Increased cholesterol biosynthesis and hypercholesterolemia in mice overexpressing squalene synthase in the liver

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Abstract **Squalene synthase (SS) is the first committed enzyme for cholesterol biosynthesis, located at a branch point in the mevalonate pathway. To examine the role of SS in the overall cholesterol metabolism, we transiently overexpressed mouse SS in the livers of mice using adenovirus-mediated gene transfer. Overexpression of SS increased de novo cholesterol biosynthesis with increased 3-hydroxy-3-methyglutaryl-CoA (HMG-CoA) reductase activity, in spite of the downregulation of its own mRNA expression. Furthermore, overexpression of SS increased plasma concentrations of LDL, irrespective of the presence of functional LDL receptor (LDLR). Thus, the hypercholesterolemia is primarily caused by increased hepatic production of cholesterol-rich VLDL, as demonstrated by the increases in plasma cholesterol levels after intravenous injection of Triton WR1339. mRNA expression of LDLR was decreased, suggesting that defective LDL clearance contributed to the development of hypercholesterolemia. Curiously, the liver was enlarged, with a larger number of Ki-67-positive cells. These results demonstrate that transient upregulation of SS stimulates cholesterol biosynthesis as well as lipoprotein production, providing the first in vivo evidence that SS plays a regulatory role in cholesterol metabolism through modulation of HMG-CoA reductase activity and cholesterol biosynthesis.**—Okazaki, H., F. Tazoe, S. Okazaki, N. Isoo, K. Tsukamoto, M. Sekiya, N. Yahagi, Y. Iizuka, K. Ohashi, T. Kitamine, R-i. Tozawa, T. Inaba, H. Yagyu, M. Okazaki, H. Shimano, N. Shibata, H. Arai, R-z. Nagai, T. Kadowaki, J-i. Osuga, and S. Ishibashi. **Increased cholesterol biosynthesis and hypercholesterolemia in mice overexpressing squalene synthase in the liver.** ***J. Lipid Res.*** **2006.** 47: **1950–1958.**

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Cholesterol biosynthesis is subject to tight regulation by a multivalent feedback mechanism at both the transcriptional and the posttranscriptional level (1). The transcriptional regulation is mediated through the action of sterol-regulatory element binding proteins (SREBPs), membrane-bound transcription factors that enhance transcription of genes encoding cholesterol biosynthetic enzymes and the LDL receptor (LDLR) (2). The translational regulation of 3-hydroxy-3-methyglutaryl-CoA (HMG-CoA) reductase (EC1.1.1.34), the rate-limiting enzyme in cholesterol biosynthesis, is mediated by nonsterol mevalonate-derived isoprenoids, which act by an undefined mechanism (3). Its degradation is regulated by both sterols and nonsterol end products of mevalonate metabolism (4, 5). The sterol-regulated degradation of HMG-CoA reductase is mediated

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Abbreviations: Ad-SS, recombinant adenovirus carrying SS cDNA under the control of cytomegalovirus promoter; apoB, apolipoprotein B; E, embryonic day; ER, endoplasmic reticulum; LDLR, LDL receptor; m.o.i., multiplicity of infection; SREBP, sterol-regulatory element binding protein; SS, squalene synthase; TC total cholesterol; TG, triglyceride; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling.

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by the binding of a pair of endoplasmic reticulum (ER) retention proteins called Insig-1 and Insig-2 to the sterol-sensing domain in membrane-spanning segments of HMG-CoA reductase (6, 7).

Inhibition of cholesterol biosynthesis in the liver by using inhibitors of HMG-CoA reductase, collectively called statins, is a widely used strategy for preventing heart attacks by lowering plasma cholesterol. However, statins have limited effects (8), partly because the deficiency of mevalonate-derived products following the inhibition of HMG-CoA reductase activity leads to a compensatory increase in HMG-CoA reductase protein, invoking the need for higher doses of statins (9), and to occasional severe adverse effects, such as rhabdomyolysis (10). Therefore, exploration of another target for the inhibition of cholesterol biosynthesis is needed.

Squalene synthase (SS, or farnesyl-diphosphate:farnesyl-diphosphate farnesyltransferase, EC2.5.1.21) catalyzes the reductive head-to-head condensation of two molecules of farnesyl diphosphate to form squalene, the first specific intermediate in the cholesterol biosynthetic pathway (11, 12). SS is unique among other enzymes in cholesterol biosynthesis in that, in addition to the sterol-mediated transcriptional control through the action of SREBPs (13), expression of the *SS* gene is regulated by lipopolysaccharide, which potently inhibits SS, whereas it stimulates *HMG-CoA reductase* expression (14). SS is an attractive target for cholesterol-lowering therapy, because the inhibition of this step theoretically may not perturb the nonsterol pathway. Indeed, several potent SS inhibitors successfully lower plasma cholesterol levels without the potential limitations reported for statins (15–17).

To elucidate the role of SS in cholesterol metabolism, we have generated SS knockout mice. *SS* knockout mice ($SS^{-/-}$) were lethal between embryonic day (E) 9.5 and E 12.5 and exhibited severe retardation of development and defective neural tube closure, indicating that SS is essential for development, particularly of the central nervous system (18). Although $SS^{+/-}$ mice expressed only 50% SS activities in the liver compared with $SS^{+/+}$ mice, their plasma lipoprotein profiles and responses to dietary challenges were indistinguishable from those of $SS^{+/+}$ mice. These observations closely resemble those seen in *HMG-CoA reductase* knockout mice: *HMG-CoA reductase*$^{-/-}$ mice were embryonic lethal, and *HMG-CoA reductase*$^{+/-}$ mice showed no significant changes in lipoprotein profiles (19). However, the role of SS in cholesterol metabolism in adult liver remains largely unknown.

