Embryonic Lethality and Defective Neural Tube Closure in Mice Lacking Squalene Synthase*

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Squalene synthase (SS)1 (farnesyl-diphosphate:farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21) 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. We used gene targeting to knock out the mouse SS gene. The mice heterozygous for the mutation (SS+/−) were apparently normal. SS+/− mice showed 60% reduction in the hepatic mRNA levels of SS compared with SS+/+ mice. Consistently, the SS enzymatic activities were reduced by 50% in the liver and testes. Nevertheless, the hepatic cholesterol synthesis was not different between SS+/− and SS+/+ mice, and plasma lipoprotein profiles were not different irrespective of the presence of the low density lipoprotein receptor, indicating that SS is not a rate-limiting enzyme in the cholesterol biosynthetic pathway. The mice homozygous for the disrupted SS gene (SS−/−) were embryonic lethal around midgestation. E9.5–10.5 SS−/− embryos exhibited severe growth retardation and defective neural tube closure. The lethal phenotype was not rescued by supplementing the dams either with dietary squalene or cholesterol. We speculate that cholesterol is required for the development, particularly of the nervous system, and that the chorioallantoic circulatory system is not mature enough to supply the rapidly growing embryos with maternal cholesterol at this developmental stage.

Squalene synthase (SS)1 (farnesyl-diphosphate:farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21) 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 (1). This enzyme is an attractive target for cholesterol-lowering therapy, because the inhibition of this step theoretically may not perturb the non-sterol pathway, which is a potential problem in the use of the inhibitors of cholesterol synthesis (2). Indeed, several potent SS inhibitors, such as squalestatins or zaragozic acids (3), 1,1-bisphosphonates (4), and quinuclidine derivatives (5), successfully lower plasma cholesterol levels without adverse effects in vivo.

Accumulating evidence supports the notion that cholesterol metabolism plays an essential role in development, particularly of a like nervous system (see Ref. 6 for review). All of the naturally occurring inborn errors in cholesterol metabolism, such as Smith-Lemli-Opitz (SLO) syndrome (7, 8), desmosterolosis (9), and mevalonate kinase deficiency (10), are associated with severe developmental abnormalities. Moreover, mice lacking apoB (11, 12), microsomal triglyceride transfer protein (13), and megalin/gp330 (14) had anomalies in the nervous system. Recently, the Hedgehog family of proteins, which are crucial for the pattern formation in the vertebrate embryogenesis (see Ref. 15 for review), was shown to undergo post-translational modification by covalent attachment of a cholesterol molecule to the biologically active amino-terminal fragment of these peptides (16). Furthermore, Patched, a putative cognate receptor for the Hedgehog protein, shares homology with cholesterol-binding proteins (17, 18). Together, it is tempting to hypothesize that the derangement of cholesterol synthesis affects the development of the nervous system through distorting Hedgehog signaling.

To test the hypothesis that cholesterol itself is essential for normal embryogenesis and whether the reduction of SS activities is associated with cholesterol-lowering effects, we have generated SS knockout mice. The SS−/− mice were lethal between E9.5 and E12.5 and exhibited severe retardation of development. The SS+/− mice expressed only 50% SS activities in the liver and testes compared with SS+/+ mice, whereas their plasma lipoprotein profiles and responses to the dietary challenge were unaffected.

EXPERIMENTAL PROCEDURES

Generation of SS Knockout Mice—A fragment (528 base pairs) of mouse SS cDNA was amplified by using primers that were designed based on reported sequences of mouse SS cDNA (sense primer, 5′-GTCGCAAGGATGGAGTTCGT-3′, and antisense primer, 5′-GTCGCACTATTGTGCAGG-3′) (19). This polymerase chain reaction product was used as a probe to clone a genomic DNA from the 129Sv mouse genomic library as described previously (20). A replacement-type targeting vector was constructed; the short arm containing a 1.2-kb PstI/VII fragment in intron 3 and the long arm containing a 8.5-kb EcoRV fragment spanning exons 6–7 were inserted into the 129Sv mouse genomic library as described previously (20). A replacement-type targeting vector was constructed; the short arm containing a 1.2-kb PstI/VII fragment in intron 3 and the long arm containing a 8.5-kb EcoRV fragment spanning exons 6–7 were inserted into the XhoI and NotI sites, respectively, of the vector pPolIIshort-neobp-HHSVTK as described previously (21). Thus, exons 4–5, which contained a putative catalytic site (22), were replaced by a neomycin-resistant cassette.

