Animal Models of Tyrosinemia

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Abstract

Hereditary tyrosinemia I (HT I) is a genetic disorder of tyrosine metabolism characterized by progressive liver damage from infancy and by a high risk for hepatocellular carcinoma. HT I is due to mutations in the fumarylacetoacetate hydrolase (Fah) gene, which encodes the last enzyme in the tyrosine catabolic pathway. Disturbances in tyrosine metabolism lead to increased levels of succinylacetone and succinylacetocarboxylic acid. However, the mechanisms causing liver failure, cirrhosis, renal tubular dysfunction, and hepatocarcinoma are still unknown. Lethal albino deletion c14CoS mice and mice with target-disrupted Fah are models for HT I. They die in the perinatal period, although with a different phenotype from that seen in HT I in humans. In addition, 2 mouse strains that carry N-ethyl-N-nitrosourea-induced mutations in the Fah gene have been described. Mice with a splice mutation exhibit the milder features of the clinical phenotype. In mice that carry both Fah and 4-hydroxyphenylpyruvate dioxygenase gene mutations, administration of homogentisate results in rapid apoptosis of hepatocytes. Simultaneously, renal tubular epithelial cells are injured, resulting in Fanconi syndrome. These are central features of visceral injury in patients with HT I. Apoptosis of hepatocyte and renal tubular cells is prevented by the caspase inhibitors acetyl-Tyr-Val-Ala-Asp-CHO or acetyl-Asp-Glu-Val-Asp-CHO. Apoptosis of hepatocytes and renal tubular epithelial cells is central features of this disease. Alterations in gene expression found in the liver of patients with HT I are responsible for the pathogenesis of this disease, for example, acute liver failure. Therefore, gene expression analysis allows a better understanding of the specific pathogenesis. Cell fusion of hematopoietic stem cells with hepatocytes leads to liver regeneration after liver injury. This finding was possible after using the liver injury model of HT I in Fah null mice. Thus, animal models of tyrosinemia are unique and useful tools to reveal mechanisms of interest to both clinical and basic science.


Hereditary tyrosinemia I (HT I) is an amino acid metabolism disorder with an autosomal recessive trait. HT I is due to defects in the fumarylacetoacetate (FAA) hydrolase (Fah) gene (Fig. 1), located in q23–q25 of chromosome 15 in humans (1,2) and in chromosome 7 in mice (3). HT I patients display a variety of clinical symptoms, such as liver damage from infancy that advances to cirrhosis; reduced coagulation factors; hypoglycemia; high concentrations of methionine, phenylalanine, and amino-levulinic acid in serum plasma; high risk of hepatocellular carcinoma; and tubular and glomerular renal dysfunction. Pathological features of the liver in HT I patients are characteristic but not diagnostic. In its severe form, a pattern of progressive liver damage begins from early infancy. In its mild form, chronic liver damage with a high incidence of hepatoma is characteristic. Renal De Toni Fanconi syndrome and developmental hypophosphatemic rickets are features of the kidney involvement. Organic acid analysis of urine demonstrates increased excretion of 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid, and 4-hydroxyphenylacetic acid, and the presence of succinylacetone is diagnostic (3). 2-(2-Nitro-4-trifluoromethylbenzoyl) -1,3-cyclohexanedione (NTBC) is an inhibitor for 4-hydroxyphenylpyruvate dioxygenase and administration of this compound is very effective in ameliorating liver and kidney damage in patients with this disease (4). Although such features are presumably related to events leading to hepatocyte injury and death, the actual process of liver damage has not been defined. The enzyme 4-hydroxyphenylpyruvate dioxygenase (Hpd) participates in the oxidation of the keto acids of tyrosine. Homogentisate (HGA) is
produced from 4-hydroxyphenylpyruvate by this enzyme and the reaction involves decarboxylation, oxidation, and rearrangement (5).