To better understand the role of SS in overall cholesterol metabolism in vivo in adult liver, we have used adenovirus-mediated gene delivery to overexpress SS in the liver and have examined changes in hepatic cholesterol biosynthesis and plasma lipoprotein profiles in vivo. We show here that increased SS activity in liver leads to increased cholesterol synthesis, as well as secretion of cholesterol-rich lipoproteins, providing the first in vivo evidence that SS plays a regulatory role in the development of hypercholesterolemia.

MATERIALS AND METHODS

Construction of recombinant adenoviruses

Recombinant adenovirus that carried murine SS cDNA under the control of the cytomegalovirus promoter, designated as Ad-SS, was constructed using the cDNA cloned by RT-PCR from mouse liver as described previously (20, 21). The recombinant adenoviruses were expanded in HEK293 cells and purified by cesium chloride ultracentrifugation. The purified viruses were stored in 10% (v/v) glycerol in PBS at −80°C. In our preparations, 1 multiplicity of infection (m.o.i.) corresponded to 25 particles of adenovirus per cell, and cells were infected at 300 m.o.i.

Animal studies

C57BL/6 male mice and LDLR knockout mice (22) that had been backcrossed into the C57BL/6 background were purchased from Charles River Japan, Inc. (Yokohama, Japan) and Jackson Laboratory (Bar Harbor, ME), respectively. Mice were maintained and cared for according to the regulations of the Animal Care Committees of the University of Tokyo and Jichi Medical University. Mice were caged separately, with 12 h light/dark cycles, and were given free access to standard chow diet containing 0.075% cholesterol (MF; Oriental Yeast Co., Ltd., Osaka, Japan). Mice were injected intravenously with 1.5×10^{11} particles (6×10^{9} plaque-forming units) of Ad-LacZ or Ad-SS. Each group contained six mice. Seven days after virus injection, food was withdrawn 4 h before collection of blood samples from the retroorbital plexus of anesthetized animals. Tissues were immediately collected, snap frozen in liquid nitrogen, and stored at −80°C.

Real-time RT-PCR

Total RNA was prepared from livers with TRIzol reagent (Life Technologies, Inc.). Two micrograms of total RNA was reverse transcribed (Taq Man Reverse Transcription Reagents; Applied Biosystems, Foster City, CA), and synthesized cDNA was quantified using Taq Man quantitative PCR analysis of each gene with the ABI Prism 7700 sequence detection system (Applied Biosystems) according to the manufacture's protocol. The specific primer pairs for SS, HMG-CoA reductase, FAS, LDLR, apolipoprotein B (apoB), CYP7A, and SREBP-1a were designed as previously described (23); the others were purchased from Applied Biosystems. The relative amounts of all mRNAs were calculated by using the comparative $2^{-\Delta\Delta CT}$ method (User Bulletin, Applied Biosystems). β-actin mRNA was used as the invariant control.

Western blot analysis

Livers were homogenized in a buffer containing 15 mM nicotinamide, 2 mM $MgCl_2$, and 100 mM potassium phosphate, pH 7.4, and centrifuged at 10,000 *g* for 20 min at 4°C. The supernatants were centrifuged at 105,000 *g* for 1 h at 4°C, and the resultant pellets, a microsome fraction, were washed, resuspended in the same buffer, and stored in aliquots at −80°C. The supernatant was used for Western blot analysis as described previously (21), using an anti-SS antibody that was raised by immunizing rabbits with glutathione S-transferase fusion protein containing amino acid sequence 174–339 of mouse SS. For Western blot of HMG-CoA reductase, we prepared and solubilized the liver membrane fraction exactly as described by Sever et al. (7) and used antibodies from Upstate Cell Signaling Solutions (Charlottesville, VA) and from Dr. Y. K. Ho at University of Texas Southwestern Medical Center at Dallas.

SS activity assay

Liver microsomal protein was obtained as described above, and stored in aliquots at −80°C. SS activities were measured according to a modified method of Cohen et al. (18, 24). In brief, the microsome fractions (~20 μg) were incubated in 50 μl of a buffer containing 20 μM [1-^{3}H]farnesyl pyrophosphate (25 μCi/μmol; NEN Life Science Products, Inc.), 1 mM NADPH, 5 mM $MgCl_2$, 6 mM glutathione, and 100 mM potassium phosphate, pH 7.4, at 37°C for 15 min. The reaction was terminated by the addition of 150 μl chloroform-methanol (1:2; v/v) containing 0.2% unlabeled squalene. After 50 μl of chloroform and 50 μl of 3 M NaOH were added, the reaction mixtures were vortexed and centrifuged. The infranatant organic phase was used for the determination of the radioactivities in the squalene produced.

Plasma lipids and lipoprotein analyses

Plasma levels of total cholesterol (TC), triglycerides (TGs), and FFAs were determined enzymatically using kits: Determiner TC555 (Kyowa Medex), Triglyceride-G, and NEFA-C (WAKO Pure Chemicals). Lipoproteins were fractionated by HPLC as described (25), and the cholesterol contents in each lipoprotein fraction were determined.

Histology

Tissues were fixed with neutral-buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Ki-67 staining was performed using rat ant-mouse Ki-67 antibody (DAKO; Glostrup, Denmark). Apoptosis was detected by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using the in situ Apoptosis Detection Kit (TaKaRa, Japan) according to the instruction manual. The number of cells positive for staining with Ki-67 or TUNEL was counted in each field of representative slides from three independent experiments at a magnification of ×100.

Tissue and cellular lipids

Lipids were extracted from tissues by the method of Folch, Lees, and Sloane Stanley (26). TC was determined by fluorometric microassay according to a modified method of Heider and Boyett (27) with the exception that 0.01% (v/v) Triton X-100 was used instead of Carbowax-600. TG was determined enzymatically using a kit (Triglyceride-G, WAKO Pure Chemicals).

