (16). We have subsequently reported that HDL subpopulations in CETP-deficient homozygotes were very large, compositionally undifferentiated HDL particles (17). Therefore, CETP activity is essential for the formation of distinguished HDL particles in the normal size range of HDL. Most importantly, CETP activity is essential for the formation of discrete LpA-I, LpA-I:A-II, and LpE HDL particles.

In the present manuscript, we document the role of LCAT in HDL metabolism and remodeling in plasma. The first important observation is that LCAT activity is not

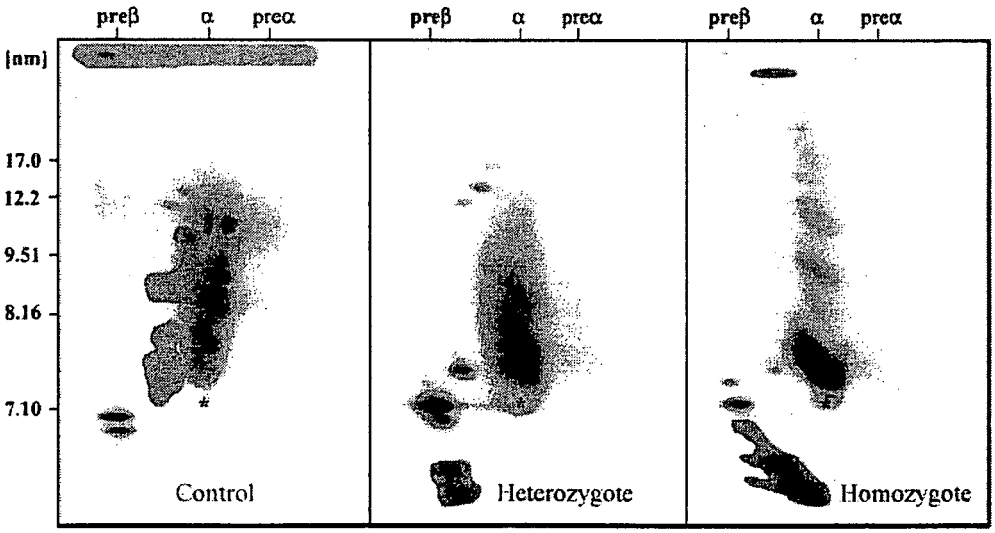

Fig. 5. ApoC-III-containing HDL subpopulations of representative control, heterozygous, and homozygous LCAT-deficient subjects superimposed on the image of apoA-I-containing subpopulations. In controls, the majority of apoC-III comigrates with apoA-I in α-1 and α-2 HDL, and some has also been found in the α-3 and α-4 size range with no comigration with apoA-I. In homozygotes and heterozygotes, practically all apoC-III has been detected in small, lipid-poor HDL particles. The asterisk represents the endogenous human serum albumin marking the α-mobility front.

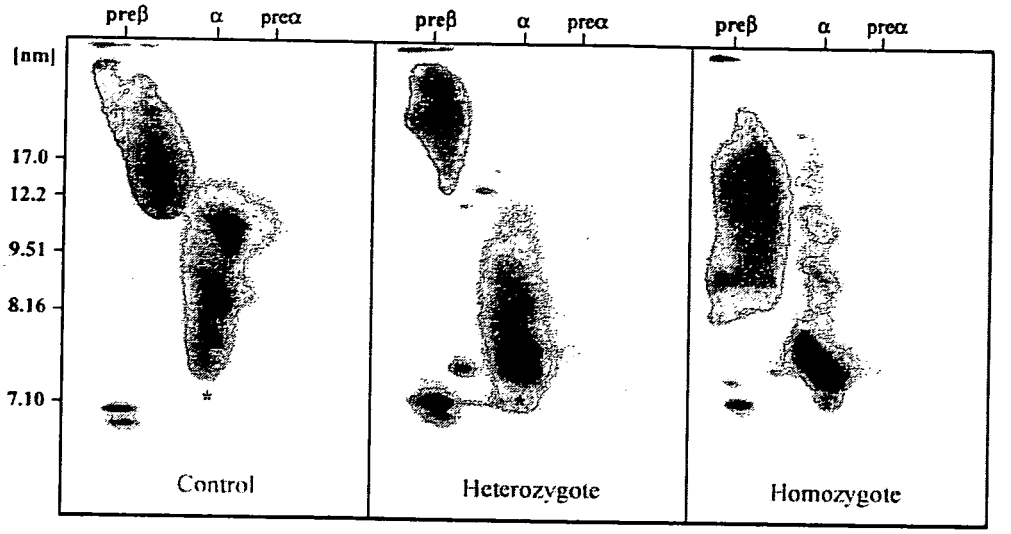

Fig. 6. ApoE-containing HDL subpopulations of representative control, heterozygous, and homozygous LCAT-deficient subjects superimposed on the image of apoA-I-containing subpopulations. There is no comigration of apoE- and apoA-I-containing particles. The asterisk represents the endogenous human serum albumin marking the α-mobility front.

necessary for the transformation of preβ-1 HDL into α-mobility HDL. Preβ-1 binds to ABCA1 and removes phospholipids and unesterified cholesterol from cells (13). During this process, there are probably changes in apoA-I conformation and electrophoretic charge. We hypothesize that α-4 HDL contains two molecules of apoA-I, as is the case for preβ-1 HDL. Larger (~8 nm–20 nm) α-mobility HDL particles have also been observed in many of the homozygotes. The tight bands of these particles suggest that these are poorly lipidated, discoidal HDL aggregates. We have no data indicating whether LCAT can act on these large, stacked disks or can use only the small α-4 HDL as a substrate. The apoA-I-containing HDL subpopulation profile of heterozygotes resembles that of low HDL-C CAD patients, inasmuch as apoA-I distribution is shifted toward the smaller particles. ApoA-II is dramatically reduced in homozygous subjects, probably because of fast catabolism (18), and, interestingly, it comigrates with preβ-1 HDLs, which normally contain only apoA-I. Some apoA-II also comigrates with preβ-1 HDL in heterozygotes; however, we do not know whether apoA-I and apoA-II are in the same particles. As a result of the presence of cholesteryl ester in the core of HDL particles, apoA-II binds to α-2 and α-3 HDL particles very early, as indicated in heterozygotes whose apoA-I/apoA-II ratios are increased in these particles. Our data also suggest that LCAT is not a key player in the formation of apoA-IV-containing particles. On the basis of these and other findings (16, 17), we hypothesize that the metabolism of apoA-IV-containing particles is independent of ABCA-1-mediated cellular cholesterol efflux, as well as of CETP and LCAT activities in humans. The majority of apoC-I comigrates with apoA-I-containing α-1 HDL in controls. About 20% of apoC-I in controls and ~35% of apoC-I in heterozygotes have α-mobility with larger than α-1 size. Homozygotes have apoC-I only in the VLDL fraction, indicating that the neutral lipid core is essential for the incorporation of apoC-I into HDL. ApoC-III has a complex pattern in controls: ~25% of apoC-III comigrates with α-1, ~50% comigrates with α-2, ~15% is found in VLDL, and the rest is in the HDL size range but does not overlap with apoA-I-containing particles. Interestingly, in both affected groups, apoC-III has been detected in small, lipid-poor form (free apoC-III), indicating that apoC-III is probably sensitive to the lipid and apolipoprotein composition of HDL. The large amount of free apoC-III in affected subjects also indicates that the fractional catabolic rate of this apolipoprotein is not increased with decreased particle size, which is clearly not the case for apoA-I and apoA-II. We clearly demonstrate that apoE-containing particles do not overlap with apoA-I-containing particles either in controls or in LCAT-deficient subjects in this study. [We have only seen apoE comigrating with apoA-I in homozygous CETP-deficient subjects where HDL size reached the size of LDL and the particles were probably loaded with excess amounts of cholesteryl ester (17)]. Although apoA-I concentrations were significantly lower in the large particles in heterozygous LCAT-deficient subjects, apoE concentrations were significantly increased in the large apoE HDL particles in these subjects. We have no explanation for this phenomenon. We do not know the chemical composition of these particles. In homozygous LCAT-deficient subjects, we observed only slightly more apoE in apparently lipid-poor particles. Therefore, LCAT activity does not seem to be a key player in supplying neutral lipids for apoE-containing HDL. Alternatively, TGs seem to be sufficient for the formation of the core of apoE HDL in homozygotes. If this is true, the questions arise as to how this TG-rich apoE HDL is metabolized and what its role in lipoprotein metabolism and CAD risk is.