After linearization by digestion with SalI, the vector was electroporated into JH1 embryonic stem cells. Targeted clones, which had been selected in the presence of G418 and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil, were identified by polymerase chain reaction
using the following primers: primer 1, 5'-ATACAGGGGAGTGCCTTCTTGTG-3' and primer 2, 5'-GATTTGGGAGAATACAGGACATGC-3' (Fig. 1). Homologous recombination was verified by Southern blot analysis after \textit{Bam}HI/EcoRI double digestion using a 0.5-kilobase pair \textit{Pst}I fragment as a probe (Fig. 1). Targeted embryonic stem clones were injected into the C57BL/6 blastocysts yielding five lines of chimeric mice, which transmitted the disrupted allele through the germline. All experiments reported here were performed using five lines of chimeric mice, which transmitted the disrupted allele.

\textbf{Breeding Experiments—SS}+/- mice were cross-bred to the LDL receptor knockout mice (\textit{LDLR}-/-) (21) to produce mice that were heterozygous for the disrupted alleles of both SS and the LDL receptor loci. An intercross of these animals was performed.

\textbf{Diets—} Three diets were used: (i) a normal chow (MF, Oriental Yeast), (ii) a 1.25% cholesterol diet, which contains 1.25% (w/w) cholesterol, 5% (w/w) cocoa butter, and 0.5% (w/w) cholic acid (23), and (iii) a 2% squalene diet, which contains 2% (w/w) squalene (WAKO) (24).

\textbf{Timed mating was performed and dietary supplementation was initiated 2 weeks before the mating.}

\textbf{SS Activity Assay—} Liver and testes were homogenized in a buffer containing 15 mM nicotinamide, 2 mM MgCl\textsubscript{2}, and 100 mM potassium phosphate, pH 7.4, and centrifuged at 10,000 \times g for 20 min at 4 °C. The supernatants were centrifuged at 105,000 \times 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. SS activities were measured according to a modified method of Cohen \textit{et al.} (25).

In brief, the microsome fractions (20 μg) were incubated in 50 μl of a buffer containing 20 μM [3\textsuperscript{H}] farnesyl pyrophosphate (25 μCi/μmol), 1 mM NADPH, 5 mM MgCl\textsubscript{2}, 6 mM glutathione, and 100 mM potassium phosphate, pH 7.4, at 37 °C for 15 min. Reaction was terminated by the addition of 150 μl of chloroform/methanol (1:2, v/v) containing 0.2% unlabeled squalene. After 50 μl of chloroform and 50 μl of 3 N NaOH were added, the reaction mixtures were vortexed and centrifuged. The infranatant organic phase was used for the determination of the radio-activities in the squalene produced.

\textbf{Measurement of Hepatic Cholesterol Synthesis—} Cholesterol synthesis in the liver was estimated by a modified method of Eisele \textit{et al.} (26). In brief, 6 \textit{SS}+/- and 6 \textit{SS}+/- mice (9–10-week-old females) were given food and water \textit{ad libitum} and injected intraperitoneally with [2-\textsuperscript{14}C]acetate (37 kBq/kg body weight). After 30 min, animals were euthanized, and two pieces of the liver (0.3 g each) were removed. [1,2-\textsuperscript{3}H]cholesterol (10,000 dpm) was added as an internal standard, and tissue samples were saponified in 2 ml of 15% KOH, 90% ethanol at 70 °C overnight. After the addition of 2 ml of water, nonsaponified lipids were extracted with n-hexane, evaporated, and dissolved in ethanol/acetone (1:1, v/v). Sterols were precipitated with digitonin. After washing the precipitates with 50% acetone, the radioactivities were determined. The results were expressed as 14C dpm/g wet weight of liver/30 min.

\textbf{Northern Blot Analyses—} Poly(A\textsuperscript{+}) RNA was isolated and pooled from the livers of six animals. 1.2 μg were subjected to 1% agarose electrophoresis in the presence of formalin. The fractionated RNA was transferred to Hybond N (Amersham Pharmacia Biotech). The filters were hybridized to 32P-labeled cDNA probes: the LDL receptor, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, and cholesterol 7a-hydroxylase.

\textbf{Plasma Lipoprotein Analyses—} After a 12-h fast, blood was collected in tubes containing EDTA. Plasma levels of total cholesterol (TC) and triglycerides (TG) were determined enzymatically using kits (Determiner TC555 and Determiner TG555, Kyowa Medex). Lipoproteins were fractionated by high performance liquid chromatography (HPLC) as described (23), and the cholesterol contents in each lipoprotein fraction were determined.