HMG-CoA reductase activity assay

Liver microsomal proteins were prepared as described above. HMG-CoA reductase activities were measured essentially as described previously (9). Briefly, the microsome fractions (50 μg) were incubated in 20 μl of a buffer containing 110 μM DL-[3-^{14}C]HMG-CoA (4.5 μCi/μmol; NEN Life Science Products, Inc.), 5 mM NADPH, 10 mM EDTA, 10 mM dithiothreitol, and 100 mM potassium phosphate, pH 7.4, at 37°C for 30 min. The reaction was terminated by the addition of 10 μl of 2 N HCl and incubated for another 30 min at 37°C to lactonize the mevalonate formed. The [^{14}C]mevalonate was isolated by TLC and counted using [^{3}H]mevalonate as an internal standard. HMG-CoA reductase activity is expressed as picomoles of [^{14}C]mevalonate formed per minute per mg of protein.

Measurement of hepatic cholesterol synthesis in vivo

Seven days after administration of adenovirus to mice, cholesterol synthesis in the liver was estimated during the mid light cycle as previously described (18, 19, 28). In brief, animals were given food and water ad libitum and injected intraperitoneally with 37 kBq/kg body weight of [2-^{14}C]acetate (NEN Life Science Products, Inc.). After 1 h, animals were euthanized and the liver was removed. Two portions of the liver (200–300 mg/each) were saponified, and the digitonin-precipitable sterols were isolated for the measurement of radioactivities. The results were expressed as ^{14}C dpm/100 mg of wet weight of liver/h.

Measurement of cellular cholesterol synthesis in vitro

Cholesterol biosynthesis in vitro was determined essentially as described previously (29, 30). Briefly, McARH7777 cells (American Type Culture Collection) were grown in DMEM containing 10% FBS and 10% fetal horse serum and were plated on day 0 at a density of 2.5×10^4 cells per square centimeter into 24-well collagen-coated plates (IWAKI). On day 1, transfection of recombinant adenovirus (Ad-LacZ or Ad-SS) was performed at the indicated m.o.i. On day 3, [2-^{14}C]acetate (51 mCi/mmol; NEN Life Science Products, Inc.) was added to a final concentration of 10 μCi per well. After incubation for 5 h, the medium was removed and centrifuged. The cells were harvested by scraping into 100 μl of 0.1 N sodium hydroxide, followed by 100 μl of water. Radiolabeled lipids were saponified by 15% KOH, extracted by petroleum ether. The content of ^{14}C-labeled squalene, ^{14}C-labeled cholesterol, and ^{14}C-labeled fatty acids in the cells and in the medium was quantified by TLC and scintillation counting. The cellular protein was measured by the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL).

Assay of microsomal ACAT activity

Liver was homogenized in buffer A (0.25 M sucrose, 1 mM EDTA, 2 μg/ml leupeptin, and 50 mM Tris-HCl, pH 7.0) and centrifuged at 100,000 *g* for 45 min at 4°C. The pellets were resuspended and used for the assay. ACAT activity in microsomes was determined by the rate of incorporation of [1-^{14}C]oleoyl-CoA (53.5 mCi/mmol; NEN Life Science Products, Inc.) into the cholesteryl ester fraction according to Yagyu et al. (25).

VLDL secretion rate

Secretion rates of TG or cholesterol in vivo were estimated by the intravenous administration of Triton WR1339 as described previously (31). Plasma levels of TG or cholesterol were measured at 1, 2, 3, and 4 h after the treatment. Aliquots of plasma were subjected to sequential ultracentrifugation to separate lipoprotein fractions using S100AT3 angle rotor for himac CS120GXL (HITACHI). In brief, 60 μl of plasma was mixed with 60 μl of saline and centrifuged at 188,000 *g* for 3 h at 16°C. Sixty microliters was aspirated from the bottom by Hamilton syringe and mixed with a KBr solution (d = 1.12) and centrifuged at 188,000 *g* for 5 h at 16°C. The remaining top fraction was used as the VLDL fraction. Sixty microliters was aspirated as described above and used as the HDL fraction, and the remaining top fraction was used as the IDL/LDL fraction.

Statistical analyses

All values are given as mean ± SE, and differences between groups were evaluated with Student's *t*-test or ANOVA, unless otherwise stated. All calculations were performed with STAT view, version 5.0, for Macintosh (SAS Institute).

RESULTS

Intravenous injection of Ad-SS elicited an 11-fold increase in mRNA expression of mouse SS in the liver as

TABLE 1. Relative amounts of mRNAs in the livers

Gene	PBS	Ad-LacZ	Ad-SS
HMG-CoA reductase	1.00 ± 0.14	1.19 ± 0.08	0.72 ± 0.13[b]
Squalene synthase	1.00 ± 0.40	1.24 ± 0.21	13.43 ± 2.20[b]
Squalene epoxydase	1.00 ± 0.18	1.26 ± 0.28[a]	0.35 ± 0.03[b]
Lanosterol 14α-demethylase	1.00 ± 0.23	1.34 ± 0.23	0.41 ± 0.07[b]
SREBP-1a	1.00 ± 0.09	0.91 ± 0.08[a]	0.54 ± 0.06[b]
Fatty acid synthase	1.00 ± 0.07	0.53 ± 0.06[a]	0.46 ± 0.04
ACAT2	1.00 ± 0.10	1.61 ± 0.24[a]	0.49 ± 0.04[b]
ApoB	1.00 ± 0.17	1.08 ± 0.06	0.55 ± 0.05[b]
LDLR	1.00 ± 0.05	0.90 ± 0.04	0.47 ± 0.04[b]
CYP7A	1.00 ± 0.37	6.75 ± 2.01[a]	3.40 ± 0.49
ABCA1	1.00 ± 0.08	0.79 ± 0.03[a]	0.52 ± 0.03[b]

Seven days after intravenous injection of adenovirus into C57BL/6 mice (n = 4), total RNA was isolated from the livers and used for real-time PCR analysis. Values are indicated as means ± SE. HMG-CoA reductase, 3-hydroxy-3-methyglutaryl-CoA reductase; SREBP, sterol-regulatory element binding protein; ACAT, acyl-CoA:cholesterol acyltransferase; apoB, apolipoprotein B; LDLR, LDL receptor; CYP7A, cholesterol 7α-hydroxylase; ABCA1, ATP binding cassette transporter A1.

