GENETIC STUDIES OF REVERSION IN HEREDITARY TYROSINEMIA TYPE I

by

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AKU—alkaptonuria

ALA—∂-aminolevulinic acid

APRT—adenine phosphoribosyltransferase

ASO—allele specific oligonucleotide

BrdU—bromodeoxyuridine

BSO—L-buthionine-(S,R)-sulfoximine

ENU—*N*-ethyl-*N*-nitrosourea

FAA—fumarylacetoacetate

FAH—fumarylacetoacetate hydrolase

GSH—glutathione

H&E—hematoxylin/eosin

HCC—hepatocellular carcinoma

HGA—homogentisic acid

HGD—homogentisate 1,2 -dioxygenase

HPD—4-hydroxyphenylpyruvate dioxygenase

HT1—hereditary tyrosinemia type I

LOH—loss of heterozygosity

MAA—maleylacetoacetate

NTBC—2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione

RT-PCR—reverse transcription-PCR

SA—succinylacetone

TAT—tyrosine aminotransferase

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Abstract

Hereditary tyrosinemia type I (HT1) is the most severe of the four inherited metabolic disorders of the tyrosine catabolic pathway, and involves both liver and kidney dysfunction as well as liver cancer. HT1 is caused by a defect in the last enzyme in the pathway, fumarylacetoacetate hydrolase (FAH), resulting in the accumulation of fumarylacetoacetate (FAA). FAA is believed to be the cause of the hepatocellular damage in HT1. Since FAH is the last enzyme in the pathway, deficiencies of any of the upstream enzymes of the pathway would inhibit the formation of the harmful metabolite, FAA. Currently, the drug 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC) is used both in patients and in FAH knock-out mice to block the pathway upstream by inhibiting 4-hydroxyphenylpyruvate dioxygenase (HPD). However, inhibiting the pathway at this step also causes an increase in blood tyrosine, which has some negative side effects.

Therefore, one aim of this thesis was to investigate the result of blocking the pathway at a different enzymatic step, by inhibiting the enzyme homogentisic acid dioxygenase (HGD) as in the disease alkaptonuria (AKU). AKU is a much more benign disorder than HT1, and in mice there is no significant phenotype. A genetic block of the pathway at HGD was studied in the background of mice with HT1 by breeding double-mutant mice, deficient in both FAH and HGD. The double mutants had normal liver and kidney function without requiring NTBC, and had no elevation in tyrosine levels.

Another interesting result was the appearance of healthy hepatocyte nodules in the livers of mice that were $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt}, after being taken off NTBC. It was then demonstrated that these nodules were due to a loss of heterozygosity event resulting in a double-mutant hepatocyte that no longer accumulates FAA. This finding is important to the field of genetics because, to our knowledge, it is the first example of an *in vivo*

suppressor mutation in a mammal. Severe kidney damage was also discovered in the *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mice, most likely due to the accumulation of HGA from the revertant nodules in the liver. This observation was also confirmed by injecting HGA into FAH mutant mice on NTBC.

These data contribute to the evidence that FAA is the cause of the liver damage in HT1. FAA is predicted to be an alkylating agent based on its chemical structure. By finding mutations in *Hgd* in the livers of *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mice, the spectrum of DNA damage resulting from FAA accumulation was illustrated. Unexpectedly, there was a wide spectrum of mutations including insertions and deletions as well as a variety of point mutations. We also found a large portion of nodules that had lost the entire wild-type *Hgd* allele, either by a large deletion or mitotic recombination. To study the mechanism of this phenomenon further, the last aim of this thesis work was to use an *in vitro* system to look at loss of heterozygosity induced by FAA, using adenine phosphoribosyltransferase (*Aprt)* as a selectable marker.

CHAPTER 1

INTRODUCTION

I. The Tyrosine Catabolic Pathway and its Diseases

The amino acid L-Tyrosine is a semi-essential amino acid that can be derived from phenylalanine, an essential amino acid, by the enzyme phenylalanine hydroxlase. Tyrosine can subsequently be broken down to fumarate (a Krebs cycle intermediate) and acetoacetate (a ketone body) as the final products in a pathway involving five enzymes (Fig. 1). Deficiencies in four of these five enzymes have been shown to cause autosomal recessive genetic diseases in man (La Du, 1995; Mitchell et al., 1999).