Our current concept of HDL remodeling in vivo in humans, presented in **Fig. 7**, is based on data generated in various genetic states associated with alterations in HDL metabolism (ABCA1, LCAT, and CETP deficiency). We are in the process of examining HDL subpopulations in other genetic disorders as well [apoA-I deficiency, apoE deficiency, abetalipoproteinemia, lipoprotein lipase (LPL) deficiency, and hepatic lipase (HL) deficiency]. On the basis of our observations, we describe the following steps in HDL metabolism.

Step 1: synthesis

ApoA-I is synthesized in the liver and small intestine, and two molecules of apoA-I form a belted structure around ~16 molecules of phospholipids to form discoidal preβ-1 HDL in the interstitial or plasma compartment.

Step 2: cellular cholesterol efflux

Preβ-1 particles pick up FC and more phospholipids from cells via the ABCA1 pathway and are transformed into small, lipid-poor, discoidal LpA-I α-4 HDL particles.

Step 3: cholesterol esterification

LCAT esterifies FC on the surface of HDL into cholesteryl esters, which move into the core with an increase in HDL particle size.

Step 4: TG hydrolysis

LPL hydrolyzes TGs in TG-rich lipoprotein (TRL), resulting in surface components (phospholipids, FC, and apolipoproteins) available and necessary for HDL particle size increase.

The concerted actions of ABCA1, LCAT, and LPL continuously increase HDL particle size (steps 2–4).

Step 5: cholesteryl ester exchange (CETP cycle)

With CETP-mediated exchange of core cholesteryl esters for TG between large HDL particles and TRL, differentiated α- and preα-migrating HDL particles form; these contain apoA-I with apoA-II, or apoA-I without apoA-II, or apoE only. CETP can also exchange cholesteryl esters for TG among HDL particles and, as a result, a substantial amount of preβ-1 forms.

Step 6: phospholipid hydrolysis

HL, endothelial lipase (EL), and secretory phospholipase A2 (sPLA2) hydrolyze TG and phospholipids on HDL, resulting in size reduction of large α-1 into α-2 HDL (HL), or disintegration of all larger HDL particles into α-4 and preβ-1 (EL), or preβ-1 and free apoA-I (sPLA2).

Step 7: hepatic cholesteryl ester uptake (SR-BI cycle)

Cholesteryl esters on α-2 and α-1 HDL particles are selectively transported from HDL particles to the liver via SR-BI for ultimate excretion of cholesterol into the bile, resulting in recycling of apolipoproteins, phospholipids, and FC from these larger HDL particles to small α-4 HDL.

The concerted actions of CETP, SR-BI, and lipases decrease HDL particle size (steps 5–7).

Step 8: apolipoprotein catabolism

Clearance of small, lipid-poor apoA-I particles: the final step in HDL particle metabolism is the uptake of whole HDL particles by the liver and cubulin/megalin-mediated clearance of free apoA-I and preβ-1 HDL in the kidney.

Based on the observations presented here, our data are consistent with the concept that LCAT plays a crucial role in the maturation of HDL particles (steps 2–4).

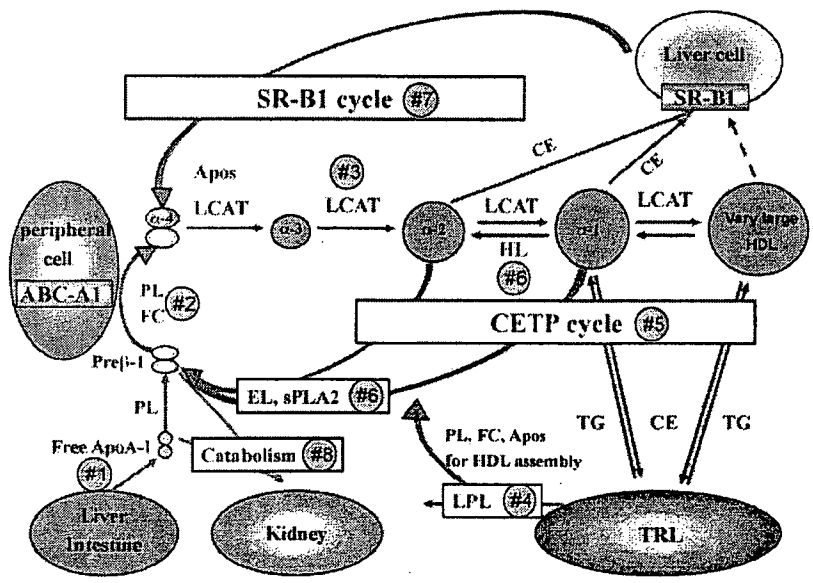

Fig. 7. Current concept of HDL remodeling in humans in vivo. Abbreviations not used in the text: phospholipids (PL), cholesteryl ester (CE).

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Adiponectin accelerates reverse cholesterol transport by increasing high density lipoprotein assembly in the liver

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Abstract

Plasma high density lipoprotein (HDL)–cholesterol levels are negatively correlated with the incidence of coronary artery disease. HDL plays an important role in protecting against atherosclerosis by removing cholesterol from atheroma and transporting it back to the liver. The ATP-binding cassette transporters (ABCA1 and ABCG1) and scavenger receptor BI (SR-BI) are thought to be one of the rate-limiting factors to generate HDL in the liver. Adiponectin (APN) secreted from adipocytes is also one of the important molecules to inhibit the development of atherosclerosis. Recently, it has been reported that plasma HDL–cholesterol levels are positively correlated with plasma APN concentrations in humans. Therefore, we investigated the association of APN with HDL assembly in the liver. Human hepatoma cell line, HepG2 cells, were incubated for 24 h in the culture medium with the indicated concentrations of recombinant APN. APN enhanced the mRNA level of apolipoprotein A-I (apoA-I) in HepG2 cells and increased the secretion of apoA-I from the cells to the medium. Furthermore, APN increased both mRNA and protein levels of ABCA1, but not ABCG1 and SR-BI, in HepG2 cells. Taken together, the current study demonstrates that APN might protect against atherosclerosis by increasing HDL assembly through enhancing ABCA1 pathway and apoA-1 synthesis in the liver.

