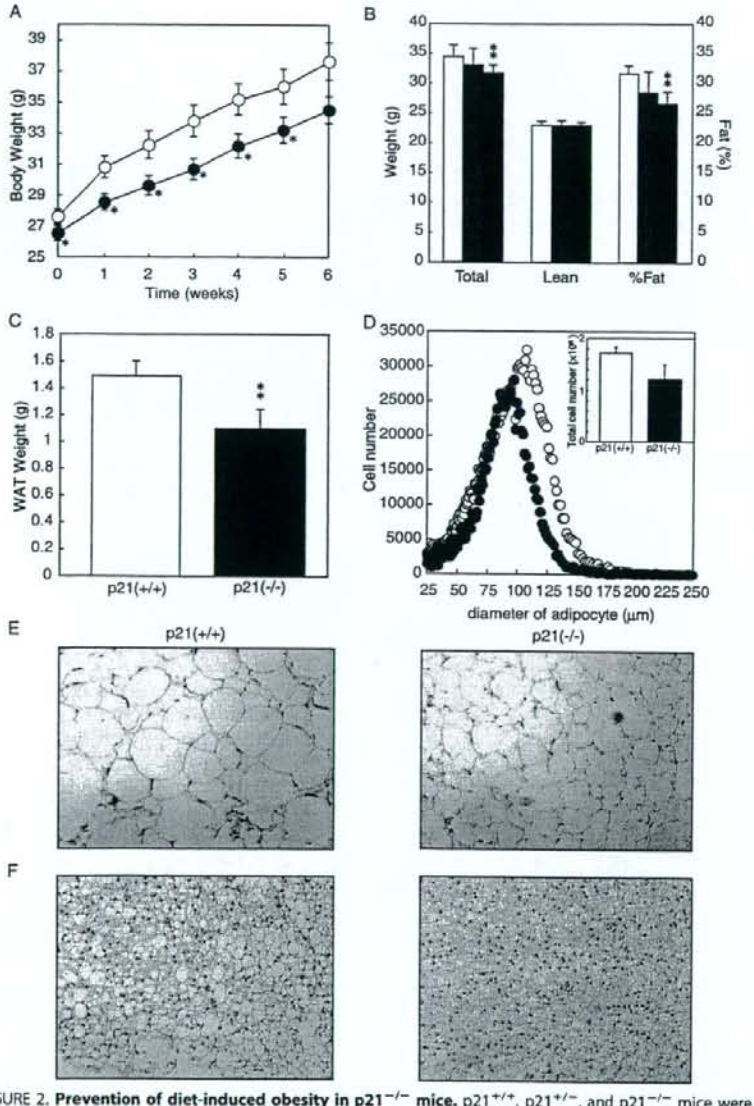


FIGURE 2. **Prevention of diet-induced obesity in $p21^{-/-}$ mice.** $p21^{+/+}$, $p21^{+/-}$, and $p21^{-/-}$ mice were placed on a high fat and high sucrose (HFHS) diet at 10 weeks of age for a total of 6 weeks. *A*, body weight curves on a HFHS diet. *B*, total and lean mass and % fat in $p21^{+/+}$ (*white bars*), $p21^{+/-}$ (*gray bars*), and $p21^{-/-}$ (*black bars*) mice as estimated by DEXA. *C*, weight of epididymal fat pads in $p21^{+/+}$ and $p21^{-/-}$ mice. *WAT*, white adipose tissue. *D*, size distribution and total cell number (*inset*) of adipocytes from white adipose tissue. *E* and *F*, microscopic examination of white (*E*) and brown (*F*) adipose tissues (hematoxylin/eosin stain). Values represent the means ± S.E. from 14 mice per group. $p < 0.05$ (*) and at $p < 0.01$ (**) for $p21^{-/-}$ compared with $p21^{+/+}$ groups.

growth in the absence of p21 (supplemental Fig. A1, *A* and *B*). Upon induction of differentiation, $p21^{-/-}$ MEF cells began to differentiate sooner; however, at terminal differentiation the size of the resultant adipocytes was smaller than differentiated wild type $p21^{+/+}$ MEFs (supplemental Fig. A1*C*). These data demonstrate that p21 contributes to, but is not indispensable, for adipocyte differentiation.

*Metabolic Effects of the Chronic Absence of p21 in Vivo*—Consistent with the results from cultured cells, adiposity of chow diet-fed $p21^{-/-}$ mice was normal. Growth curves were nearly identical between $p21^{-/-}$ and $p21^{+/+}$ mice (supplemental Fig. A2*A*). Body weight, total fat weight (by DEXA), and adipocyte size did not significantly changed with only a minimal trend toward a decrease in $p21^{-/-}$ mice (supplemental Fig. A2, *B* and *C*, and A3, A–C). There were no significant changes in plasma glucose, insulin, lipids, or leptin levels (supplemental Table A1). Insulin sensitivity and secretion did not change in $p21^{-/-}$ mice as assessed by glucose and insulin tolerance tests (supplemental Fig. A2, *D* and *E*, and Table A1).

Next, the role of p21 in adipogenesis in diet-induced obesity was evaluated. Our preliminary data indicated that gene expression of p21 was highly induced by high fat diet, suggesting a role of this protein in the hypertrophic change of adipocytes (data not shown). When $p21^{-/-}$ mice were fed the HFHS diet, which rapidly induces obesity and insulin resistance, the degree of body weight gain was ameliorated compared with strain-matched C57BL/6 $p21^{+/+}$ controls (Fig. 2*A*). The retarded body weight gain in $p21^{-/-}$ mice was due to decreased fat mass as estimated by DEXA (Fig. 2*B*). The weight of epididymal fat pads was also significantly decreased in $p21^{-/-}$ mice (Fig. 2*C*). Total fat weight of $p21^{+/-}$ mice was between those of $p21^{+/+}$ and $p21^{-/-}$ mice, demonstrating the dose-dependent effect of p21 on adiposity (Fig. 2*B*). The size distribution of adipocytes from the epididymal adipose tissue, as estimated by a Coulter counter, also confirmed a significant decrease in the size of $p21^{-/-}$ adipocytes (Fig. 2*D*). Consistently, histological analysis of adipose tissue demonstrated that the marked enlargement of adipocytes in HFHS-fed mice was suppressed in $p21^{-/-}$ mice (Fig. 2*E*). The presence of large lipid vacuoles, which emerged in the cytosol of brown fat cells of HFHS diet-fed $p21^{+/+}$ mice, was decreased in $p21^{-/-}$ mice (Fig. 2*F*). In fact, brown adipose tissue of $p21^{-/-}$ mice fed HFHS diets appeared similar to

$p21^{+/+}$ mice on a chow diet (compare Fig. 2*F* and supplemental Fig. A3*D*).

The amelioration of obesity was accompanied by marked improvement of insulin resistance (Fig. 3). Improved insulin sensitivity in HFHS-fed $p21^{-/-}$ mice was supported by decreased plasma insulin levels despite similar or slight trends of decreasing plasma glucose levels in fasted and fed conditions and in intravenous glucose tolerance tests (supplemental Table A2, Fig. 3, *A* and *B*). Insulin tolerance tests showed a more prominent decrease in plasma glucose levels after injection of insulin in $p21^{-/-}$ mice than in control mice (Fig. 3*B*). These data indicate that gene disruption of p21 causes a partial protection from diet-induced obesity and a marked improvement in insulin resistance. This protection from diet-induced obesity was not due to differences in food intake (2.95 ± 0.10 *versus* 2.86 ± 0.05 g/day for $p21^{+/+}$ and $p21^{-/-}$ mice, respectively; $n = 6$; data were collected over a 4-week period).

*Gene Expression in Metabolic Tissues of $p21^{-/-}$ Mice*—Expression of various genes in white adipose tissue of HFHS-fed $p21^{+/+}$ and $p21^{-/-}$ mice were estimated by Northern blot analysis (Fig. 4*A*). Adipogenic transcription factors such as PPARγ, C/EBPα, and C/EBPβ were not different between $p21^{+/+}$ and $p21^{-/-}$ mice. Expression of leptin and ADRP, known to be up-regulated in white adipose tissue in obesity, was suppressed in $p21^{-/-}$ mice on a HFHS diet, whereas adipsin, known to be suppressed in obesity, was up-regulated in $p21^{-/-}$ adipose tissues to varying degrees. As estimated by reverse transcription-PCR (supplemental Fig.A5*A*), decreased leptin and increased adiponectin were observed, consistent with amelioration of obesity in $p21^{-/-}$ mice on a HFHS diet (supplemental Fig. A5*A*). Expression of monocyte chemoattractant protein 1 and tumor necrosis factor α was also decreased, indicating that macrophage infiltration could be suppressed by the absence of p21 (supplemental Fig. A5*B*). PGC1α in brown adipose tissue, SCD-1 in the liver, and PPARδ in skeletal muscle are all known to be involved in energy consumption. These genes were decreased in diet-induced obesity in $p21^{+/+}$ mice, and their levels were normalized in $p21^{-/-}$ mice, indicating a shift of energy balance toward to expenditure (Fig. 4*B*).

*Potential Involvement of Apoptosis in Mechanisms by Which p21 Deficiency Protects against Diet-induced Obesity*—To determine the mechanism by which the absence of p21 protects against diet-induced obesity and insulin resistance, p21 was suppressed in 3T3-L1 cells after they had already differentiated into mature adipocytes. In contrast to infection of adenoviral RNAi before the differentiation protocol (Fig. 1, *C–E*), acute suppression of p21 at day 6 caused inhibition of lipid accumulation, detachment of the lipid-containing cells, and a marked change in color of the medium (Fig. 5*A*). TUNEL staining confirmed that these changes significantly involve apoptosis (Fig. 5, *B* and *C*). Supporting these observations, immunoblot analysis indicated that inhibition of p21 was followed by a time-dependent induction of caspase-3, the terminal player of apoptosis, but no change in expression of Bcl-2, a gene that plays a protective role from apoptosis (Fig. 6*B*). p53 phosphorylated at Ser-46, associated with apoptosis (26), was induced at day 8, 48 h after infection with p21RNAi adenovirus (Fig. 6*B*). These data suggest that p21 inhibition during the terminal differentiation of

