3-Hydroxy-3-methylglutaryl-CoA lyase (HL): gene targeting causes prenatal lethality in HL-deficient mice

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3-Hydroxy-3-methylglutaryl-CoA lyase (HL, EC 4.1.3.4) catalyses the last step of ketogenesis from leucine and fatty acids. HL deficiency in humans is one of the many inborn errors of CoA ester metabolism. By gene targeting, we created a strain of HL-deficient mice. Heterozygous HL-deficient mice are clinically normal and fibroblasts from homozygous HL-deficient embryos grow normally despite absence of HL activity. In contrast, homozygous HL-deficient embryos die at ~11.5 days post-coitum. Histologically, HL-deficient embryos show marked vacuolization, particularly in liver. Ultrastructural studies of hepatocytes obtained before death from HL-deficient embryos reveal abnormal dilated mitochondria. HL-deficient mice are the first mammalian example of a disease primarily affecting CoA ester metabolism with abnormal prenatal development.

INTRODUCTION

The pathophysiology of HL deficiency, and of the many other organic acidemias in which CoA esters are thought to accumulate abnormally, is incompletely understood. CoA esters are exclusively intracellular. Patients are therefore followed clinically and by measurement of metabolites like organic acids and carnitine esters, which are related to the CoA ester upstream of the metabolic block (2). The events leading to metabolic crises and the status of cell metabolism between crises are largely unknown, because non-invasive assessment of key metabolites and enzyme activities is not possible in humans.

In the hope of studying these events directly, we created a mouse model of HL deficiency. To our surprise, HL-deficient mice died in utero. This observation challenges current dogma about the pathophysiology of HL deficiency and related organic acidemias in humans.

RESULTS

Creation of HL-deficient mice

We obtained 228 G418-resistant clones, five (2.2%) of which contained targeted HL alleles (Fig. 1b). These lines were used to produce 21 chimeric pups, 16 of which were male. Ten of 16 (63%) had >60% coat color chimerism. One of these (60% chimerism) was bred and transmitted the targeted HL allele to 18/32 (56%) offspring.

Transmission of the targeted HL allele in heterozygous intercrosses

The offspring of matings of HL+/- heterozygotes are shown in Table 1. The ratio of heterozygotes to wild-type animals was not statistically different from the expected 1:2 ratio (P > 0.10). However, no HL–/– homozygotes were observed, suggesting embryonic lethality. To screen for a possible major deleterious effect specific to the 129 strain background, we bred HL+/- progeny of crosses between heterozygous HL+/- 129sv and either C57BL/6J or CD1 mice. No HL–/– progeny were observed (Table 1).

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Figure 1. Targeting strategy used to disrupt the mouse HL gene. (a) The upper diagram shows the introduction of a neomycin construct into HL exon 2. The lower panel demonstrates the sizes of diagnostic restriction fragments in the normal and targeted HL genes. Figures in parentheses represent the fragment lengths generated from the targeted allele. H, HindIII; X, XbaI. (b) Genomic Southern blot to detect targeting of the HL locus in clones of ES cells. Lanes 1–6, HindIII + XbaI digest of DNA from five targeted clones (1–5) plus a normal control (6); lanes 7–12, HindIII digest of the same targeted clones (7–11) and a control line (12).

Table 1. Progeny of HL+/– × HL+/– matings: HL genotype

<table>
<thead>
<tr>
<th>Parental lines</th>
<th>Total offspring</th>
<th>HL genotype of offspring</th>
<th>+/+</th>
<th>+/-</th>
<th>–/-</th>
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<tr>
<td>129 × C57BL/6J a</td>
<td>151</td>
<td></td>
<td>56 (37%)</td>
<td>95 (63%)</td>
<td>0 (0%)</td>
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<tr>
<td>129 × 129</td>
<td>19</td>
<td></td>
<td>4 (21%)</td>
<td>15 (79%)</td>
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<tr>
<td>129 × CD1 a</td>
<td>24</td>
<td></td>
<td>10 (42%)</td>
<td>14 (58%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>194</td>
<td></td>
<td>70 (36%)</td>
<td>124 (64%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Table 2. Embryo HL genotypes at different times of gestation, HL+/– × HL+/– matings