Keywords: ABCA1; ABCG1; Adiponectin; Apolipoprotein A-I; Apolipoprotein B; HDL; Liver; Metabolic syndrome; Reverse cholesterol transport; SR-BI

Plasma high density lipoprotein (HDL)–cholesterol levels are negatively correlated with the incidence of coronary artery disease (CAD). HDL plays an important role in preventing the development of atherosclerosis. One of the major mechanisms by which HDL protects against atherosclerosis is postulated to be so-called reverse cholesterol transport (RCT) [1]. In RCT system, HDL is thought to remove excess cholesterol from atheroma and transport it back to the liver.

The ATP-binding cassette transporters (ABCA1 and ABCG1) and scavenger receptor BI (SR-BI) are thought

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to be rate-limiting factors to generate HDL [2–5]. ABCA1, the defective gene in Tangier disease [6–8], promotes cholesterol efflux to lipid-poor apolipoprotein A-I (apoA-I), decreasing cholesterol accumulation in macrophages and initiating HDL formation in the liver [2]. ABCG1 also stimulates cholesterol efflux from macrophages to mature HDL particles, but not to lipid-poor apoA-I [3]. SR-BI has two functions to accelerate the selective uptake of cholesteryl ester from HDL in the liver and remove cholesterol from macrophages to generate HDL [4,5].

Adiponectin (APN) secreted from adipocytes is one of the important molecules to inhibit the development of atherosclerosis. Recently, Ryo et al. reported that the subjects with hypoadiponectinemia had multiple risk factors for CAD, such as visceral fat obesity, hypertension, impaired glucose tolerance and dyslipidemia, including low HDL–cholesterol [9]. Furthermore, Kumada et al. showed that male patients with hypoadiponectinemia had an increase in CAD prevalence, independent of well-known CAD risk factors [10]. We and other groups have reported that plasma HDL–cholesterol levels are positively correlated with plasma APN concentrations in humans [9,11,12]. However, the underlying mechanism for the correlation between plasma HDL-cholesterol and APN levels has not been clarified yet. Therefore, we have tested a hypothesis that APN might be associated with HDL assembly in the liver to increase plasma HDL–cholesterol level *in vivo*. In the present study, we demonstrate, for the first time, that APN might protect against atherosclerosis by increasing HDL assembly through enhancing ABCA1 pathway and apoA-1 synthesis in the liver.

Methods

Cells. A human hepatoma cell line, HepG2 cells were cultured in 12-well cell culture plates (FALCON, Becton–Dickinson Labware, USA), according to the standard condition in Dulbecco's modified Eagle's medium (DMEM) containing 10% of fetal calf serum (FCS) in a humidified 5% CO_2 controlled incubator at 37 °C. For the assay, HepG2 cells were incubated for 24 h in DMEM containing 0.5% FCS with the indicated concentrations of recombinant human APN prepared as previously reported [13]. TO-901317 [liver X receptor (LXR) agonist, Sigma–Aldrich, USA] was added to the medium with a concentration of 3 μM to induce ABCA1 or ABCG1 in HepG2 cells.

Cell protein isolation and Western blot analysis. HepG2 cells were solubilized with 0.5% CHAPS [3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, Pierce Chemical, USA] and 1 mM EDTA in phosphate buffered saline (PBS). Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Germany). Incubations of antibodies with the membranes were performed in TBS including 0.1% Tween 20 and 2% skimmed milk at 4 °C overnight. Detection of the immune complexes was carried out by ECL Advance Western Blot Detection System (Amersham Biosciences, UK). Anti-human ABCA1 (Novus Biologicals, USA), anti-human ABCG1 antibody (Novus Biologicals), anti-human SR-BI antibody as previously used [14], anti-human apoA-I antibody (Academy Bio-Medical Company, Inc., USA), and anti-human apolipoprotein B (apoB) antibody (Santa Cruz, USA) were used for the assay.

RNA isolation, cDNA synthesis, and quantitative PCR. Total RNA from HepG2 cells was purified by using RNeasy Mini Kit (Qiagen, USA) followed by treatment with DNase I (Qiagen). One microgram of total RNA was primed with 50 pmol of oligo(dT) 20 and reverse-transcribed with SuperScript III (Invitrogen, USA) for first strand cDNA synthesis, according to the protocol of the manufacturer. Real-time quantitative PCR was performed according to the protocol of DyNamo HS SYBR Green quantitative PCR kit.

Primers used in this study. The primes for human ABCA1 were ABCA1-forward: 5′-GCACTGAGGAAGATGCTGAAA-3′ and AB CA1-reverse: 5′-AGTTCCTGGAAGGTCTTGTTCAC-3′, for human ABCG1, ABCG1-forward: 5′-GCCTACTGCAGACTCGTGTA-3′ and ABCG1-reverse: 5′-GTCGAAGCTGACGAAGAACC-3′, for human SR-BI, SR-BI-forward: 5′-CTGTGGGTGAGATCATGTGG-3′ and SR-BI-reverse: 5′-GCCAGAAGTCAACCTTGCTC-3′, for human apoA-I, apoA-I-forward: 5′-CTGGCCACTGTGTACGTGGATG-3′ and apoA-I-reverse: 5′-TGGCGGTAGAGCTCCATCTCCT-3′, for human apoB, apoB-forward: 5′-CTGGGAAAACTCCCACAGCAAG-3′ and apoB-reverse: 5′-CCACATTTTGAATCCAGGATGCAG-3′, for human GA

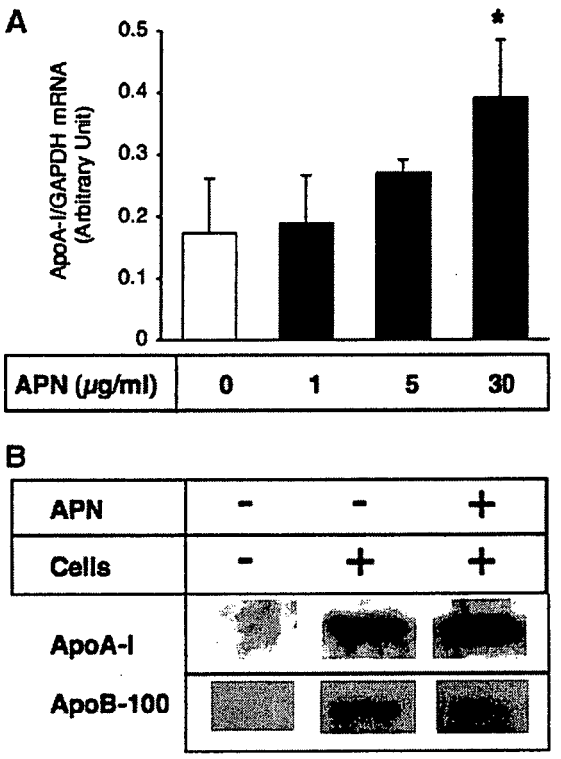

Fig. 1. APN increased the expression level of apoA-I in HepG2 cells and enhanced the secretion of apoA-I from the cells to the medium. HepG2 cells were incubated for 24 h in DMEM containing 0.5% FCS with the indicated concentrations (0–30 μg/ml in the medium) of recombinant human APN. The mRNA level of apoA-I in HepG2 cells was analyzed by real-time quantitative PCR. ApoA-I or apoB mass in the medium was determined by western blot. (A) APN increased the mRNA level of apoA-I in HepG2 cells. Values are mean ± SD of triplicate determinations. $^{*}P < 0.05$ vs 0 μg/ml of APN. (B) APN increased the secretion of apoA-I from HepG2 cells, while it reduced the release of apoB-100. A representative result of three independent experiments is shown. APN (−), the medium without APN; APN (+), the medium containing 30 μg/ml of APN; cells (−), the medium incubated without HepG2 cells; cells (+), the medium incubated with cells.

