surfaces *(10)*. In other words, the surface structures of remnant lipoproteins differ from those of other lipoproteins and are thought to be modified by LPLs. Because there are no fundamental differences in the overall structure of CMs, VLDLs, CMRs, and VLDLRs, however, it has been difficult to differentiate these lipoproteins. By releasing apo E, apo C-III, and phospholipids from TRL remnants, the reagent containing CHER, CHOD, POE-POB, and PL-D may promote cholesterol reactivity specifically in these modified particles.

Although a high correlation was observed between the immunoseparation and proposed assay methods in healthy individuals, there were cases with discrepancies among diabetic patients, who displayed increased concentrations of IDLs. These sera were analyzed by Lipophor, in which IDLs were detected as a "midband" *(14)*. We found that such sera contained more IDLs rather than large VLDLs. This result suggested the possibility that the proposed assay can quantify IDLs with high sensitivity.

There is a spectrum of sizes for CMRs and VLDLRs depending on their degree of lipolysis. Therefore, further studies need to be performed to determine the specificity of their assays to the various-sized remnant particles produced in the postprandial state.

The above findings indicate that this method detects not only TG-rich large particle size lipoproteins, such as CMRs, but also smaller remnants, such as VLDLRs and IDLs, with high sensitivity. Therefore, the proposed assay can quantify the VLDLR and IDL fractions, which reflect CAD progression, as reported in the Montreal Heart Study *(6)*. Furthermore, the proposed assay does not require any sample pretreatment and can be performed in a short period of time with the use of an autoanalyzer. It may therefore be useful for risk assessment of CAD and the diagnosis of type III hyperlipoproteinemia and other dyslipidemias characterized by accumulation of TRL remnants.

Grant/funding support: This study was supported by Kyowa Medex Co., Ltd.
Financial disclosures: None declared.
Acknowledgments: We thank Dr. Richard Havel (University of California, San Francisco, Medical Center) and Dr. G. Russell Warnick (Berkeley HeartLab, Inc.) for a critical review of an earlier draft of this manuscript.

References

1. Havel RJ. Determination and clinical significance of triglyceride-rich lipoprotein remnants. In: Rifai N, Warnick GR, Dominiczak MH, eds. Handbook of Lipoprotein Testing, 2nd ed. Washington, DC: American Association of Clinical Chemistry Press, 2000:565–80.
2. Zilversmit DB. Atherosclerosis: a postprandial phenomenon. Circulation 1979;60:473–85.
3. Karpe F, Steiner G, Uffelman K, Olivecrona T, Hamsten A. Postprandial lipoproteins and progression of coronary atherosclerosis. Atherosclerosis 1994;106:83–97.
4. Havel RJ. Postprandial hyperlipidemia and remnant lipoproteins. Curr Opin Lipidol 1994;5:102–9.
5. Tanaka A. Postprandial hyperlipidemia and atherosclerosis. J Atheroscler Thromb 2004;11:322–9.
6. Phillips NR, Waters D, Havel RJ. Plasma lipoproteins and progression of coronary artery disease evaluated by angiography and clinical events. Circulation 1993;88:2762–70.
7. McNamara JR, Shah PK, Nakajima K, Cupples LA, Wilson PW, Ordovas JM, et al. Remnant-like particle (RLP) cholesterol is an independent cardiovascular disease risk factor in women: results from the Framingham Heart Study. Atherosclerosis 2001;154: 229–36.
8. Eisenberg S. Remnant lipoprotein metabolism. In: Crepaldi G, Tiengo A, Manzato E, eds. Diabetes, Obesity and Hyperlipidemia: V. The Plurimetabolic Syndrome. New York: Elsevier Science Publishers, 1993:7–14.
9. Sata T, Havel RJ, Jones AL. Characterization of subfractions of triglyceride-rich lipoproteins separated by gel chromatography from blood plasma of normolipemic and hyperlipemic humans. J Lipid Res 1972;13:757–68.
10. Mjøs OD, Faergeman O, Hamilton RL, Havel RJ. Characterization of remnants produced during the metabolism of triglyceride-rich lipoproteins of blood plasma and intestinal lymph in the rat. J Clin Invest 1975;56:603–15.
11. Pagnan A, Havel RJ, Kane JP, Kotite L. Characterization of human very low density lipoproteins containing two electrophoretic populations: double pre-beta lipoproteinemia and primary dysbetalipoproteinemia. J Lipid Res 1977;18:613–22.
12. Marcoux C, Tremblay M, Nakajima K, Davignon J, Cohn JS. Characterization of remnant-like particles isolated by immunoaffinity gel from the plasma of type III and type IV hyperlipoproteinemic patients. J Lipid Res 1999;40:636–47.
13. Cohn JS, Marcoux C, Davignon J. Detection, quantification, and characterization of potentially atherogenic triglyceride-rich remnant lipoproteins. Arterioscler Thromb Vasc Biol 1999;19:2474–86.
14. Kameda K, Matsuzawa Y, Kubo M, Ishikawa K, Maejima I, Yamamura T, et al. Increased frequency of lipoprotein disorders similar to type III hyperlipoproteinemia in survivors of myocardial infarction in Japan. Atherosclerosis 1984;51:241–9.
15. Tatami R, Mabuchi H, Ueda K, Ueda R, Haba T, Kametani T, et al. Intermediate-density lipoprotein and cholesterol-rich very low density lipoprotein in angiographically determined coronary artery disease. Circulation 1981;64:1174–84.
16. Carlson LA, Ericsson M. Quantitative and qualitative serum lipoprotein analysis. Part 1. Studies in healthy men and women. Atherosclerosis 1975;21:417–33.
17. Nakajima K, Saito T, Tamura A, Suzuki M, Nakano T, Adachi M, et al. Cholesterol in remnant-like lipoproteins in human serum using monoclonal anti apo B-100 and anti apo A-I immunoaffinity mixed gels. Clin Chim Acta 1993;223:53–71.
18. Nakajima K, Okazaki M, Tanaka A, Pullinger C, Wang T, Nakano T, et al. Separation and determination of remnant-like particles in human serum using monoclonal antibodies to apoB-100 and apoA-I. J Clin Ligand Assay 1996;19:177–83.
19. Nauck M, Wiebe D, Warnick GR. Measurement of high-density lipoprotein cholesterol. In: Rifai N, Warnick GR, Dominiczak MH, eds. Handbook of Lipoprotein Testing, 2nd ed. Washington, DC: American Association of Clinical Chemistry Press, 2000:221–44.
20. Bachorik PS. Measurement of low-density lipoprotein cholesterol. In: Rifai N, Warnick GR, Dominiczak MH, eds. Handbook of Lipoprotein Testing, 2nd ed. Washington, DC: American Association of Clinical Chemistry Press, 2000:245–64.

21. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955;34:1345–53.

22. Hatch FT, Lees RS. Practical methods for plasma lipoprotein analysis. Adv Lipid Res 1968;6:1–68.

23. Kieft KA, Bocan TM, Krause BR. Rapid online determination of cholesterol distribution among plasma lipoproteins after high-performance gel filtration chromatography. J Lipid Res 1991;32: 859–66.

24. Okazaki M, Usui S, Hosaki S. Analysis of plasma lipoproteins by gel permeation chromatography. In: Rifai N, Warnick GR, Dominiczak MH, eds. Handbook of Lipoprotein Testing, 2nd ed. Washington, DC: American Association of Clinical Chemistry Press, 2000: 647–69.

25. Kitamura T, Ito S, Moriyama H, Kato Y, Sasamoto K, Okazaki M. Quantitative analysis of serum lipoproteins (CM, VLDL, LDL and HDL) by high-performance gel filtration chromatography. Chromatography 1996;17:33–7.

26. Yanai H. Statcel: The useful add-in software forms on Excel, 2nd ed. Tokyo: OMS, 2004.

27. Juhász J, Lenaerts V, Bellemare M, Pimienta C, Ong H. Stability and absorption to polymeric surfaces of rat ANF in poloxamer 407 solutions. Int J Pharm 1991;77:309–13.

28. Cappel MJ, Kreuter J. Effect of nonionic surfactants on transdermal drug delivery: II. Poloxamer and poloxamine surfactants. Int J Pharm 1991;69:155–67.

