Early Embryonic Lethality Caused by Targeted Disruption of the 3-Hydroxy-3-methylglutaryl-CoA Reductase Gene*

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The endoplasmic reticulum (ER) enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which converts HMG-CoA to mevalonate, catalyzes the rate-limiting step in cholesterol biosynthesis. Because this mevalonate pathway also produces several non-sterol isoprenoid compounds, the level of HMG-CoA reductase activity may coordinate many cellular processes and functions. We used gene targeting to knock out the mouse HMG-CoA reductase gene. The heterozygous mutant mice (Hmgcr+/−) appeared normal in their development and gross anatomy and were fertile. Although HMG-CoA reductase activities were reduced in Hmgcr+/− embryonic fibroblasts, the enzyme activities and cholesterol biosynthesis remained unaffected in the liver from Hmgcr+/− mice, suggesting that the haploid amount of Hmgcr gene is not rate-limiting in the hepatic cholesterol homeostasis. Consistently, plasma lipoprotein profiles were similar between Hmgcr+/− and Hmgcr+/+ mice. In contrast, the embryos homozygous for the Hmgcr mutant allele were recovered at the blastocyst stage, but not at E8.5, indicating that HMG-CoA reductase is crucial for early development of the mouse embryos. The lethal phenotype was not completely rescued by supplementing the dams with mevalonate. Although it has been postulated that a second, peroxisome-specific HMG-CoA reductase could substitute for the ER reductase in vitro, we speculate that the putative peroxisomal reductase gene, if exists, does not fully compensate for the lack of the ER enzyme at least in embryogenesis.

The mevalonate pathway produces isoprenoids that are essential for diverse cellular functions, ranging from cholesterol synthesis to growth control. The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), which catalyzes the conversion of HMG-CoA to mevalonate, is the rate-limiting enzyme in the mevalonate pathway (1). Because of its major role in cholesterol biosynthesis, the regulation of HMG-CoA reductase has been intensely studied. To ensure a steady mevalonate supply, the non-sterol and sterol end-products of mevalonate metabolism exert feedback regulation on the activity of this enzyme through multivalent mechanisms, including inhibition of transcription of the HMG-CoA reductase mRNA, blocking of translation, and acceleration of protein degradation, thus regulating the amount of reductase protein over a several hundred-fold range (reviewed in Refs. 1–3).

Inhibitors of HMG-CoA reductase, statins, are potent hypocholesterolemic agents that exhibit some cholesterol-independent, or so-called pleiotropic, effects, that involve improving or restoring endothelial function, enhancing the stability of atherosclerotic plaque, and decreasing oxidative stress and vascular inflammation (4). Benefits of statins also extend beyond cardiovascular diseases, including a reduction in the risk of dementia (5), Alzheimer’s disease (6), ischemic stroke (7), and osteoporosis (8, 9). Most of these pleiotropic effects of statins are shown to be mediated by their ability to block the synthesis of non-sterol isoprenoid intermediates. However, it remains to be unraveled how the mevalonate pathway is affected in those disease processes, particularly in cells involved in atherogenesis. In addition, some of the collateral effects of statins have been found to be independent of HMG-CoA reductase (10–12).

In mammals, only one gene has been found to encode HMG-CoA reductase (13). In yeast, fungi, and plants, on the other hand, more than one gene encode the enzyme. Yeast, for example, contains two functional genes for HMG-CoA reductase, HMG-CoA reductase 1 (HMG1) and HMG2. HMG1 and HMG2 are differentially expressed, and, when HMG1 is deleted, HMG2 can replace the function of HMG1 (14). Given the major role of HMG-CoA reductase in the mevalonate pathway, it is tempting to hypothesize that mammalian cells also have a second gene for the enzyme. In fact, although the classic form of the enzyme is a transmembrane protein anchored to the endoplasmic reticulum (ER), recent studies using a mutant cell line that lacks...
the ER isofrom of the enzyme indicate the existence of a second
isofrom of the reductase exclusively localized in peroxisomes
and that the peroxisomal activity might be due to a second
isoform (15–17).

We have previously shown that the targeted disruption of the
gene for squalene synthase, the first committed enzyme of
sterol synthesis, results in embryonic death at mid gestation
with growth retardation and defective neural tube closure (18).
In mice, the non-sterol pathways are presumed to be
retained, whereas de novo cholesterol synthesis is blocked. To
further determine the physiological consequences of perturba-
tion of the mevalonate pathway and gain some insights into
a putative second gene for HMG-CoA reductase, we have gener-
ated and characterized mice defective in mevalonate synthesis
by disrupting the gene for HMG-CoA reductase.