In mammals, genetic deficiency of Hpd has been detected by measuring Hpd activity. Human HT type III (HT III) is caused by lack of Hpd activity. Model mice for HT III (III mice) are a strain that exhibits high levels of serum tyrosine. The mice lack the entire exon 7 sequence of the Hpd gene and have no activity of Hpd enzyme (6,7). Thus, HT III mice are an animal model for human HT III. The mice have no evidence of liver or kidney damage, probably because the accumulation of 4-hydroxyphenylpyruvate does not cause any specific pathogenesis. Meanwhile, lethal albino c14cos mice are an animal model of HT I (3,4,8,9). They have a large deletion on chromosome 7, including the albinoc locus and the Fah gene. We will discuss here the apoptosis of hepatocytes and renal tubular epithelial cells in animal models that are characteristic features of HT and recent approaches to establishing the genetic and pathophysiological feature of this disease.

Animal models of tyrosinemia

There are 2 strains of mutant mice that carry Fah deficiency. One is an albino lethal c14CoS mouse, which is neonatally lethal (10). A transgenic experiment revealed the lethal phenotype of these mice was caused by a deficiency of Fah. These mice have a large deletion on chromosome 7, including the albinoc locus and the Fah gene (11,12). The lethal phenotype of the homozygous c14CoS mice is related to impaired expression of hepatocyte-specific genes in the liver during the perinatal period (12–14). The other Fah-deficient mice were generated by targeted disruption of the Fah gene and these knockout mice are neonatally lethal (15). A study on these Fah-deficient mice suggested that neonatal death is probably due to hypoglycemia (11). There are no gross abnormalities in the histology of the liver of the Fah-deficient mice; however, ultrastructural investigations of homozygous c14CoS mice revealed altered membranous components. In the kidney of homozygous mice, dilatation and vesiculation of proximal tubular epithelium, and aggregates of cytoplasmic microfilaments have all been observed (17).

Alternatively, rescue of Fah-deficient mice has been achieved by the introduction of a mutant Hpd (EC 1.13.11.27) gene into homozygous c14CoS mice (Fig. 2); the double mutant mice (Fah−/−Hpd−/−) are viable (18). The double mutants carry mutant Hpd gene from III mice, a model for HT III. The phenotype of III mice is hyper-tyrosinemia without visceral injury (19) and there is a C to T transition at nucleotide +7 on exon 7 of the Hpd gene on chromosome 5, the result being premature termination of translation (20). As the formation of HGA is exclusively dependent on Hpd activity, a complete block of tyrosine catalysis at this step would result in depletion of HGA and its oxidative products. The complete block of tyrosine catalysis was expected to obliterate any clinical symptoms related to Fah deficiency.

Phenotype of the double mutant

The clinical phenotype of the double mutant (Fah−/−Hpd−/− mice) was indistinguishable from that of the III mice. The findings in liver and kidney sections from Fah−/−Hpd−/− mice were completely normal. Long-term investigations of Fah−/−Hpd−/− mice (12–18 mo) revealed no evidence of hepatocellular carcinomas or preneoplastic lesions. Thus, the mutant Hpd alleles from the III mice not only rescued the lethal phenotype of the Fah−/−mice but also eliminated the critical visceral phenotype of Fah deficiency (19). These data suggest that liver carcinomas in the target-disrupted Fah-deficient mice under NTBC treatment could be caused by small amounts of the oxidative product(s) of HGA or their derivatives, produced by an incomplete block of the oxidation of 4-hydroxyphenylpyruvate by this drug.

Induction of visceral injuries in the double mutants

In the double mutant, we expected that recovery of the activity of Hpd in the liver would induce liver damage and that the investigations of the induced damage would provide some clues to the mechanism of cellular injury in Fah deficiency. At first, administration of recombinant adenovirus-expressing human

![FIGURE 1](https://example.com/figure1.png)

**FIGURE 1** Tyrosine catabolic pathway and related diseases. Enzymes that catalyze each step are listed (right) opposite the disorders caused by the deficiency of the individual enzyme (left). As shown, NTBC is an inhibitor for Hpd. In our study, we used mice with a deficiency of Hpd and mice with a deficiency of Fah. The double-mutant (Fah−/−Hpd−/−) mice carry metabolic blocks at the steps of Hpd and Fah.