29. Sugiuchi H, Irie T, Uji Y, Ueno T, Chaen T, Uekama K, et al. Homogeneous assay for measuring low-density lipoprotein cholesterol in serum with triblock copolymer and α-cyclodextrin sulfate. Clin Chem 1998;44:522–31.

30. Takayama M, Itoh S, Nagasaki T, Tanimizu I. A new enzymatic method for determination of serum choline-containing phospholipids. Clin Chim Acta 1977;79:93–8.

31. Havel RJ. Origin, metabolic fate, and metabolic function of plasma lipoproteins. In: Steinberg D, JM Olefsky, eds. Contemporary Issues in Endocrinology and Metabolism. Vol. 3. New York: Churchill Livingstone, 1987:117–41.

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Senescent phenotypes of skin fibroblasts from patients with Tangier disease

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Received 20 March 2007
Available online 9 April 2007

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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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.03.172

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

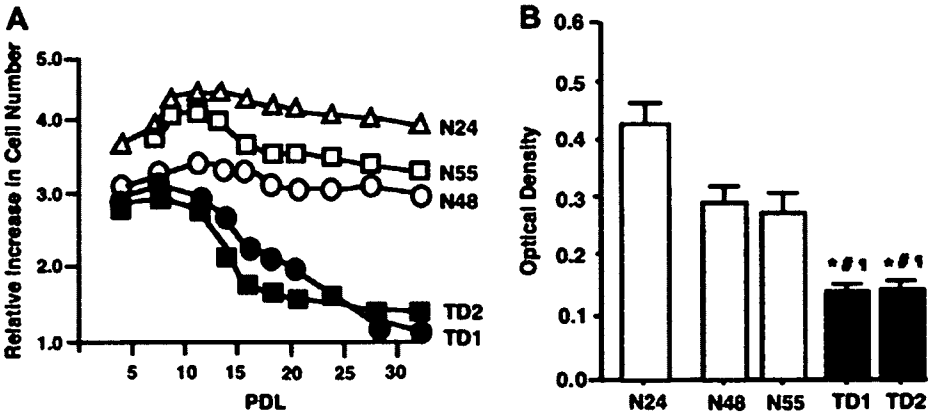

Fig. 1. Decreased proliferating rate and DNA synthesis of TD fibroblasts. (A) Fibroblasts (3×10^5 cells) were plated onto 9 cm-plastic dishes. Every 4 days, cells were trypsinized, counted with a Coulter counter, and reseeded at a constant density. The proliferating rate of cells was determined as the 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 $^\$P < 0.005$ 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.

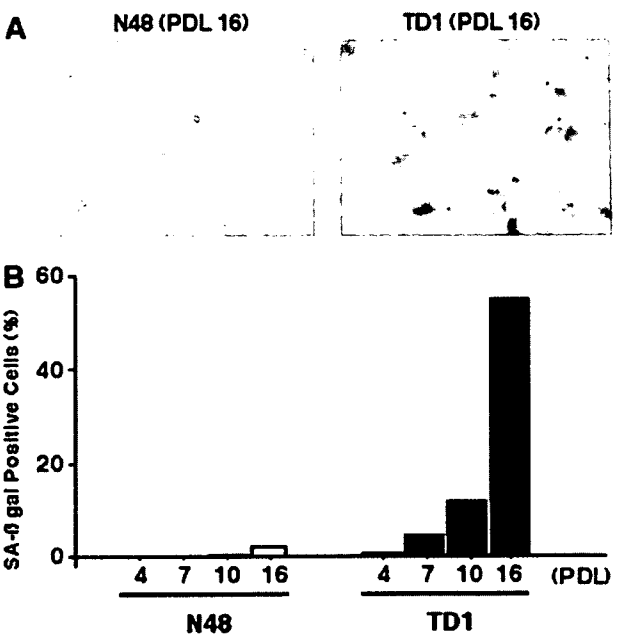

Fig. 2. Expression of the senescence marker (senescence-associated β-galactosidase: SA-β-gal) in fibroblasts from a patient with Tangier disease. Fibroblasts were fixed at 70–80% of cellconfluence with PBS containing 2% formaldehyde and 0.2% glutaraldehyde at the indicated PDLs. Cells were then stained 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.

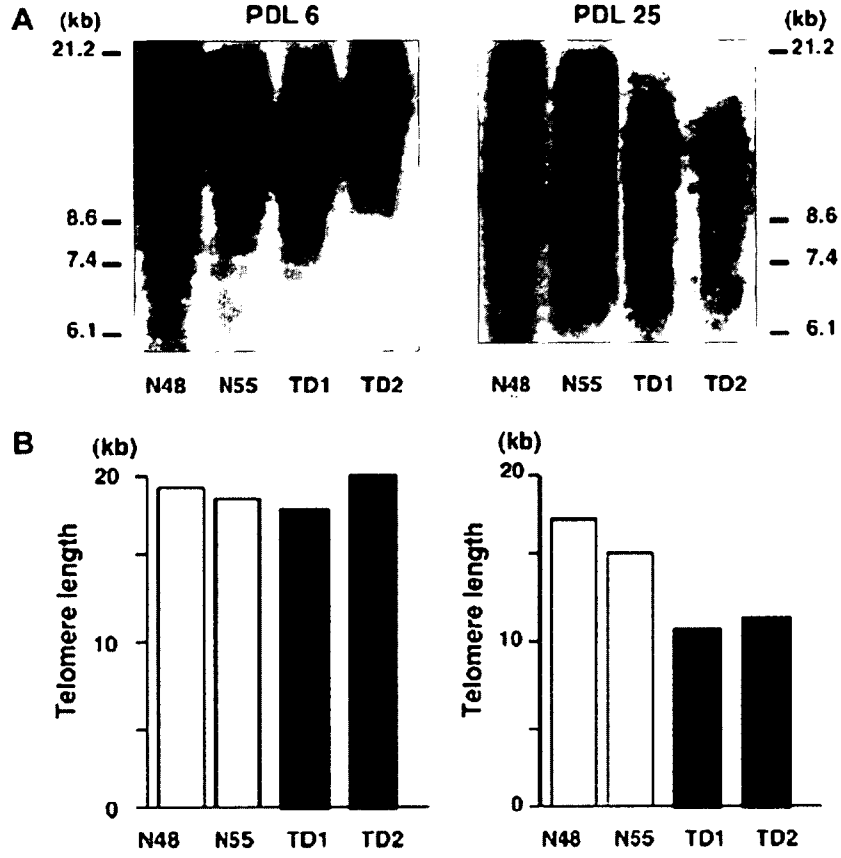

Fig. 3. Telomere length in fibroblasts from TD patients. Genomic DNAs from human fibroblasts were extracted for the determination of telomere length at the indicated PDL. The DNAs were digested by an optimized mixture of frequently cutting restriction enzymes (*Hinf*1 and *Rsa*1) followed by Southern 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

This work was supported by research grants from the Study Group of Molecular Cardiology (Japan), from Japan Heart Foundation (Japan), from Osaka Heart Club (Japan), Japan Heart Foundation/Pfizer Grant for Research on Hypertension and Vascular Metabolism (Japan), and from Tanabe Medical Frontier Conference (TMFC) (Japan) to K. Hirano. The authors gratefully thank Drs. Yuji Matsuzawa (Sumitomo Hospital, Osaka, Japan) and Alan R. Tall (Columbia University, NY, USA) for their helpful comments and discussions. This work was supported by Grants-in-Aid (Nos. 11557055 and 10671070) from the Japanese Ministry of Education, Science, Sports, and Culture, an International HDL Research Awards Program grant from Pfizer, a grant from the Takeda Medical Research Foundation, and a grant from the ONO Medical Research Foundation, and the Novartis Foundation for Gerontological Research to S. Yamashita.

References

[1] G. Assmann, A. von Eckardstein, H.B. Brewer Jr., Familial analphalipoproteinemia: Tangier disease, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, eighth ed., McGraw-Hill, New York, 2001, pp. 2937–2960 (Chapter 122).