PDH, GAPDH-forward: 5′-GAGTCAACGGATTTGGTCGT-3′ and GAPDH-reverse: 5′-TTGATTTTGGAGGGATCTCG-3′.

Statistical analysis. Results were expressed as mean ± SD. Statistical significance was assessed by Student's *t* test for paired values and set at $P < 0.05$.

Results

APN increased the mRNA expression level of apoA-I in HepG2 cells and enhanced the secretion of apoA-I and apoB-100 from the cells to the medium

A human hepatoma cell line, HepG2 cells, were incubated for 24 h in DMEM including 0.5% FCS with the indicated concentrations of recombinant APN. The mRNA levels and the secretions of apolipoproteins (apoA-I and apoB-100) were analyzed by real-time quantitative PCR and western blot, respectively. APN increased the mRNA level of apoA-I in a dose dependent manner (Fig. 1A). Furthermore, APN (30 μg/ml in the medium) slightly increased the release of apoA-I from the cells to the medium (Fig. 1B). On the contrary, although the mRNA level of apoB-100 was not influenced (data not shown), APN reduced the release of apoB-100 from HepG2 cells (Fig. 1B).

APN enhanced the expression level of ABCA1, but not ABCG1 and SR-BI, in HepG2 cells

After incubation with the indicated concentrations of APN, the expression levels of ATP transporters (ABCA1 and ABCG1) and SR-BI in the HepG2 cells were analyzed by real-time quantitative PCR and Western blot. APN increased the mRNA level of ABCA1, but not ABCG1 and SR-BI, in a dose dependent manner (Fig. 2A). APN (30 μg/ml in the medium) enhanced the cellular ABCA1 protein level, however, the protein levels of ABCG1 and SR-BI were not influenced (Fig. 2B). These data suggest that APN might promote HDL assembly through enhancing ABCA1 expression in the liver.

Discussion

In the present study, we for the first time demonstrated that human APN increased the secretion of apoA-I from

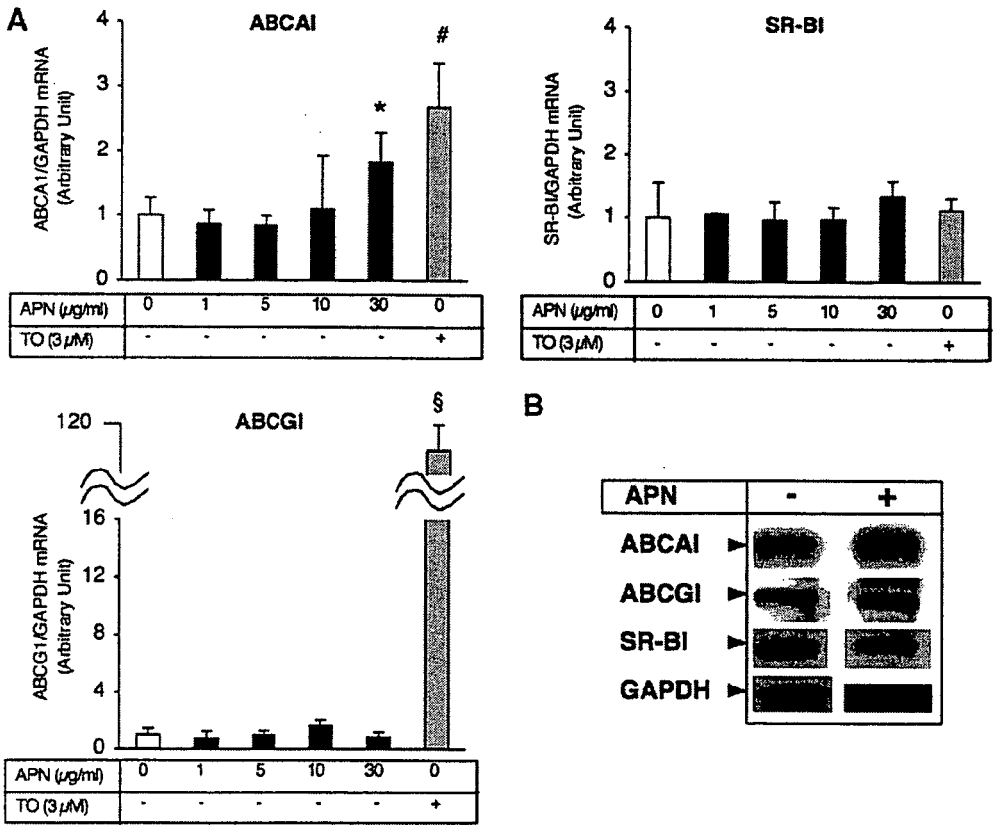

Fig. 2. APN enhanced the expression level of ABCA1, but not ABCG1 and SR-BI, in HepG2 cells. HepG2 cells were incubated for 24 h in DMEM containing 0.5% FCS with the indicated concentrations of APN or with 3 μM of TO-901317 (TO). The expression levels of ABCA1, ABCG1, and SR-BI were analyzed by real-time quantitative PCR or western blot. (A) APN increased the mRNA level of ABCA1 in a dose dependent manner, but not ABCG1 and SR-BI. Values are mean ± SD of triplicate determinations. $^{*}P < 0.05$, $^{\#}P < 0.005$, $^{\S}P < 0.0005$ vs 0 μg/ml of APN in the medium. (B) APN (30 μg/ml in the medium) enhanced the cellular ABCA1 protein level, while the protein levels of ABCG1 and SR-BI were not influenced. A representative result of three independent experiments is shown.

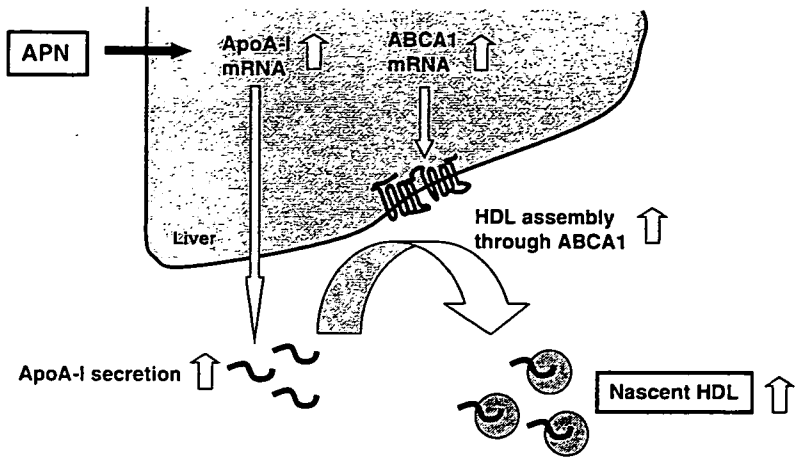

Fig. 3. APN and HDL assembly. APN increases HDL assembly through enhancing ABCA1 pathway and apoA-I synthesis in the liver.