EXPERIMENTAL PROCEDURES

Generation of HMG-CoA Reductase Knockout Mice—A replace-
ment-type targeting vector was constructed; the 0.8-kb short arm spanning
exons 14–15, and the 10-kb long arm fragment encompassing exons
2–12 were generated by PCR using genomic DNA from the 129/Sv
mouse as template. Primers used were as follows: 5′-CCGTCGACGA-
AGAAGGCCCTTTTGATAGCACCAGCA-3′ (exon 14) and 5′-CCGTCGAC-
AGCTTAGAGATCATGTCATGCCCATCCCGTG-3′ (exon 15) for the short
arm and 5′-GCGGCGCTTGGCTTGGGCTCCCTGAGTTATTTTATT-
GTT-3′ (exon 2) and 5′-GGGGGGCGCTTTGATAGCACCAGCA-3′
(exon 15) for the long arm. Integrity of the amplified
fragments was verified by Southern blot analysis and partial sequen-
cing of the subcloning into pBluescript I vector (Invitrogen, Carlsbad, CA), the
fragments were cut out and inserted into the XhoI and NotI sites,
respectively, of the pPolII-short-neo-bpa-HSVTK as described previ-
ously (19). Thus, a 1.2-kb region spanning the exons 12–14 of
the HMG-CoA reductase gene was replaced by a neomycin-resistant
con cassette, which was expected to abolish translation of the entire carboxyl
half of the protein containing the catalytic activity (20). After linear-
ization by digestion with SalI, the vector was electroporated into J1H1
embryonic stem cells (A gift from Dr. J. Herz at the University of Texas
Southwestern Medical Center at Dallas). Targeted clones, which had been
selected in the presence of G418 and 1-(2-deoxy-2-fluoro-
inosine) (21).

To test whether supplementation of
mevalonic acid lactone (Sigma, St. Louis, MO) was infused via
a miniosmotic pump (ALZET model 2004 osmotic pump, ALZA, Palo Alto,
CA), which was implanted subcutaneously in female heterozygous
mice. About 1 week after surgery, the mice were subjected to timed matings
with heterozygous males. The pumps are designed to release the con-
stantly for 4 weeks, long enough to cover the entire pregnancy
period. In a pilot experiment, plasma concentration of mevalonate rose
from 25–35 ng/ml in non-treated females to 60,000–70,000 ng/ml
in infused animals.

Mouse Embryonic Fibroblasts—Primary mouse embryonic fibro-
basts (MEFs) were prepared from 14.5-day-old embryos and cultured in
Dulbecco’s modified Eagle medium (DMEM) supplemented with 10%
fetal calf serum (FCS). MEFs were used within three passages. All
experiments were done when the cells reached confluence. Each
monolayer was washed three times with phosphate-buffered saline,
after which fresh media containing either 10% (v/v) FCS or 5 mg/ml
human lipoprotein-deficient serum (LPDS) with or without 100 nM
simvastatin (Wako, Osaka, Japan) were added. Cells were incubated for
6 h and subjected to the following analyses. Simvastatin was activated
by alkaline hydrolysis prior to use.

**HMG-CoA Reductase Activity Assay**—For preparation of liver
microsomes, animals were sacrificed during the early dark cycle, at a time

**FIG. 1. Targeted disruption of the Hmgcr gene. A, a map of the
Hmgcr locus, together with the sequence replacement gene-targeting
vector and the targeted Hmgcr allele. Only relevant exons (closed boxes)
and introns (thick lines) are shown. The gene-targeting event replaced
the region spanning exons 12–14 with a cassette containing a neomycin
resistance gene (neo) driven by the RNA polymerase II promoter.
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when HMG-CoA reductase activity was at its peak of diurnal rhythm (21). Livers were homogenized in a buffer containing 15 mm nicotinamide, 2 mm MgCl₂, 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 20 min at 4 °C, and the resultant pellets, comprising a microsome fraction, were washed, resuspended in the same buffer, and stored in aliquots at −80 °C. HMG-CoA reductase activities were measured essentially as described previously (24).