<table>
<thead>
<tr>
<th>Gestation (d.p.c.)</th>
<th>HL genotype</th>
<th>+/-</th>
<th>–/-</th>
<th>P-value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.5</td>
<td>12 (27%)</td>
<td>22 (49%)</td>
<td>11 (24%)</td>
<td>NS</td>
</tr>
<tr>
<td>11.5</td>
<td>12 (22%)</td>
<td>29 (53%)</td>
<td>13 (24%)</td>
<td>NS</td>
</tr>
<tr>
<td>12.5</td>
<td>9 (27%)</td>
<td>23 (70%)</td>
<td>1 (3%)</td>
<td>&lt; 0.025</td>
</tr>
<tr>
<td>13.5</td>
<td>7 (24%)</td>
<td>21 (73%)</td>
<td>1 (3%)</td>
<td>&lt; 0.025</td>
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</tr>
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*Calculated by the χ² method, in comparison with the expected 1:2:1 ratio among HL+/+, +/- and –/- genotypes. NS, not significant.

Phenotype of the HL–/- embryos

To determine the time of embryonic death, embryos from crosses of HL+/– heterozygotes were analyzed between 10.5 and 13.5 days post-coitum (d.p.c.) (Table 2). At 10.5 and 11.5 d.p.c., the observed genotype ratio did not deviate from the expected 1:2:1 distribution (P > 0.5). At both 12.5 and 13.5 d.p.c., only one HL–/- embryo was found, significantly different from the expected 1:2:1 ratio (P < 0.025).

Most HL-deficient embryos were visibly smaller than their littermates (Fig. 2). Two HL–/- embryos were absent, with only a yolk sac remaining to permit genotyping. None of the 33
heterozygous or HL-normal fetuses was small but three HL+/– embryos were resorbed. No localized abnormalities were detected macroscopically in HL–/– embryos.

**HL mRNA and activity in embryonic fibroblasts**

Fibroblasts from HL–/– and +/– embryos grew normally. To determine whether HL-null embryos lack intact HL mRNA, RT–PCR was performed using total RNA from the embryonic fibroblasts. With primers specific to normal murine HL, a fragment was detected by cDNA amplification from HL+/+ and +/– fibroblasts, but not HL–/– fibroblasts. Conversely, with a 5′ primer within the murine HL cDNA and a 3′ primer complementary to the Neo sequence, an amplified fragment was produced from HL+/– and –/– fibroblast cDNA but not from cDNA of wild-type cells (Fig. 3). On PCR of HL cDNA using oligonucleotide pairs 4+6 and 3+6 (Fig. 3), a fragment was also identified (data not shown). HL activities in the three fibroblast lines, expressed as units per mg protein, were: HL+/+, 22.3; HL+/–, 13.2; HL–/–, 0.0.

**Pathological examination**

For histological analysis, we studied 22 embryos from eight litters (HL+/+, 8; HL+/–, 8; HL–/–, 6). Three HL–/– embryos showed signs of autolysis, suggesting death several hours before sampling. Liver, heart, brain and placenta were identifiable in most 11.5 d.p.c. embryos and histological evaluation focused on these organs. No specific histological finding differentiated HL–/– embryos from other embryos. The principal abnormality was vacuolization, which was frequent in liver, heart and placenta of HL–/– embryos, but was also observed in some HL+/– and +/+ embryos. The degree of vacuolization was more severe in the liver of HL–/– embryos than in HL+/+ and +/+ embryos. Samples of three non-autolyzed 11.5 d.p.c. HL–/– embryos contained identifiable hepatic tissue. Moderate to marked vacuolization was present in livers of all three HL–/– embryos. In contrast, only two of eight HL+/+ embryos had identifiable vacuolization, which was focal and mild. None of eight HL+/+ embryos had identifiable hepatic vacuoles by light microscopy. The liver

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**Figure 2.** Photograph of two 11.5 d.p.c. mouse embryos. Left, HL-deficient embryo (HL–/–) which has reduced vascularity and is growth retarded; right, a normal sized HL+/– littermate.

**Figure 3.** HL expression in mouse embryo fibroblasts containing the targeted HL allele. (a) Diagram of HL cDNA amplification of transcripts from the mutant and the normal alleles. The positions and orientations of primers 1–6 are indicated by arrowheads. The sizes of diagnostic amplified fragments are shown for the normal (120 bp) and targeted (300 bp) transcripts. (b) HL mRNA amplification in three embryonal fibroblast lines. The genotype of the cell line is indicated above each lane.
vacuoles of the HL−/− embryos were negative on Oil Red-O staining for neutral lipid. Some vacuolization was observed in heart, brain and placenta, but this was not greater in HL−/− than in other embryos.