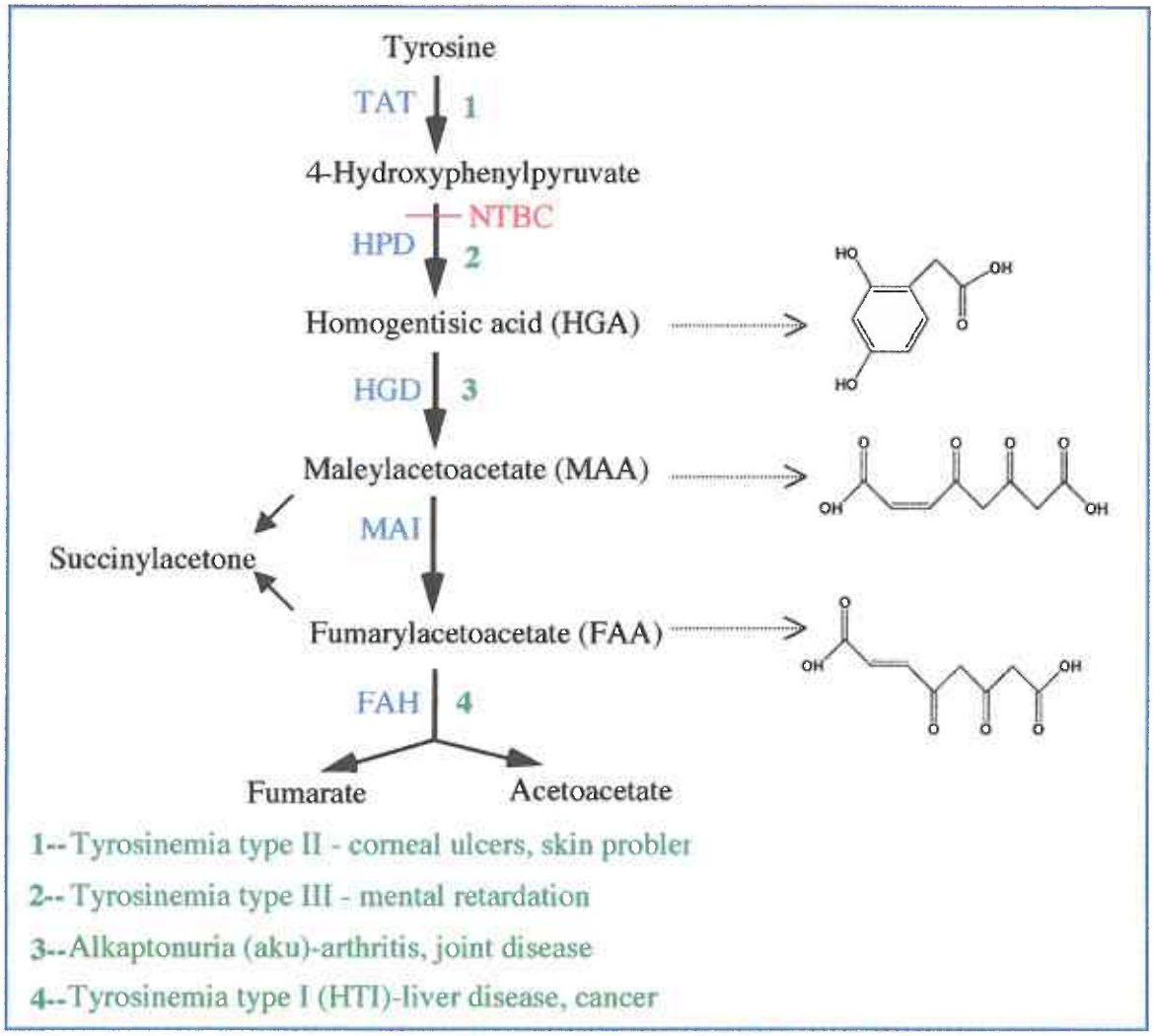

Figure 1. Tyrosine degradation pathway and associated diseases (structures of substrates/products are shown for the distal part of the pathway)

Hereditary tyrosinemia type I is the most severe of these disorders and is the focus of this thesis work. It is discussed in more detail below.

Tyrosinemia type II is associated with the loss of tyrosine aminotransferase (TAT), the first enzyme in the pathway. Patients with this disease have symptoms mainly affecting the skin, eyes, and central nervous system most likely due to the buildup of tyrosine (Fois et al., 1986)(Mitchell et al., 1999).

Tyrosinemia type III is caused by the lack of 4-hydroxyphenylpyruvate dioxygenase (HPD), the second enzyme in the pathway. These patients have symptoms involving the central nervous system, including mental retardation, but do not accumulate as much tyrosine as type II patients (Cerone et al., 1997).

There has not yet been a human disorder identified by the loss of the fourth enzyme in the pathway, maleylacetoacetate isomerase (MAI).

Alkaptonuria

Alkaptonuria (AKU) is due to a deficiency of homogentisate 1,2 -dioxygenase (HGD), the third enzyme in the pathway. AKU was the first human disease described as having an autosomal recessive mode of inheritance (Garrod, 1902) and is identifiable by a darkly colored urine. This is due to oxidation of homogentisic acid (HGA), which accumulates in patients with this disease (Zannoni et al., 1969). This dark pigment is also deposited in tissues causing ochronosis and arthritis in adults (La Du, 1995). The gene mutated in AKU was first cloned in a fungus (Fernández-Cañón and Peñalva, 1995) and subsequently the human gene was found, allowing for mutation detection (Fernández-Cañón et al., 1996). Several mutations have now been identified, including two common alleles in the Slovakian population, where there is the highest occurrence of AKU (Muller et al., 1999).

A mouse model for the disease, created by random *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis, was discovered. Mutant mice did not show any evidence of joint disease, although HGA was excreted in the urine. This mild phenotype may be due to the ability of mice to synthesize ascorbate (Montagutelli et al., 1994). Ascorbic acid was found to decrease the binding of HGA to connective tissue in rats (Lustberg et al., 1970), but it has not been successful as a treatment for human patients. Adequate tissue concentrations of ascorbic acid to prevent ochronosis may not be obtainable by exogenous treatment (La Du, 1995). The identification of the mutation in this mouse model is discussed in Chapter 2.