Briefly, the microsome fractions (~50 μg) were incubated in 20 μl of a buffer containing 110 μM tris-3-[14C]HMG-CoA (4.5 μCi/μmol), 5 mm NADP, 10 μM EDTA, 10 μM dithiothreitol, and 100 mm potassium phosphate, pH 7.4, at 37 °C for 30 min. 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 [14C]mevalonate was isolated by thin-layer chromatography and counted using [3H]mevalonate as an internal standard. For MEFs, cellular extracts were prepared as described (25). Extracts (~50 μg of protein) were incubated in 50 μl of a buffer containing 30 μM tris-[3-14C]HMG-CoA (20 μCi/μmol), 2.5 mm NADP, 5 μM EDTA, 5 μM dithiothreitol, and 100 μM potassium phosphate, pH 7.4, at 37 °C for 120 min, then processed as in the assay of liver microsomes. HMG-CoA reductase activity is expressed as picomoles of [14C]mevalonate formed per minute per mg of protein.

Measurement of Hepatic Cholesterol Synthesis—Cholesterol synthesis in the liver was estimated in littermate 12-week-old male mice (n = 6) during the mid light cycle as previously described (18, 26). In brief, animals were given food and water ad libitum and injected intraperitoneally with [2-14C]acetate (37 kBq/kg body weight). 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 14C dpm/100 mg of wet weight of liver.

Northern Blot Analysis—Poly(A⁺) RNA was isolated and pooled from the livers of five animals. 1.2 μg were subjected to 1%-agarose gel electrophoresis in the presence of formalin. The fractionated RNA was transcribed to cDNA and hybridized to a HMG-CoA reductase, HMG-CoA synthase, mevalonate kinase, farnesyl diphosphate synthase, and squalene synthase, showed a significant change in the hepatic HMG-CoA reductase activities. Consistently, the amounts of [14C]acetate incorporated into digitonin-precipitable sterols in the liver were not different between the wild-type and heterozygous mice (Table III).

Analysis of hepatic lipids demonstrated that both cholesterol and triglyceride content were unchanged. Plasma levels of total cholesterol and triglycerides did not differ in the two groups of mice. Plasma lipoprotein analysis by high-performance liquid chromatography revealed no discernible difference in the amount of each lipoprotein fraction (data not shown).

Hmgr+/− MEFs—To investigate the regulation of HMG-CoA reductase in Hmgr+/− mice further, we isolated MEFs from Hmgr+/− and Hmgr+/+ embryos. Their growth in culture media was comparable. Fig. 4 summarizes HMG-CoA reductase activities of MEFs maintained for 6 h in the presence of either 10% FCS, or 5 mg/ml LPDS with or without 100 nM simvastatin, a competitive inhibitor of HMG-CoA reductase, which induces up-regulation of HMG-CoA reductase in vivo and in vitro. As expected, the enzyme activity in MEFs was
HMG-CoA Reductase Deficiency in Mice

FIG. 2. Genotyping of blastocyst. A, scheme for PCR detection of wild-type and disrupted Hmgcr alleles. Exon 14 (solid box), the neo expression cassette (open box), and the location of primers 1, 3, and 4 are depicted. B, representative Southern blot analysis of PCR products amplified from blastocyst DNA. Wild-type (300 bp) and mutant (220 bp) bands are indicated.

TABLE II
Effects of maternal mevalonate supplementation on the genotypes of offspring from heterozygous intercrosses

| Age        | Appearance | Genotype | Total |
|------------|------------|----------|-------|
| 3 weeks    | Normal     | Abnormal* | +/+   | +/−   | −/−   | 31    |
| E9.5–10.5  | 39         | 10       | 10    | 21    | 0     | 49    |

*Abnormal embryos were with egg cylinder stage appearance at E9.5–10.5 and their precise genotyping was not successful.

FIG. 3. Hepatic expression of the genes involved in cholesterol homeostasis in Hmgcr+/− mice. Northern blot for HMG-CoA reductase, HMG-CoA synthase, farnesyl diprophosphate synthase, squalene synthase, LDL receptor (upper panels), and GAPDH (lower panels) as a loading control using liver poly(A) RNA. W, wild-type; H, heterozygote.

up-regulated by the addition of LPDS and further induced by concomitant treatment with simvastatin. In each experimental condition, the HMG-CoA reductase activity was reduced by −50% in Hmgcr+/− MEFs. Increases in the enzymatic activities over the levels observed in the presence of FCS were approximately 2- and 12-fold in the presence of LPDS and in the presence of LPDS plus simvastatin, respectively, and did not differ significantly between heterozygous and wild-type MEFs.