We questioned whether non-specific hypoxic/ischemic changes might be responsible for the histological findings. This seemed unlikely because several histologically normal HL+/+ and +/- embryos had undergone fixation after HL−/− littermates that showed marked liver vacuolization. However, for subsequent ultrastructural analysis, we studied only normal sized embryos in which cardiac function was present at the time of sampling.

We performed electron microscopy on samples from three 11.5 d.p.c. littermates, two HL−/− and one +/- . The HL+/+ embryo had been isolated and fixed after the HL−/− embryos. Hepatocytes of the two HL−/− embryos had marked vacuolization, in particular in mitochondria (Fig. 4a). In contrast, mild focal vacuolization was present in hepatocytes of the HL+/+ embryo (Fig. 4b). Cytoplasmic glycogen was present to a similar extent in hepatocytes of HL−/− and +/- embryos. In the cytoplasmic vacuoles of these mice, no accumulation of lipid or other stored materials was present. The ultrastructural appearance of Kupffer cells, hematopoetic cells and cells of limb buds and brain was similar in the two HL-deficient embryos and the control.

We studied a limited number of embryos at ages other than 11.5 d.p.c. At 10.5 d.p.c., two of three HL-deficient embryos were visibly smaller than three HL+/− or +/- littermates. No conclusions could be drawn from histological examination, due to the early stage of organogenesis at 10.5 d.p.c. (3). In early studies in which histological examinations were not performed, we discovered one HL-deficient fetus at each of 12.5 and 13.5 d.p.c. In comparison with HL+/+ and +/- littermates, the 12.5 d.p.c. HL−/− fetus was of normal size but the 13.5 d.p.c. HL−/− fetus was smaller.

**DISCUSSION**

Unexpectedly, HL deficiency is lethal at midgestation in mice. Although HL−/− fibroblasts grow normally, most affected embryos show runting and maceration by 11.5 d.p.c. To date, our attempts to avoid prenatal lethality in HL−/− fetuses by outbreeding (Table 1) or with l-carnitine and/or pantothenic acid supplementation (data not shown) have been unsuccessful.

We considered whether a bicistronic neomycin resistance–HL mRNA could produce functional HL. A transcript spanning the neomycin resistance cassette plus at least part of HL is detectable. However, there are no in-frame methionine codons upstream of the HL-related sequences of the transcript. The HL sequence itself contains a methionine codon (Met26 in the HL cDNA), which could initiate synthesis of an enzymatically active peptide that would not enter mitochondria but could be sequestered in peroxisomes (1,4). The efficiency of translation from this codon is predicted to be minimal, since the sequence preceding this Met codon lacks the purine in the −3 position that is typical of functional start codons (5) and the efficiency of translation of 3′ open reading frames is usually low (6). We cannot formally eliminate the presence of some HL in peroxisomes of HL−/− mice but HL activity was undetectable in fibroblasts from affected fetuses, demonstrating that if any peroxisomal HL is present, its amount is minimal.

**Figure 4.** Electron micrographs of liver from 11.5 d.p.c. embryos. (a) HL−/− embryo, showing marked focal hydropic swelling of mitochondria (arrows at the top). There is moderate cytoplasmic vacuolization. Adjacent hematopoetic cells (lower part) are normal. (b) HL+/+ embryo, showing mild cytoplasmic vacuolization and normal mitochondrial ultrastructure.

Possibly, disruption of transcripts other than HL may explain the prenatal demise of HL−/− mice. The genomic region has not been studied extensively in mice, but some humans with typical hereditary HL deficiency have large homozygous deletions.
encompassing the entire targeted region (7). Therefore, there is no evidence that non-HL genes explain the phenotype of HL/−/− mice, although this possibility cannot be excluded with the available data.

Marked mitochondrial vacuolization is present in hepatocytes from HL/−/− embryos prior to death or the development of growth deficiency. Although some vacuolization was also seen in HL+/− or +/+ fetuses, the changes in HL/−/− fetuses were more severe. The ultrastructural changes were non-specific but it is notable that they involve mitochondria, which are predicted to be the site of HMG-CoA accumulation in HL deficiency. Placental histology and architecture were comparable in embryos of all genotypes. Unfortunately, the mechanism by which HL deficiency results in prenatal death in mice cannot be directly studied in HL/−/− embryos by currently available techniques [10–12 d.p.c. mouse embryos weigh only 10–100 mg (3)].