Hereditary tyrosinemia type I

A defect in the final enzyme in the pathway, fumarylacetoacetate hydrolase (FAH), causes hereditary tyrosinemia type I (HT1). HT1 is the most severe disease associated with the pathway and is characterized by liver and kidney disease, and often liver cancer. There are both acute and chronic forms of the disease where patients with the acute form have liver failure in infancy, and patients with the chronic form have a longer progression of liver and renal proximal tubular disease, developing liver cirrhosis and hypophosphatemic rickets (Russo and O'Regan, 1990). Risk for hepatocellular carcinoma (HCC) can be as high as 37% early in life (Weinberg et al., 1976). They can also have episodes of porphyria-like neurologic crises (Mitchell et al., 1990). Biochemically, patients show increased serum α-fetoprotein (AFP) and increased plasma amino acids including tyrosine and methionine (Mitchell et al., 1999). Patients with HT1 accumulate succinylacetoacetate (SAA) and succinylacetone (SA) (see Fig. 1); the latter is easily detectable in urine and is diagnostic for the disease (Lindblad et al., 1977; Mitchell et al., 1999).

HT1 is most prevalent in the French Canadian population where the carrier frequency is about 1 in 20 (De Braekeleer and Larochelle, 1990). Elsewhere, the incidence is about 1 in 100,000 live births (Mitchell et al., 1999). The FAH gene has been cloned (Phaneuf et al., 1991) and many mutations have been identified, including the predominant mutation in French Canada (Grompe et al., 1994; St-Louis and Tanguay, 1997).

Treatments

The first treatment used for HT1 was dietary restriction of phenylalanine and tyrosine (Bodegard et al., 1969). Although disease progression was slowed, it was not prevented by diet restriction alone (Mitchell et al., 1999). Because of the severe liver disease and high risk of HCC associated with the disorder (Russo and O'Regan, 1990), liver transplant was the first option for a cure and has been successful in many patients (Mohan et al., 1999).

In 1991, 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC) was first used in a clinical trial for an infant with HT1 (Lindstedt et al., 1992). NTBC, a bleaching herbicide, was discovered as an inhibitor of HPD (Schulz et al., 1993) and was proposed as a treatment for HT1. Inhibition of HPD blocks the pathway upstream and thus prevents the formation of fumarylacetoacetate (FAA) and maleylacetoacetate (MAA), which accumulate in the absence of FAH and are thought to be the cause of liver damage in this disease (see Fig. 1) (Mitchell et al., 1999). Over 220 patients have been treated with NTBC with good results, including improved liver function and a largely decreased risk for HCC (Holme and Lindstedt, 1998). However, one drawback of inhibiting the pathway at HPD is that there is an increase in blood tyrosine which can cause corneal ulcers and possibly neurological problems as seen in tyrosinemia type III (Giardini et al., 1983). Some patients on NTBC have experienced corneal lesions and in

such cases patients would require a restricted diet of low phenylalanine and tyrosine (Holme and Lindstedt, 1998). For this reason, inhibition of HGD may be an alternate strategy since there is no increase of tyrosine in AKU. This approach was tested in mice as discussed in Chapter 3.

Mouse models

There are two mouse models for HT1. The first is the lethal albino mouse, c^{14CoS}, which was created by X-ray-induced deletion (Russell et al., 1979). The gene encoding FAH was mapped to the deletion interval in these mice and later suggested to be the primary cause of lethality in this mouse model (Klebig et al., 1992) (Ruppert et al., 1992). This was later proven by the creation of the second mouse model, by targeted disruption of exon 5 of *Fah*. The $Fah^{\Delta exon5}$ mice had an identical phenotype to the c^{14CoS} mice, including death in the neonatal period from hypoglycemia and liver dysfunction (Grompe et al., 1993). After the discovery of NTBC, this drug was used in the $Fah^{\Delta exon5}$ mice to successfully rescue the lethal phenotype and more closely mimic HT1 in humans. When taken off NTBC, the mice quickly (within two weeks) developed liver disease with the hallmarks of HT1 such as elevated α-fetoprotein (AFP) and accumulation of SA in blood and urine. The mice also demonstrated renal tubular disease and had elevated amino acid levels including tyrosine (Grompe et al., 1995). Even on high doses of NTBC, the mice may still develop HCC (see Figure 2) (Grompe, unpublished observations).

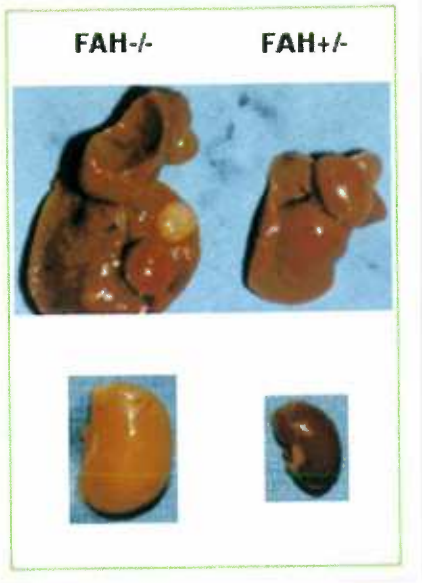

Figure 2. Liver and kidney phenotype of adult FAH mutant mice after NTBC withdrawal. Liver and kidney of an FAH mutant mouse off NTBC is shown on the left, with a normal, heterozygous mouse on the right. The FAH mutant liver is cancerous, and the kidney is pale and enlarged in comparison to the heterozygote.