![FIGURE 2](https://example.com/figure2.png)

**FIGURE 2** Mutations and metabolic defects in the double mutant. Rescue of Fah-deficient mice is achieved by introduction of mutant Hpd gene into homozygous c14CoS mice; the double mutant mice (Fah−/−Hpd−/−) are viable. The double mutants carried mutant Hpd gene from HT III mice, a model for HT III.
Hpd was attempted. Administration of this recombinant adenovirus into the 8-wk-old III mice resulted in a rapid appearance of the enzyme protein and activity in the liver within 1 h (21). Normalization of blood tyrosine levels was achieved within 12 h. When the recombinant virus was administered into the double mutant, they became evidently ill within 12 h after the injection. Mice had reduced mobility and lost appetite, and most often the mice died within 30 h after injection of the recombinant virus at a dose of 5 × 10⁸ plaque forming units (PFU). However, the clinical symptoms in the control mice did not differ, indicating that the symptoms are caused by the appearance of Hpd activity in the liver of the double mutant. The changes in the histology of liver were striking in the double mutants administered the recombinant adenovirus (19). There was death of massive numbers of hepatocytes and no infiltration of inflammatory cells, but there were small areas of bleeding. Some of the damaged cells showed evidence of chromatic condensation, suggesting apoptosis as a cause of the cell death. When the liver sections were investigated with the TUNEL method, <15–25% of the hepatocytes were positive for the signals, indicating apoptosis, at a dose of 5 × 10⁸ PFU. Examination of nuclear DNA of the livers by gel electrophoresis from the recombinant virus-treated Fah−/−Hpd−/− mice revealed fragmentation, with sizes corresponding to typical nucleosomes. Thus, the most striking changes of the liver after the recovery of Hpd function in Fah−/−Hpd−/− mice was the massive apoptosis of hepatocytes. This result suggests that apoptosis is a central feature of the cellular damage observed in Fah deficiency. HGA, an intermediate metabolite of the tyrosine catabolic pathway, had similar effects on hepatocytes with Fah deficiency (19). When 400 mg/kg of neutralized HGA was injected intraperitoneally into 8-wk-old Fah−/−Hpd−/− mice, all of the mice died within 16 h. There are no clinical symptoms or histological changes in the liver sections of the control mice or the III mice after injection of 400–4000 mg/kg intraperitoneally, suggesting HGA is a relatively innocuous chemical in the control mice and III mice.

On the other hand, the liver sections from the HGA-treated Fah−/−Hpd−/− mice had massive numbers of dead hepatocytes with fragmentation of nuclei. Ultrastructural analyses revealed that the nuclear chromatin was condensed peripherally, many mitochondria were swollen, and cytoplasmic vacuolation was evident. With the TUNEL method, ~20–30% of the hepatocytes gave positive signals by in situ detection of DNA fragmentation after the administration of 400 mg/kg of neutralized HGA in the double mutants. Apoptosis was demonstrated by experiments using primary cultured hepatocytes obtained from the double mutants after the addition of the recombinant adenovirus-expressing human Hpd or addition of HGA into the medium. In such experiments, caspase inhibitors, acetyl-Tyr-Val-Ala-Asp-CHO (YVAD), which is a potent, selective, cell-permeating inhibitor of caspase-1, and acetyl-Asp-Glu-Val-Asp-CHO (DEVD), which specifically inhibits caspase-3 and caspase-3, effectively prevent apoptosis of the Fah-deficient hepatocytes (22). These results suggest that the apoptosis seen in Fah-deficient hepatocytes involves mitochondria and the caspase 3 pathway. In vivo administration of YVAD or DEVD effectively prevented apoptosis of hepatocytes in the double mutant treated with HGA; when the Fah−/−Hpd−/− mice were given 200 mg/kg of YVAD or DEVD intraperitoneally 2 h before they were killed, <1% of hepatocytes were apoptotic (22).