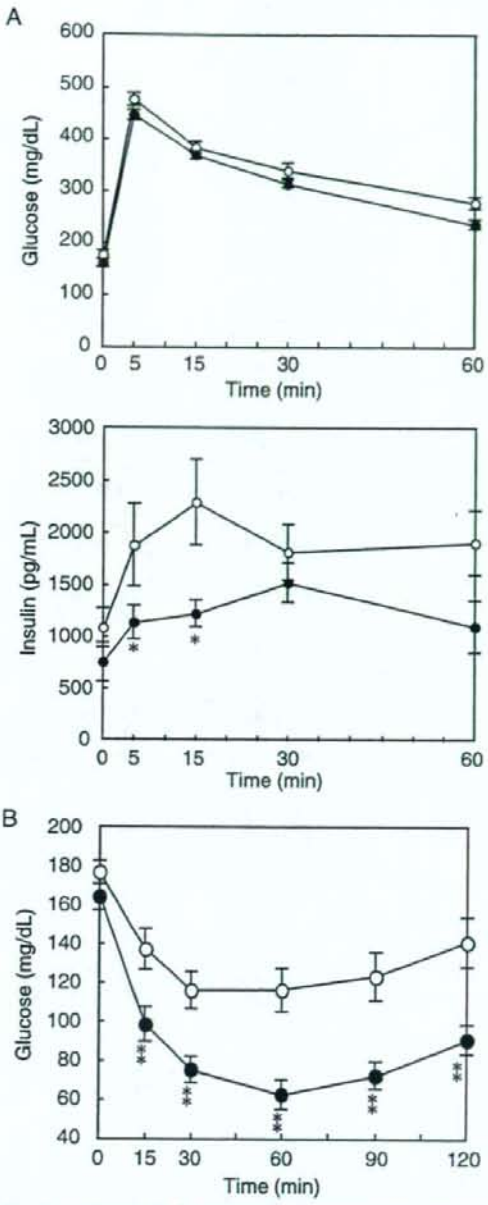


FIGURE 3. **Protection of $p21^{-/-}$ mice from insulin resistance caused by diet-induced obesity.** Glucose tolerance test (*A*) and insulin tolerance test (*B*) on $p21^{+/+}$ (*white circles*) and $p21^{-/-}$ mice (*black circles*). Mice were fed the HFHS diet for 4 weeks and injected with 2 mg/kg D-glucose (*A*) or 0.75 units/kg insulin for 5 weeks under the HFHS diet condition (*B*). The panels show serum glucose concentration (*upper panel* of *A* and *B*) and insulin concentration (*lower panel* of *A*) at the indicated time (min). Values represent the means ± S.E. from 14 mice per group. $p < 0.05$ (*) and at $p < 0.01$ (**) for $p21^{-/-}$ compared with $p21^{+/+}$ groups.

adipocytes could induce apoptosis, suppressing the hypertrophic changes required for completion of differentiation. p53, the most potent inducer of p21, is known to be activated in

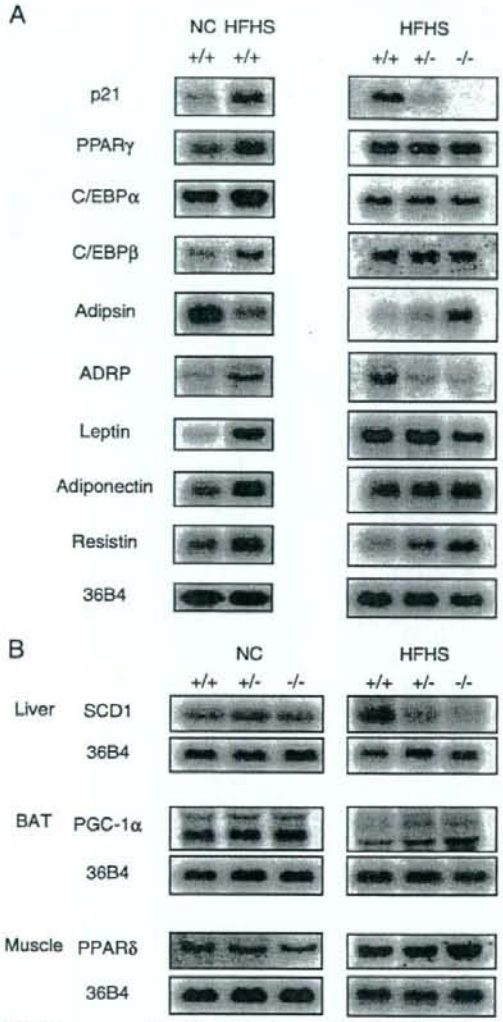


FIGURE 4. **Gene expression of p21$^{-/-}$ mice on a HFHS diet.** *A*, Northern blot analysis in epididymal fat pads in p21$^{+/+}$ and p21$^{-/-}$ mice fed a HFHS diet for 6 weeks. *Left panel*, p21$^{+/+}$ mice on a normal chow (*NC*) diet as a control and HFHS diet. *Right panel*, p21$^{+/+}$, p21$^{+/-}$, and p21$^{-/-}$ mice on a HFHS diet for 6 weeks. *B*, Northern blot analysis of livers, blown adipose tissues (*BAT*) and skeletal muscles in p21 knock-out mice was shown. *Left panel*, p21$^{+/+}$, p21$^{+/-}$, and p21$^{-/-}$ mice on a normal chow diet as a control. *Right panel*, p21$^{+/+}$, p21$^{+/-}$, and p21$^{-/-}$ mice fed a HFHS diet for 6 weeks. All mice were sacrificed at fasted state for 12 h. Total RNA was extracted from the organs of six mice. Equal aliquots of the RNA samples were pooled for each organ (10 μg) and subjected to Northern blot analysis for hybridization with the indicated cDNA probes (36B4 as a loading control).

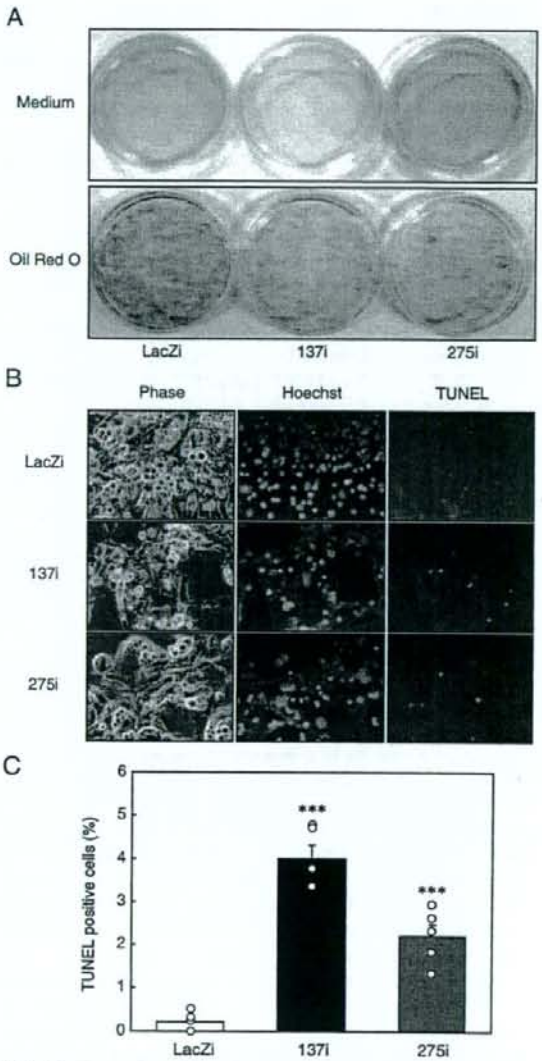


FIGURE 5. **p21 gene knockdown induces apoptosis in 3T3-L1 adipocytes.** *A*, 3T3-L1 cells were differentiated into adipocytes, and p21 RNAi adenovirus was infected at day 6. Media (*upper panel*) and Oil Red O staining of 3T3-L1 cells (*lower panel*) 4 days after infection of p21 RNAi (*137i* or *275i*), or control LacZi adenovirus (day 10). *B*, apoptosis of 3T3-L1 adipocytes after p21 knockdown at day 10. Microscopic examinations of the cells with light phase contrast for morphology (phase (*left*)), Hoechst 33342 for nuclear staining (Hoechst (*middle*)), and TUNEL staining for apoptotic cells (TUNEL (*right*)) are shown at a magnification of ×400. *C*, emergence of apoptotic cells was determined by the ratio of TUNEL positive cells to Hoechst 33342-positive cells. Values represent the mean ± S.E. from six dishes per group. ***, at $p < 0.0001$ for 137i and 275i *versus* LacZi adenovirus-infected cells.

response to cellular stresses. p53 protein has been detected in 3T3-L1 cells and remains constant during intracellular accumulation of lipids in Fig. 6*A*, consistent with the previous report (27, 28). However, phosphorylation of p53 protein at Ser-15 and Ser-20 is the key process for p21 trans-activation (29, 30). In accordance with p21 induction, the phosphorylation of these sites was elevated during the adipocyte differentiation. In contrast, phosphorylation of p53 at Ser-46 was reported to be associated with apoptosis (26). This apoptosis-related change of p53 was induced at the late stage of differentiation in the 3T3-L1 adipocytes after the Ser-15 and Ser-20 phosphorylation (Fig. 6*A*).

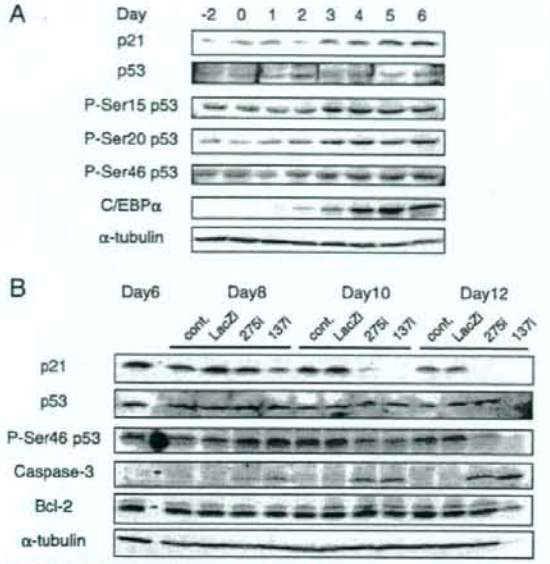


FIGURE 6. **Activation of caspase-3 by p21 knockdown in 3T3-L1 adipocytes after intrinsic activation of p21/p53.** *A*, time-course changes of p53 and p21 activation in 3T3-L1 cells during adipocyte differentiation. 3T3-L1 fibroblasts were differentiated into adipocytes according to the standard protocol (see "Experimental Procedures"). The level of p53, p21 proteins, and phosphorylation (*P*) of p53 at Ser-15, Ser-20, and Ser-46 crucial for p21 transactivation were analyzed at the indicated days by immunoblot analysis. *B*, 3T3-L1 cells were induced to differentiate into adipocytes and infected with p21 RNAi adenovirus at day 6. Changes of p21, p53, Ser-46 phosphorylation of p53, caspase-3, Bcl-2, and α-tubulin (as a loading control) proteins in 3T3-L1 adipocytes at the indicated days after the induction for adipocyte differentiation were determined by immunoblot analysis.

To examine the involvement of apoptosis in the suppression of p21 *in vivo*, adenoviral RNAi for p21 was injected directly into epididymal adipose tissue of HFHS-fed mice. Significant inhibition of p21 expression was obtained 4 days after the infection (Fig. 7*A*), causing a trend to loss of body weight (Fig. 7*B*) accompanied by decreased glucose and insulin levels in insulin and glucose tolerance tests, respectively (Fig. 7, *C* and *D*). TUNEL staining demonstrated induction of adipocyte apoptosis by p21 knockdown (Fig. 7*E*). These data implicated that p21 could be induced by diet-induced obesity and contributes to protection from apoptosis during adipocyte hypertrophy.