Our goal of creating an animal model to study the pathophysiology of HL deficiency during and between crises has not been met and will require the development of other models in which HL expression can be suppressed in a development- and/or tissue-specific manner.

Increasingly, prenatal abnormalities have been recognized in association with human inborn errors of metabolism like Smith–Lemli–Opitz syndrome (8), lysosomal storage diseases (9), non-ketotic hyperglycemia (10), peroxisomal disorders like Zellweger syndrome (11) and disorders of terminal energy degradation like HL deficiency are felt to permit normal prenatal development. To our knowledge HL-deficient mice are the first mammals with an inborn error of CoA ester metabolism and abnormal prenatal development.

MATERIALS AND METHODS

Production of targeting constructs

We made a replacement vector using a 7.5 kb HindIII subclone (HLMG7.5) of the previously described 129 strain mouse HL genomic clone (7). The subclone contains mouse HL exons 2–5 and ends 1130 bp 5′ of exon 2 (Fig. 1).

We replaced the 1130 bp 5′-region by a 797 bp amplified fragment, introducing a NotI site at the 5′-extremity and XhoI and BamHI sites in exon 2. Amplification was performed using oligonucleotides HLM18 (5′-AAGCGGCGCTCTGGAGAATCAA-3′, corresponding to residues 780–797 upstream of the exon 2 acceptor splice site) and HLM23 (5′-AACCATGGATCCTCTAGACTCGAGAATTCG- GCTCACCTTGGTTT-3′, complementary to nine intronic and eight exonic nucleotides at the intron 1 acceptor splice junction plus cloning XhoI, XbaI, BamHI and Neol sites). We cloned this fragment into naturally occurring NotI and Neol sites of the vector, then inserted a bacterial neomycin resistance (Neo) gene oriented in the direction of HL transcription and driven by the herpes simplex thymidine kinase promoter (pMC1neo poly A; Stratagene) into exon 2 at the XhoI and BamHI sites. In the resulting transcript, HL sequences terminate at codon 23 and the neomycin resistance stop codon occurs ~1000 nt downstream. The Neo cassette is flanked by −0.8 (5′) and 6 kb (3′) of HL gene sequence.

Targeting and culture of embryonic stem cells (ES cells)

Electroporation was performed using 10 μg linearized vector and 107 G8R8 ES cells (13) at 240 V and 500 μF in a 0.4 cm Gene Pulse cuvette (Bio-Rad). Cells were cultured for 24 h in Glasgow minimum essential medium (MEM; Gibco BRL) with 0.1 mM MEM non-essential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.2 mM β-mercaptoethanol, 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 1000 U/ml leukemia inhibiting factor (Gibco BRL) in gelatinized (0.1%) tissue culture dishes (Nunclon). Selection was performed for 10–12 days in the same medium with 200 μg/ml G418 (Gibco BRL).

HL genotyping by PCR and Southern blotting

Pools of four G418-resistant colonies were screened by PCR. ES cell lysates were digested with 2 μg proteinase K in a volume of 50 μl at 50°C for 90 min. Amplification was performed in 100 μl containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, 0.5% DMSO, 0.2 mM each dNTP and 2.5 U Taq DNA polymerase. The sense primer HLM28 (5′-CCGTTCCGTTCAATACGAGG-3′), which is outside the targeting construct, corresponds to residues 1034–1054 upstream of exon 2; the antisense primer Neo-1 (5′-ATCCATCTTGTTCAATGCGCATCC-3′) is complementary to nt 735–759 of the neomycin resistance cDNA in the pMC1neo poly A vector. Forty cycles of amplification were performed as follows: 94°C, 45 s; 60°C, 45 s; 72°C, 90 s. Amplification of a 1.4 kb fragment was diagnostic for the presence of a targeted allele.

For Southern blots, mouse genomic DNA (5 μg) was digested with HindIII or HindIII and XhoI. Following electrophoresis in a 0.8% agarose gel in 1× TAE buffer (14), the samples were transferred to a nylon membrane (Hybond-N; Amersham). Probes were used either an amplified fragment spanning intron 1 residues 799–1130 upstream of the acceptor splice site or the Neo cDNA. Hybridizations were carried out in 4× SSC, 5× Denhardt’s solution (1× Denhardt’s is Ficoll, bovine serum albumin and polyvinyl pyrrolidone, each at 0.2 mg/ml), 10% (w/v) dextran sulfate, 1% (w/v) SDS and 100 mg/ml sheared salmon sperm DNA. The membranes were washed three times at 65°C, once in 2× SSC, 0.5% SDS for 30 min; once in 1× SSC, 0.5% SDS for 30 min; once in 0.5× SSC, 0.5% SDS for 20 min.