The HT1 mouse model has also been used as a model system for liver gene therapy (Overturf et al., 1998; Overturf et al., 1997; Overturf et al., 1996). Because of the liver's high regenerative potential and the toxicity of FAA, the HT1 mouse provides an excellent model for developing gene therapy as a potential treatment for HT1 or other liver diseases in the future (Grompe et al., 1999). Our laboratory has shown that as few as 1000 donor cells were sufficient to repopulate the majority of a mutant mouse liver with transplanted, wild-type cells. Remaining mutant cells did not appear to negatively affect transplanted cells, indicating that the hepatocellular damage in this disease is cell autonomous (Overturf et al., 1996). However, the potential for developing HCC still exists since the repopulation is not 100 percent (Grompe et al., 1998). These repopulation studies have demonstrated the powerful selection for hepatocytes expressing

FAH over mutant cells in the liver. This phenomenon is also evident in the cases of somatic reversion seen in HT1 patients, discussed later in this chapter. These observations are important in the development of better therapies for HT1, but understanding the cellular damage that occurs with FAH deficiency is also essential since the potential for developing HCC has not been eradicated by any therapies, other than whole organ transplant, thus far.

Hereditary Tyrosinemia Type I Pathophysiology

Many symptoms of HT1 have been shown to be caused by either of the accumulating metabolites, SA or FAA, through multiple lines of evidence. Table 1 summarizes the mechanisms thought to lead to some of the major symptoms of HT1, caused by the buildup of those molecules.

Symptom	Causative Agent	Mechanism
Neurologic crises	SA	Inhibition of ALA dehydratase
Renal Fanconi syndrome	SA	?
Increased blood tyrosine	FAA	Inhibition of HPD or TAT
Hepatocyte transformation (HCC)	FAA	DNA mutations
Hepatocyte death	FAA	Apoptosis, cytochrome c release

Table 1. Summary of HT1 pathophysiology. Symptoms and mechanisms are discussed sequentially in the text.

Succinylacetone

Although FAA and MAA are not found in the blood or urine of patients with HT1, which may be because of a short intracellular half-life (Grompe et al., 1993), SA is found at high levels in the circulation. However, the only cell types that express the full tyrosine catabolic pathway are hepatocytes and renal proximal tubule cells (Mitchell et al., 1999). Therefore, SA is the more likely cause for the major non-liver or kidney phenotype of the disease, the neurologic crises. In proof of this, SA was found to inhibit ∂-aminolevulinic acid (ALA) dehydratase, involved in heme biosynthesis, causing a

buildup of ALA which can lead to porphyria-like neurologic crises (Sassa and Kappas, 1983). These crises can be a cause of death in HT1 (Mitchell et al., 1999).

SA is also thought to be the cause of the renal Fanconi syndrome associated with the disease. This was evidenced by several studies in rats where SA was injected intraperitoneally and showed a subsequent inhibition of sodium-dependent phosphate uptake and impaired sugar and amino acid uptake in renal tubule cells (Roth et al., 1991; Roth et al., 1989). However, this kidney dysfunction was not accompanied by structural damage to the kidney (Wyss et al., 1992). This suggests that the tubular epithelial damage and dilatation seen in HT1 kidneys may be due to FAA, rather than SA, as discussed in chapter 4. The precise mechanism of the disturbance of renal tubular transport caused by SA is not known.

FAA: evidence for a disease-causing agent

It has long been thought that FAA was primarily responsible for the tissue damage in HT1 (Lindblad et al., 1977). Based on their structure, both MAA and FAA are predicted to be alkylating agents (Michael reaction acceptors) (Fig. 3) (Nebert et al., 2000). It has been suggested that the liver and kidney damage in HT1 is due to the alkylation of sulfhydryl groups of proteins and the alteration of the redox state of cells through the effects of these metabolites (Mitchell et al., 1999; Nebert et al., 2000).

Figure 3. Michael reaction acceptor. Demonstrates nucleophilic attack at the electron-withdrawing heteroatom. Adapted from (Nebert et al., 2000).

Altered gene expression in mouse models

Several lines of evidence have supported the above idea. First, in both mouse models of HT1—first found in the lethal albino mouse and later confirmed in the FAH knock-out—several genes were found to have altered expression levels. The changes in mRNA levels were found only in hepatocytes and renal proximal tubule cells (Ruppert et al., 1990). Interestingly, genes that have an increased level of expression include NADPH: menadione oxidoreductase (NMO-1) and some *gadd* (growth arrest and DNA damage inducible) genes, which are collectively involved in DNA or oxidative damage. The NMO-1 gene product protects against quinone toxicity, and has been shown to be induced by other Michael reaction acceptors (Joseph et al., 2000; Talalay et al., 1988). The *gadd* gene transcripts shown to be increased with FAH deficiency, including *gadd153*, are also induced by other alkylating agents (Fornace et al., 1989). Additionally, there is a severely decreased expression of cAMP-responsive genes in HT1 such as phosphoenolpyruvate carboxykinase (PEPCK) and TAT (Ruppert et al., 1992).