HepG2 cells and enhanced the cellular expression of ABCA1. APN might accelerate RCT by increasing HDL assembly in the liver through enhancing ABCA1 pathway and apoA-I synthesis (Fig. 3).

Recently, Neumeier et al. reported that APN did not influence the expression of ABCA1 and the secretion of apoA-I in human primary hepatocytes [15]. They examined with lower concentrations of APN (maximal dose: 20 μg/ml in the medium), compared with the maximal APN concentration (30 μg/ml) in our experiments. In the current study, the increased apoA-I secretion and ABCA1 expression in HepG2 cells were observed with 30 μg/ml of APN. Furthermore, Neumeier et al. used human primary hepatocytes, while we utilized in human hepatoma cell line, HepG2 cells. Therefore, the discrepancy in the results between our and their study might be due to the difference of maximal APN concentration in the medium or a cell line used for the assay.

In the present study, we could not clarify the mechanism of increasing apoA-I production and ABCA1 expression by APN in HepG2 cells. It is known that apoA-I production in the liver is regulated through peroxisome proliferators-activated receptor alfa (PPAR-α), which is one of nuclear receptors [16]. ABCA1 is also a target gene of other nuclear receptor, LXR [17]. APN might regulate the expression of target genes of these nuclear receptors in the liver. However, although ABCG1 is also one of the target genes of LXR, the mRNA level of ABCG1 was not enhanced by APN. Therefore, the expression of ABCA1 in HepG2 cells might be upregulated by APN through a pathway independent of LXR. This issue needs to be investigated in the future studies.

Sahoo et al. reported that ABCA1-dependent depletion of cholesterol by apoA-I in murine hepatocytes decreased the secretion of apoB-100-containing lipoprotein, such as very low density lipoprotein (VLDL) [18]. Furthermore, Sniderman et al. also showed that there was an inverse relation between the amount of apoA-I added to the medium and the secretion of apoB-100 from HepG2 cells [19]. As observed in our study, the reduction of apoB-100 secretion from HepG2 cells by APN might be a secondary effect by which the release of apoA-I from the cells was increased.

The metabolic syndrome (MetS), a clustering of cardiovascular risk factors, is a powerful predictor of CAD. Ryo et al. reported that plasma APN levels are decreased in the subjects with MetS and that plasma APN concentrations are negatively correlated with visceral fat mass, but positively with plasma HDL–cholesterol level [9]. The visceral fat accumulation might increase risk for CAD by the reduced HDL assembly in the liver, which is associated with the decreasing plasma APN levels.

Taken together, the current study demonstrates that APN might accelerate RCT and protect against atherosclerosis by increasing HDL assembly through enhancing ABCA1 pathway and apoA-I synthesis in the liver.

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Senescent phenotypes of skin fibroblasts from patients with Tangier disease

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Abstract

Tangier disease (TD) is characterized by a deficiency of high density lipoprotein (HDL) in plasma and patients with TD have an increased risk for coronary artery disease (CAD). Recently, we reported that fibroblasts from TD exhibited large and flattened morphology, which is often observed in senescent cells. On the other hand, data have accumulated to show the relationship between cellular senescence and development of atherosclerotic CAD. The aim of the present study was to investigate whether TD fibroblasts exhibited cellular senescence. The proliferation of TD fibroblasts was gradually decreased at population doubling level (PDL) ~10 compared with control cells. TD cells practically ceased proliferation at PDL ~30. DNA synthesis was markedly decreased in TD fibroblasts. TD cells exhibited a higher positive rate for senescence-associated β-galactosidase (SA-β-gal), which is one of the biomarkers of cellular senescence *in vitro*. These data showed that TD cells reached cellular senescence at an earlier PDL compared with controls. Although, there was no difference in the telomere length of fibroblasts between TD and controls at the earlier passage (PDL 6), the telomere length of TD cells was shorter than that of controls at the late passage (PDL 25). Taken together, the current study demonstrates that the late-passaged TD fibroblasts showed senescent phenotype *in vitro*, which might be related to the increased cardiovascular manifestations in TD patients.

Keywords: ABCA1; Cdc42; SA-β-gal; Senescence; Tangier disease; Telomere

Tangier disease (TD) is characterized by a marked deficiency of high density lipoprotein (HDL) in plasma and the accumulation of cholesteryl esters in many tissues such as tonsils, liver, spleen, intestinal mucosa, peripheral nerves, and cornea. The patients with TD have an increased risk for coronary artery disease (CAD) [1]. This disorder is caused by the mutations in the ATP-binding cassette transporter-A1 (ABCA1) gene [2–4]. Obviously, the higher susceptibility for CAD is to some extent attributed to the deficiency of HDL, which is the major player in the reverse cholesterol transport. However, the pathophysiology of this disorder has not been completely understood yet.

Cells from patients with TD were known to have some cell-biological abnormalities such as defective cholesterol efflux and development of Golgi apparatus [1]. We recently presented that the expression of small G protein [5],

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Cdc42Hs, which has various cell-biological functions such re-arrangement of actin–cytoskeletons and vesicular transport [5], was markedly decreased in skin fibroblasts and monocyte-derived macrophages from TD patients [6]. We found that Cdc42 was decreased in aged fibroblasts *in vitro* and *in vivo* [7]. Furthermore, we raised a hypothesis that Cdc42 plays a role in intracellular transport and export of lipids from the cells [5,7]. We and others have reported TD cells were characterized by the presence of enlarged morphology with altered actin–cytoskeletons [5,8], which is often observed in aged cells *in vitro* and *in vivo* [9].

It is known that human cultured skin fibroblasts ceased proliferation and exhibited senescence after serial passaging *in vitro*, which is termed replicative senescence. Cells from various types of aging syndromes such as Werner syndrome and Hutchinson-Gilford progeria had a reduced proliferative capacity *in vitro* and exhibited cellular senescence at the earlier passage [11]. On the other hand, although the underlying mechanisms still need to be examined, data have accumulated to show that cellular senescence *in vitro* may be related to many human disorders including cardiovascular diseases other than aging syndromes [12].

From the above observations, we have tested a hypothesis that cells from patients with TD may exhibit senescent phenotype *in vitro*. In the present study, we demonstrate that TD fibroblasts showed senescent phenotypes *in vitro*, such as accelerated replicative senescence at earlier PDL in association with shortening of telomere.

Materials and methods

Cells and culture. Skin fibroblasts were obtained from two unrelated TD patients (TD1 and TD2 showed in Table 1) who were diagnosed by both classical TD phenotypes and mutations in the ABCA1 gene [11,13]. Both patients had an apparent CAD. The following three fibroblasts cell lines served as controls. Human normal fibroblasts from a 55-year-old male (N55) were obtained from KURABO. Co. Ltd. (Japan), when the PDL was 6. The other normal human fibroblasts from 24-, and 48-year-old female (N24 and N48) were obtained with an informed consent. These subjects had no clinical complications. For the study of aging, all fibroblast cell lines were carefully established at Health Science Research Resources Bank (HSRRB, Japan) and Osaka University [7]. The cells were cultured according to the standard conditions in modified Eagle's medium (MEM) supplemented with L-glutamine, nonessential amino acids, and 10% of fetal calf serum in a humidified 5% CO_2 controlled incubator at 37 °C. The cells were passaged by 1:4 split to increase cumulative cell population doubling level (PDL) by 2 on passage.