Production and analysis of HL-deficient mice

Targeted ES cell clones were injected into C57BL/6J blastocysts and transferred to pseudopregnant recipient ICR strain females. The targeted HL allele was bred onto C57BL/6J, 129 and CD1 backgrounds. HL+/− heterozygotes were crossed and their offspring were genotyped. All animals studied were anesthetized with methoxyflurane (Janssen Pharmaceutica). Dissections were performed rapidly after decapitation.

Isolation of genomic DNA

Mouse tails (~0.5 cm) or yolk sacs were incubated overnight at 65°C in 400 μl 4 M urea, 100 mM Tris–HCl, pH 8.8, 0.5% N-lauroylsarcosine and 10 mM EDTA. Then 20 μl proteinase K (2 μg/ml) were added and incubation was continued for 5 h at 65°C. DNA was extracted with phenol, precipitated with ethanol and suspended in 10 mM Tris–HCl, pH 8.6, 1 mM EDTA.
Fibroblast lines from HL-deficient mouse embryos

Mouse fibroblast lines were generated as described (15). Briefly, 10.5 d.p.c. embryos were washed in phosphate-buffered saline solution and forced from a 3 ml syringe through an 18 gauge needle. The homogenate was placed in a gelatinized (0.1%) DMEM containing 0.37% sodium bicarbonate plus 10% fetal calf serum, 15% fetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin. After reaching confluence (‘passage 0’), cells were cultured in DMEM containing 37% sodium bicarbonate plus 10% fetal calf serum, 50 µ/ml penicillin and 50 µg/ml streptomycin.

RT-PCR analysis of HL-deficient mRNA

Transcripts from the endogenous and targeted alleles were detected as follows. Reverse transcription of 10 µg total fibroblast RNA was initiated with HLM20 (5′-ACTTTAGCGGGAGAG-3′), complementary to residues 539–556 of the mouse HL cDNA. The reaction was carried out in a 30 µl volume containing 140 ng primer, 500 µM each dNTP, 17 µM dithiothreitol, 200 U Superscript RT RNase H′-free reverse transcriptase (Gibco BRL), 40 U RNasin (Promega) and 1× First Strand Buffer (Gibco BRL) at 42°C for 30 min and then at 95°C for 7 min. Amplification was carried out using the same buffer as described above and 50 µM dNTP with two pairs of primers: 5′-AGTGGAAGATGGCGTCAGTGA-3′ (nt 7–13, primer 1, Fig. 2a) and 5′-GACTTCCAGATCCACCTGCTTT-3′ (nt 90–105, primer 5 or primers 1 and 2; Neo-1 above). One microliter of the above cDNA mixture was amplified for 30 cycles as follows: 94°C, 30 s; 62°C, 30 s; 72°C, 90 s.

For detection of transcripts containing 3′ sequences from both neomycin and HL, we performed reverse transcription as above except that we used a poly(T) primer. Then we amplified as above using primer 3 (5′-GAAGATGGCGTCAGTGA-3′) and primer 6 (5′-ACCAGGTCCCTCAGTAGCCCA-3′). HL cDNA residues 845–826 or primer 4 (5′-TGCCCAAAGCGAGGTGAAGATGC-3′). HL cDNA residues 86–106 and primer 6.

HL assay

HL was assayed spectrophotometrically using HMG-CoA as substrate (16). One unit of enzyme activity is the amount which produces 1 µmol acetoacetate/min. Protein was assayed as described (17) with bovine serum albumin (Sigma) as the standard.

Microscopy

Conventional techniques were used for sample preparation. For histology, paraffin-embedded sections fixed in formalin (pH 7.0) were stained with hematoxylin/eosin or Oil Red-O. For electron microscopy, epon-embedded sections were stained with osmium tetroxide.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

DMEM, Dulbecco’s modified Eagle’s medium; ES cells, embryonal stem cells; HL, 3-hydroxy-3-methylglutaryl-CoA lyase; HMG-CoA, 3-hydroxy-3-methylglutaric acid-CoA; MEM, Glasgow minimum essential medium.

REFERENCES