Another manifestation in HT1 patients--increased plasma tyrosine levels --is thought to be caused by the secondary inhibition of upstream enzymes in the pathway, HPD and/or TAT. A direct accumulation of the amino acid can be ruled out by the fact that AKU patients do not have increased blood tyrosine. In humans, HPD enzyme levels were shown to be reduced in the livers of HT1 patients (Lindblad et al., 1977; Whelan and Zannoni, 1974). As mentioned, decreased mRNA levels of TAT were found in both lethal albino and FAH knock-out mice, due to a failure to express some hormone-dependent genes (Grompe et al., 1995; Ruppert et al., 1992). A similar transcriptional inhibition may cause the decreased HPD levels in humans. Reduction of either enzyme could cause an accumulation of tyrosine as seen in patients with tyrosinemia types II and III.

FAA and oxidative stress

Other evidence of the toxic nature of FAA has been found in patients with HT1. One report showed low levels of glutathione (GSH) in both the liver and erythrocytes. Low levels of mixed function oxidase activity (7-ethoxycoumarin deethylase and aryl hydrocarbon hydroxylase) were also found, which may impair detoxification processes in the liver (Stoner et al., 1984). FAA was previously shown to form adducts with GSH (Edwards and Knox, 1956), which may explain the decreased levels of GSH found in patients. GSH has many important functions in the cell including the maintenance of thiol groups and the detoxification of electrophiles and oxygen radicals (Lu, 1999). Therefore, a reduced amount of glutathione could potentially contribute to the liver damage seen in HT1. A reduced amount of liver protein thiol groups was also shown in HT1 patients (Lloyd et al., 1995).

Another report from two HT1 patients showed that their plasma total antioxidant activity (TAA) was low compared to control levels. This test measures the level of free radical scavenging in the patient's plasma. These studies also showed that one patient's TAA increased to within the normal range after starting NTBC therapy (Bird et al., 1995). Increased chromosome breakage was also reported in a skin fibroblast culture from a patient who died from HT1 (Gilbert-Barness et al., 1990). These findings provide evidence that the cellular damage found in HT1 could be due to a general state of oxidative stress and/or the depletion of GSH due to the buildup of FAA. Additional evidence from the FAH knock-out mouse showed a large increase in the 8-hydroxy-2'deoxyguanine (8-OH-dG) content in liver DNA of mice taken off NTBC (Grompe, unpublished data). 8-OH-dG is generally found as a result of oxidative damage (Kasai, 1997).

FAA as a mutagen

HCC is a frequent outcome of HT1 (Russo and O'Regan, 1990). The development of cancer is thought to begin by DNA mutations (Weinberg, 1996), which in the case of HT1, are likely caused by FAA. The first direct demonstration of the mutagenic effects of FAA was reported by exposing tissue culture cells to FAA and looking for 6-thioguanine resistance due to mutations in hypoxanthine-guanine phosphoribosyl transferase (HPRT). FAA and MAA were both tested in this assay, with and without first depleting the cells of GSH. The ability of FAA itself to form adducts with GSH resulting in a reduction in cellular GSH levels was also confirmed using this system. While MAA was not shown to have a mutagenic effect, FAA did cause an increased mutation frequency, and had an even greater effect in combination with GSH depletion (Jorquera and Tanguay, 1997). This finding confirmed the idea that FAA causes damage to cells and that this damage may somehow involve cellular GSH depletion. It also suggested that MAA is not as mutagenic as FAA. This had not previously been known since there are no known patients with a defect in MAI.

As an alkylating agent, FAA may be expected to cause mainly point mutations as seen with other mutagens such as ENU (Vogel and Nivard, 1994). To further investigate the mutagenic properties of FAA, this thesis work examined the effects of FAA both *in vivo* (chapter 3) and *in vitro* (chapter 4) and revealed the types of mutations found as a result of exposure to FAA, in an effort to further understand the mechanism of FAA toxicity.

FAA and apoptosis

Recently, a number of studies showed that FAA (by acute application) caused hepatocyte and renal tubule cell death by induction of apoptosis. This was shown both *in vitro* in a cellular system (Jorquera and Tanguay, 1999), and *in vivo* in another mouse

model developed to study the defects in HT1 (Endo et al., 1997)(Kubo et al., 1998). Endo *et al.* created mice doubly mutant for HPD (tyrosinemia type III) and FAH (c^{14CoS}), which exhibited the symptoms of tyrosinemia when given HGA by reopening the pathway and allowing the production of MAA and FAA (Endo et al., 1997). Both models showed that FAA caused the release of cytochrome *c* from the mitochondria, and that the apoptotic cell death could be prevented by caspase inhibitors. In the mouse model, apoptosis was shown to occur in both hepatocytes and renal proximal tubule cells (Sun et al., 2000). Additionally, as with the mutagenic properties of FAA, it was also found that GSH depletion enhanced the apoptotic effects of FAA (Jorquera and Tanguay, 1999).

III. Somatic Reversion in Genetic Disease

Reversion in genetic disease, by correction of the original genetic mutation or by a secondary DNA sequence change resulting in a functional gene product, can result in partial or complete restoration of the wild-type phenotype. If the reversion occurs in a somatic cell in a given tissue, only that tissue (likely just a portion of the tissue) will show the reverted phenotype leading to a mosaic expression of the functional gene product, or "revertant mosaicism" (Jonkman, 1999).