[2] A. Brooks-Wilson, M. Marcil, S.M. Clee, L.H. Zhang, K. Roomp, M. van Dam, L. Yu, C. Brewer, J.A. Collins, H.O. Molhuizen, O. Loubser, B.F. Ouelette, K. Fichter, K.J. Ashbourne-Excoffon, C.W. Sensen, S. Scherer, S. Mott, M. Denis, D. Martindale, J. Frohlich, K. Morgan, B. Koop, S. Pimstone, J.J. Kastelein, M.R. Hayden, Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency, Nat. Genet. 22 (1999) 336–345.

[3] M. Bodzioch, E. Orso, J. Klucken, T. Langmann, A. Bottcher, W. Diederich, W. Drobnik, S. Barlage, C. Buchler, M. Porsch-Ozcurumez, W.E. Kaminski, H.W. Hahmann, K. Oette, G. Rothe, C. Aslanidis, K.J. Lackner, G. Schmitz, The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease, Nat. Genet. 22 (1999) 347–351.

[4] S. Rust, M. Walter, H. Funke, A. von Eckardstein, P. Cullen, H.Y. Kroes, R. Hordijk, J. Geisel, J. Kastelein, H.O. Molhuizen, M. Schreiner, A. Mischke, H.W. Hahmann, G. Assmann, Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1, Nat. Genet. 22 (1999) 352–355.

[5] K. Hirano, F. Matsuura, K. Tsukamoto, Z. Zhang, A. Matsuyama, K. Takaishi, R. Komuro, T. Suehiro, S. Yamashita, Y. Takai, Y. Matsuzawa, Decreased expression of a member of the Rho GTPase family, Cdc42Hs, in cells from Tangier disease—the small G protein may play a role in cholesterol efflux, FEBS Lett. 484 (2000) 275–279.

[6] Y. Takai, T. Sasaki, T. Matozaki, Small GTP-binding proteins, Physiol. Rev. 81 (2001) 153–208.

[7] K. Tsukamoto, K. Hirano, S. Yamashita, N. Sakai, C. Ikegami, Z. Zhang, F. Matsuura, H. Hiraoka, A. Matsuyama, M. Ishigami, Y. Matsuzawa, Retarded intracellular lipid transport associated with reduced expression of Cdc42, a member of Rho-GTPases, in human aged skin fibroblasts: a possible function of Cdc42 in mediating intracellular lipid transport, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 1899–1904.

[8] W. Drobnik, G. Liebisch, C. Biederer, B. Tr mbach, G. Rogler, P. Muller, G. Schmitz, Growth and cell cycle abnormalities of fibroblasts from Tangier disease patients, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 28–38.

[9] P. van Gansen, N. van Lerberghe, Potential and limitations of cultivated fibroblasts in the study of senescence in animals. A review on the murine skin fibroblasts system, Arch. Gerontol. Geriatr. 7 (1988) 31–74.

[10] Y. Nishida, K. Hirano, K. Tsukamoto, M. Nagano, C. Ikegami, K. Roomp, M. Ishihara, N. Sakane, Z. Zhang, K. Tsujii, A. Matsuyama, T. Ohama, F. Matsuura, M. Ishigami, N. Sakai, H. Hiraoka, H. Hattori, C. Wellington, Y. Yoshida, S. Misugi, M.R. Hayden, T. Egashira, S. Yamashita, Y. Matsuzawa, Expression and functional analyses of novel mutations of ATP-binding cassette transporter-1 in Japanese patients with high-density lipoprotein deficiency, Biochem. Biophys. Res. Commun. 290 (2002) 713–721.

[11] W. Klapper, R. Parwaresch, G. Krupp, Telomere biology in human aging and aging syndromes, Mech. Age. Dev. 122 (2001) 695–712.

[12] N.F. Mathon, D.S. Malcolm, M.C. Harrisingh, L. Cheng, A.C. Lloyd, Lack of replicative senescence in normal rodent glia, Science 291 (2001) 872–875.

[13] G.P. Dimri, X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, A biomarker that identifies senescent human cells in culture and in aging skin in vivo, Proc. Natl. Acad. Sci. USA 92 (1995) 9363–9367.

[14] R.J. Aiello, D. Brees, O.L. Francone, ABCA1-deficient mice: insights into the role of monocyte lipid efflux in HDL formation and inflammation, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 972–980.

[15] K. Tsukamoto, K. Hirano, K. Tsujii, C. Ikegami, Z. Zhongyan, Y. Nishida, T. Ohama, F. Matsuura, S. Yamashita, Y. Matsuzawa, ATP-binding cassette transporter-1 induces rearrangement of actin cytoskeletons possibly through Cdc42/N-WASP, Biochem. Biophys. Res. Commun. 287 (2001) 757–765.

[16] M.F. Olson, A. Ashworth, A. Hall, An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1, Science 269 (1995) 270–1272.

[17] A.C. Lloyd, Limits to lifespan, Nat. Cell. Biol. 4 (2002) E25–E27.

[18] A. Ahmed, T. Tollefsbo, Telomeres and telomerase: basic science implications for aging, J. AM. Geriatr. Soc. 49 (2001) 1105–1109.

[19] C.B. Harley, A.B. Futcher, C.W. Graider, Telomere shorten during aging of human fibroblasts, Nature 345 (1990) 458–460.

[20] M. Walter, N.R. Forsyth, W.E. Wright, J.W. Shay, M.G. Roth, The establishment of telomerase-immortalized Tangier disease cell lines indicates the existence of an apolipoprotein A-I-inducible but ABCA1-independent cholesterol efflux pathway, J. Biol. Chem. 279 (2004) 20866–20873.

[21] R. Komuro, S. Yamashita, S. Sumitsuji, K. Hirano, T. Maruyama, M. Nishida, F. Matsuura, A. Matsuyama, T. Sugimoto, N. Ouchi, N. Sakai, T. Nakamura, T. Funahashi, Y. Matsuzawa, Tangier disease with continuous massive and longitudinal diffuse calcification in the coronary arteries: demonstration by the sagittal images of intravascular ultrasonography, Circulation 101 (2000) 2446–2448.

[22] N.J. Samani, R. Boultby, R. Butler, Telomere shortening in atherosclerosis, Lancet 358 (2001) 472–473.

[23] M. Ogami, Y. Ikura, M. Ohsawa, T. Matsuo, S. Kayo, N. Yoshimi, E. Hai, N. Shirai, S. Ehara, R. Komatsu, T. Naruko, M. Ueda, Telomere shortening in human coronary artery diseases, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 546–550.
[24] T. Minamino, H. Miyauchi, T. Yoshida, K. Tateno, T. Kunieda, I. Komuro, Vascular cell senescence and vascular aging, J. Mol. Cell. Cardiol. 36 (2004) 175–183.
[25] S.W. Brouilette, J.S. Moore, A.D. McMahon, J.R. Thompson, I. Ford, J. Shepherd, C.J. Packard, N.J. Samani, West of Scotland Coronary Prevention Study Group. Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study, Lancet 369 (2007) 107–114.
[26] A. Smogorzewska, B. van Steensel, A. Bianch, S. Oelmann, M.R. Schefer, G. Schnapp, T. de Lange, Control of human telomere length by TRF1 and TRF2, Mol. Cell. Biol. 20 (2000) 1659–1668.
[27] K. Ancelin, M. Brunori, S. Bauwens, C.E. Koering, C. Brun, M. Ricoul, J.P. Pommier, L. Sabatier, E. Gilson, Targeting assay to study the cis functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2, Mol. Cell. Biol. 22 (2002) 3474–3487.