**Release of cytochrome c from liver mitochondria**

Cytochrome c is localized in the intermembrane space and on the surface of the inner mitochondrial membrane. Cytochrome c released from the mitochondria interacts with Apaf-1, and caspase-9, pro-caspase-3, to activate caspase-3 and the caspase cascade, leading to fragmentation of the nucleus. The apoptosis of hepatocytes in Fah deficiency seems to involve the caspase 3 pathway, so it was postulated that cytochrome c plays some role in the apoptosis related in Fah deficiency. There was a considerable release of cytochrome c into the cytosol of hepatocytes in the HGA-treated Fah−/−Hpd−/− mice but no significant release in the HGA-treated III mice. The release of cytochrome c occurred as early as 1 h after the administration of HGA, and apparently preceded the onset of apoptotic hepatocyte death and liver failure. These results suggest that the release of cytochrome c is the trigger leading to death of Fah-deficient hepatocytes following the administration of HGA. Release of cytochrome c from mitochondria was observed in a cell free system when mitochondria from control mice were incubated with soluble fractions from HGA-treated Fah−/−Hpd−/− mice (22). The release did not differ when the soluble fraction from the control mice was added to the incubation medium. These investigations indicate that there is a low molecular weight substance(s) in the cytosol of the liver of HGA-treated Fah−/−Hpd−/− mice that induces the release of cytochrome c from mitochondria. HPLC analysis showed that FAA, the substrate for Fah enzyme, was predominantly present in the soluble fraction. In the experiments in which purified FAA was incubated with control mitochondria, a considerable amount of cytochrome c was released into the medium. Thus, it is highly likely that FAA causes the apoptosis of hepatocytes in Fah-deficient mice via the caspase 3 pathway. The release of cytochrome c from the mitochondria seems to be an essential step in initiating the process of apoptosis in Fah deficiency.

**Apoptosis of renal tubular cells**

Apoptosis was seen in the proximal renal tubular epithelial cells from the double mutants after the administration of HGA (23). Pathological features included bleeding in vast areas, accumulation of mononuclear cells at the interstitium, proximal tubular dilatation, and cytoplasmic vacuolation. The number of apoptotic cells in the proximal renal tubules from the HGA-treated Fah−/−Hpd−/− mice depended on the dose of HGA administered. Electron microscopic analysis of proximal tubular cells revealed that droplets of fat and large lysosomes were present and many mitochondria were swollen (23). The breakage and vacuolization were present at the brush border. In some cells, compaction and degradation of chromatin were present in association with convolution of the nuclear profile. Thus, severe cellular damage and apoptosis might be central features of proximal renal tubular cells in Fah deficiency.

The urinary excretion of glucose and phosphate in the double mutants was used to evaluate the renal tubular function (23). The urinary glucose/creatinine ratio was markedly increased in the Fah−/−Hpd−/− mice after the administration of 100–400 mg/kg of HGA. In contrast, the urinary glucose/creatinine ratio was essentially unchanged in the control. These results suggest that, in the Fah−/−Hpd−/− mice, the administration of HGA resulted in a reduced reabsorption of glucose by the renal tubules. The caspase inhibitor YVAD effectively prevents apoptosis of renal tubular epithelial cells when administered 2 h prior to the administration of HGA (23). Preadministration of YVAD did not alter the urinary levels of glucose and phosphate. In hepatocytes, pretreatment with YVAD did not have any influence on the release of cytochrome c from mitochondria in the double mutant after the administration of HGA. A similar phenomenon is likely to occur in the renal tubular cells. The
Microarray analysis of tyrosinemia model mice