Mitotic reversion in HT1

Examination of livers from two HT1 patients receiving liver transplants led to the unexpected discovery of FAH-positive staining nodules that also showed a high amount of FAH enzyme activity. These findings led the authors to speculate that a somatic reversion had occurred in a fraction of hepatocytes that then proliferated and formed clones (Kvittingen et al., 1993). Such nodules of FAH-positive hepatocytes were found in a total of 20 out of 23 liver samples (all obtained during liver transplant) studied by immunohistochemistry (Kvittingen et al., 1994; Kvittingen et al., 1993). Mutation analysis was done in four of the patients showing a mosaic FAH staining pattern in their liver tissue. Two of these patients were homozygous for the same HT1 causing mutation, and two were compound heterozygotes. All were found to have reverted back to the wild-type sequence in one allele in the FAH-positive staining sections of liver tissue, but retained the mutant sequences in the FAH-negative staining sections (Kvittingen et al., 1994).

Reversion in other diseases

The finding of somatic reversion in HT1 was one of the first demonstrations of this phenomenon in a human genetic disease. As shown with the HT1 mouse model, the selective advantage of a FAH positive hepatocyte is high and can allow this cell to divide and even repopulate the liver (Overturf et al., 1996). There are now several other examples of reversion in genetic diseases affecting other tissues capable of regeneration, namely skin and blood (Table 2). As shown in the table, a number of disorders have shown reversion in a population of cells in the blood. In both Fanconi anemia and in the case of severe combined immunodeficiency (SCID) caused by mutation in adenosine deaminase (ADA), reversion has resulted in a milder phenotype due to the presence of corrected lymphocytes (Hirschhorn et al., 1996)(Lo Ten Foe et al., 1997). Also in the skin disease, generalized atrophic benign epidermolysis bullosa (GABEB), there were visible patches of clinically normal skin due to a gene conversion event in a compound heterozygous patient (Jonkman et al., 1997). In HT1, it has not been shown whether patients with some FAH expression in their liver due to reversion events have a milder phenotype, but there are phenotypic differences among some patients within the same family that could be explained by this phenomenon (Mitchell et al., 1999).

In other examples, such as Duchenne muscular dystrophy, Wiscott-Aldrich syndrome, and Bloom syndrome, the revertant cells were detected either as patches of muscle fibers that stain positive for dystrophin in Duchenne muscular dystrophy patients (Thanh et al., 1995), or by looking at the genomic DNA from blood lymphocytes (Ariga et al., 1998; Ellis et al., 1995). However, none of the patients showed any changes symptomatically.

Genetic mechanisms

Different genetic mechanisms have been demonstrated to cause somatic reversion. In both HT1 and ADA deficiency the disease mutation itself was reverted to wild-type sequence. This could be caused by the mutagenic activities of the metabolites that accumulate in these disorders, FAA and deoxyadenosine respectively. Deoxyadenosine, like FAA, is thought to be responsible for the cellular damage in ADA deficiency and has been shown to cause DNA strand breaks *in vitro* (Brox et al., 1984)(Hirschhorn et al., 1996). Although a point mutation at the specific base that was originally mutated must be an extremely rare event, the high selection pressure in both diseases would allow any such events to be detected.

TISSUE	DISEASE	METHOD OF REVERSION	REFERENCE
BLOOD	Bloom Syndrome	Intragenic mitotic recombination	(Ellis et al., 1995)
	SCID—Adenosine deaminase deficiency —X-linked	Back mutation	(Hirschhorn et al., 1996)
		Back mutation	(Stephan et al., 1996)
	Fanconi Anemia	Frameshift correction/ Second missense change	(Waisfisz et al., 1999)
		Gene conversion/ Intragenic mitotic recombination	(Lo Ten Foe et al., 1997)
	Wiscott-Aldrich Syndrome	Frameshift correction	(Ariga et al., 1998)
LIVER	Tyrosinemia Type I	Back mutation	(Kvittingen et al., 1994)
SKIN	Generalized atrophic benign epidermolysis bullosa	Gene conversion	(Jonkman et al., 1997)
		Frameshift correction	(Darling et al., 1999)
MUSCLE	Duchenne Muscular Dystrophy	Frameshift correction	(Thanh et al., 1995)

Table 2. Genetic diseases showing somatic reversion

Another reported mechanism of reversion is the correction of a frameshift mutation by either deletion or insertion of additional nucleotides. This has occurred in several of the examples shown in Table 2. The secondary changes that corrected the frameshift vary from an additional one base insertion to correct a one base deletion (Ariga et al., 1998), to deletions of surrounding exons to restore the correct reading frame (Thanh et al., 1995). In Fanconi anemia patients, a one base deletion was corrected by two additional one base deletions, and a one base insertion was corrected by a five base insertion to restore the reading frame. The mechanism for such frame-restoring mutations was suggested to be DNA polymerase slippage because of the surrounding sequences in these cases (Darling et al., 1999; Waisfisz et al., 1999).

Patients that are compound heterozygotes and have a different disease causing mutation on each chromosome have the possibility of undergoing gene conversion or mitotic recombination as a mechanism of reversion. With these mechanisms the wild-type sequence is completely restored as opposed to some of the above mechanisms where a second sequence change downstream may result in amino acid changes. Gene conversion was shown to be the cause of the revertant cells in both Fanconi anemia (Lo Ten Foe et al., 1997) and GABEB (Jonkman et al., 1997). In these cases, a small amount of DNA surrounding a mutation on one chromosome was replaced with DNA from the other chromosome that contained wild-type sequence for the gene involved. This is distinguished from mitotic recombination because in gene conversion there are regions of heterozygosity surrounding the reverted sequence on the same chromosome. A double recombination event could also show such a result but this event is unlikely to occur in such a small segment of DNA (see Figure 4).