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

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Received 1 May 2007
Available online 15 May 2007

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-I 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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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.05.040

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 ABCA1-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

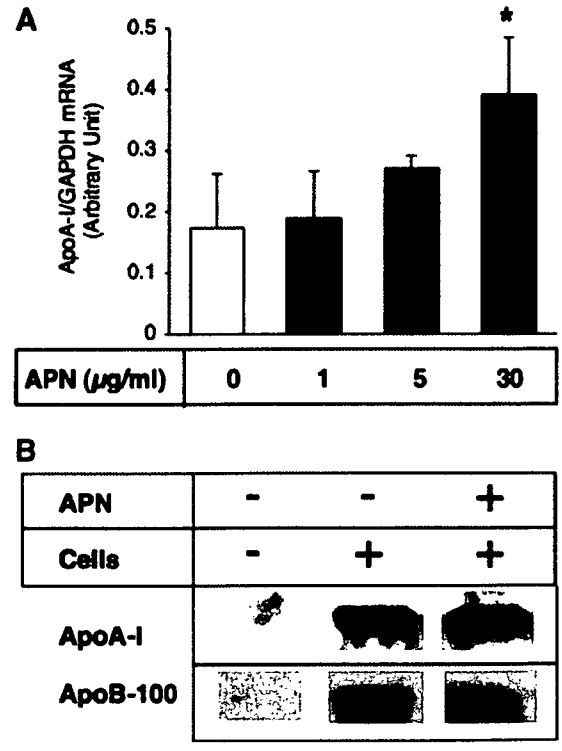

Fig. 1. APN increased the expression level of apoA-I in HepG2 cells and enhanced the secretion of apoA-I from the cells to the medium. HepG2 cells were incubated for 24 h in DMEM containing 0.5% FCS with the indicated concentrations (0–30 μg/ml in the medium) of recombinant human APN. The mRNA level 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

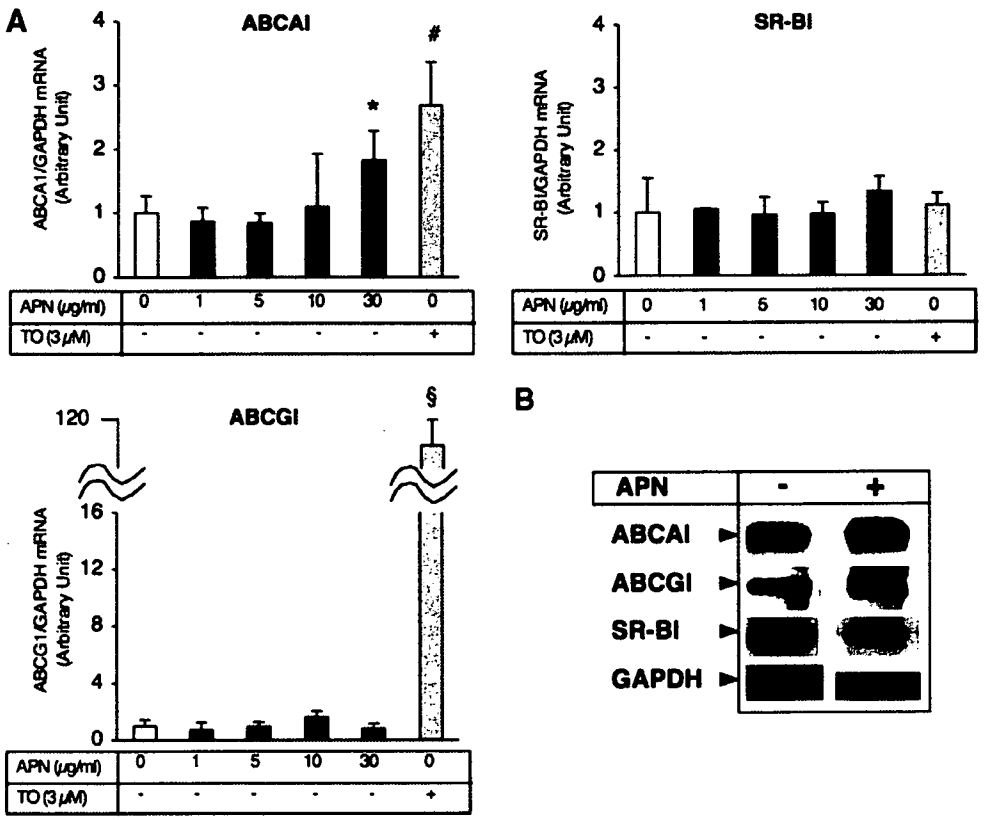

Fig. 2. APN enhanced the expression level of ABCA1, but not ABCG1 and SR-BI, in HepG2 cells. HepG2 cells were incubated for 24 h in DMEM containing 0.5% FCS with the indicated concentrations of APN or with 3 μM of TO-901317 (TO). The expression levels of ABCA1, ABCG1, and SR-BI were analyzed by real-time 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.

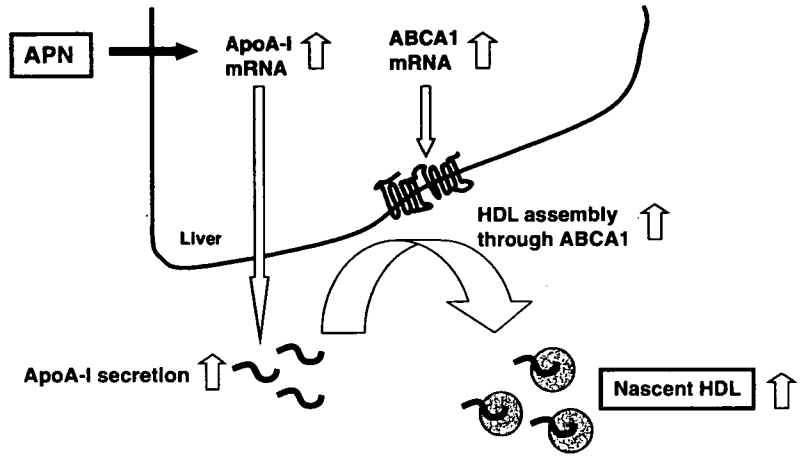

Fig. 3. APN and HDL assembly. APN increases HDL assembly through enhancing ABCA1 pathway and apoA-I synthesis in the liver.

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-1 synthesis in the liver.

Acknowledgments

This work was supported by Grants-in-Aid (No. 11557055 and No. 10671070) from the Japanese Ministry of Education, Science, Sports, and Culture, an International HDL Research Awards Program grant from Pfizer, a grant from the Takeda Medical Research Foundation, and a grant from the ONO Medical Research Foundation, and the Novartis Foundation for Gerontological Research to S. Yamashita. This work was also supported by research grants from the Study Group of Molecular Cardiology (Japan), from Japan Heart Foundation (Japan), from Osaka Heart Club (Japan), Japan Heart Foundation/Pfizer Grant for Research on Hypertension and Vascular Metabolism (Japan), and from Tanabe Medical Frontier Conference (TMFC) (Japan) to K. Hirano.

References

[1] N.E. Miller, A. La Ville, D. Crook, Direct evidence that reverse cholesterol transport is mediated by high density lipoprotein in rabbit, Nature 314 (1985) 109–111.

[2] S. Yokoyama, Assembly of high density lipoprotein by the ABCA1/apolipoprotein pathway, Curr. Opin. Lipidol. 16 (2005) 269–279.