## DISCUSSION

*Role of p21 in Adipocyte Differentiation and Apoptosis*—Our current study clearly demonstrates that p21 plays a crucial role in later stages of differentiation and hypertrophy of adipocytes. p21 is induced during adipogenesis and sustained during terminal differentiation. p21 knockdown experiments demonstrated that p21 contributes to but is not absolutely required for adipocyte differentiation. Emergence of vigorous apoptosis upon acute p21 knockdown during the terminal phase of 3T3-L1 differentiation and in hypertrophic adipose tissue during diet-induced obesity in mice reveals that p21 is crucial for adipocyte hypertrophy rather than for the initial stages of differentiation. *In vivo*, p21 was highly induced in enlarged adipose tissue of diet-induced obese mice, and its absence ameliorated obesity. In contrast to acute knockdown experiments of p21, apoptosis was not detected in adipose tissue from $p21^{-/-}$ mice on a HFHS diet or in $p21^{-/-}$ MEF throughout adipocyte differentiation (data not shown). We speculate that in the absence of p21, adipocytes might not become hypertrophic enough to cause apoptosis.

In normal physiology, it is conceivable that the increased accumulation of intracellular lipids, which occurs during differentiation, is a cellular stress to large adipocytes, leading to activation of the p53/p21 pathway. In this situation p21 might be required for survival of hypertrophic adipocytes. This potential of p21 is mediated through its anti-apoptotic actions rather than its growth arrest properties. It has long been speculated that adipocyte hypertrophy involves apoptosis (31, 32), but evidence of apoptosis was not appreciably observed. Our data provide evidence that in the presence of p21, hypertrophic adipocytes are relatively protected against apoptosis, possibly explaining this discrepancy.

*Role of p53 in Sustained p21 Expression in Adipocytes*—Previous studies have shown that the transcription factors Foxo1, SREBP-1c/ADD1, and C/EBP family are involved in adipocyte differentiation, and all three have strong transcriptional activity for the p21 promoter (4, 13, 33, 34). Each of these factors likely contributes to the induction of p21 during the initial stages of differentiation in normal adipocytes. Although expression of p53, a dominant regulator of p21, remains stable at both mRNA and protein levels during adipocyte differentiation, we found that Ser-15 and Ser-20 of p53, crucial sites for trans-activation of p21 (29, 35) were hyperphosphorylated during the later stages of differentiation. It can be speculated that sustained p21 expression during terminal differentiation or obesity-related activation is mediated through p53 activation. We previously observed that the p53/p21 pathway is activated in hypertrophied adipocytes, such as from leptin-deficient ob/ob mice, and also in fatty livers (15, 16). This is consistent with our current observation in adipose tissue of HFHS diet-induced obese mice. p53 is the key sensor of indications of cellular stress such as DNA damage and ultimately determines whether the cell will go through cell growth arrest or apoptosis. p21 is an established cell cycle arrest inducer but has been also implicated to be involved in inhibition of apoptosis (12). In 3T3L1 adipocytes, p53 activation, as indicated by phosphorylation at Ser-15 and Ser-20, could result from lipid accumulation. Furthermore, phosphorylation of p53 at Ser-46 at the later stage of differentiation indicated the potential risk of apoptosis (26) that was masked by p21. Our current data suggest that hypertrophic adipocytes in obesity are also under considerable stress and are vulnerable to apoptosis, which under normal conditions, could be inhibited by activation of p21. Thus, we postulate that p21 plays a role in adipogenesis by halting cell cycle but, more importantly, by preventing the apoptosis of hypertrophic adipocytes.

*Effect of p21 on Obesity and Insulin Resistance*—Of clinical relevance, amelioration of high fat diet-induced obesity in

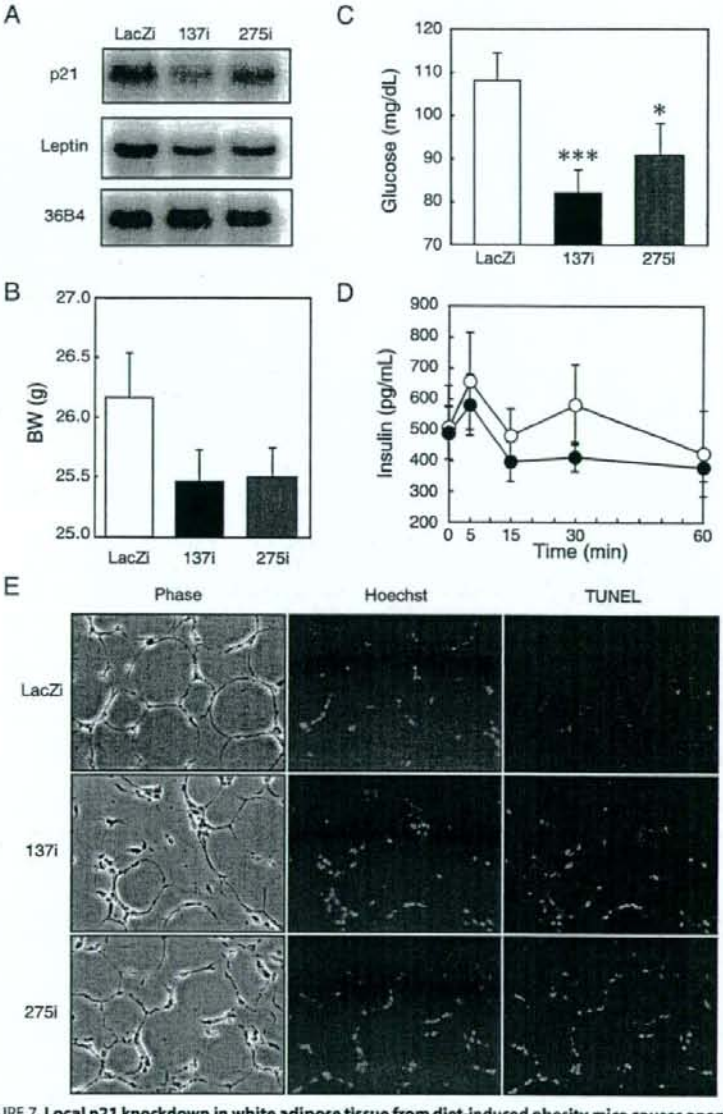


FIGURE 7. **Local p21 knockdown in white adipose tissue from diet-induced obesity mice causes apoptosis.** C57BL/6J mice (6 weeks of age) were fed a HFHS diet for 3 weeks. Adenoviral p21 RNAi (137i (*black*) or 275i (*gray*)) or LacZi (*white*) as a control was directly injected into epididymal white fat pads. After injection of adenovirus, these mice were fed a HFHS diet. Four days after the local infection, mice were sacrificed, and adipose tissues were collected. Total RNA of equally pooled aliquots (10 $\mu$g) extracted from the fat pads of six mice was subjected to Northern blot analysis for p21 and leptin (*A*). Body weights (*BW*), blood glucose concentrations 5 min after intraperitoneal injection of insulin 4 days after the local infection (*C*), and plasma insulin concentrations on an intravenous glucose tolerance test 6 days after the local infection (*D*) are shown in the diet-induced obesity mice after p21 knockdown in white adipose tissue ($n = 12$). Shown are TUNEL assays for apoptotic cells with light phase contrast microscopy and Hoechst 33342 staining for nuclear staining 4 days after the local infection (*E*). Values represent the means ± S.E. from 12 mice per group. $p < 0.05$ (*) and at $p < 0.001$ (***) for 137i and 275i *versus* LacZi adenovirus infected mice.

$p21^{-/-}$ mice was associated with improved insulin sensitivity. Adiposity in $p21^{-/-}$ mice was originally reported to be slightly increased as estimated by epididymal fat pads (22). The cause of this discrepancy with our data is currently unknown; however, we speculate that it could be strain and/or sex differences in which we used male $p21^{-/-}$ mice backcrossed to C57BL6 *versus* the original work on female mice on the undescribed back ground. We presume that p21 in adipocytes plays the major role in obesity and insulin resistance since even partial knockdown of p21 in epididymal fat pads effectively reproduced the apoptosis and decreased the size of adipocytes as observed in $p21^{-/-}$ mice on HFHS diet. Because decreased size of adipose tissue was associated with reduction in monocyte chemoattractant protein 1 and tumor necrosis factor $\alpha$ expression (supplemental Fig. A5*B*), macrophage infiltration and subsequent proinflammatory responses in obesity were suppressed in $p21^{-/-}$ mice. The absence of p21 in macrophages could contribute to inhibition of inflammation in adipose tissues as reported in atherosclerosis (36). There were only mild changes in the expression of genes related to energy metabolism in liver and skeletal muscle of $p21^{-/-}$ mice on HFHS diet, indicating that they are not the primary organs responsible for anti-obesity in the absence of p21. It should be noted that in brown adipose tissue, PGC1$\alpha$ was slightly restored in $p21^{-/-}$ mice. Diet-induced obesity is usually accompanied by fat accumulation and decreased burning function in this tissue (6), which was prevented in HFHS-fed $p21^{-/-}$ mice Considering the similar food intake between $p21^{+/+}$ and $p21^{-/-}$ mice, it is possible that p21 deficiency contributed to amelioration of diet-induced obesity and insulin resistance at least partially by restoring the fat-burning function of brown fat tissue.

*A New Link between Cell Growth and Metabolism*—Our study provides evidence that factors responsible for the regulation of cell growth and survival are also involved in metabolism. A recent report on a direct interaction between p21 and Akt substantiates this novel link between a CDK inhibitor and metabolism (37). The p53/p21 pathway might play a determining role in the fate of hyper-

trophic adipocytes in situations of excess energy. In the context of obesity-related diseases including insulin resistance and diabetes, inhibition of p21 in adipocytes might have a therapeutic effect by suppressing adipose tissue expansion. However, it is also possible p21-mediated adipocyte differentiation is important for proper storage of excess lipids in adipose tissue, thereby protecting against ectopic lipid storage and insulin resistance. Further studies are needed to clarify the precise mechanism by which p53/p21 pathway is regulated and involved in obesity and insulin resistance in adipose tissue. Recent reports on another CDK inhibitor, p27, as well as Skp2, the F-box protein that controls ubiquitin-mediated degradation of p27, implicates the Skp2/p27 pathway in determining the cell number of adipocytes and pancreatic $\beta$ cells (5, 22, 38–40). Mice that lack CDK4 develop insulin-dependent diabetes as a result of a reduction in islet mass, whereas deletion of p27 ameliorates hyperglycemia in diabetic mice by maintaining compensatory islet hyperplasia and hyperinsulinemia, suggesting that these factors are involved in cell cycle regulation and could also play a role in $\beta$ cell mass and function (38, 41). However, p21 did not have a significant impact on $\beta$ cell function in our study (supplemental Table A3) as recently reported (42). Taken together, these data implicate p21 in controlling adipocyte cell size and p27 in determining cell number, reflecting distinct regulations and adipose tissue growth by both of the major CDK inhibitors.