[a] $P < 0.05$ vs. PBS by ANOVA and Fisher's protected least significant difference (PLSD) test.

[b] $P < 0.05$ vs. Ad-LacZ by ANOVA and Fisher's PLSD.

compared with Ad-LacZ (**Table 1**). In parallel, mouse SS protein was markedly expressed in liver of Ad-SS-injected mice (**Fig. 1A**). Hepatic SS activities were also remarkably increased in the liver, by 52-fold (Fig. 1B).

To determine whether the overexpressed SS accompanied increased cholesterol synthesis, we measured the amounts of cholesterol synthesized from acetyl-CoA in the liver (**Fig. 2A**). Although the use of acetic acid as a substrate for cholesterol biosynthesis poses several limitations to the interpretation of the results (32), we presume that overexpression of SS is unlikely to affect other potential confounding factors such as transmembrane permeability of acetic acid and/or differential dilution by the intracellular pool of acetyl-CoA. Hepatic cholesterol synthesis was increased 2-fold. Given that HMG-CoA reductase is the

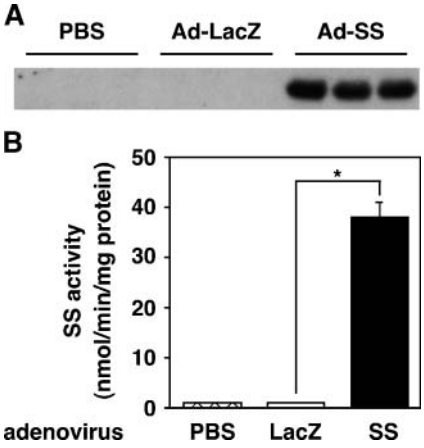

Fig. 1. Expression of squalene synthase (SS). Seven days after intravenous injection of adenovirus into C57BL/6 mice (n = 6), livers were excised and used for Western blot analysis of SS (A) and for measurements of SS activities (B). *, $P < 0.0001$ vs. Ad-LacZ. Three representative samples were used for Western blot analysis. Error bars represent standard deviation.

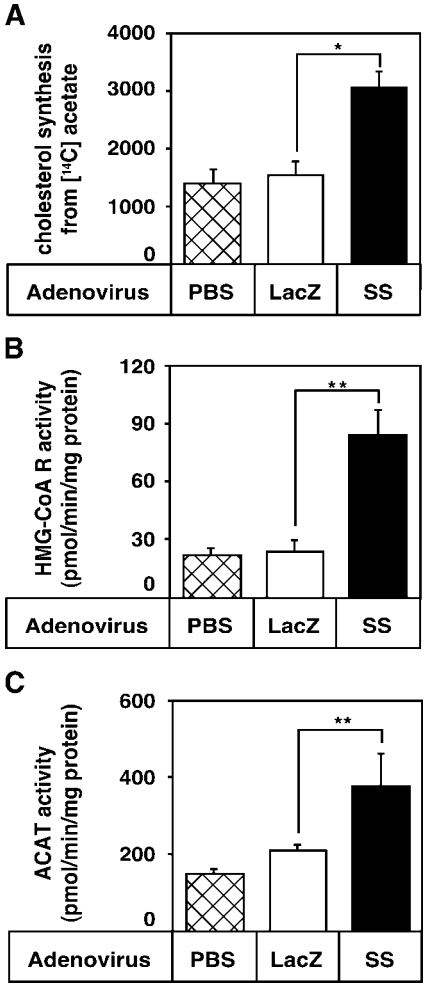

Fig. 2. Cholesterol synthesis, HMG-CoA reductase, and ACAT activities in the liver. Seven days after intravenous injection of adenovirus into C57BL/6 mice (n = 6), rates of hepatic cholesterol synthesis (A) and HMG-CoA reductase (B) and ACAT activities (C) in the livers were measured. *, $P < 0.05$ and **, $P < 0.0001$ vs. Ad-LacZ. Error bars represent standard deviation.

rate-limiting enzyme of cholesterol synthesis, we measured the activities of HMG-CoA reductase in the microsome fraction of the liver (Fig. 2B). There was a 3.6-fold increase in the HMG-CoA reductase activities in the Ad-SS-injected mice compared with Ad-LacZ-injected mice. To determine whether the increase in the HMG-CoA reductase activities was associated with induction of the mRNA expression, we performed RT-PCR of HMG-CoA reductase along with other enzymes in the cholesterol synthetic pathway (Table 1). The hepatic mRNA level of HMG-CoA reductase was rather decreased, by 39%, in the Ad-SS-treated mice, indicating that the increased enzymatic activity was at posttranscriptional level. More notably, the relative mRNA expression of enzymes catalyzing the steps downstream of HMG-CoA reductase, such as squalene epoxidase and lanosterol 14α-demethylase, was significantly suppressed in the Ad-SS-injected mice by 72% and 69%, respectively. To estimate the protein expression of HMG-CoA reductase in the liver membrane fraction, we performed Western blot analysis. Although we tried to

detect HMG-CoA reductase protein expression by using two different antibodies, we failed to detect a 97 kDa band that reportedly corresponds to HMG-CoA reductase (data not shown).