[3] N. Wang, D. Lan, W. Cheng, F. Matsuura, A.R. Tall, ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins, Proc. Natl. Acad. Sci. USA 101 (2004) 9774–9779.
[4] Y. Ji, B. Jian, N. Wang, Y. Sun, M.L. Moya, M.C. Phillips, G.H. Rothblat, J.B. Swaney, A.R. Tall, Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux, J. Biol. Chem. 272 (1997) 20982–20985.
[5] S. Xu, M. Laccotripe, X. Huang, A. Rigotti, V.I. Zannis, M. Krieger, Apolipoproteins of HDL can directly mediate binding to the scavenger receptor SR-BI, an HDL receptor that mediates selective lipid uptake, J. Lipid. Res. 38 (1997) 1289–1298.
[6] A. Brooks-Wilson, M. Marcil, S.M. Clee, L.H. Zhang, K. Roomp, M. van Dam, L. Yu, C. Brewer, J.A. Collins, H.O. Molhuizen, O. Loubser, B.F. Ouelette, K. Fichter, K.J. Ashbourne-Excoffon, C.W. Sensen, S. Scherer, S. Mott, M. Denis, D. Martindale, J. Frohlich, K. Morgan, B. Koop, S. Pimstone, J.J. Kastelein, M.R. Hayden, Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency, Nat. Genet. 22 (1999) 336–345.
[7] M. Bodzioch, E. Orso, J. Klucken, T. Langmann, A. Bottcher, W. Diederich, W. Drobnik, S. Barlage, C. Buchler, M. Porsch-Ozcurumez, W.E. Kaminski, H.W. Hahmann, K. Oette, G. Rothe, C. Aslanidis, K.J. Lackner, G. Schmitz, The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease, Nat. Genet. 22 (1999) 347–351.
[8] S. Rust, M. Walter, H. Funke, A. von Eckardstein, P. Cullen, H.Y. Kroes, R. Hordijk, J. Geisel, J. Kastelein, H.O. Molhuizen, M. Schreiner, A. Mischke, H.W. Hahmann, G. Assmann, Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1, Nat. Genet. 22 (1999) 352–355.
[9] M. Ryo, T. Nakamura, S. Kihara, M. Kumada, S. Shibazaki, M. Takahashi, M. Nagai, Y. Matsuzawa, T. Funahashi, Adiponectin as a biomarker of the metabolic syndrome, Circ. J. 68 (2004) 975–981.
[10] M. Kumada, S. Kihara, S. Sumitsuji, T. Kawamoto, S. Matsumoto, N. Ouchi, Y. Arita, Y. Okamoto, I. Shimomura, H. Hiraoka, T. Nakamura, T. Funahashi, Y. MatsuzawaOsaka CAD Study Group, Coronary artery disease. Association of hypoadiponectinemia with coronary artery disease in men, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 85–89.
[11] C. Zoccali, F. Mallamaci, G. Tripepi, F.A. Benedetto, S. Cutrupi, S. Parlongo, L.S. Malatino, G. Bonanno, G. Seminara, F. Rapisarda, P. Fatusso, M. Buemi, G. Nicocia, S. Tanaka, N. Ouchi, S. Kihara, T. Funahashi, Y. Matsuzawa, Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease, J. Am. Soc. Nephrol. 13 (2002) 134–141.
[12] M. Cnop, P.J. Havel, K.M. Utzschneider, D.B. Carr, M.K. Sinha, E.J. Boyko, B.M. Retzlaff, R.H. Knopp, J.D. Brunzell, S.E. Kahn, Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex, Diabetologia 46 (2003) 459–469.
[13] N. Ouchi, S. Kihara, Y. Arita, M. Nishida, A. Mastsuyama, Y. Okamoto, M. Ishigami, H. Kuriyama, K. Kishida, H. Nishizawa, K. Hotta, M. Muraguchi, Y. Ohmoto, S. Yamashita, T. Funahashi, Y. Matsuzawa, Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages, Circulation 103 (2001) 1057–1063.
[14] K. Hirano, S. Yamashita, Y. Nakagawa, T. Ohya, F. Matsuura, K. Tsukamoto, Y. Okamoto, A. Matsuyama, K. Matsumoto, J. Miyagawa, Y. Matsuzawa, Expression of human scavenger receptor class B type I in cultured human monocyte-derived macrophages and atherosclerotic lesions, Circ. Res. 85 (1999) 108–116.
[15] M. Neumeier, A. Sigruener, E. Eggenhofer, J. Weigert, T.S. Weiss, A. Schaeddler, H.J. Schlitt, C. Aslanidid, P. Psio, T. Langmann, G. Schmitz, J. Scholmerich, C. Buechler, High molecular weight adiponectin reduces apolipoprotein B and E release in human hepatocytes, Biochem. Biophys. Res. Commun. 352 (2007) 543–548.
[16] H. Duez, B. Lefebvre, P. Poulain, I.P. Torra, F. Percevault, G. Luc, J.M. Peters, F.J. Gonzalez, R. Gineste, S. Helleboid, V. Dzavik, J.C. Fruchart, C. Fievet, P. Lefebvre, B. Staels, Regulation of human apoA-I by gemfibrozil and fenofibrate through selective peroxisome proliferator-activated receptor alpha modulation, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 585–591.
[17] J.J. Repa, S.D. Turley, J.A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, R.A. Heyman, J.M. Dietschy, D.J. Mangelsdorf, Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers, Science 289 (2000) 1524–1529.
[18] D. Sahoo, T.C. Trischuk, T. Chan, V.A.B. Drover, S. Ho, G. Chimini, L.B. Agellon, R. Agnihotri, G.A. Francis, R. Leher, ABCA1-dependent lipid efflux to apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes, J. Lipid Res. 45 (2004) 1122–1131.
[19] A.D. Sniderman, Z. Zhang, J. Genest, K. Cianflone, Effects on apoB-100 secretion and bile acid synthesis by redirecting cholesterol efflux from HepG2 cells, J. Lipid Res. 44 (2003) 527–532.

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Adiponectin deficiency suppresses ABCA1 expression and ApoA-I synthesis in the liver

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Received 20 August 2007; revised 18 September 2007; accepted 18 September 2007

Available online 29 September 2007

Edited by Laszlo Nagy

Abstract Plasma high density lipoprotein (HDL)-cholesterol levels are inversely correlated with the incidence of cardiovascular diseases. HDL is mainly assembled in the liver through the ATP-binding cassette transporter (ABCA1) pathway. In humans, plasma HDL-cholesterol levels are positively correlated with plasma adiponectin (APN) concentrations. Recently, we reported that APN enhanced apolipoprotein A-I (apoA-I) secretion and ABCA1 expression in HepG2 cells. In the present study, we investigated HDL assembly in APN-knockout (KO) mice. The apoA-I protein levels in plasma and liver were reduced in APN-KO mice compared with wild-type-mice. The ABCA1 expression in liver was also decreased in APN-KO mice. APN deficiency might cause the impaired HDL assembly by decreasing ABCA1 expression and apoA-I synthesis in the liver.

Keywords: ABCA1; Adiponectin; ApoA-I; ApoB-100; HDL; Reverse cholesterol transport

1. Introduction

Plasma high density lipoprotein (HDL)-cholesterol levels are negatively correlated with the incidence of coronary artery disease (CAD). It is thought that HDL prevents the development of atherosclerosis by removing excess cholesterol from atheroma and transporting it back to the liver in the protective system, so-called "reverse cholesterol transport" (RCT) [1].

The ATP-binding cassette transporters (ABCA1 and ABCG1), which are expressed in the liver, small intestine and peripheral tissues, are thought to be rate-limiting factors for HDL assembly in RCT system [2,3]. ABCA1, the responsible gene for familial HDL deficiency including Tangier disease [4–6], promotes apoA-I-mediated cholesterol efflux, which is the initial step in RCT system, decreasing cholesterol accumulation in macrophages and initiating HDL formation in the liver [2]. ABCG1 also stimulates cholesterol efflux to mature HDL in macrophages [3].

Adiponectin (APN), a bioactive peptide secreted from adipocytes is one of the important molecules to inhibit the development of atherosclerosis. Several clinical studies have demonstrated that plasma levels of APN are extremely low in patients with the metabolic syndrome which clusters risk factors for CAD such as visceral obesity, dyslipidemia, impaired glucose tolerance and hypertension. Plasma APN concentrations are positively correlated with plasma HDL-cholesterol levels [7–11]. Although these findings suggest that APN might have an ability to prevent the development of atherosclerosis by the acceleration of RCT system, the underlying mechanisms for it has not been clarified yet. Recently, we reported that human recombinant APN enhanced the expression of ABCA1 and accelerated the synthesis of apoA-I in a human liver cell line, HepG2 cells, suggesting that APN might increase HDL assembly in the liver [12]. Therefore, in the present study, we investigated the HDL assembly in APN knockout (APN-KO) mice.