In conclusion, our data demonstrate that p21 plays dual roles in both adipocyte differentiation through cell cycle arrest and in adipocyte hypertrophy through its anti-apoptotic action. Via these mechanisms mutually connected, p21 supports adipose tissue expansion during obesity linking to insulin resistance. Our data suggest that a cell cycle regulator can be involved in a wide range of physiology and pathophysiology in adipocytes, providing a new link between cell growth and metabolism.

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# Adiponectin prevents atherosclerosis by increasing cholesterol efflux from macrophages

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ABSTRACT

Plasma high density lipoprotein (HDL)-cholesterol levels are inversely correlated to the risk of atherosclerotic cardiovascular diseases. Reverse cholesterol transport (RCT) is one of the major protective systems against atherosclerosis, in which HDL particles play a crucial role to carry cholesterol derived from peripheral tissues to the liver. Recently, ATP-binding cassette transporters (ABCA1, ABCG1) and scavenger receptor (SR-BI) have been identified as important membrane receptors to generate HDL by removing cholesterol from foam cells. Adiponectin (APN) secreted from adipocytes is one of the important molecules to inhibit the development of atherosclerosis. Epidemiological studies have revealed a positive correlation between plasma HDL-cholesterol and APN concentrations in humans, although its mechanism has not been clarified. Therefore, in the present study, we investigated the role of APN on RCT, in particular, cellular cholesterol efflux from human monocyte-derived and APN-knockout (APN-KO) mice macrophages. APN up-regulated the expression of ABCA1 in human macrophages, respectively. ApoA-I-mediated cholesterol efflux from macrophages was also increased by APN treatment. Furthermore, the mRNA expression of LXRα and PPARγ was increased by APN. In APN-KO mice, the expression of ABCA1, LXRα, PPARγ, and apoA-I-mediated cholesterol efflux was decreased compared with wild-type mice. In summary, APN might protect against atherosclerosis by increasing apoA-I-mediated cholesterol efflux from macrophages through ABCA1-dependent pathway by the activation of LXRα and PPARγ.



Plasma HDL levels are inversely correlated to the risk of atherosclerotic cardiovascular diseases. High density lipoprotein (HDL) plays a crucial role to remove excess cholesterol in atherosclerotic plaques and transport it back to the liver in the protective system, so-called "reverse cholesterol transport (RCT)" [1]. The cellular cholesterol efflux by HDL or a major apolipoprotein of HDL, apolipoprotein A-I (apoA-I) is the initial phase in RCT system. The ATP-binding cassette transporters (ABCA1 and ABCG1), which are expressed in the liver, small intestine and peripheral tissues, have been identified as key regulator molecules of cellular cholesterol efflux from macrophages [2–4]. ABCA1 promotes both free cholesterol and phospholipids efflux from macrophages to apoA-I [2]. ABCG1 also facilitates cellular cholesterol efflux to large, mature HDL, but not lipid poor apoA-I [3]. Scavenger receptor, class B, typeI (SR-BI) is expressed in hepatocytes to mediate selective cholesteryl ester uptake. It is also expressed in macrophages and is thought to be associated with HDL-mediated cholesterol efflux [4].

Adiponectin (APN) secreted from adipose tissues is the important molecule to prevent from development of atherosclerosis *in vivo*. Numerous epidemiological studies have revealed that male patients with hypoadiponectinemia had an increase in coronary artery disease (CAD) prevalence [5]. Furthermore, we and other

*Abbreviations:* *ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; APN, adiponectin; APN-KO, adiponectin knockout; ApoA-I, apolipoprotein A-I; BSA, bovine serum albumin; CAD, coronary artery disease; HDL, high density lipoprotein; LDL, low density lipoprotein; PCR, polymerase chain reaction; PBS, phosphate buffered saline; RCT, reverse cholesterol transport; SR-BI, scavenger receptor, class B, type I; LXRα, liver X receptor α; PPARγ, peroxisome proliferator-activated receptor γ.*

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laboratories have demonstrated that plasma HDL-cholesterol levels are positively correlated to APN concentrations in humans [6,7]. Although these findings suggest that APN might have an ability to prevent the development of atherosclerosis by the acceleration of RCT system, the underlying mechanism for it has not been clarified yet. It has been reported that *in vitro* APN suppresses atherosclerotic vascular changes, for example, the expression of adhesion molecules in vascular endothelial cells [8,9] and inhibits the formation of foam cells [10]. Recently, we reported that human recombinant APN enhanced the expression of ABCA1 and accelerated the synthesis of apoA-I in a human hepatoma cell line, HepG2 cells, suggesting that APN might increase the HDL assembly through ABCA1 in the liver [11]. Furthermore, we demonstrated that the expression levels of apoA-I in plasma and the liver were decreased in APN-knockout (APN-KO) mice and that the ABCA1 expression was also reduced in APN-KO mice, compared to wild-type mice [12].

In the present study, to clarify further the role of APN in RCT system, we investigated the effect of APN on cellular cholesterol efflux from human monocyte derived- and APN-KO or wild-type mice peritoneal macrophages.

## Materials and methods

*Cell culture.* Human mononuclear cells (monocytes and lymphocytes) were isolated from peripheral venous blood by density gradient centrifugation using NycoPrep™, with a density of 1.077 g/ml (Nycomed Pharma, Oslo, Norway). The isolated cells were plated in 24-well culture plates (FALCON, Becton-Dickinson Labware, USA), according to the standard condition in RPMI1640 medium containing 10% human AB-type serum in a humidified 5% $CO_2$ controlled incubator at 37 °C. The medium was changed twice a week, and the attached cells obtained after 7 days were used as human monocyte-derived macrophages. For the assay, macrophages were incubated for 24 h in RPMI1640 medium containing 0.1% BSA with the indicated concentrations of recombinant human APN as previously prepared [11]. Liver X receptor (LXR) agonist [TO-901317 (TO), Sigma–Aldrich, USA] was added to the medium with the concentration of 3 μM to induce ABCA1 and ABCG1 in macrophages. Peritoneal macrophages of APN-KO and wild-type mice were isolated after injection of 4% thioglycollate.

*RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (PCR).* Total RNA from macrophages 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 primers for human ABCA1 were ABCA1-forward: 5′-GCACTGAGGAAGATGCTGAAA-3′ and ABCA1-reverse: 5′-AGTTCCTGGAAGGTCTTGTTCAC-3′, for human LXRα, LXRα-forward: 5′-CTGTGCCTGACATTCCTCCT-5′ and LXRα-reverse: 5′-CATCCTGGCTTCCTCTCTGA-3′, for human PPARγ, PPARγ-forward: 5′-AACTCTGGGAGATTCTCCTGTTGA-3′ and PPARγ-reverse: 5′-TGGTAATTTCTTGTGAAGTGCTCATA-3′, for human GAPDH, GAPDH-forward: 5′-GAGTCAACGGATTTGGTCGT-3′ and GAPDH-reverse: 5′-TTGATTTTGGAGGGATCTCG-3′, for mouse LXRα, LXRα-forward: 5′-GCTCTGCTCATTGCCATCAG-3′, and LXRα-reverse: 5′-TGTTGCAGCCTCTCTACTTGGA-3′, for mouse PPARγ, PPARγ-forward: 5′-TTCCCCTGGAGATGAGTGTC-3′, and PPARγ-reverese: 5′-CAGTAGGCCACAGGGAACAT-3′, for mouse GAPDH, GAPDH-forward: 5′-ACTCCACTCACGGCAAATTC-3′ and GAPDH-reverse: 5′-TCTCCATGGTGGTGAAGACA-3′

*Western blotting.* Macrophages 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 Bioscience, UK). Anti-ABCA1 (Novus Biologicals, USA), anti-ABCG1 antibody (Novus Biologicals, USA) and anti-SR-BI antibody were used for the assay as previously described [11].

*Cellular cholesterol efflux.* Human macrophages were cultured in RPMI1640 medium (serum free) containing [$^3$H]-cholesterol (12 h), and then incubated in RPMI1640 medium containing 0.1% BSA with the indicated concentrations of human-recombinant APN (24 h) and in the presence of acceptor [apoA-I (10 μg/ml) or HDL (50 μg/ml)]. Cellular cholesterol efflux was determined as the percentage of radioactivity of [$^3$H]-cholesterol in the efflux medium to total cell-labeled radioactivity. The radioactivity was determined by liquid scintillation counting. The background for the efflux was in the presence of 0.1% BSA alone. Specific apoA-I or HDL-mediated cholesterol efflux was calculated by subtracting the background from total cellular cholesterol efflux. Mouse peritoneal macrophages were cultured for 3 h in DMEM medium (serum free) containing [$^3$H]-cholesterol, and then incubated in DMEM medium containing 0.1% BSA with the acceptor [apoA-I (10 μg/ml) or HDL (50 μg/ml)].

*Statistical analysis.* Values were expressed as the means ± SD. Statistical significance was assessed by Student's $t$ test for paired values and set at $p < 0.05$.

## Results

### *Effects of APN on ABCA1 expression level in and apoA-I-mediated cholesterol efflux from human macrophages*

To investigate the effect of APN on ABCA1 expression level, human monocyte-derived macrophages were incubated for 24 h in RPMI1640 medium containing 0.1% BSA with the indicated concentrations of recombinant APN. The mRNA and the protein levels of ABCA1 were analyzed by real-time quantitative PCR and Western blot. APN increased the mRNA level of ABCA1 in a dose-dependent manner (Fig. 1A) and also enhanced the protein level of ABCA1 (Fig. 1B). To study the effect of APN on apoA-I-mediated cellular cholesterol efflux, the macrophages were incubated for 24 h in RPMI1640 medium containing apoA-I (10 μg/ml). As expected, APN accelerated apoA-I-mediated cholesterol efflux by 5-fold compared with that without treatment (Fig. 1C).

### *Effects of APN on ABCG1 and SR-BI expression levels in and HDL-mediated cholesterol efflux from human macrophages*

The protein levels of ABCG1 and SR-BI were analyzed by Western blot after incubation. The protein level of ABCG1 was slightly, but not significantly, increased by APN (Fig. 2A). Similarly, HDL-mediated cholesterol efflux was not significantly enhanced by APN (Fig. 2B). Regarding SR-BI expression, the protein levels of SR-BI were not significantly influenced by APN (Fig. 2A).