To determine whether the increased cholesterol synthesis in the liver overexpressing SS affected hepatic cholesterol contents, we measured lipid contents in the liver (**Table 2**). The weight of the liver was increased by 37% in Ad-SS-injected mice compared with Ad-LacZ-injected mice. Cholesterol contents per weight of wet tissue were not different between the Ad-LacZ- and Ad-SS-injected mice, whereas TG contents per weight of wet tissue were decreased by 35% in Ad-SS-injected mice. Thus, cholesterol content in the whole liver was increased in proportion to the increase in liver weight. To determine the reasons for the hepatomegaly in Ad-SS-injected mice, we performed histology (**Fig. 3**). Hematoxylin and eosin staining showed negligible infiltration of inflammatory cells; there were no differences between the Ad-LacZ- and Ad-SS-injected mice. The number of Ki-67-positive cells, which are correlated with cell proliferation activity (33), was significantly increased in the livers of Ad-SS-injected mice compared with Ad-LacZ-injected mice (17.7 ± 2.3 vs. 0 ± 0 cells/field; $P < 0.01$), whereas the number of TUNEL-positive cells was not different between the Ad-LacZ- and Ad-SS-injected mice. These results indicate that overexpression of SS stimulates cell proliferation of hepatocytes, thereby leading to hepatomegaly.

Plasma lipoprotein profiles were determined 7 days after the injection of the virus into wild-type mice (**Fig. 4A** and **Table 3**). Plasma cholesterol levels were significantly increased, by 66%, in Ad-SS-injected mice compared with Ad-LacZ-injected mice, whereas there were no significant differences in plasma levels of either TG or FFA. HPLC lipoprotein analyses revealed that LDL- and HDL-cholesterol levels were increased by 1.9- and 1.3-fold, respectively.

To determine how the hepatic overexpression of SS led to hypercholesterolemia, we determined hepatic VLDL secretion rates (**Fig. 5**). Rates of increases in plasma cholesterol levels after the injection of Triton WR1339 were significantly increased in Ad-SS-injected mice compared with Ad-LacZ-injected mice (Fig. 5B), whereas those in plasma TG levels were not different between the two groups (Fig. 5A). These results indicate that the increase in plasma LDL-cholesterol levels resulted primarily from the secretion of cholesterol-rich VLDL from the liver. Indeed, the

TABLE 2. Lipid contents of the livers

	Ad-LacZ	Ad-SS
Body weight (g)	24.1 ± 0.8	25.1 ± 0.6
Liver weight (g)	1.465 ± 0.61	2.003 ± 0.059[a]
TC (mg/g wet tissue)	1.101 ± 0.038	1.310 ± 0.132
TG (mg/g wet tissue)	3.672 ± 0.228	2.387 ± 0.310

Seven days after intravenous injection of adenovirus into C57BL/6 mice (n = 6), lipids were extracted from the livers by the method of Folch, Lees, and Sloane Stanley (26). TC and TG were measured enzymaticaly. TC, total cholesterol; TG, triglyceride. Values are indicated as means ± SD.

[a] $P < 0.0001$ vs. Ad-LacZ.

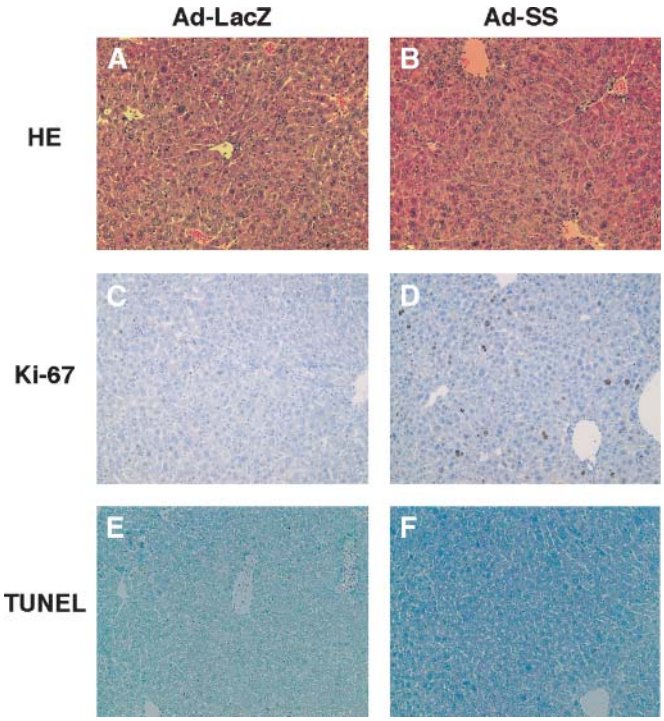

Fig. 3. Histology of the liver. Seven days after intravenous injection of adenovirus into C57BL/6 mice, liver samples from mice overexpressing Ad-LacZ (A, C, E) or Ad-SS (B, D, F) were fixed in neutral-buffered formalin and embedded in paraffin. A, B: Tissue sections were stained with hematoxylin and eosin (HE). C, D: Ki-67 staining was performed using rat anti-mouse Ki-67 antibody. Ki-positive cells are brown. E, F: Apoptosis was detected by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using the in situ Apoptosis Detection Kit. TUNEL-positive cells are brown.

cholesterol/protein ratio of VLDL from Ad-SS-injected mice was 1.6-fold higher than that of VLDL from Ad-LacZ-injected mice at 4 h after the injection of Triton WR1339 (**Table 4**). Cholesterol in the IDL/LDL fraction did not increase at 4 h after the injection of Triton WR1339 (Ad-LacZ, from 10.5 ± 0.8 mg/dl to 9.8 ± 0.7 mg/dl; Ad-SS, from 30.3 ± 3.6 mg/dl to 28.4 ± 2.7 mg/dl). Thus, the contribution of increased secretion of LDL to the development of hypercholesterolemia was negligible. Consistent with the production of cholesterol-rich VLDL, ACAT activities in the liver microsome were significantly increased, by 81% (Fig. 2C). However, mRNA expression levels of ACAT-2 or apoB in Ad-SS-injected mice were rather suppressed (Table 1).