\textbf{Morphological Studies—} Embryonic tissues were examined by standard histological techniques.
RESULTS

SS Knockout Mice—Two genomic clones were isolated from a library with a cDNA probe. The restriction mapping revealed that one genomic clone (Fig. 1A) consisted of 8 exons spanning more than 26 kb. A replacement-type vector, which allowed the deletion of exon 4–5, was constructed and used to generate heterozygous SS knockout mice (SS+/–). SS+/– mice, which were viable and fertile, were intercrossed to obtain homozygous SS knockout mice (SS–/–). No viable SS–/– mice were identified among 149 weaned offspring, although 99 were heterozygous (Table I). To determine the developmental stage where the embryos were lethal, the embryos were genotyped at four different embryonic stages: 9.5, 10.5, 12.5, and 13.5 days postcoitum (Table I, Fig. 1B). At E9.5–10.5, SS–/– was identified at the frequency significantly lower than the expected mendelian frequency (χ² = 8.35, p < 0.05). All SS–/– embryos were significantly smaller than SS+/+ embryos with comparable developmental age. SS–/– embryos exhibit a wide variety of morphology. Among 13 SS–/– embryos, only 1 at E9.5 showed a nearly normal shape with a small forebrain (Fig. 2C), 6 had a shape that is similar to E8.5 embryos, early somite-stage embryos, with an open head fold (Fig. 2D). Their microscopic examination showed necrotic neuronal cells with condensation of nuclei, i.e., apoptotic cells (Fig. 2, F and G). Four were much smaller and ambiguous and did not have somites (Fig. 2B). At E12.5–13.5, however, no SS–/– were identified (Table I).

Reduction in mRNA Levels and Activities of SS in Heterozygotes of SS Knockout Mice—The SS mRNA levels in the SS+/– mice were reduced by 60% in the liver (Fig. 3A). Consistently, their SS activities were significantly reduced by 50% both in the liver and testes (Fig. 3B). In the liver, the mRNA levels of the LDL receptor appeared to be increased by 20% in the SS+/– mice (Fig. 3A). On the other hand, the mRNA of cholesterol 7α-hydroxylase appeared to be decreased by 20%. There was no significant change in the mRNA levels of HMG-CoA reductase between the SS+/+ and SS+/– mice.

Cholesterol Synthesis in the Liver—If SS is the rate-limiting step for TC synthesis, cholesterol synthesis is expected to be reduced in the liver. However, the amounts of [14C]acetate incorporated into cholesterol in the liver were not different between the wild-type and SS+/– mice (10,173 ± 6444 versus 13,992 ± 10,276 dpm/g liver/30 min, p > 0.05, n = 6).

Plasma Lipoprotein Analyses—To determine whether the reduction in the SS activities in the liver is associated with the changes in plasma lipoprotein profile, we determined the plasma lipid levels. Neither plasma TC nor TG levels were different between the wild-type and SS+/– mice, which were fed a normal chow (Fig. II). There was no significant difference in the amounts of VLDL, LDL, or HDL cholesterol as measured by HPLC (Table III). To evaluate the effects of the suppression of SS activities in the liver on the plasma lipoprotein profile in the setting of LDL deficiency, we further cross-bred these animals with LDL receptor knockout mice to generate SS+/+;LDLR–/– mice. Neither plasma TC nor TG levels were different between the SS+/+;LDLR–/– and SS+/–;LDLR–/– mice (Table II).

Attempt to Rescue the Heterozygotes by Feeding with Squalene or Cholesterol—If the embryonic lethality of the heterozygotes results from the cholesterol deficiency, it is theoretically possible that dietary supplementation of squalene or cholesterol would reverse the phenotype. To test this hypothesis, we fed the pregnant SS+/– mice with the diets supplemented with either 2% squalene or 1.25% cholesterol. No viable SS–/– mice were identified among 21 offspring for squalene rescue (4
yolk sac is drastically increased (30). Evidence suggests that several lipoprotein receptor systems mediate the transfer of maternal lipoprotein: the LDL receptor, megalin/gp330, VLDL receptor, and low density lipoprotein receptor-related protein for the uptake of lipoproteins containing either apoB-100 or apoE (reviewed in Refs. 6 and 31), and scavenger receptor B-1 for the binding of apoA-I containing lipoproteins (32). Because mice lacking the LDL receptor (21) or VLDL receptor (33) are fertile, there may be some redundancy in the receptor function. On the other hand, mice lacking low density lipoprotein receptor-related protein (34) or megalin/gp330 (14) are embryonic lethal, suggesting the pivotal role of these receptor in embryogenesis.