### *Expression of ABCA1, ABCG1, SR-BI in and apoA-I-mediated or HDL-mediated cholesterol efflux from peritoneal macrophages of APN-KO and wild-type mice*

The mRNA and the protein levels of ABCA1, ABCG1, SR-BI were analyzed by real-time quantitative PCR and Western blot. The

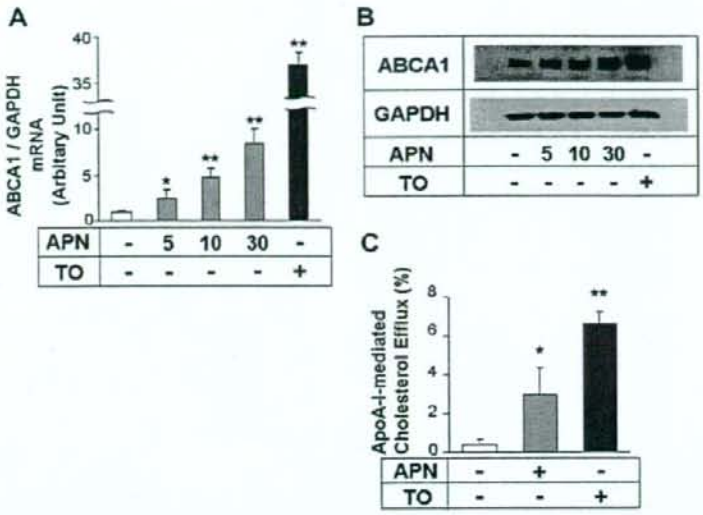


**Fig. 1.** Effects of APN on the expression of ABCA1 and apoA-I-mediated cholesterol efflux from human macrophages. Human monocyte-derived macrophages were incubated for 24 h in RPMI1640 medium containing 0.1% BSA with the indicated concentrations (0–30 μg/ml) of recombinant human adiponectin or with 3 μM of TO. (A) APN increased the mRNA level of ABCA1. Data are expressed as means ± SD of triplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ vs 0 μg/ml of APN. (B) APN enhanced the protein level of ABCA1 in a dose dependent manner. (C) For cholesterol efflux assay, macrophages labeled with [$^{3}$H]-cholesterol were incubated in RPMI1640 medium containing 0.1%BSA with APN (10 μg/ml) and apoA-I (10 μg/ml) for 24 h. APN significantly increased apoA-I-mediated cholesterol efflux from cells. Values are means ± SD of triplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ vs 0 μg/ml of APN.

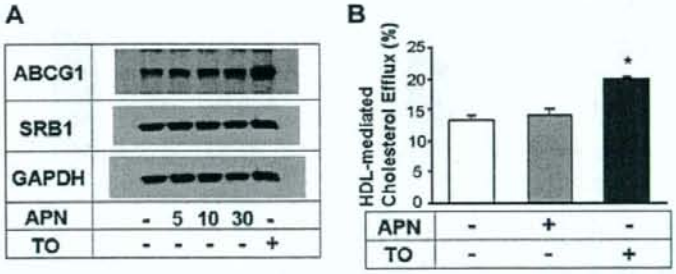


**Fig. 2.** Effects of APN on the expression of ABCG1 and HDL-mediated cholesterol efflux from human macrophages. Macrophages were incubated for 24 h in RPMI containing 0.1% BSA with the indicated concentrations (0–30 μg/ml) of recombinant human adiponectin or with 3 μM of TO. (A) APN slightly enhanced the protein level of ABCG1, but not SR-BI. (B) Macrophages labeled with [$^{3}$H]-cholesterol were incubated for 24 h in RPMI1640 medium containing 0.1%BSA with APN (10 μg/ml) and HDL (50 μg/ml). APN did not increase HDL-mediated cholesterol efflux from macrophages. Values are means ± SD of triplicate determinations. $^{*}P < 0.05$ vs 0 μg/ml of APN.

mRNA levels of ABCA1 and ABCG1, but not SR-BI were decreased in macrophages of APN-KO mice macrophages compared with wild-type mice (data not shown). The protein levels of ABCA1 and ABCG1 were also decreased in APN-KO mice compared with wild-type mice (Fig. 3A). To study apoA-I-mediated and HDL-mediated cellular cholesterol efflux, the macrophages were incubated for 24 h in DMEM medium containing apoA-I (10 μg/ml) and HDL (50 μg/ml). As expected, apoA-I-mediated cholesterol efflux was decreased in APN-KO compared with wild-type mice (Fig. 3B). In contrast, HDL-mediated cholesterol efflux was slightly but not significantly reduced in APN-KO mice (Fig. 3C).

*Expression of LXRα and PPARγ*

To investigate the mechanism of APN on cellular cholesterol efflux, we examined the mRNA levels of LXRα and PPARγ, analyzed by real-time quantitative PCR. In human monocyte-derived macrophages, APN significantly increased the mRNA levels of LXRα and PPARγ (Fig. 4A,B). As expected, in APN-KO mice the expressions of LXRα and PPARγ were significantly decreased compared with those of wild-type mice (Fig. 4C,D).

## Discussion

In the present study, we for the first time demonstrated that APN induced ABCA1 expression and enhanced apoA-I-mediated cellular cholesterol efflux in human monocyte-derived macrophages. APN might accelerate RCT system by increasing cellular cholesterol efflux from macrophages through ABCA1 pathway.

It is well known that both ABCA1 and ABCG1 are target genes of a nuclear receptor, LXR. However, unexpectedly, the protein level of ABCG1 was not significantly changed by APN. Furthermore, HDL-mediated cholesterol efflux was not significantly influenced by APN as well. Recently, Ranalletta et al. reported

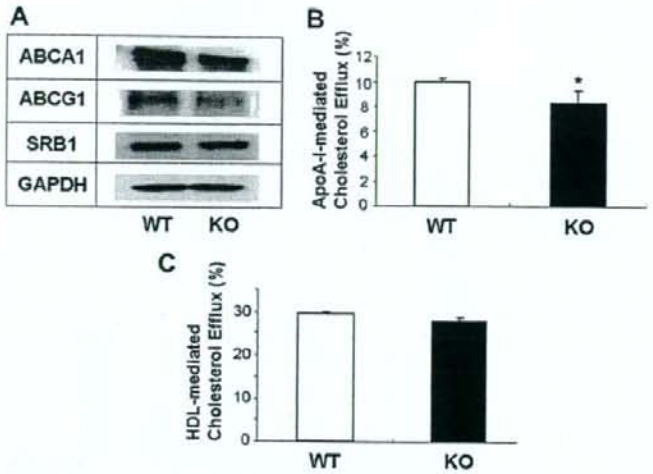


**Fig. 3.** Expression of ABCA1, ABCG1, SR-BI, and apoA-I- or HDL-mediated cholesterol efflux in peritoneal macrophages of APN-KO (KO) and wild-type (WT) mice. Peritoneal macrophages of APN-KO (KO) and wild-type (WT) mice were isolated after injection of 4% thioglycollate. (A) The protein levels of ABCA1 and ABCG1 were also decreased in APN-KO mice. (B,C) Macrophages labeled with [$^3$H]-cholesterol were incubated for 24 h in DMEM medium containing 0.1%BSA with apoA-I (10 μg/ml) and HDL (50 μg/ml). (B) ApoA-I-mediated cholesterol efflux was decreased in APN-KO compared with wild-type mice. Data are expressed as means ± SD of triplicate determinations. $^*P < 0.05$ vs wild-type mice. (C) HDL-mediated cholesterol efflux was slightly but not significantly reduced in APN-KO mice.

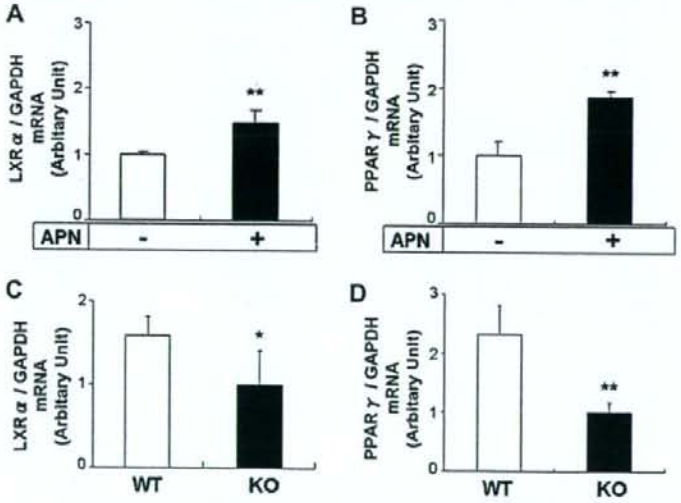


**Fig. 4.** Expression of LXRα and PPARγ mRNA in monocyte-derived macrophages (A,B) Human monocyte-derived macrophages were incubated for 24 h in RPMI1640 medium containing 0.1% BSA with recombinant human adiponectin (10 μg/ml). Data are expressed as means ± SD of triplicate determinations. $^*P < 0.05$, $^{**}P < 0.01$ vs 0 μg/ml of APN. (A) APN increased the mRNA levels of LXRα. (B) APN enhanced the mRNA levels of PPARγ. (C,D) Peritoneal macrophages of APNKO and wild-type mice were isolated after injection of 4% thioglycollate. Values are expressed as means ± SD of triplicate determinations. $^*P < 0.05$, $^{**}P < 0.01$ vs wild-type mice. (C) The mRNA level of LXRα was reduced in APN-KO compared with wild-type mice. (D) The mRNA level of PPARγ was also decreased in APN-KO mice compared with wild-type mice.

that the protein level of ABCA1 was definitely increased in ABCG1-deficient mouse macrophages [13]. Furthermore, Charvet et al. also showed that apoA-I-mediated cholesterol efflux was significantly increased in the peritoneal macrophages from ABCG1 knockout mice compared with wild-type mice and that HDL-mediated cholesterol efflux was enhanced in the macrophages from ABCA1-knockout mice [14]. These data suggested that the deficiency of ABCG1 in macrophages might lead to the compensatory induction of ABCA1. There might be an inverse relationship between ABCA1 and ABCG1 expressions in macrophages. Therefore, in this study, although the protein level of ABCA1 in human macrophages was up-regulated by APN, the level protein of ABCG1 might not be increased by the mechanism of compensatory reduction. These issues need to be investigated in future studies. Otherwise, Ling Tian et al. have recently reported that HDL-mediated cholesterol efflux was significantly induced by APN [15]. In their report, the adiponectin-conditioned medium, in which adiponectin gene transfected THP-1 cells were

incubated, was used to study the HDL-mediated cholesterol efflux from THP-1 foam cells. Therefore, the discrepancy in the results between ours and their study might be due to the difference of APN concentration in the medium or cell lines used for the assay.

In our previous report, it has been shown that APN did *not* enhance the expression level of SR-BI in HepG2 cells [11]. In the current study, APN did *not* increase the expression of SR-BI nor accelerate HDL-mediated cholesterol efflux from human macrophages. Although SR-BI is known to be one of the very important molecules to promote cellular cholesterol efflux, APN might not enhance the SR-BI-mediated cholesterol efflux.

Recently, we have reported that APN deficiency might impair the HDL synthesis in the liver [11,12]. In APN-KO mice, the synthesis of apoA-I in the liver was reduced compared with wild-type mice. Furthermore, the ABCA1 expression in the liver was also decreased in APN-KO mice. *In vivo*, HDL is mainly synthesized in the liver. However, partially, the cellular cholesterol efflux from foam cells is also associated with HDL generation. In this study, the ABCA1 expression in macrophages and apoA-I cholesterol efflux were also decreased in APN-KO mice.

We demonstrated that APN influenced the expression of ABCA1 and ABCG1 in macrophages. The expression of these transporters was reported to be regulated by LXR. In the present study, the mRNA level of LXRα was significantly enhanced by APN. APN also increased the expression of PPARγ. Furthermore, in APN-KO mice, the expression of LXRα and PPARγ mRNA was significantly reduced compaired with that in wild-type mice. Therefore, APN might enhance the expression of ABCA1 and ABCG1 in macrophages by the activation of LXRα and PPARγ.