Table 1
Clinical profile of the patients with Tangier disease

	TD1	TD2	Normal range
Sex	Male	Male	
Age (yr)	51	57	
TC (mmol/l)	0.72	0.78	(<6.85)
TG (mmol/l	2.60	2.00	(<1.80)
HDL-C (mmol/l)	0.16	0.13	(0.80–1.80)

Age: age when skin fibroblasts were obtained.
TC: plasma total cholesterol level.
TG: plasma triglyceride level.
HDL-C: plasma high density lipoprotein-cholesterol level.

Cell proliferating rate. Cell proliferating rate was analyzed by the method as described by Mathon et al. [12]. Human fibroblasts (3×10^5 cells) were plated onto 9 cm-plastic dishes (SUMILON, Sumitomo Bakelite Co. Ltd., Japan) under conditions as detailed above. Every 4 days, cells were trypsinized, counted with a Coulter counter, and reseeded at a constant density. The proliferating rate of cells was determined as the ratio of total cell number trypsinized to the initial cell number seeded before 4 days.

DNA synthesis analysis. Human fibroblasts (10^4 cells/well) were seeded in 96-well plastic plates (FALCON culture plate, Becton–Dickinson Labware). DNA synthesis of cells was analyzed by bromodeoxyuridine (BrdU) incorporation assay method, using commercial kit (Cell proliferation ELISA system, Amersham).

Staining for senescence-associated β-galactosidase (SA-β-gal). Cells were washed twice with PBS and fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde. Cells were then stained with SA-β-gal staining solution (1 mg/mL of X-gal, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanidine, 5 mM potassium ferricyanide, and 40 mM citric acid/sodium phosphate buffer, at pH 6.0) for overnight [13].

Telomere length analysis. Purified genomic DNA (1 μg) from human fibroblasts (at PDL 6 and 25) was digested by an optimized mixture of frequently cutting restriction enzymes (*Hin*fI and *Rsa*I). Following DNA digestion, the DNA fragments were electrophoresed through a 0.8% agarose gel and transferred onto a nylon membrane for Southern blotting. Telomere length was analyzed by using *Telo* TAGGG telomere length assay kit (Roche, Germany). The exposed X-ray film was scanned with a densitometer. The mean of telomere length was calculated as $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is the the chemiluminescent signal and L_i is the length of the terminal restriction fragments at position i.

Statistical analysis. Results are expressed as means ± SD. Statistical significance was assessed by Student's t test for paired values and set at $P < 0.05$.

Results

Decreased proliferating rate and DNA synthesis of TD fibroblasts

To investigate the ability of proliferation of TD fibroblasts, we examined the proliferating rate at various PDLs (Fig. 1A). Although, the proliferating rates of three controls cell lines (N24: a young-aged normal cell line, N48 and N55: age-matched cell lines for TD patients) remained constant with continual passaging (PDL ~30), the proliferation of TD cells (TD1 and TD2) became slower at PDL ~10. Both TD1 and TD2 cells practically ceased proliferation at PDL ~30. Next, we examined DNA synthesis by BrdU incorporation assay method. Fig. 1B shows that DNA syntheses were significantly decreased at PDL 18 in TD cells, compared with those of the normal controls. It is known that the passaged fibroblasts had limited life span *in vitro* called "replicative senescence". These results suggest that TD cells might reach the replicative senescence at an earlier PDL.

Increased number of senescence-associated β-galactosidase (SA-β-gal) positive cells in Tangier disease

In order to further characterize TD cells from the viewpoints of cellular senescence, we analyzed the expression of

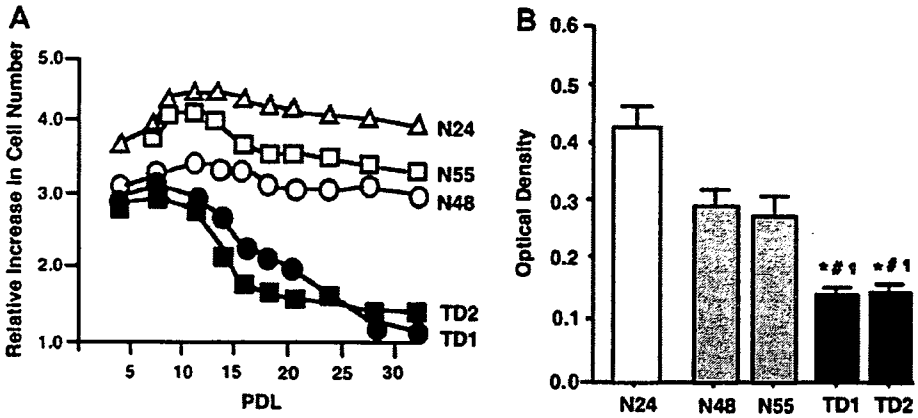

Fig. 1. Decreased proliferating rate and DNA synthesis of TD fibroblasts. (A) Fibroblasts (3×10^5 cells) were plated onto 9 cm-plastic dishes. Every 4 days, cells were trypsinized, counted with a Coulter counter, and reseeded at a constant density. The proliferating rate of cells was determined as the ratio of total cell number trypsinized to the initial cell number seeded before 4 days. Each of points is the mean of triplicate counts. TD1 and TD2, cells from Tangier disease patients; N24, control cells from a 24-year-old normal subject; N48, control cells from a 48-year-old normal subject; N55, control cells from a 55-year-old normal subject. (B) Fibroblasts (10^4 cells/well at passage 12, PDL 18) from two patients with TD (TD1 and TD2) and the controls (N24, N48, and N55) were incubated in 96-well plastic plates with modified Eagle's medium (MEM) supplemented 10% of fetal calf serum in a humidified 5% CO_2 controlled incubator at 37 °C. DNA synthesis of cells was analyzed by bromodeoxyuridine (BrdU) incorporation assay method. Each data represents means ± SD of triplicate determinations. $^*P < 0.001$ vs. N24, $^\#P < 0.005$ vs. N48 and $^\P P < 0.005$ vs. N55, respectively.

a senescent marker: a particular isoform of β-galactosidase (senescence-associated β-galactosidase, SA-β-gal). Fig. 2A shows a representative pattern of staining for SA-β-gal in TD fibroblasts (TD1) and control cells (N48) at PDL 16. Although, the fibroblasts from N48 were not stained with SA-β-gal at PDL 7, the fibroblasts from TD1 were mildly stained with SA-β-gal (0% vs. 4%, respectively). Furthermore, the percentage of SA-β-gal positive cells was extremely increased in TD1 cells at PDL 16, compared with N48 cells (Fig. 2B). Similar results were obtained in TD2 cells (data not shown).

Shortening of telomeres in TD fibroblasts

Telomeres are thought to serve as a replicometer for replicative senescence. It is thought that telomere shortening is responsible for entry into senescence in human fibroblasts. Therefore, we investigated the length of telomeres in TD cells. As shown in Fig. 3, although there was no difference in the telomere length of fibroblasts at the early passage (PDL 6) between TD and controls, the telomere lengths of TD cells was shorter than that of controls at the late passage (PDL 25). These data suggest that the telomere shortening in TD cells might be closely related to accelerated replicative senescence at an earlier PDL, as shown in Fig. 1.