2. Materials and methods

2.1. Animals

Adiponectin-knockout (APN-KO) mice were generated as described previously and backcrossed to wild-type (WT) C57BL/6J mice [13]. Both APN-KO and WT mice (male) were housed in temperature and humidity controlled facility with a 12-h light/dark cycle and fed a normal chow diet (MF, OrientalBio Laboratories, Chiba, Japan) and sacrificed for analysis at the age of 8–10 weeks old. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

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Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; APN, adiponectin; apoA-I, apolipoprotein A-I; apoB-100, apolipoprotein B-100; CAD, coronary artery disease; CM, chylomicron; FC, free cholesterol; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; KO, knockout; LDL, low density lipoprotein; MTP, microsomal triglyceride transfer protein; PL, phospholipids; RCT, reverse cholesterol transport; TC, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein; WT, wild-type

0014-5793/$32.00
doi:10.1016/j.febslet.2007.09.038

2.2. Lipid profile by high performance liquid chromatography (HPLC) analysis

One hundred microliter of blood from anesthetized mice (at the age of 8–10 weeks) were drawn from retro-orbital plexus *ad libitum* and plasma was immediately isolated from the collected blood by centrifugation at 4 °C. The lipid profile of plasma was analyzed by an online dual enzymatic method using high performance liquid chromatography (HPLC) at Skylight Biotech Inc. (Akita, Japan), according to the procedure as described by Usui et al. [14]. The plasma concentrations of total cholesterol (TC), triglyceride (TG), free cholesterol (FC) and phospholipids (PL) of four fractioned groups [chylomicron (CM): lipoprotein particle size >80 nm, very low density lipoprotein (VLDL): 30 < particle size <80 nm, low density lipoprotein (LDL): 16 < particle size <30 nm and HDL: 8 < particle size < 16 nm] were determined by using enzymatic reagents (Kyowa Medex, Tokyo, Japan).

2.3. Western blot analysis

Mice plasma or proteins isolated from the liver 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% skim milk at 4 °C overnight. Detection of the immune complexes was carried out by ECL Advance Western Blot Detection System (Amersham Biosciences, UK). Anti-mouse apoA-I antibody (Biodesign, USA), anti-mouse apolipoprotein B48/100 (apoB) antibody (Biodesign, USA), anti-mouse ABCA1 antibody (Novus, USA) and anti-mouse ABCG1 antibody (Santa Cruz, USA) were used for the assay.

2.4. cDNA synthesis and quantitative PCR

One microgram of total RNA isolated from tissues 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. Relative gene expression was quantified using GAPDH as an internal control.

2.5. Primers used in this study

The primes for mouse ABCA1 were ABCA1-forward: 5′-TGGGAACTCCTGCTAAAAT-3′ and ABCA1-reverse: 5′-CCATGTGGTGTGTAGACA-3′, for mouse apoA-I, apoA-I-forward: 5′-GTGGCTCTGGTCTTCCTGAC-3′ and apoA-I-reverse: 5′-ACGGTTGAACCCAGAGTGTC-3′, for mouse apoB, apoB-forward: 5′-TGGGATTCCTCTGCCATCTCGAG-3′ and apoB-reverse: 5′-GTAGAGATCCATCACAGGACAATG-3′, for mouse GAPDH, GAPDH-forward: 5′-ACTCCACTCACGGCAAATTC-3′ and GAPDH-reverse: 5′-TCTCCATGGTGGTGAAGACA-3′.

2.6. Statistical analysis

Values were expressed as means ± S.D. Statistical significance was assessed by Student's *t*-test for paired values and set at $P < 0.05$.

3. Results

3.1. Plasma VLDL-TG levels were increased in APN-KO mice, while there was no significant difference in plasma levels of HDL-cholesterol between WT and APN-KO mice

Blood samples from anesthetized APN-KO ($n = 6$) and WT ($n = 6$) mice at the age of 8–10 weeks were drawn from retro-orbital plexus ad libitum and plasma was immediately isolated by centrifugation at 4 °C. The lipid profile of plasma was analyzed by automatic HPLC and enzymatic methods. The plasma levels of TG, in particular VLDL-TG, were significantly increased in APN-KO mice compared with WT mice (Fig. 1). However, there was no significant difference in plasma TC, HDL-cholesterol and PL levels between APN-KO and WT mice.

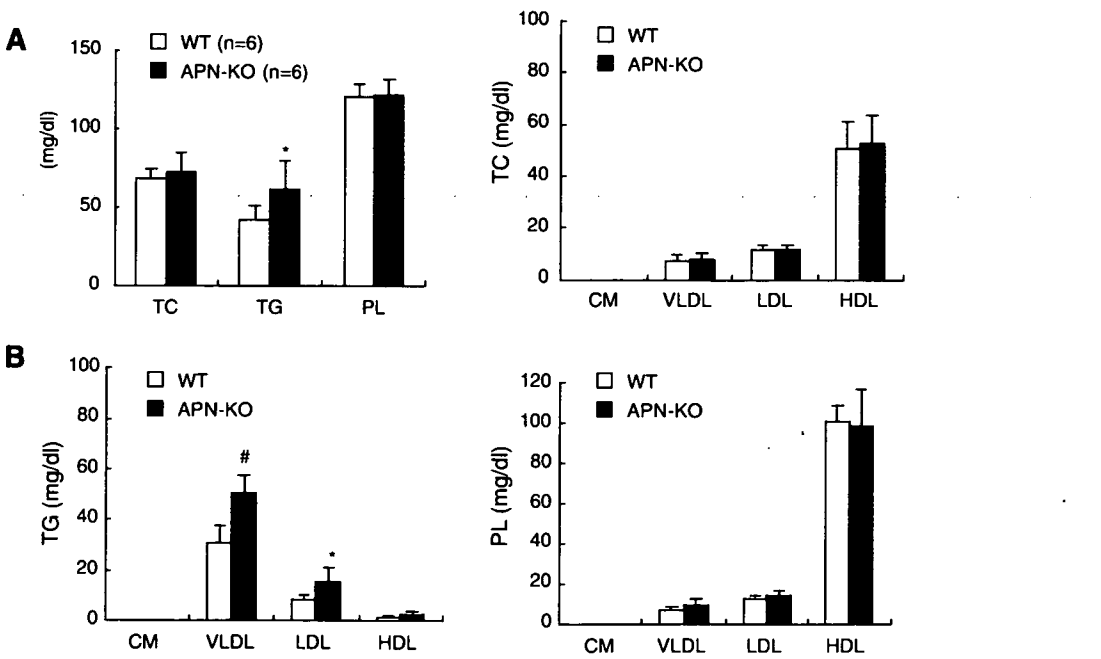

Fig. 1. Plasma lipid profile of APN-KO mice. Blood samples from anesthetized APN-KO ($n = 6$) and WT mice ($n = 6$) at the age of 8–10 weeks were drawn from retro-orbital plexus *ad libitum*. (A) Plasma TC, TG and PL levels. Plasma TG levels of APN-KO mice were significantly higher than those of WT mice. (B) Lipid composition of lipoproteins (CM, VLDL, LDL, and HDL). VLDL-TG concentrations were significantly increased in APN-KO mice compared with WT mice. Values are expressed as means ± S.D. $^{*}P < 0.05$, $^{\#}P < 0.01$, vs. WT mice.

3.2. ApoA-I levels in plasma and the liver were decreased in APN-KO mice

Recently, we reported that APN increased the secretion of apoA-I and decreased the release of apolipoprotein B-100 (apoB-100) from HepG2 cells. Therefore, first, plasma levels of apolipoproteins (apoA-I and apoB-100) in APN-KO mice were investigated by Western blot. As shown in Fig. 2A, plasma levels of apoA-I were slightly decreased in APN-KO mice compared with WT mice, while plasma concentrations of apoB-100 were increased in APN-KO mice. Furthermore, both the mRNA and protein levels of apoA-I in the liver were definitely reduced in APN-KO mice compared to WT mice (Fig. 2B and C). However, there was no significant difference in the mRNA levels of apoB-100 in the liver between APN-KO and WT mice.

3.3. ABCA1 expressions were reduced in APN-KO mice

Finally, we investigated in APN-KO mice the expression levels of ABC transporters (ABCA1 and ABCG1) to generate HDL in the liver. Both the protein (Fig. 3A) and mRNA (Fig. 3B) levels of ABCA1 were significantly decreased in the liver of APN-KO mice compared with WT mice. However, there was no significant difference in the levels of ABCG1 protein between APN-KO and WT mice (Fig. 3A).