Taken together, the current study has demonstrated that APN might have an ability to accelerate RCT and protect against atherosclerosis by increasing apoA-I-mediated cholesterol efflux through enhancing ABCA1 pathway in macrophages, as well as enhancing the HDL assembly in the liver.

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Original Article

# Long-Term Probucol Treatment Prevents Secondary Cardiovascular Events: a Cohort Study of Patients with Heterozygous Familial Hypercholesterolemia in Japan

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***Aim***: The POSITIVE study assessed whether long-term treatment with probucol, a potent anti-oxidant and cholesteryl ester transfer protein (CETP) activator, is associated with a lowered risk of cardiovascular events in a very high-risk population: familial hypercholesterolemia (FH).

***Methods***: The study cohort included 410 patients with heterozygous FH, diagnosed between 1984 and 1999 by cardiovascular and metabolic experts at fifteen centers. Traceable patients were screened using predefined eligibility criteria. The primary outcome measure for comparison between probucol exposure and non-exposure was the time to the first cardiovascular event involving hospitalization.

***Results***: Analysis revealed significant differences in baseline characteristics and follow-up treatment between exposure and non-exposure. An observed indication bias was the use of probucol in more severe FH at diagnosis, both for primary and secondary prevention. When the multivariate Cox regression procedure was used after adjustment for possible confounding factors, probucol lowered the risk (hazard ratio [HR], 0.13; 95% confidence interval [CI], 0.05–0.34) in secondary prevention ($n$=74) and was statistically significant ($p$<0.001), although not significant (HR, 1.5; 95% CI, 0.48–4.67; $p$=0.49) in primary prevention ($n$=233). Safety assessment found no specific difference between exposure and non-exposure.

***Conclusion***: Long-term probucol treatment may prevent secondary attack in a higher cardiovascular risk population of heterozygous FH.

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**Key words**; Atherosclerosis, Antioxidants, CETP activator, Dyslipidemia

## Introduction

Cardiovascular (CV) diseases, including coronary heart disease and stroke, are the leading cause of death in Japan. Prevention of fatal CV events is therefore the final goal as well as the rationale of cholesterol-lowering therapy.

Probucol, a conventional cholesterol-lowering drug, originated with the report by Barnhart in 1970 [1]. The drug has been used clinically in Japan since 1985. Nearly 60,000 Japanese patients still take probucol; western countries discontinued probucol use after

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the original manufacturer's withdrawal notice to the United States FDA in 1995 after 18 year's use of the drug. Probucol's cholesterol-lowering mechanism has not yet been clearly established, but it is thought to increase catabolic excretion of cholesterol into bile[2]. Later studies[3-5] have described new mechanisms of probucol, including anti-atherogenic and anti-oxidant actions. Another controversial and anti-atherogenic feature of probucol is its paradoxical effect of lowering high-density lipoprotein cholesterol (HDL-C). This action reflects, most likely, its molecular mechanisms: promoting cholesterol efflux, and enhancing reverse cholesterol transport by activation of cholesteryl ester transfer protein (CETP)[6-8] and class B type 1 scavenger receptor[9, 10]. Matsuzawa and his colleagues reported an observed close correlation between the extent of regression in Achilles' tendon xanthoma and probucol-induced decrease in HDL-C levels in patients with familial hypercholesterolemia (FH)[11].

No large-scale, randomized, double blind comparative study has been conducted to justify the use of probucol in the prevention of CV events or diseases. however, clinical studies as well as pre-clinical data have been accumulating evidence of the clinical worth of probucol in arteriosclerotic diseases. Numerous clinical results, including a reduction in Achilles' tendon xanthoma thickness after long-term treatment for FH[12, 13], reduced rates of restenosis after angioplasty[14-16], and a decrease in carotid artery intima-media thickness[17, 18] support the therapeutic and preventative effects of probucol on arteriosclerotic lesions and plaque. To evaluate the risk and benefit of long-term probucol treatment, we conducted a cohort study to determine whether probucol treatment is associated with the risk reduction of CV events in patients with heterozygous FH, a very high-risk population.

## Methods

### Study Cohort

We registered patients with FH who received treatment between January 1, 1984 and December 31, 1999 at 15 centers specializing in CV and metabolic diseases, including FH, nationwide. Patients were traceable by medical recend and met the diagnostic criteria for heterozygous FH under the Japan Atherosclerosis Society Guidelines (2002) for the Diagnosis and Treatment of Atherosclerotic CV Diseases[19]. Definite heterozygous FH was defined as having at least two of the major features: total cholesterol (TC) of 260 mg/dL and above; tendon xanthoma or xanthoma tuberosum; reduced or abnormal receptor activity noted by LDL receptor analysis. Probable heterozygous FH was defined as having at least one each of the major (as above) and minor features: palpebral xanthoma; arcus juvenilis (< 50 years); juvenile (< 50 years) ischemic heart disease. For other eligibility criteria, we excluded patients with possible homozygous FH or with severe ventricular arrhythmias (polymorphic premature ventricular contractions). Possible homozygous FH was defined as having any one of the clinical features: defect of homozygous or hetero-polymeric LDL receptors confirmed by gene analysis; no LDLR activity observed by receptor analysis, severe elevation of plasma TC higher than 500 mg/dL; xanthoma or atherosclerotic vascular lesions including symptoms of juvenile ischemic heart disease; hypercholesterolemia confirmed in both parents; history of ischemic heart disease confirmed in both parents; or poor response to any 3-hydroxy-3methyl-glutaryl-coenzyme A reductase inhibitor (statin).

During the study period between June, 2004 and September, 2005, we collected anonymous case report forms with the patients' baseline data, including medical history, findings at clinical examination, medication data, and laboratory data. The investigators transcribed the data on to case report forms (identified by a code) from the stored medical charts of the patients. The observation period was the period for which each patient's clinical course could be traced. The longest observation period exceeded 20 years for patients on stable doses of probucol.

We required a sample size of 200 in both the probucol exposure and non-exposure groups, supposing a difference of 10% in the incidence of CV events for 5 years (15% in exposure and 25% in non-exposure). A least 400 subjects were needed to detect the difference with 80% power and a type I error of 5% at the 5% significance level with two-sided log-rank test based on normal approximation. The study protocol was approved through the process of ethics committee or institutional review board at each center.

### Definitions and Endpoints

The primary outcome measure was the time to the first CV event, defined as acute myocardial infarction (MI), angina pectoris (AP), heart failure (HF), stroke, transient ischemic attack (TIA) or arteriosclerotic peripheral artery diseases (PAD) leading to hospitalization or death as well as sudden death within 24 hours of an observed intrinsic event. The obtained baseline data at the first visit of each patient included demographic characteristics: sex, date of diagnosis at the participant medical center, age, height, weight, and habits of smoking and drinking. Body mass index (BMI) was calculated as weight in kilograms divided

by the square of height in meters. The other collected characteristic factors at diagnosis were the presence of xanthoma and its location, prior CV event, onset date if any prior CV event, treatment for the event, and other possible risk factors for CV events, including the presence of hypertension, diabetes, ventricular arrhythmia, and PAD. We collected data on cholesterol-lowering therapy (with or without probucol) and other concomitant therapy with anti-platelet, antihypertensive or diabetic drugs. Dates of drug initiation, discontinuation, re-administration, and termination were entered as elemental information. Treatment period was defined as the length from initiation until medication termination, or until the occurrence of the defined CV event, whichever came first. A lipid profile of TC, triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and HDL-C, blood pressure, level of fasting blood sugar (FBS), hemoglobin$A_{1c}$ ($HbA_{1c}$), and thickness of tendon xanthoma in both feet were variables of interest, seen as potential predictors of CV events. We obtained measurements of those variables on a yearly basis after each patient was diagnosed. LDL-C levels were calculated from TC and HDL-C measurements with the Friedewald formula in TG $<400$ mg/dL. For TG of 400 mg/dL and more than 400 mg/dL, the expression of 0.16 X TG was applied in stead of 0.2 X TG[20]. Most patients had fasted compliantly at periodic checkups of their lipid levels. We set a follow-up period of 10 years for the measurements.

**Statistical Analyses**

The primary objective of analysis was a comparison between probucol exposure and non-exposure to evaluate whether treatment with probucol (500 mg to 1,000 mg daily) for FH provided CV benefits. The analysis was based on intent-to-treat principles. The secondary objective was to assess whether changes in the lipid profile after probucol treatment predicted CV events in the cohort. Event-free survival, defined as the time from diagnosis to the first CV event, was determined as a response variable. Statistical analysis was performed to evaluate clinical outcomes separately for secondary and primary prevention groups; that is, patients with or without a history of CV events at diagnosis.

Baseline characteristics of each group were explored to detect risk factors for CV events because potential confounders, including indication bias, were anticipated. For baseline comparison, Wilcoxon's rank sum test and Fisher's exact test were used for continuous variables and categorical variables respectively. For detection of risk factors, univariate Cox proportional hazards regression with a baseline variable as covariate was used as a screening step to determine the relationship with CV events. Variables that achieved significance at the level of 20% in univariate analysis were subsequently included in a multivariate Cox proportional hazards regression using backward variable selection. Variables proving significant at the 10% significance level were selected as risk factors to be adjusted. Consequently, probucol treatment effect was evaluated using the multivariate Cox model with adjustment for the selected baseline variables. Finally, the other observed treatment factors: cholesterol-lowering drugs other than probucol, LDL-apheresis, anti-platelet drugs, anti-hypertensive drugs, and diabetic drugs were entered into that model to assess their effects.

For the association between changes in lipid profile after probucol treatment and the risk of CV events, pre-treatment values of TG, LDL-C, HDL-C as well as TC, and each lipid reduction ratio after treatment were used as covariates. Multivariate analyses of time from probucol start to the first CV event used multivariate Cox's proportional hazards models. Statistical analysis was peerformed with SAS version 8.2.

## Results

**Patient Characteristics**

We collected data from the medical records of 541 patients, and excluded the data of 131 patients that did not meet eligibility predefined in the protocol.

The flow diagram (**Fig. 1**) gives reasons for the exclusion. A substantial fraction of probucol-exposed patients, 80.0% and 93.2%, took probucol within two years after diagnosis for in primary and secondary prevention groups, respectively. Baseline characteristics at diagnosis are given for each group (**Table 1**, 2). The secondary prevention group (**Table 2**) had prior diseases of AP, MI, stroke, HF, and TIA. This group was found to have significant higher proportions of men (60.2%, $p<0.01$), smokers (50.0%, $p<0.01$), hypertension (40.9%, $p<0.001$) diabetes (15.9%, $p=0.02$), and older median age (52 years, $p=0.01$) than the primary prevention group. Moreover, the group tended to have hypo-HDL cholesterolemia of median 42 (20–90) mg/dL, and to receive combined treatments with anti-platelet drugs (56.8%), anti-hypertensive drugs (53.4%), and LDL-apheresis (14.8%).