DISCUSSION

In the present study, we have demonstrated that HMG-CoA reductase is essential for the early development of the embryos. Null embryos were recovered at the blastocyst stage, but not at E8.5, indicating that loss of HMG-CoA reductase activity through targeted disruption of the gene in the germ line leads either to implantation failure or to embryonic death prior to implantation. Moreover, the lethal phenotypes of Hmgcr−/− embryos were not completely reversed by supplementation with mevalonate to the dams.

Mammalian cells accelerate their growth rate dramatically upon implantation. Cholesterol synthesis in the embryos appears to begin at the peri-implantation stage (around E4–E5 in mice) (30, 31). Cholesterol plays an essential role in mammalian embryonic development, including the covalent modification of the morphogenic sonic hedgehog signal pathway during early gestation (32). Nevertheless, defective cholesterol synthesis is unlikely to explain the peri-implantational lethality of Hmgcr−/− embryos, because mice lacking squalene synthase (Ss−/−), the first committed enzyme of sterol synthesis in the mevalonate pathway, are viable until around E9.5 even with gross growth retardation and defective neural tube closure (18). Observations in Ss−/− embryos suggest that, even in the complete absence of endogenous cholesterol biosynthesis, maternally supplied cholesterol could, even although incompletely, support the embryonic growth by E9.5.

The phenotype of Hmgcr null mice is strikingly more severe than that of Ss−/− mice. In addition to loss of de novo cholesterol synthesis, disruption of mevalonate synthesis has many ramifications, including loss of non-sterol isoprenoids essential for protein isoprenylation modifications and potential perturbation on N-linked glycosylation through inhibition of dolichol synthesis (1). Non-sterol isoprenoids serve as lipid attachment for a variety of intracellular signaling molecules, including small GTP-binding proteins, such as Rho, Ras, and Rac, whose proper membrane localization and function are dependent on isoprenylation (33). Given the role that these proteins play in pathways regulating cell survival, proliferation, differentiation, and cytoskeletal organization, it seems likely that the altered expression will manifest as markedly abnormal function during embryogenesis. Furthermore, studies with mice deficient in dolichol-mediated N-glycan formation suggest that N-glycosylation is also essential for the peri-implantation stage embryos (34). In addition, recent studies in Drosophila, a species without sterol synthesis, revealed another critical developmental function of HMG-CoA reductase in providing spatial information to guide migrating primordial germ cells (35). Indeed, HMG-CoA reductase is highly expressed during the development in humans (36), mice (37), sea urchin (38), and Drosophila (39). Studies with in situ hybridization revealed that HMG-CoA reductase mRNA is highly expressed in the post-implantation rat embryos, from the egg cylinder stage through gastrulation, neurulation, and early organogenesis, with strong signal observed in primitive ectoderm and neural tube, which may reflect developmental requirements for products of the mevalonate pathway in these organs (30). In Hmgcr−/− mice, production of those non-sterol metabolites was hindered, while it may remained unaffected in Ss−/− mice. Therefore, perturbation of non-sterol isoprenoid synthesis would probably underlie the severer phenotypes of Hmgcr−/− embryos. Whether the lethal phenotype observed in the HMG-CoA reductase deficiency results from deficiency of some specific isoprenoids or from a more global deficiency of isoprenoids is uncertain at present.

A requirement for mevalonate-derived isoprenoids in early embryogenesis was reported two decades ago, in studies of cultured embryos where compactin, a classic HMG-CoA reductase inhibitor, interrupted their pre-implantation development after 32-cell stage in vitro, the effect which was reversed by mevalonate supplementation (37). Interestingly, ultrastructural examination of growth-arrested embryos revealed a predominance of nuclei with highly condensed chromatin, a hall-
of the mevalonate pathway and not at more proximal steps (Reviewed in Refs. 46 and 47). This indicates that many of the intermediates of the cholesterol biosynthetic pathway are essential for fundamental cellular processes and that the loss of these important pathways would result in cell death and early embryonic lethality. The absence of human genetic disease caused by HMG-CoA reductase deficiency suggests that the defect would cause prenatal death also in humans as observed in Hmgcr-null mice. In this regard, it is interesting to note that HMG-CoA reductase activity in the liver of human anencephalic fetus was reported to be 10-fold less than in the liver of normal fetus (48), although the causal relationship was not implied. On the other hand, several subjects with partial absence of mevalonate kinase, the enzyme immediately following HMG-CoA reductase, have been described (49). These patients are characterized by mevalonic aciduria and a broad range of phenotypic findings, including malformations of the central nervous system, facial dysmorphism, psychomotor retardation, and recurrent fever episodes. Plasma cholesterol levels are normal in subjects with mevalonate kinase deficiency, suggesting that compensatory mechanisms, such as increased activities of HMG-CoA reductase and the LDL receptor pathway (50), are sufficient to overcome the enzyme deficiency and provide adequate synthesis of cholesterol as well as non-sterol intermediates for embryogenesis.