4. Discussion

In the present study, we demonstrated for the first time that the expression levels of apoA-I in plasma and the liver were decreased in APN-KO mice as expected from our previous report [12]. Furthermore, we found that the ABCA1 expressions were also reduced in APN-KO mice. These data suggest that low plasma APN concentrations might suppress HDL assembly in the liver, suggesting that the subjects with low serum APN show low plasma HDL-cholesterol levels. However, there was no significant difference in plasma HDL-cholesterol levels between APN-KO and WT mice fed a normal chow diet despite the decrease of apoA-I levels in plasma and the liver of APN-KO mice. There might be some difference in the lipid composition of HDL particles, for example, in the apoA-I mass of HDL particles between APN-KO and WT mice. Abnormal HDL particles with low apoA-I concentrations might have a decreased ability in promoting cholesterol efflux to prevent against atherosclerosis.

Recently, Otabe et al. reported that overexpression of human APN in transgenic mice results in suppression of visceral fat accumulation and reduction of plasma fasting glucose, insulin and leptin levels compared with WT mice [15]. However, these differences were observed only when mice were

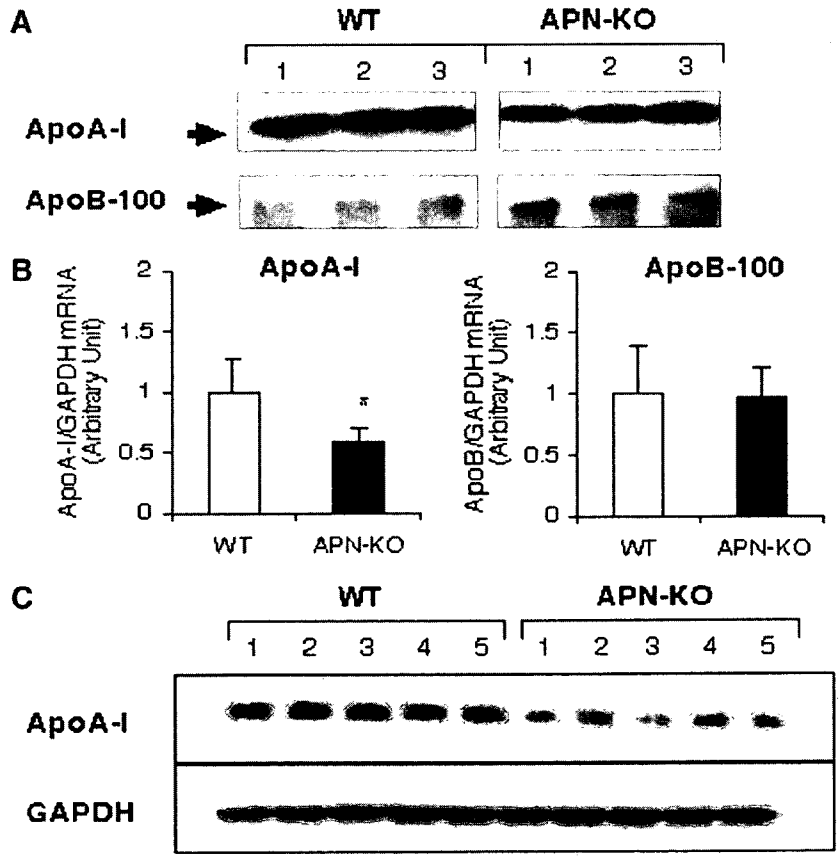

Fig. 2. ApoA-I levels in plasma and the liver were decreased in APN-KO mice. (A) Analysis of plasma apolipoprotein (apoA-I and apoB-100) levels by western blot. Plasma apoA-I levels were slightly decreased in APN-KO mice ($n = 3$) compared with WT mice ($n = 3$), while plasma apoB100 levels were increased in APN-KO mice. (B) The mRNA and (C) the protein expression levels of apoA-I or apoB-100 in the liver. Both the mRNA and protein of apoA-I levels were significantly reduced in the liver of APN-KO mice ($n = 5$) compared with WT mice ($n = 5$). Relative gene expression determined by quantitative PCR was quantified using GAPDH as an internal control. Values are expressed as means ± S.D. $^*P < 0.05$, vs. WT mice.

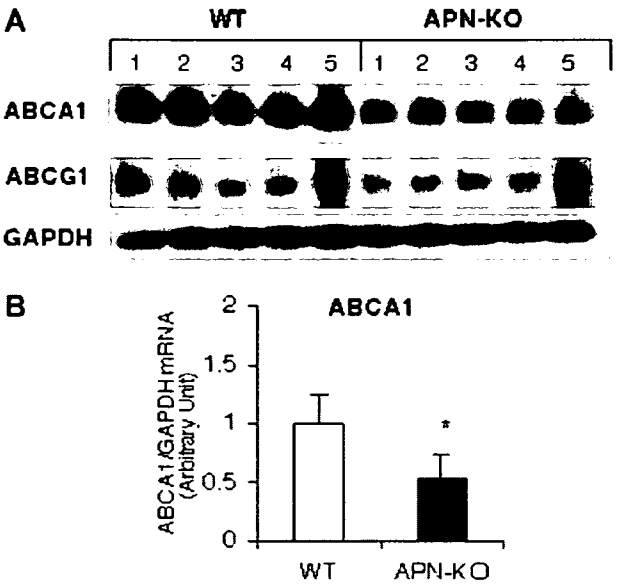

Fig. 3. ABCA1 expressions were reduced in the liver of APN-KO mice. Both the protein (in A) and mRNA (in B) levels of ABCA1 were significantly reduced in the liver of APN-KO mice ($n = 5$) compared with WT mice ($n = 5$). Relative gene expression determined by quantitative PCR was quantified using GAPDH as an internal control. Values are expressed as means ± S.D. $^*P < 0.05$, vs. WT mice.

fed a high fat/high sucrose diet, but not a normal chow diet. In our study, the lipid profiles of APN-KO mice were examined with feeding only a normal chow diet. The effect of APN on some parameters including HDL-cholesterol levels might be dependent on the nutritional condition. Therefore, possibly, low plasma HDL-cholesterol might be observed in APN-KO mice fed with over nutrition like a high cholesterol/high fat diet. These issues will be studied in the near future.

We found that apoB-100-containing lipoproteins (VLDL and LDL)-TG, in particular, VLDL-TG levels were increased in APN-KO mice. Recently, it is clinically focused that the accumulation of TG-rich lipoprotein like VLDL in plasma is also strongly linked to CAD as well as that of an atherogenic lipoprotein, LDL [16]. Therefore, plasma VLDL accumulation might be in part associated with the development of atherosclerosis in APN deficiency.

Although plasma VLDL-TG levels were significantly increased in APN-KO mice, there was no significant difference in the apoB-100 expression in the liver between APN-KO and WT mice. As shown in our previous and Neumeier's reports [12,17], in vitro, the apoB secretion from HepG2 cells or primary human hepatocytes was enhanced by recombinant APN, while the mRNA expression levels of apoB were not influenced by APN. Therefore, APN might be involved in the assembly or secretion of VLDL in the liver, but not in apoB-100 synthesis. Microsomal triglyceride transfer protein (MTP) is well known to be an intracellular lipid transfer protein, closely associated with VLDL output from the liver [18,19]. We need to examine the effect of APN on MTP expression (or activity) in the liver.

In summary, we clarified that apoA-I synthesis and ABCA1 expression in the liver were suppressed in APN-KO mice. APN might play an important role in preventing the development of atherosclerosis by the acceleration of HDL assembly in RCT system.

Acknowledgements: The authors thank Hiromi Kogo-Nishinaka for excellent technical assistance. This work was supported by Grants-in-Aid (Nos. 11557055 and 10671070) from the Japanese Ministry of Education, Science, Sports, and Culture, an International HDL Research Awards Program Grant from Pfizer, a grant from the Takeda Medical Research Foundation, and a Grant from the ONO Medical Research Foundation, and the Novartis Foundation for Gerontological Research to S. Yamashita. This work was also supported by a Grant from Astellas Pharma Inc. to F. Matsuura.