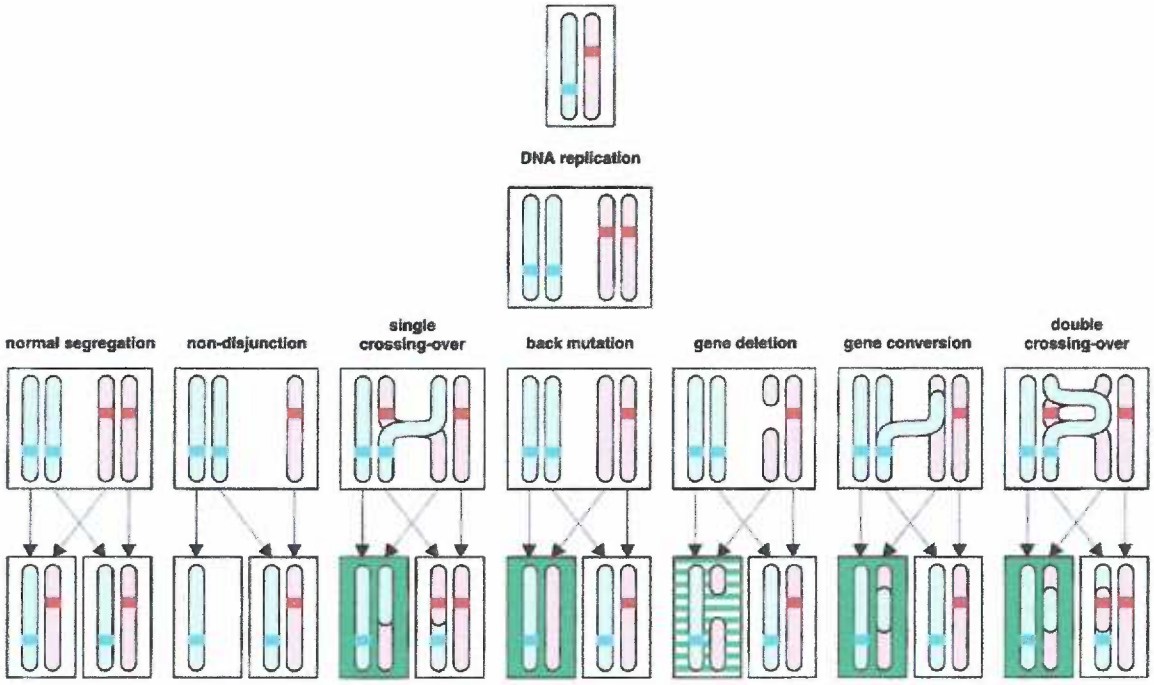

Figure 4. Methods of reversion in a compound heterozygote. Green boxes result in a corrected cellular phenotype due to loss of the second mutation (red); striped box may not be fully corrected due to deletion. Other products would still express the mutant phenotype (adapted from (Jonkman et al., 1997)).

A single crossover between two homologous chromosomes during mitosis can also cause a reversion event in a compound heterozygote. Formal evidence of mitotic recombination in normal mammalian cells *in vivo* was not provided until the recent studies of reversion in both mice and in humans. In mice, mitotic recombination was shown to be the cause of some of the reversions seen at the *W/Kit* locus that gave rise to spots of wild-type coat color on an otherwise white coat background (De Sepulveda et al., 1995). In Bloom syndrome, it was demonstrated that intragenic mitotic recombination

was responsible for the mosaic population of cells found in 5 out of 11 individuals studied by looking at polymorphic loci surrounding the gene (Ellis et al., 1995).

As first demonstrated in retinoblastoma as a model of tumor formation, mitotic recombination has been shown to cause loss of heterozygosity (LOH) in tumor suppressor genes (Cavenee et al., 1983), but it has only recently been shown to occur spontaneously in normal cells at a high frequency (Shao et al., 1999). This suggests that mitotic recombination may play an important role in generating somatic cell mosaicism in humans. It also suggests that a population of cells that has had a reversion event may be a more common finding than previously expected, especially in individuals that are compound heterozygotes for a genetic disease that affects regenerating tissues such as skin, liver, or blood.

IV. Thesis Rationale

Rationale for Chapter 2—Identification of the mutation in the alkaptonuria mouse model (Manning et al., 1999)

As discussed in the introduction, the current therapy for HT1, NTBC, has had good results. However, increased levels of tyrosine have caused problems in some patients on NTBC, and HCC can still occur (Holme and Lindstedt, 1998). To see whether inhibition of the tyrosine catabolic pathway at a different enzymatic step may have better results as a therapy for HT1, we obtained the aku mouse model from Xavier Montagutelli at the Pasteur Institute (Montagutelli et al., 1994). By breeding the aku mice with FAH knockout mice, the phenotype of the double-mutants would demonstrate the effect of inhibiting HGD in the FAH mutant mice. These data are presented in chapter 3.

In order to carry out these experiments, we first identified the mutation in the aku mice since this was not previously known. The discovery of this mutation allowed the creation of a genotyping assay using genomic DNA. This assay was also useful for other experiments in chapter 3. Jose Manuel Fernandez-Canon in the Grompe laboratory contributed mouse *Hgd* sequence information and some PCR primers for these experiments.