Several mutant cell lines deficient in HMG-CoA reductase have been characterized (51). One such cell line, designated as UT-2 cells, is a mutant clone of Chinese hamster ovary cells that expresses only 2–5% of the HMG-CoA reductase activity of parental Chinese hamster ovary cells and requires exogenous mevalonate for growth (52). Recently, Engfelt et al. (16) identified the responsible mutations in the structural gene for reductase. By growing UT-2 cells in the absence of mevalonate, they have established a new cell line, designated as UT-2* cells, and demonstrated that UT-2* cells had up-regulated HMG-CoA reductase activity, which was localized exclusively to peroxisomes and the peroxisomal enzyme in these cells was sufficient for survival without mevalonate (15, 17). Based on the detailed characterization of UT-2* cells, they suggested the existence of a second, peroxisome-specific gene for the reductase. The presence of multiple genes encoding HMG-CoA reductase in lower organisms such as yeast, fungi, and plants also appeared to support the notion of a second reductase gene in mammals.

Contrary to these observations, the lethal phenotype of Hmgcr−/− mice argues against the presence of a second gene, irrespective of whether it encodes a peroxisomal or ER reductase, which could functionally replace the classic ER reductase, at least in the very early stage of development. Consistently, in a recent review Breitling and Krisans (53) noted that comprehensive analysis of genome sequence of human and mouse through homology search revealed no evidence of a second gene in mammals. They concluded that the peroxisomal activity is

**Table III**

Phenotypic comparison of Hmgcr+/+ and Hmgcr−/− mice

| Parameter                                      | Number of mice | Genotype          |
|------------------------------------------------|----------------|-------------------|
| HMG-CoA reductase activity (pmol/mg protein/min) | 5              | Wild-type         |
|                                                |                | Heterozygote      |
| [14C]Acetate incorporation into sterols (dpm/100 mg liver/h) | 6              | 129.6 ± 78.7      |
|                                                |                | 117.5 ± 59.3      |
| Liver cholesterol content (mg/g)               | 5              | 3.5 ± 0.47        |
|                                                |                | 3.0 ± 0.44        |
| Liver triglyceride content (mg/g)               | 5              | 15.2 ± 2.2        |
|                                                |                | 11.6 ± 6.3        |
| Plasma total cholesterol (mg/dl)                | 14             | 94.0 ± 19.4       |
|                                                |                | 100.5 ± 30.9      |
| Plasma triglycerides (mg/dl)                    | 20             | 82.3 ± 24.7       |
|                                                |                | 84.3 ± 35.8       |
due to alternative targeting of the ER enzyme to peroxisomes by an as yet uncharacterized mechanism. Establishment of Hmgcr−/− ES cell lines, if possible, would provide another opportunity to address these issues.

In Hmgcr+−+ mice, the HMG-CoA reductase activities and rate of cholesterol biosynthesis in the liver remained unaffected, indicating that the haploid amount of Hmgcr gene is not rate-limiting in hepatic cholesterol homeostasis. This is not surprising, in light of multivalent control of HMG-CoA reductase activities to retain cholesterol homeostasis. On the other hand, Hmgcr+−− MEFs exhibited 50% reduction in enzymatic activities in either basal or activated condition examined, suggesting that HMG-CoA reductase activities in MEFs may simply reflect the gene dosage, whereas that in the liver is controlled by a more complex mechanism.

In conclusion, we have generated a murine model of HMG-CoA reductase deficiency. This model should provide the basis for understanding the roles of the mevalonate pathway in the embryonic development as well as the pleiotropic effects of statins.

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