It is conceivable that increased cholesterol synthesis suppresses the expression of LDLR, the major pathway for LDL removal, and thereby delays the plasma clearance of LDL. To explore this possibility, we measured relative mRNA expression of LDLR in the liver. It was decreased by 48% in the Ad-SS-injected mice compared with the Ad-LacZ-injected mice (Table 1).

Cholesterol could be eliminated from the liver as bile acids. The mRNA level of cholesterol 7α-hydroxylase, the rate-limiting enzyme for bile acid synthesis, however, was not increased (Fig. 1). Cholesterol could be secreted into

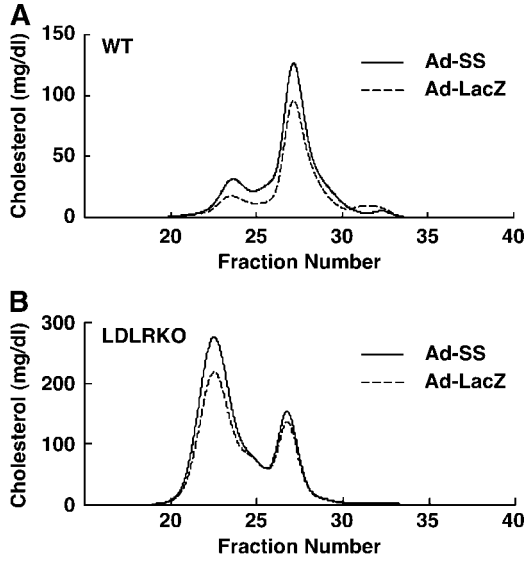

Fig. 4. Plasma lipoprotein profiles. Seven days after intravenous injection of adenovirus into C57BL/6 mice (A) (n = 6) or LDL receptor (LDLR) knockout mice (B) (n = 6), blood was collected after a 4 h fast. Pooled plasma was subjected to HPLC lipoprotein analyses. WT, wild type; LDLRKO, LDLR knockout.

circulation directly from the liver via ABCA1, for example. However, mRNA expression of ABCA1 was decreased by 34% (Table 1).

Secretion of cholesterol-rich VLDL as a major cause of hypercholesterolemia was confirmed by an experiment in which Ad-SS was injected into LDLR knockout mice. In the absence of LDLR, plasma cholesterol levels are determined primarily by the VLDL production rate, because the difference in the LDL clearance is negligible. TC levels were increased by 1.3-fold (Table 3). HPLC lipoprotein analyses revealed that cholesterol was increased mainly in the non-HDL fraction (Fig. 4B, Table 3).

To further verify the notion that the overexpression of SS is associated with increased cholesterol synthesis and

TABLE 3. Changes in plasma lipids and lipoproteins in wild-type or LDLR knockout mice

Mice	Lipids and Lipoproteins	Ad-LacZ	Ad-SS
Wild-type	TC (mg/dl)	83.1 ± 3.9	137.6 ± 4.4[a]
	TG (mg/dl)	50.7 ± 1.9	44.2 ± 2.3
	FFA (μM)	684.2 ± 101.5	583.8 ± 56.4
	VLDL (mg/dl)	3.50 ± 0.39	4.77 ± 0.40
	LDL (mg/dl)	15.68 ± 1.61	29.58 ± 3.62[a]
	HDL (mg/dl)	63.25 ± 2.81	84.07 ± 0.83[a]
LDLRKO	TC (mg/dl)	260.5 ± 9.6	334.7 ± 22.3[a]
	TG (mg/dl)	111.3 ± 6.6	117.8 ± 6.2
	FFA (μM)	421.6 ± 20.0	383.0 ± 23.9
	VLDL (mg/dl)	53.4	71.6
	LDL (mg/dl)	184.5	213.3
	HDL (mg/dl)	93.8	104.0

Seven days after intravenous injection of adenovirus into wild-type C57BL/6 mice (n = 6) or LDL receptor knockout (LDLRKO) mice (n = 6), blood was collected after a 4 h fast. Plasma was subjected to HPLC lipoprotein analyses individually. Pooled plasma was used to obtain lipoprotein data for LDLRKO mice. Values are indicated as means ± SD.

[a] $P < 0.05$ vs. Ad-LacZ.

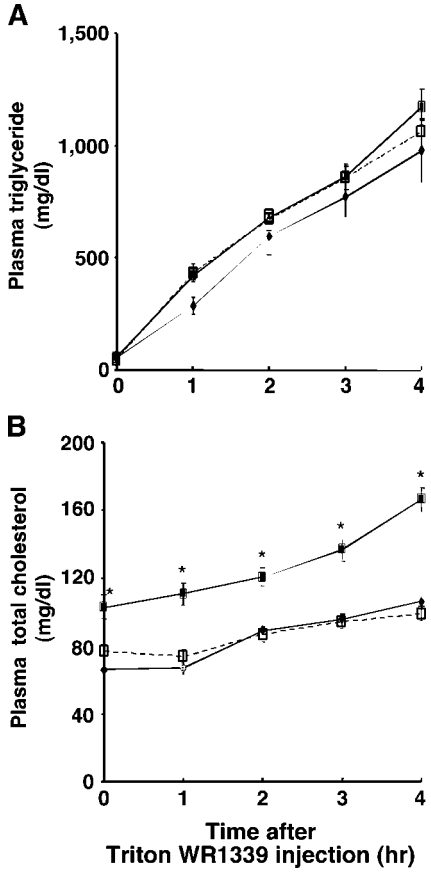

Fig. 5. Hepatic VLDL secretion. Seven days after intravenous injection of PBS (n = 4), Ad-LacZ (n = 6), or Ad-SS (n = 6) into C57BL/6 mice, VLDL secretion rates were determined by the increments of plasma levels of triglyceride (A) or cholesterol (B) after the intravenous injection of Triton WR1339. VLDL-cholesterol secretion rate in Ad-SS-injected mice was larger than that in Ad-LacZ-injected mice. *, $P < 0.05$ vs. Ad-LacZ by 2-way ANOVA and Fisher's protected least significant difference test. Dotted lines with open squares denote Ad-LacZ; solid lines with closed diamonds denote PBS; solid lines with closed squares denote Ad-SS.