Comparison between probucol-exposed and non-exposed groups revealed significant differences in some baseline characteristics and treatments, which showed a confounding indication that patients with more severe FH took probucol. For baseline characteristics, the exposed group for primary prevention had more

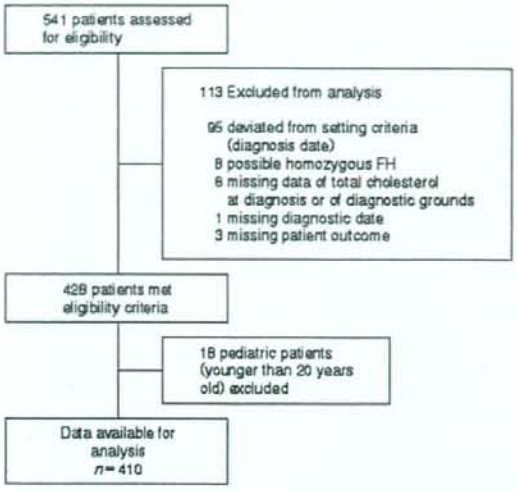



**Fig. 1.** Patient Flowchart.

We collected data from the medical records of 541 patients, and excluded the data of 131 patients who did not meet the eligibility predefined in the protocol. The flow diagram gives reasons for the exclusion.

palpebral xanthoma (13.4%, $p=0.05$), thicker median measurement of tendon xanthoma (12.5 mm, $p<0.01$), higher median $HbA_{1c}$ (5.8%, $p=0.03$), and more use of antihypertensive drugs (25.3%, $p<0.01$). Their lipid profile was more severe with a higher median baseline TC (325 mg/dL, $p=0.001$), a higher median LDL-C level (253 mg/dL, $p<0.001$), and a lower HDL-C level (47 mg/dL, $p<0.001$) than the unexposed group. The exposed group for secondary prevention had a higher prevalence of post-MI (44.6%, $p<0.01$) than the unexposed group. Observed medications were also significantly different between the exposed and unexposed groups. The exposed group used anti-hypertensive drugs concomitantly at a higher rate (25.3% vs. 11.2%, $p<0.01$) for primary prevention.

Descriptive analysis of baseline characteristics and treatments during observation implies that in both primary and secondary prevention, the exposed groups tended to include patients with more severe FH at diagnosis. Arguably, patients considered more severe at diagnosis would receive more intensive treatment, including probucol.

### Outcomes

We present the absolute number of CV events requiring hospitalization by prevention group with details of the events (**Table 3**). The incidence of CV events without consideration of confounding factors was 11.6% in the exposed group and 4.5% in the unexposed group for primary prevention. For secondary prevention, the incidence was 27.0% in the exposed group and 64.3% in the unexposed group. The event-free survival curve of the secondary prevention group is given (**Fig. 2**).

To identify risk factors for CV events, we determined the relationship between the incidence and every baseline variable using univariate Cox regression at a significant level of 20%. Variables proving significant at the 10% significance level in multivariate Cox regression were selected as risk factors to be adjusted. We estimated the effect of treatment after adjusting the selected risk factors. We calculated hazard ratios (HRs) with 95% confidence interval (CI) for binary variables, BMI ≥25 vs BMI<25, drinking vs no drinking, for example, and the indicated HRs corresponded to a 1 standard deviation increase for continuous variables, including TC. Estimated results are given (**Table 4**).

In the primary prevention group, significant variables were BMI ≥25 (HR 1.86, 95% CI 0.87–3.98; $p=0.11$), drinking (HR 2.17, 95% CI 1.02–4.63; $p=0.05$), tendon xanthoma (HR 2.17, 95% CI 0.76–6.23; $p=0.15$), prior diseases other than CV events (HR 1.87, 95% CI 0.87–3.99; $p=0.11$), PAD (HR 5.23, 95% CI 0.70–39.2; $p=0.11$), diabetes (HR 2.27, 95% CI 0.79–6.50; $p=0.13$), TC (HR 1.37, 95% CI 0.99–1.89; $p=0.06$), HDL-C (HR 0.75, 95% CI 0.50–1.12, $p=0.16$), SBP (HR 1.48, 95% CI 1.00–2.18; $p=0.05$), and the thickness of tendon xanthoma (HR 1.50, 95% CI 1.06–2.14; $p=0.02$). Three of these variables, drinking, TC, and PAD were selected for adjustment at the 10% significance level as a result of a multivariate Cox regression with backward variable selection. After adjustment for these three baseline variables, we found no significant effect by probucol at the 5% significant level. The estimated hazard ratio of probucol use for CV events was 1.50 (95% CI 0.48–4.67; $p=0.49$).

In the secondary prevention group, significance variables were drinking (HR 1.74, 95% CI 0.80–3.79; $p=0.17$), presence of palpebral xanthoma (HR 5.34, 95% CI 2.26–12.61, $p<0.001$), TIA (HR 4.16, 95% CI 0.54–32.21; $p=0.17$), history of coronary artery bypass graft (HR 0.31, 95% CI 0.11–0.90; $p=0.03$), hypertension (HR 0.58, 95% CI 0.26–1.28; $p=0.18$), diabetes (HR 2.89, 95% CI 1.30–6.42; $p<0.01$), and fasting blood sugar (HR 1.31, 95% CI 0.91–1.89; $p=0.15$). Two of these variables, palpebral xanthoma and diabetes, were selected for adjustment at the 10% sig-

**Table 1.** Baseline characteristics of patients in primary prevention group[†]

| Characteristics | Primary prevention No. (%) of patients | | | *p* |
|---|---|---|---|---|
| | All $n=322$ | Exposed $n=233$ (72.4) | Unexposed $n=89$ (27.6) | |
| Age, mean (range) | 49 (27–74) | 50 (20–74) | 47 (20–72) | 0.18 |
| Men, No. (%) | 134 (41.6%) | 96 (41.2%) | 38 (42.7%) | 0.90 |
| BMI ≥25 | 71 (22.5%) | 49 (21.4%) | 22 (25.6%) | 0.45 |
| Smoker | 99 (33.2%) | 74 (34.1%) | 25 (30.9%) | 0.68 |
| Drinker | 124 (42.2%) | 93 (43.7%) | 31 (38.3%) | 0.43 |
| Xanthoma | 259 (80.7%) | 190 (81.9%) | 69 (77.5%) | 0.43 |
| Tendon xanthoma | 245 (76.3%) | 181 (78.0%) | 64 (71.9%) | 0.30 |
| Nodular xanthoma | 28 (8.7%) | 22 (9.5%) | 6 (6.7%) | 0.51 |
| Palpebral xanthoma | 36 (11.2%) | 31 (13.4%) | 5 (5.6%) | 0.05 |
| PAD | 4 (1.2%) | 1 (0.4%) | 3 (3.4%) | 0.07 |
| Hypertension | 54 (16.8%) | 40 (17.2%) | 14 (15.7%) | 0.87 |
| Diabetes | 22 (6.9%) | 17 (7.3%) | 5 (5.6%) | 0.81 |
| Lipid profile, mg/dL | | | | |
| TC[§] | 320 (188–493) | 325 (188–493) | 307 (194–464) | 0.001 |
| TG[§] | 120 (28–1289) | 121 (34–1068) | 120 (28–1289) | 0.96 |
| HDL-C[§] | 49 (20–108) | 47 (20–90) | 52 (27–108) | <0.001 |
| LDL-C[§] | 244 (45–425) | 253 (98–425) | 223 (45–403) | <0.001 |
| Blood Pressure, mmHg | | | | |
| SBP[§] | 129 (82–190) | 128 (82–190) | 131 (90–190) | 0.57 |
| DBP[§] | 0 (48–120) | 80 (48–120) | 80 (56–120) | 0.91 |
| FBS (mg/dL)[§] | 95 (63–276) | 94 (63–140) | 95 (81–276) | 0.41 |
| $HbA_{1c}$ (%)[§] | 5.7 (4.1–12.4) | 5.8 (4.1–9.7) | 5.3 (4.3–12.4) | 0.03 |
| Tendon xanthoma thickness (mm)[§] | 12.1 (7.5–49.0) | 12.5 (7.5–49.0) | 10.5 (8.0–20.0) | <0.01 |
| Treatment | | | | |
| Cholesterol-lowering drugs (non-probucol) | 302 (93.8%) | 219 (94.0%) | 83 (93.3%) | 0.80 |
| LDL-apheresis | 7 (2.2%) | 6 (2.6%) | 1 (1.1%) | 0.68 |
| Anti-platelet drugs | 49 (15.2%) | 41 (17.6%) | 8 (9.0%) | 0.06 |
| Anti-hypertensive drugs | 69 (21.4%) | 59 (25.3%) | 10 (11.2%) | <0.01 |
| Diabetic drugs | 15 (4.7%) | 12 (5.2%) | 3 (3.4%) | 0.37 |

[†]Continuous variables compared by Wilcoxon's rank sum test, distribution of categorical variables by Fisher's exact test. [§]Data are median (range). All data are number (%) unless otherwise indicated. Each percentage shown is related to the total number with measurement data. BMI, body mass index; PAD, peripheral artery disease; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; $HbA_{1c}$, hemoglobin $A_{1c}$. LDL-C was calculated with the Friedewald formula.

nificance level as a result of multivariate Cox regression analysis using a backward variable selection. After adjustment for these two baseline variables, the hazard ratio of probucol use for CV events was estimated to be 0.13 (95% CI 0.05–0.34) and significant ($p<0.001$). In sensitivity analyses, we also obtained similar estimation results on probucol for various sets of baseline covariates for adjustment.