Discussion

In the present study, we for the first time demonstrated that the fibroblasts from TD patients express the phenotype associated with cellular senescence such as slow cell proliferation and increased staining for SA-β-gal. Furthermore, we have showed the shortened telomeres in TD cells.

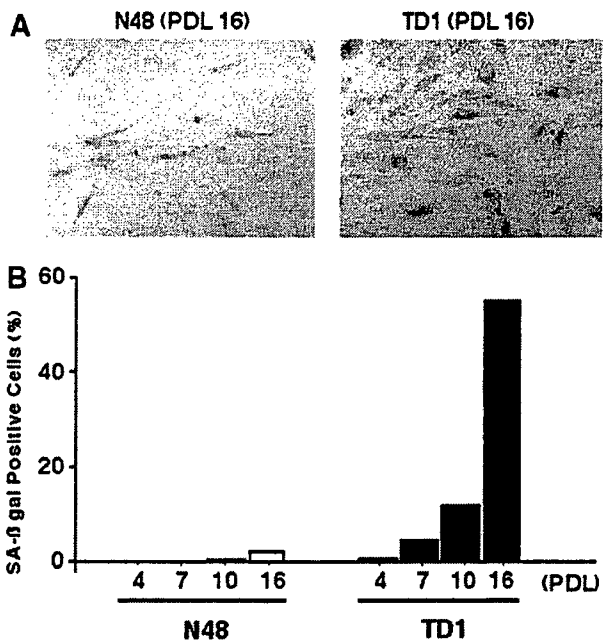

Fig. 2. Expression of the senescence marker (senescence-associated β-galactosidase: SA-β-gal) in fibroblasts from a patient with Tangier disease. Fibroblasts were fixed at 70–80% of cellconfluence with PBS containing 2% formaldehyde and 0.2% glutaraldehyde at the indicated PDLs. Cells were then stained with SA-β-gal staining solution (1 mg/mL of X-gal, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanidine, 5 mM potassium ferricyanide, 40 mM citric acid/sodium phosphate buffer, at pH 6.0) for overnight. The expression of SA-β-gal was determined as the percentage of SA-β-gal positive cells to total plated cells. (A) Representative pictures for SA-β-gal positive fibroblasts. Fibroblasts of TD1 and N48 were stained for SA-β-gal activity at PDL16. The activities were manifested as a blue coloration of cytoplasm. (B) Percentage of SA-β-gal positive cells. The percentages of SA-β-gal positive cells to total cells were determined in TD1 and N48 at the indicated PDLs.

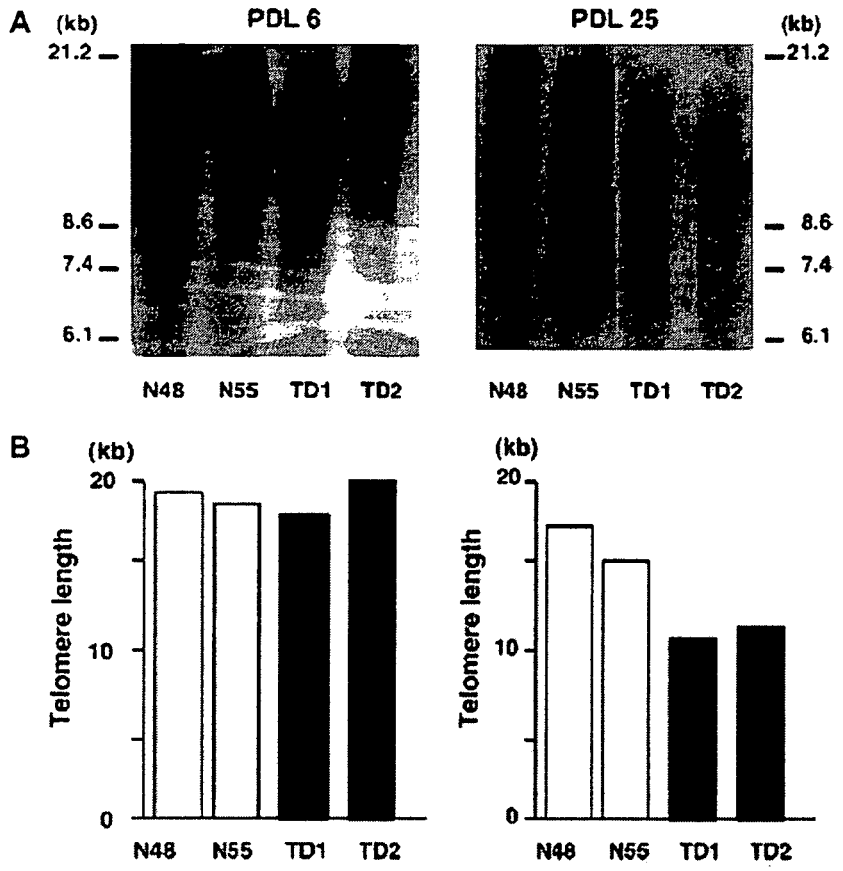

Fig. 3. Telomere length in fibroblasts from TD patients. Genomic DNAs from human fibroblasts were extracted for the determination of telomere length at the indicated PDL. The DNAs were digested by an optimized mixture of frequently cutting restriction enzymes (*Hinf*I and *Rsa*I) followed by Southern blotting onto a nylon membrane. The detection was performed according to the manufacturer's protocol. (A) Representative images at early (PDL 6) and late passages (PDL 16). (B) The mean telomere length was measured by densitometric scanning the image. Telomere lengths were shorter in TD cells at late passage, compared with controls.

It is well known that TD is caused by the mutations in the ABCA1 gene. We previously reported that the patients in the present study had mutations in the ABCA1 gene and that the mutated ABCA1 lost the function for cholesterol efflux from the cells over-expressing the mutant genes [5,10]. It was reported that ABCA1 has multiple functions [14] to regulate not only cholesterol export but also actin–cytoskeletons [15]. The deficiency of ABCA1 might cause the cellular senescence with abnormalities of lipid transport and cytoskeletons *in vitro*. Furthermore, we previously presented that the expression of small G protein, Cdc42Hs, which is related to various cell-biological functions such as intracellular lipid transport, actin–cytoskeleton formation, and cell cycle [16], was markedly decreased in skin fibroblasts from patients with TD. Olson et al. reported that the expression of activated form of Cdc42 induces the progression of adherent quiescent fibroblasts into S phase of cell cycle [16]. Therefore, the abnormal expression of Cdc42 might be associated with the decreased DNA synthesis in TD cells.

Telomere is one of the major determinants for cell cycle and cellular senescence [17]. It was reported that telomeres were shortened in the aging process [18,19]. In the present study, we showed that telomeres were shortened at an earlier PDL in TD fibroblasts compared with the age-matched control cells. It would be of importance to clarify the relationship between telomere and cellular senescence. Recently, Walter et al. have reported that induction of telomerase successfully immortalized TD fibroblasts [20]. These results strongly supported our finding.

The patients with TD have a moderately increased risk for CAD and TD patients in the present study have already suffered from CAD [5,21]. On the other hand, data appear to be accumulated to show that cellular senescence and telomere shortening may be related to atherosclerosis [22–24]. Samani et al. reported the relationship between the formation of atherosclerosis and telomere shortening [24]. They showed that telomere length in leukocytes of patients with CAD was significantly shorter than that of controls without CAD. Furthermore, Brouilette also described in the West of Scotland Primary Prevention Study that leukocyte telomere length was a predictor of future CAD events in middle-aged, high-risk men [25]. It would be of great importance to know whether the observed cellular senescence may be related to the clinical phenotype of TD, especially CAD.