VLDL secretion, we performed in vitro experiments using McARH7777 cells (**Fig. 6**). Cultured McARH7777 cells were infected with either Ad-LacZ or Ad-SS. After 48 h, [^{14}C]acetate was added to the culture, and the cells were incubated for 5 h. Radiolabeled lipids were extracted from the cells and medium, and the radioactivities incorporated into the sterols and fatty acids (unesterified plus esterified) were determined after they were resolved by TLC. As expected, Ad-SS-infected cells synthesized larger amounts of cholesterol than did the control cells infected with Ad-LacZ. Synthesis of fatty acids was also increased. Furthermore, secretion of cholesterol and fatty acids into the culture medium was also stimulated in Ad-SS-infected cells more than in Ad-LacZ-infected cells.

DISCUSSION

In the present study, we showed that overexpression of SS in the liver causes increased cholesterol biosynthesis

TABLE 4. Lipid contents in VLDL and IDL/LDL before and after intravenous injection of Triton WR1339

Lipid	h	PBS	Ad-LacZ	Ad-SS
			μg/mg protein	
Triglyceride				
VLDL	0	33.7 ± 2.5	31.2 ± 4.7	30.7 ± 1.8
	4	2,755 ± 559	2,311 ± 101	2,457 ± 167
IDL/LDL	0	27.2 ± 2.6	38.3 ± 7.7	38.3 ± 3.7
	4	79.9 ± 11.8	78.1 ± 12.8	110 ± 17
Cholesterol				
VLDL	0	26.2 ± 1.6	23.0 ± 1.7	30.3 ± 1.9[a]
	4	225 ± 29	176 ± 10	277 ± 17[a]
IDL/LDL	0	40.7 ± 6.3	32.9 ± 4.5	77.5 ± 7.9[a]
	4	81.4 ± 9.5	48.6 ± 5.7	156 ± 17[a]

Seven days after intravenous injection of PBS (n = 4), Ad-LacZ (n = 6), or Ad-SS (n = 6) into wild-type C57BL/6 mice, Triton WR1339 was injected intravenously. Blood was collected before and 4 h after the injection. Plasma was subjected to sequential analytical ultracentrifugation. Concentrations of protein, TG, and cholesterol were determined. Values are indicated as means ± SE.

[a] $P < 0.05$ vs. Ad-LacZ by ANOVA and Fisher's PLSD.

and hypercholesterolemia, which is the first in vivo evidence that SS plays a regulatory role in cholesterol metabolism, and is a mirror image of previous observations by others that SS inhibitors possess cholesterol-lowering activity primarily through inhibition of hepatic VLDL production (16, 17, 34). Other new findings in the current paper are as follows: *1*) HMG-CoA reductase activity is upregulated in spite of downregulation of its own mRNA expression. *2*) Secretion of cholesterol-rich VLDL and possibly downregulation of LDLR are responsible for the development of hypercholesterolemia. *3*) Increased cholesterol synthesis is associated with hepatomegaly due to increased proliferation of hepatocytes. *4*) Genes of enzymes in the cholesterol synthetic pathway, including the enzymes downstream of SS, such as squalene epoxidase and lanosterol 14α-demethylase, are downregulated.

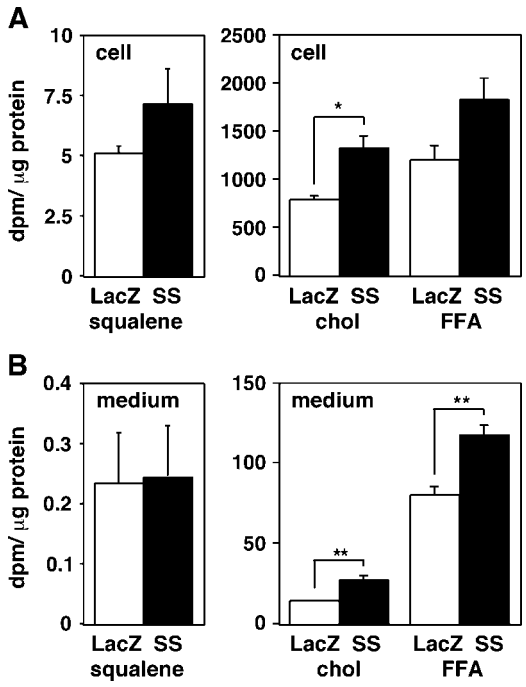

Fig. 6. Lipid synthesis and secretion in McARH7777 cells. McARH7777 cells were transfected with recombinant adenovirus [Ad-LacZ (open bars) and Ad-SS (closed bars)], and after 48 h, [^{14}C]acetate was added to a final concentration of 28.6 μCi/ml. After 5 h of incubation, the media (B) were removed and the cells (A) were harvested. Radiolabeled sterols and fatty acids were extracted and resolved by TLC, and the content of ^{14}C-labeled sterol and fatty acids in cells and media were measured. *, $P < 0.05$ and **, $P < 0.01$ vs. Ad-LacZ. Chol, cholesterol. Error bars represent standard deviation.