In this concern, it is noteworthy that SS−/− embryos develop to the stage around E9.5. Results indicate that cholesterol required for growth is exclusively supplied from the dams in SS−/− mice. Indeed, it has not been reported that pharmacological doses of HMG-CoA reductase inhibitors or SS inhibitors cause preimplantation lethality (35). It is known that nonpolar squalene, an immediate product of squalene synthase, is transported in association with VLDL in the plasma (36) and subsequently by a squalene and sterol carrier protein (37). Therefore, embryos may utilize squalene that is diffused from extra embryonic tissues for cholesterol synthesis. Furthermore, in the rodents, the nutritional supply of the embryo during the immediate postimplantation period is dependent on the resorptive and synthetic capacity of the yolk sac (38). Presumably, cholesterol transported across this maternal fetal barrier is utilized for development.

The embryonic lethality of the SS−/− mice may result from either cholesterol deficiency, toxic precursor buildup, or both. The cholesterol-supplying system in the early gestational period may no longer be effective beyond midgestation when a functional choioallantoic circulatory system is established (38). It is conceivable that the placental system is too immature to fully compensate for the complete deficiency of the de novo cholesterol synthesis around this developmental stage. Belknap and Dietschy (27) have shown that the de novo cholesterol synthesis remains highly active during the whole midgestational period and that the contents of newly synthesized sterols are maximal at E17 in the rat fetus. Similar failure to catch up with the increasing demand may underlie the lethal phenotype of the SS−/− embryos (39). It is conceivable that the placental system is too immature to fully compensate for the complete deficiency of the de novo cholesterol synthesis around this developmental stage.

During the early development of animals, particularly during fetal stage, there is a marked demand for new sterol. Cholesterol is required for new membrane synthesis, for maintenance of existing membranes, and for the synthesis of hormone and bile acids. As in the adult, this cholesterol is supplied by either de novo synthesis within the fetal compartment or by transfer from the maternal compartment to the fetus through uptake of cholesterol carried in lipoproteins. Previous studies have shown that sterol synthesis is markedly increased in the developing fetus including a preimplantation embryo (27–29). In addition, transport of maternal LDL and HDL to the fetal
sive urinary excretion of farnesol-derived dicarboxylic acids (41), indicating the elevation of the plasma concentration of farnesol and its metabolites. Farnesol has recently been found to be a biologically active substance; it inhibits arterial vasoconstriction through blocking L-type Ca²⁺ channels in vascular smooth muscle cells (42, 43). Other studies have reported the existence of a farnesol-specific, orphan nuclear receptor in vertebrate cells, the farnesoid X-activated receptor, but its precise functions remain unknown (44). Therefore, it is possible that farnesol or its metabolites may have toxic effects on the embryos. This toxic precursor buildup hypothesis may be more plausible, because supplementation of the dams with dietary squalene failed to rescue the lethal phenotype.

As mentioned above, neural developmental anomalies are common in mice with the induced mutations in cholesterol transport systems (11–14), humans with genetic defects in the enzymes of the cholesterol biosynthetic pathway (7–9), and fetuses born to animals treated with the inhibitors of these enzymes (36, 40). These observations lead us to the speculation that SS−/− mice develop anomalies in the nervous system. In this regard, it is noteworthy that SS−/− had an open neural plate (Fig. 2D). Because the general growth of SS−/− was severely impaired, the defective neural tube closure may simply reflect general growth retardation. Interestingly, the phenotypes of SS−/− are different from those of infants with SLO syndrome, despite the fact that the responsible enzymes are involved in the proximal part of the developmental anomalies of SLO syndrome (8). In the SLO syndrome, Δ⁷-reductase activity is partially compensated by Δ⁶-reductase, an alternative enzyme involved in the terminal step of cholesterol biosynthesis, thereby leading to a milder phenotype (9). The differences may also arise from either species differences or build up of different precursors.

Recent studies have suggested that cellular cholesterol metabolism and signaling molecules that are crucial for early development, the Hedgehog proteins (16–18), Farnesol and its metabolites. Farnesol has recently been found (41), indicating the elevation of the plasma concentration of farnesol-derived dicarboxylic acids (41), indicating the elevation of the plasma concentration of farnesol and its metabolites. Farnesol has recently been found to be a biologically active substance; it inhibits arterial vasoconstriction through blocking L-type Ca²⁺ channels in vascular smooth muscle cells (42, 43). Other studies have reported the existence of a farnesol-specific, orphan nuclear receptor in vertebrate cells, the farnesoid X-activated receptor, but its precise functions remain unknown (44). Therefore, it is possible that farnesol or its metabolites may have toxic effects on the embryos. This toxic precursor buildup hypothesis may be more plausible, because supplementation of the dams with dietary squalene failed to rescue the lethal phenotype.

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