References

[1] Miller, N.E., La Ville, A. and Crook, D. (1985) Direct evidence that reverse cholesterol transport is mediated by high density lipoprotein in rabbit. Nature 314, 109–111.
[2] Yokoyama, S. (2005) Assembly of high density lipoprotein by the ABCA1/apolipoprotein pathway. Curr. Opin. Lipidol. 16, 269–279.
[3] Wang, N., Lan, D., Cheng, W., Matsuura, F. and Tall, A.R. (2004) ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins, Proc. Natl. Acad. Sci. USA 101, 9774–9779.
[4] Brooks-Wilson, A., Marcil, M., Clee, S.M., Zhang, L.H., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J.A., Molhuizen, H.O., Loubser, O., Ouelette, B.F., Fichter, K., Ashbourne-Excoffon, K.J., Sensen, C.W., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S., Kastelein, J.J. and Hayden, M.R. (1999) Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. Nat. Genet. 22, 336–345.
[5] Bodzioch, M., Orso, E., Klucken, J., Langmann, T., Bottcher, A., Diederich, W., Drobnik, W., Barlage, S., Buchler, C., Porsch-Ozcurumez, M., Kaminski, W.E., Hahmann, H.W., Oette, K., Rothe, G., Aslanidis, C., Lackner, K.J. and Schmitz, G. (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. Nat. Genet. 22, 347–351.
[6] Rust, S., Walter, M., Funke, H., von Eckardstein, A., Cullen, P., Kroes, H.Y., Hordijk, R., Geisel, J., Kastelein, J., Molhuizen, H.O., Schreiner, M., Mischke, A., Hahmann, H.W. and Assmann, G. (1999) Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. Nat. Genet. 22, 352–355.
[7] Ryo, M., Nakamura, T., Kihara, S., Kumada, M., Shibazaki, S., Takahashi, T., Nagai, M., Matsuzawa, Y. and Funahashi, T. (2004) Adiponectin as a biomarker of the metabolic syndrome. Circ. J. 68, 975–981.
[8] Kumada, M., Kihara, S., Sumitsuji, S., Kawamoto, T., Matsumoto, S., Ouchi, N., Arita, Y., Okamoto, Y., Shimomura, I., Hiraoka, H., Nakamura, T., Funahashi, T. and Matsuzawa, Y.Osaka CAD Study Group (2003) Coronary artery disease, Association of hypoadiponectinemia with coronary artery disease in men. Arterioscler. Thromb. Vasc. Biol. 23, 85–89.
[9] Zoccali, C., Mallamaci, F., Tripepi, G., Benedetto, F.A., Cutrupi, S., Parlongo, S., Malatino, L.S., Bonanno, G., Seminara, G., Rapisarda, F., Fatusso, P., Buemi, M., Nicocia, G., Tanaka, S., Ouchi, N., Kihara, S., Funahashi, T. and Matsuzawa, Y. (2002) Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease. J. Am. Soc. Nephrol. 13, 134–141.
[10] Cnop, M., Havel, P.J., Utzschneider, K.M., Carr, D.B., Sinha, M.K., Boyko, E.J., Retzlaff, B.M., Knopp, R.H., Brunzell, J.D. and Kahn, S.E. (2003) Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia 46, 459–469.
[11] Ouchi, N., Kihara, S., Arita, Y., Nishida, M., Mastsuyama, A., Okamoto, Y., Ishigami, M., Kuriyama, H., Kishida, K., Nishizawa, H., Hotta, K., Muraguchi, M., Ohmoto, Y., Yamashita, S., Funahashi, T. and Matsuzawa, Y. (2001) Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and

class A scavenger receptor expression in human monocyte-derived macrophages. Circulation 103, 1057–1063.
[12] Matsuura, F., Oku, H., Koseki, M., Sandoval, J.C., Yuasa-Kawase, M., Tsubakio-Yamamoto, K., Masuda, D., Maeda, N., Tsujii, K., Ishigami, M., Nishida, M., Hirano, K., Kihara, S., Hori, M., Shimomura, I. and Yamashita, S. (2007) Adiponectin accelerates reverse cholesterol transport by increasing high density lipoprotein assembly in the liver. Biochem. Biophys. Res. Commun. 358, 1091–1095.
[13] Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Mastuda, M., Nagaretani, H., Furuyama, N., Kondo, H., Takahasshi, M., Arita, Y., Komuro, R., Ouchi, N., Kihara, S., Tochino, Y., Okutomi, K., Horie, M., Takeda, S., Aoyama, T., Funahashi, T. and Matsuzawa, Y. (2002) Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. Nat. Med. 8, 731–737.
[14] Usui, S., Hara, Y., Hosaki, S. and Okazaki, M. (2002) A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. J. Lipid Res. 43, 805–814.
[15] Otabe, S., Yuan, X., Fukutani, T., Wada, N., Hashinaga, T., Nakayama, H., Hirota, N., Kojima, M. and Yamada, K. (2007) Overexpression of human adiponectin in transgenic mice results in suppression of fat accumulation and prevention of premature death by high-calorie diet. Am. J. Physiol. Endocrinol. Metab. 293, 210–218.
[16] Byrne, C.D. (1999) Triglyceride-rich lipoproteins: are links with atherosclerosis mediated by a procoagulant and proinflammatory phenotype? Atherosclerosis 145, 1–15.
[17] Neumeier, M., Sigruener, A., Eggenhofer, E., Weigert, J., Weiss, T.S., Schaeffler, A., Schlitt, H.J., Aslanidis, C., Piso, P., Langmann, T., Schmitz, G., Scholmerich, J. and Buechler, C. (2007) High molecular weight adiponectin reduces apolipoprotein B and E release in human hepatocytes. Biochem. Biophys. Res. Commun. 352, 543–548.
[18] Gordon, D.A., Jamil, H., Sharp, D., Mullaney, D., Yao, Z., Gregg, R.E. and Wetterau, J. (1994) Secretion of apolipoprotein B-containing lipoproteins from HeLa cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availability. Proc. Natl. Acad. Sci. USA 91, 7628–7632.
[19] Lin, M.C., Arbeeny, C., Bergquist, K., Kienzle, B., Gordon, D.A. and Wetterau, J.R. (1994) Cloning and regulation of hamster microsomal triglyceride transfer protein. The regulation is independent from that of other hepatic and intestinal proteins which participate in the transport of fatty acids and triglycerides. J. Biol. Chem. 269, 29138–29145.

Geriatr Gerontol Int 2007; 7: 205–214

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

Accepted for publication 12 March 2007.

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doi: 10.1111/j.1447-0594.2007.00403.x

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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.

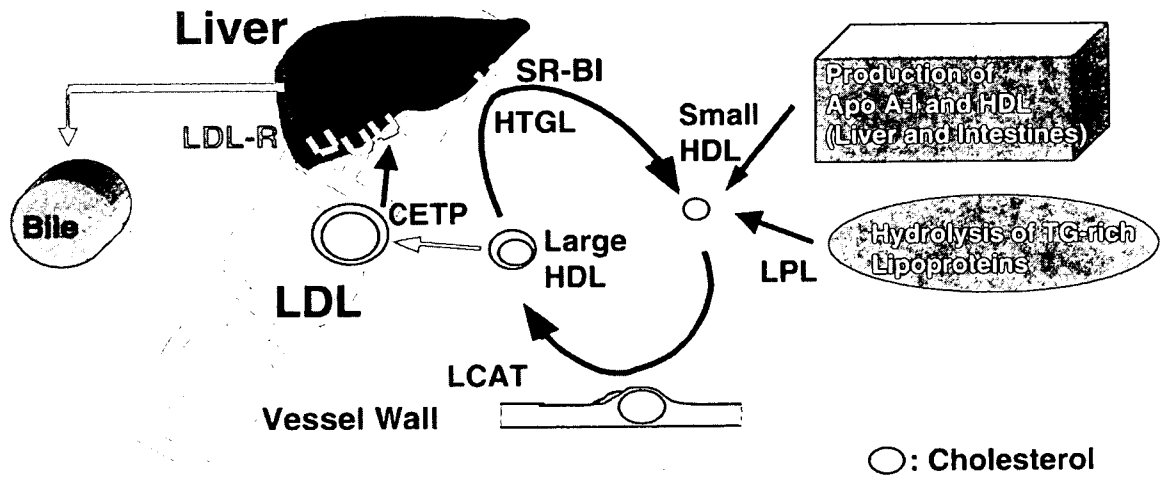

Figure 1 Molecular mechanisms of reverse cholesterol transport as a protective system against atherosclerosis.