In the present study, we could not clarify the mechanism of shortened telomere length in TD fibroblasts. It is known that telomere capping proteins, TRF1 and TRF2, regulate telomere length in human cells [26]. While TRF1 represses the telomerase pathway, TRF2, on the contrary, appears to activate the telomeric degradation pathway [27]. The abnormal expressions of TRF1 or TRF2 might be observed in TD cells. This issue needs to be investigated in the future studies.

In summary, we have shown that the skin fibroblasts from patients with TD showed senescent phenotype in association with shortening telomere length *in vitro*. This finding could have important implications for our understanding of the pathophysiology of CAD with aging.

Acknowledgments

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REVIEW ARTICLE

Impaired efflux of cholesterol from aged cells and its molecular mechanism: A basis for age-related enhancement of atherosclerosis

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Aging is one of the risk factors for atherosclerotic cardiovascular diseases, however, its molecular mechanism is currently unknown. Many types of cells in the atherosclerotic lesions are considered to have various biological abnormalities such as impaired lipid homeostasis and slow cell proliferation, which may be related to senescence at cellular levels. One of the common characteristics of senescent cells *in vitro* is the alteration of actin cytoskeletons, which were reported to be involved in the intracellular transport of lipids. Cholesterol efflux from the cells is the initial step of reverse cholesterol transport, a major protective system against atherosclerosis. Recently, we demonstrated that Cdc42, a member of the *Rho*-GTPase family, might be crucial for cellular lipid transport and cholesterol efflux based upon studies of Tangier cells that are deficient in *ABCA1* gene. In the current review, we also indicate that the expression of Cdc42 is decreased in the cells from aged subjects in close association with the retarded intracellular lipid transport. Furthermore, the Cdc42 expression is reduced by culturing fibroblasts *in vitro* for a long duration. Werner syndrome (WS) is characterized by the early onset of senescent phenotypes including premature atherosclerotic cardiovascular diseases, although the underlying molecular mechanism for the enhanced atherosclerosis has not been fully understood yet. We examined the intracellular lipid transport and cholesterol efflux and the expression levels of cholesterol efflux-related molecules in skin fibroblasts obtained from patients with WS. Cholesterol efflux was markedly reduced in the WS fibroblasts in association with an increased cellular cholesterol content. Fluorescent recovery after photobleaching technique revealed that intracellular lipid transport around Golgi apparatus was markedly reduced when using a C6-NBD-ceramide as a tracer. Cdc42 protein and its guanosine 5′-triphosphate-bound active form were markedly reduced in the WS fibroblasts. The adenovirus-mediated complementation of wild-type Cdc42 corrected the impaired cholesterol efflux, intracellular lipid transport and cellular cholesterol levels in the WS fibroblasts. These data indicate that the reduced expression of Cdc42 might be responsible for

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the abnormal lipid transport, which in turn might be related to the accelerated cardiovascular manifestations in WS patients. The current review focuses on the impaired efflux of cholesterol from aged cells and its molecular mechanism as a basis for age-related enhancement of atherosclerosis.

Keywords: aging, aging, Cdc42, intracellular lipid transport, senescence, vesicular transport, Werner syndrome.

Introduction

Atherosclerotic cardiovascular disease is one of the major causes of death all over the world. The development of atherosclerotic lesions is caused by various cellular dysfunctions as well as modifications of plasma lipoproteins.[1] The lesions are believed to have an increased local cellular turnover in response to inflammatory changes and most somatic cells can undergo a finite number of cell divisions before reaching cellular senescence. Therefore, it was speculated that many cells in the lesions might have experienced biological aging. It is well known that many cell types, such as macrophages and smooth muscle cells, in atherosclerotic lesions have the following senescence-related characteristics: slow cell proliferation, apoptotic changes and impaired lipid homeostasis.[2–4] These changes are thought to be closely associated with the rupture of cholesterol-rich atherosclerotic plaque, leading to the onset of acute coronary syndrome, one of the major research foci in basic and clinical cardiology.

High-density lipoprotein (HDL) particle or free apolipoprotein (apo) A-I removes cholesterol from lipid-laden cells, which is called "cholesterol efflux", and delivers it to the liver, a terminal of reverse cholesterol transport (RCT) (Fig. 1).[5,6] Therefore, cholesterol efflux from the cells is the initial and crucial step of RCT. We have continued to elucidate the significance of RCT by analyzing the pathophysiological mechanisms of disorders with abnormal HDL metabolism including Tangier disease (TD), which is a genetic deficiency of HDL characterized by an absence of plasma HDL and deposition of CE in the reticuloendothelial system with splenomegaly and enlargement of tonsils and lymph nodes. TD is a model for defective apo A-I-mediated cholesterol efflux due to mutations in the *ABCA1* gene.[7] The detailed molecular mechanism for cholesterol efflux has become focused, because the investigation of cholesterol efflux is believed to be one of the major strategies for the development of treatment against atherosclerosis. The first step of RCT is the interaction between HDL and peripheral cells such as fibroblasts and macrophages, which is postulated to include aqueous diffusion, *ABCA1*-mediated cholesterol efflux, and SR-BI or SR-BIII-mediated cholesterol efflux.[8] Figure 2 summarizes the current status of the molecular mechanisms for the cholesterol efflux from cells.

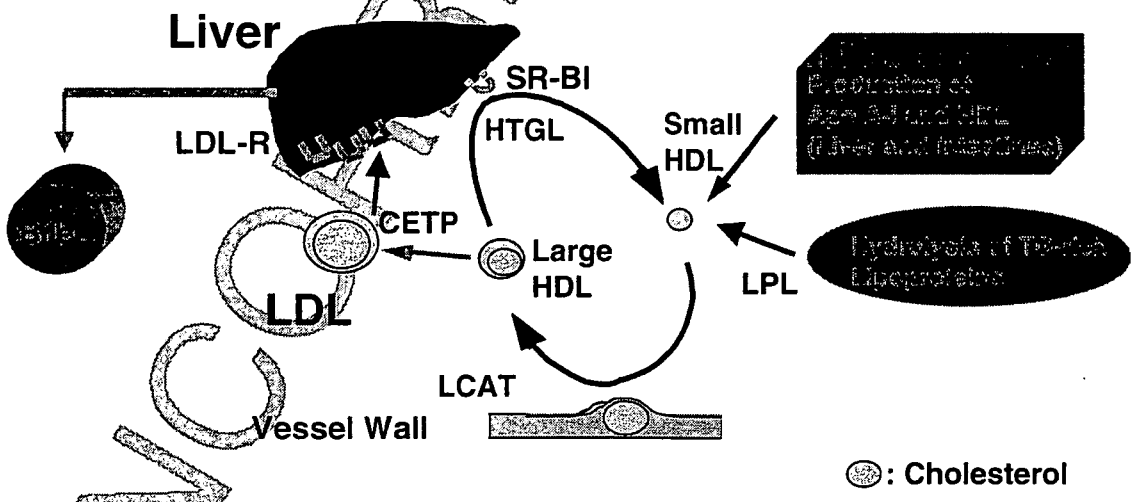

Figure 1 Molecular mechanisms of reverse cholesterol transport as a protective system against atherosclerosis.