Recently, microarrays have become established as a valuable tool to measure gene expression. A microarray is an application of thousands of target sequences representing individual genes onto a substrate that is investigated with a sample labeled to enable detection after hybridization in a single experiment (24). It is useful in finding new candidate genes related to metabolic disorders (24,25). Microarray analysis was applied to investigate the pathogenesis of HT I. Numerous expressed genes have been identified and validated in the liver of HT I model mice that induced liver damage upon HGA administration (26). These genes were related to apoptosis, tyrosine metabolism, and hepatocytic regeneration and were not expected to be involved in the liver dysfunction of HT I (Table 1). The fact that aminolevulinate dehydratase is downregulated in HGA-treated HT I model mice may suggest a role for this enzyme in the liver dysfunction and neuropathy of the HT I patient. It was found that 3 genes representing liver function were downregulated in the HT I model mice during the liver dysfunction process. The systematic approach used in the study provides an effective and efficient method for identifying genes involved in the pathogenesis of the HT I patient.

Liver regeneration in tyrosinemia model mice

Fah-deficient mice are utilized for a model of persistent liver damage and regeneration for the hepatocyte replacement. Administration of NTBC prevents progression of liver lesions in Fah-deficient mice (17,27). Withdrawal of NTBC leads to liver injury by apoptosis. After the liver damage, liver regeneration proceeds during the administration of NTBC while the progression of the damage is arrested. Thus, the regulation of liver damage and regeneration enables the replacement of hepatocytes in the Fah-deficient liver (27). The model revealed that cell fusion of hematopoietic stem cells with hepatocytes leads to liver regeneration after liver injury (28).

<table>
<thead>
<tr>
<th>Genes downregulated</th>
<th>Genes upregulated</th>
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<tr>
<td>Amino acid metabolism</td>
<td>Apoptosis-related genes</td>
</tr>
<tr>
<td>HGA 1,2-dioxgenase</td>
<td>Caspase 2</td>
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<tr>
<td>S-adenosyl homocysteine hydase</td>
<td>Caspase 3</td>
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<tr>
<td>Histidine ammonia lyase</td>
<td>Caspase 8</td>
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<td>Pyruvate metabolism</td>
<td>Caspase 9</td>
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<tr>
<td>Pyruvate carboxylase</td>
<td>Caspase 12</td>
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<td>Pyruvate dehydrogenase E1 α</td>
<td>TGF β</td>
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<tr>
<td>Liver function-related genes</td>
<td>Tyrosine metabolism-related genes</td>
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<tr>
<td>Coagulation factor VII p450 3a16</td>
<td>Tyrosine aminotransferase</td>
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<tr>
<td>Flavin-containing monooxygenase</td>
<td>Hpd</td>
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<td>Liver regeneration</td>
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<td>HGF activator precursor</td>
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<td>Others</td>
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<tr>
<td>Liver carboxylase precursor</td>
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<td>Nuclear hormone receptor isoform CAR2</td>
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<td>Δ-Aminolevulinate dehydratase</td>
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Double mutant (Fah−/−/Hpd−/−) mice were used to study the mechanism of visceral injury in HT I. These investigations have provided important information concerning the disease process. The knowledge provided by this model mouse is summarized as follows. The Fah−/−/Hpd−/− mice appear normal, at least until the age of 18 mo, and there is no evidence of liver or kidney disease. The complete block of the tyrosine catabolic pathway at the step of Hpd effectively prevents the development of clinical phenotypes. Apoptosis of hepatocytes and renal tubular epithelial cells is a central feature of the visceral injury observed in patients with HT I. Apoptosis of hepatocyte and renal tubular cells is prevented by either of the caspase inhibitors, YVAD or DEVD. However, the inhibitors do not prevent the release of cytochrome c. A systematic approach using microarrays provides an effective and efficient method for identifying the genes involved in the pathogenesis of the HT I patient. HT I model mice were used to show that cell fusion of hematopoietic stem cells with hepatocytes leads to liver regeneration after liver injury. Fah null mice enable this finding after using the liver injury model of HT I. Thus, animal models of tyrosinemia are unique and useful tools to reveal the mechanisms of interest to both clinical and basic science investigation.

### Literature Cited