The lipid levels of TC, LDL-C and HDL-C were lowered after probucol treatment both in primary and secondary prevention. In the primary prevention group, the median (range) levels of TC, TG, LDL-C and HDL-C closest to before treatment were respectively 305 (165–493), 119 (35–1068), 228 (107–425) and 48 (25–96) mg/dL, and those at 10-year treatment were, respectively, 222 (141–371), 94 (43–335), 157 (91–311) and 39 (17–81) mg/dL. In the secondary prevention, the median levels of TC, TG, LDL-C and HDL-C closest to before atment were, respectively, 320 (191–469), 129 (37–636), 240 (117–381) and 44 (24–90) mg/dL, and those at 10-year treatment were, respectively, 211(135–305), 71 (48–475),

**Table 2.** Baseline characteristics of patients in secondary prevention group

| Characteristics | Secondary prevention No. (%) of patients | | | $p$ |
|---|---|---|---|---|
| | All $n=88$ | Exposed $n=74$ (84.1) | Unexposed $n=14$ (15.9) | |
| Age, mean (range) | 52 (23–71) | 51 (29–70) | 53 (23–71) | 0.62 |
| Men, No. (%) | 53 (60.2%) | 46 (62.2%) | 7 (50.0%) | 0.55 |
| BMI ≥25 | 21 (25.3%) | 17 (24.3%) | 4 (30.8%) | 0.73 |
| Smoker | 42 (50.0%) | 38 (53.5%) | 4 (30.8%) | 0.23 |
| Drinker | 39 (46.4%) | 33 (46.5%) | 6 (46.2%) | 1.00 |
| Xanthoma | 75 (85.2%) | 63 (85.1%) | 12 (85.7%) | 1.00 |
| Tendon xanthoma | 71 (80.7%) | 61 (82.4%) | 10 (71.4%) | 0.46 |
| Nodular xanthoma | 7 (8.0%) | 6 (8.1%) | 1 (7.1%) | 1.00 |
| Palpebral xanthoma | 8 (9.1%) | 5 (6.8%) | 3 (21.4%) | 0.11 |
| PAD | 2 (2.3%) | 2 (2.7%) | 0 (0.0%) | 1.00 |
| Hypertension | 36 (40.9%) | 30 (40.5%) | 6 (42.9%) | 1.00 |
| Diabetes | 14 (15.9%) | 9 (12.2%) | 5 (35.7%) | 0.04 |
| Lipid profile, (mg/dL) | | | | |
| TC† | 332 (191–469) | 334 (191–469) | 322 (229–444) | 0.41 |
| TG† | 128 (37–636) | 128 (37–636) | 136 (63–318) | 0.85 |
| HDL-C† | 42 (20–90) | 42 (20–90) | 39 (26–73) | 0.91 |
| LDL-C† | 249 (117–381) | 256 (117–381) | 245 (138–354) | 0.57 |
| Blood Pressure, mmHg | | | | |
| SBP† | 129 (90–180) | 128 (96–180) | 136 (90–166) | 0.97 |
| DBP (mmHg)† | 80 (52–114) | 80 (52–114) | 78 (60–104) | 0.33 |
| FBS (mg/dL)† | 96 (72–252) | 97 (72–197) | 94 (79–252) | 0.96 |
| HbA1c (%)† | 5.8 (4.1–10.6) | 5.5 (4.1–8.1) | 6.4 (5.3–10.6) | 0.06 |
| Tendon xanthoma thickness (mm)† | 14.5 (5.8–25.0) | 15.0 (5.8–25.0) | 10.0 (8.5–18.8) | 0.09 |
| Prior CV events | | | | |
| Angina Pectoris | 45 (51.1%) | 36 (48.6%) | 9 (64.3%) | 0.39 |
| Myocardial Infarction | 34 (38.6%) | 33 (44.6%) | 1 (7.1%) | <0.01 |
| Stroke | 7 (8.0%) | 4 (5.4%) | 3 (21.4%) | 0.08 |
| Heart failure | 2 (2.3%) | 2 (2.7%) | 0 (0.0) | 1.00 |
| TIA | 2 (2.3%) | 1 (1.4%) | 1 (7.1%) | 0.29 |
| Treatment | | | | 0.08 |
| Cholesterol-lowering drugs (non-probucol) | 81 (92.0%) | 70 (94.6%) | 11 (78.6%) | |
| LDL-apheresis | 13 (14.8%) | 11 (14.9%) | 2 (14.3%) | 1.00 |
| Anti-platelet drugs | 50 (56.8%) | 44 (59.5%) | 6 (42.9%) | 0.38 |
| Anti-hypertensive drugs | 47 (53.4%) | 42 (56.8%) | 5 (35.7%) | 0.24 |
| Diabetic drugs | 6 (6.8%) | 3 (4.1%) | 3 (21.4%) | 0.05 |

†Data are the median (range). All data are numbers (%) unless otherwise indicated. Each percentage is related to the total number with measurement data. TIA indicates transient ischemic attack.

147 (124–197) and 33 (17–70) mg/dL. Sub-analysis of changes in the lipid profile after probucol treatment detected significant three predictors of CV event risk: higher baseline TC (HR 2.74, 95% CI 1.05–7.16; $p=0.04$) in the primary prevention group; reduction in TG (HR 0.22, 95% CI 0.06–0.86; $p=0.03$); and reduction in LDL-C (HR 0.17,95% CI 0.03–0.90; $p=0.04$) after treatment in the subset of the secondary prevention group on stable doses of probucol. Neither TC nor HDL-C after treatment was associated with CV event risk in the probucol-exposed group, which indicates that reduction of the HDL-C level after probucol treatment is not related to CV event risk for probucol-exposed patients.

We evaluated the safety of probucol for all collected data from 541 patients, and found 56 adverse

**Table 3.** Incidence of cardiovascular events

| | | Cardiovascular Event | | No event | Total | *p* |
|---|---|---|---|---|---|---|
| Primary prevention ($n$=322) | Exposed ($n$=233) | | 27 (11.6%) | 206 | 233 | |
| | | MI | 4 | | | |
| | | AP | 18 | | | |
| | | Str. | 3 | | | |
| | | TIA | 1 | | | |
| | | PAD | 1 | | | 0.058 |
| | Unexposed ($n$=89) | | 4 (4.5%) | 85 | 89 | |
| | | AP | 1 | | | |
| | | Str. | 2 | | | |
| | | TIA | 1 | | | |
| Secondary prevention ($n$=88) | Exposed ($n$=74) | | 20 (27.0%) | 54 | 74 | |
| | | MI | 6 | | | |
| | | AP | 12 | | | |
| | | HF | 1 | | | |
| | | Str. | 1 | | | 0.012 |
| | Unexposed ($n$=14) | | 9 (64.3%) | 5 | 14 | |
| | | MI | 2 | | | |
| | | AP | 6 | | | |
| | | Str. | 1 | | | |

MI, myocardial infarction; AP, angina pectoris; HF, heart failure; Str., stroke; TIA, transient ischemic attack; PAD, peripheral artery disease.
†One of the 4 patients died after 12 months of probucol termination.

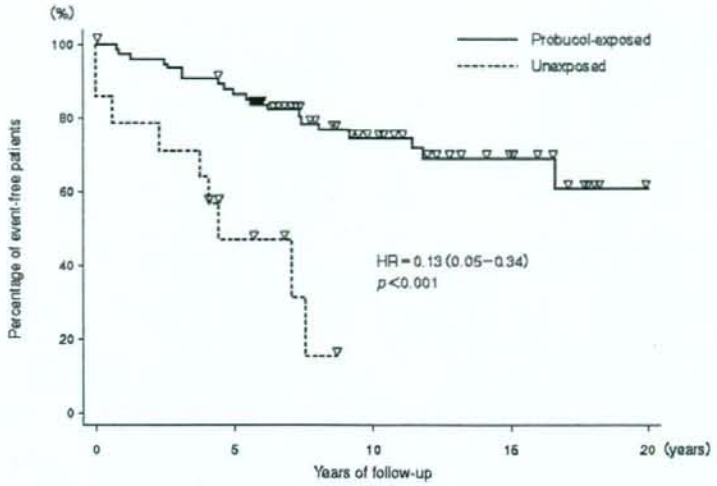
events in 18 patients. Malaise, pruritus, macrocytic anemia and pain in the extremities were recorded as adverse drug reactions associated with probucol. We noted and reported gastric cancer stage III immediately to the Ministry of Health and Welfare as an unexpected serious event, because of an unknown drug relation due to many concomitant drugs, although probucol was found to be non-carcinogenic alone[21]. Six deaths were observed in the population not taking probucol or stopping probucol. There was no other difference in the incidence of adverse events, including serious events, between probucol exposure and non-exposure.

## Discussion

Many data from large-scale randomized controlled trials have overwhelmingly demonstrated the clinical benefits of lowering cholesterol with statins[22, 23], yet the rapid and extensive prophylactic use of cholesterol-lowering drugs remains controversial. Few studies have addressed the clinical risks and benefits of long-term treatment of hyperlipidemia among women[24] or elderly patients[25]. The safety of long-term cholesterol-lowering therapy, including the issue of associated cancer risk or benefit, remains inconclusive because of conflicting clinical evidence[20]. More importantly, conclusions from the results of randomized controlled trials are limited by their relatively short follow-up periods (generally less than 5 years) in the analyzed studies.

In long-term treatment for FH, probucol was used with other cholesterol lowering drugs in over 80% of the secondary prevention group–those with a more severe clinical outlook than the primary prevention group: a higher prevalence of hypertension and diabetes, significant thicker tendon xanthoma, more combined therapy with LDL-apheresis, anti-platelet drugs, and anti-hypertensive drugs. The high rate of probucol use in FH was surprising, different from expected. This might partly reflect the prescription behavior of experts with the result that intractable patients responded to the regimen.

In the secondary prevention, the higher-risk group, probucol exposure was associated with a reduction in the risk of cardiovascular events (HR 0.13; 95% CI 0.05–0.34) with high significance ($p<0.001$), while it was not significant in the primary prevention group. This result was also contrary to our expectation that probucol exposure would likely be associated with increased event risk due to a confounding indication–that patients considered more severe at diagnosis would receive more treatment, including probucol. We did not collect the details of non-probucol drugs




Number at risk

| Years | 0 | | | | 5 | | | | 10 | | | | 15 | | | | 20 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Exposed | 74 | 71 | 70 | 68 | 66 | 62 | 54 | 50 | 42 | 38 | 34 | 30 | 25 | 19 | 17 | 13 | 12 | 8 | 3 | 1 | 0 |
| Unexposed | 14 | 11 | 11 | 10 | 9 | 5 | 4 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Estimates of event-free rates are according to whether patients received probucol. The cumulative probability of remaining without events was higher in patients treated with probucol ($p<0.001$; log-rank test).

**Fig. 2.** Kaplan-Meier Estimates of Event-free Rate.

For secondary prevention, the incidence of cardiovascular events was 27.0% in the exposed group and 64.3% in the unexposed group. An event-free survival curve for the secondary prevention group is given.

**Table 4.** The results of multivariate analysis using Cox regression procedure

| Factor | Primary prevention | | | Secondary prevention | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | $p$ | HR | 95% CI | $p$ |
| Baseline variables | | | | | | |
| Total cholesterol | 1.58 | 1.06–2.33 | 0.02 | – | – | – |
| Drinking | 2.43 | 1.09–5.44 | 0.03 | – | – | – |
| Peripheral artery disease | 5.27 | 0.51–54.63 | 0.16 | – | – | – |
| Palpebral xanthoma | – | – | – | 2.94 | 1.02–8.47 | 0.05 |
| Diabetes | – | – | – | 2.58 | 0.76–8.76 | 0.13 |
| Treatment in follow-up | | | | | | |
| Probucol use | 1.50 | 0.48–4.67 | 0.49 | 0.13 | 0.05–0.34 | <0.001 |
| Anti-platelet drug use | – | – | – | 2.48 | 1.00–6.17 | 0.05 |

to simplify the study procedure. However, we would likely exclude underused statins because of the reduced use of non-probucol drugs from the possible factors of the higher event rate in the unexposed group, because statins were available when all of the 9 recurrent patients (**Table 3**) started and the patients continued on cholesterol-lowering drugs. We suppose, therefore, that the reasons for this unanticipated great risk reduction include some antioxidant and anti-atherogenic actions[3, 4, 27] of probucol. The finding in second prevention may be suggested by the report[27] that probucol significantly decreased *in vitro* LDL oxidizability measured under typically strong oxidative conditions, and that long-term treatment with probucol had an