The mRNA expression of enzymes in the cholesterol biosynthetic pathway, such as HMG-CoA reductase, squalene epoxidase, and lanosterol 14α-demethylase, was downregulated (Table 1). The downregulation of these genes might be mediated through a negative feedback regulation by the end product, cholesterol, which was overproduced by the liver (Fig. 2). The downregulation of the genes for squalene epoxidase and lanosterol 14α-demethylase suggests that there are no other rate-limiting steps in the cholesterol biosynthetic pathway past SS. Interestingly, cholesterol biosynthesis was increased in spite of the overall downregulation of genes in the cholesterol biosynthetic pathway. This puzzling phenomenon might be explained by an increase in HMG-CoA reductase activity, the rate-limiting enzyme in cholesterol biosynthesis. The induction of HMG-CoA reductase activity should be through its post-transcriptional regulations; both translation and degradation of HMG-CoA reductase protein are subject to feedback regulation by nonsterol mevalonate metabolites, as mentioned above. In the state of overwhelmingly increased activity of SS, most of the mevalonate metabolites may be utilized to produce squalene, leaving little substrates for nonsterol pathways such as geranyl diphosphate and farnesyl diphosphate. Under this condition, it is expected that translation of HMG-CoA reductase is increased and that its proteosomal ER degradation is suppressed, because farnesol (35, 36) and/or granylgeraniol (6) stimulate degradation of this enzyme. To verify this possibility, we need to examine the protein stability of HMG-CoA reductase and the cellular contents of isoprenoids. Unfortunately, we failed to detect HMG-CoA reductase protein in the liver membrane fractions by Western blot analysis, although we followed the established protocol and used two different

antibodies against HMG-CoA reductase. Our standard diet containing 0.075% of cholesterol might suppress the HMG-CoA reductase protein to a level below the sensitivity (37). A similar deficiency of mevalonate metabolites may underlie the marked upregulation of HMG-CoA reductase in cells from mevalonate kinase-deficient patients, where HMG-CoA reductase activity is increased by 6-fold despite the absence of the increases in its expression level (38). In addition to the substrate pool size of isoprenoids in the cholesterol biosynthetic pathway, flux to the isoprenoid pathway might be regulated by a separate isoprenoid transport network that selectively extracts some of these compounds and distributes them to other synthetic enzymes.

There are two potential explanations for the hypercholesterolemia in mice infected with Ad-SS: *i*) secretion of cholesterol-rich VLDL and *ii*) defective plasma clearance of LDL. Involvement of the secretion of cholesterol-rich VLDL is supported by the following findings: *1*) LDL-cholesterol was increased even in the LDLR knockout mice in which the LDL clearance was maximally suppressed (Fig. 4B). *2*) Cholesterol-rich VLDL was accumulated in the plasma of mice after the intravenous injection of Triton WR1339 (Fig. 5B). *3*) Hepatic ACAT activity, which is tightly associated with VLDL secretion (39–41), was increased. Supporting this, an in vitro study using McARH7777 cells showed that the secretion of cholesterol and fatty acids into the medium was increased when the cells overexpressed SS (Fig. 6). In this regard, it is interesting to note that some SS inhibitors, but not statins, inhibit VLDL secretion from the liver (17, 34, 42). These considerations suggest the intriguing possibility that nonsterol mevalonate metabolites, for example, farnesol, or newly synthesized cholesterol regulate VLDL secretion (42); this awaits further investigation. Defective LDL clearance may also contribute to the development of hypercholesterolemia, because the mRNA expression of the LDLR gene is downregulated (Table 1). The downregulation of the LDLR gene might result from the feedback regulation by the increased cholesterol biosynthesis. Consistently, SS inhibitors stimulate the LDLR activity (16, 17).

It is of note that cholesterol contents per weight of wet tissue did not significantly increase in the liver despite the increased cholesterol synthesis (Table 2). This resistance to cholesterol accumulation was not observed in transgenic mice overexpressing SREBP-1a, -1c, or -2 under the control of the phosphoenolpyruvate carboxykinase promoter in which cholesterol synthesis is stimulated by the overexpression of the active form of SREBPs and which exhibit massive accumulation of cholesterol in the liver (43–45). One explanation for this may be that the degree of the increase in cholesterol synthesis was not large enough to achieve a detectable buildup of cholesterol in the liver, possibly because the time span (7 days) was not long enough. An increased cholesterol flux into the lipoprotein assembly without an increased hepatic uptake may be another explanation. SREBP may promote the transcription of genes that participate in lipid droplet formation, rather than genes involved in VLDL secretion. A third possibility is that cholesterol is efficiently eliminated out of the liver into the bile in compensation for the increased synthesis, although the expression of genes responsible for this pathway, such as cholesterol 7α-hydroxylase, was not significantly changed (Table 1). A final but most interesting possibility is that newly synthesized cholesterol may be utilized for the plasma membranes of dividing cells. The liver was heavier, with an increased number of cells positive for Ki-67, after infection with Ad-SS (Table 2, Fig. 3), indicating that the hepatocytes were in a hyperproliferative state. Although the precise mechanisms are currently unknown, changes in the expression of Ras and Ras-related proteins (46) or in the cholesterol content of caveolae (47) may be intriguing possibilities.

The present study shows that selective upregulation of SS may lead to hypercholesterolemia. Mutations in the SS gene that confer stimulation of enzymatic activity may underlie some forms of hypercholesterolemia. In combination with liver-specific knockout of SS, which is being generated in this lab, manipulation of hepatic SS activity should provide the basis for understanding the complex regulatory mechanisms in the cholesterol biosynthetic pathway and for developing novel therapeutic modalities for hyperlipoproteinemia.

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