Rationale for Chapter 3—In vivo suppressor mutations correct a murine model of hereditary tyrosinemia type I (Manning et al., 1999)

As mentioned above, aku and FAH $^{\Delta exon5}$ ($Fah^{-/-}$) mice were bred to determine whether inhibition of HGD would be a better therapy for HT1 than NTBC. Double-mutant mice (Hgd^{aku}/Hgd^{aku}, $Fah^{-/-}$) were created that were deficient in HGD protein and therefore could not synthesize the downstream products, MAA and FAA. All of the

different mouse genotypes, including HGD and FAH heterozygotes, were compared for liver and kidney function by biochemical and pathological analysis.

The laboratory of Nancy Kennaway (OHSU biochemical genetics laboratory) determined plasma amino acids. The laboratory of Ching-Nan Ou at Texas Children's Hospital determined alanine aminotransferase (ALA) and conjugated bilirubin (BC). The laboratory of Milton Finegold at Texas Children's Hospital performed all of the histological analysis. Mouse handling and tissue preparation were done with the assistance of Muhsen Al-Dhalimy in the Grompe laboratory. I performed the rest of the experiments including the DNA and mutation analysis.

Rationale for Chapter 4—Effects of HGA on HT1 mouse kidneys

Long term investigation of the mice from the aku/HT1 breedings, discussed in chapter 3, led to the discovery that the kidney phenotype of mice that were FAH deficient and HGD heterozygous was worse than in mice that were deficient in only one of the enzymes (HT1 or aku mice). To study this phenomenon further, we looked at the effect of exogenous HGA on the kidneys of FAH knock-out mice. Kidney pathology was examined to determine the effects of HGA.

The laboratory of Milton Finegold at Texas Children's Hospital performed all of the histological analysis. Mouse handling and tissue preparation was done with the assistance of Muhsen Al-Dhalimy in the Grompe laboratory. Inge Van Den Berg at the University Medical Center Utrecht in The Netherlands also assisted with the determination of HGA amounts tolerated by the HT1 mice.

Rationale for Chapter 5—Analysis of mutations induced by fumarylacetoacetate at the APRT locus

In order to further investigate the mechanism of LOH induced by FAA, as shown in chapter 3, we chose to use an *in vitro* system established by the laboratory of Mitchell Turker at OHSU (Turker et al., 1999). This system uses mouse kidney cells that are heterozygous for adenine phosphoribosyltransferase (*Aprt*) and allows the study of mitotic recombination by analysis of polymorphic markers on mouse chromosome 8. Mutation frequency and mechanism of loss of *Aprt* could be analyzed after treating the cells with FAA, and then compared to what is seen in untreated cells. Cells were plated in selection media that selected for loss of *Aprt* and clones were picked for analysis. Clones that have lost *Aprt* were analyzed for 11 polymorphic markers along chromosome 8 to look for chromosome loss, deletions, or recombination. For this experiment we collaborated with Drs. Petra Jakobs, Mike Litt, and Ray Hershberger to use the ABI Prism Genotyper for analysis of microsatellite markers using fluorescently labeled PCR primers.

Jose Manuel Fernandez-Canon provided additional help with preparation of FAA. Members of the Turker laboratory at OHSU, including Chi Shin and Olga Ponomareva, provided the cells used in the experiment, the 2-fluoroadenine, *APRT* primer sequences and PCR conditions, and general technical assistance. Yasmine Akkari also provided technical assistance in the Grompe laboratory.

CHAPTER 2

IDENTIFICATION OF THE MUTATION IN THE ALKAPTONURIA MOUSE MODEL

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In Human Mutation, Mutation in Brief #216 (1998) Online

(Manning et al., 1999)

Abstract

Alkaptonuria (*aku*), an inborn error of metabolism caused by the loss of homogentisate 1,2-dioxygenase (HGD), has been described in a mouse model created by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis but the mutation in these mice has not previously been identified. We used RT-PCR to amplify the *Hgd* cDNA from Hgd^{aku}/Hgd^{aku} mice. Two products shorter than the wild-type product were amplified. Restriction mapping and DNA sequencing were then used to identify the Hgd^{aku} mouse mutation, found to be a single base change in a splice donor consensus sequence, causing exon skipping and frame-shifted products. This base change allowed us to create a non-radioactive genotyping assay for this allele.

Introduction

Alkaptonuria (*aku*) was the first human disease to be described as having autosomal recessive inheritance (Garrod, 1902). Patients with *aku* lack the enzyme homogentisate 1,2-dioxygenase (HGD) which converts homogentisic acid to maleylacetoacetate in the tyrosine catabolic pathway (La Du et al., 1958). HGD deficiency results in an accumulation of its substrate, homogentisic acid (HGA). Excretion of HGA in the urine is generally the first sign of the disease since it can spontaneously be oxidized to a black pigment (Zannoni et al., 1969), causing a darkly colored urine. This pigment is also deposited in many tissues causing a black pigmentation (ochronosis) and leading to connective tissue damage and arthritis in adulthood (La Du, 1995).

A mouse model of *aku* which was created by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis has previously been described (Montagutelli et al., 1994). Hgd^{aku}/Hgd^{aku} mice have high levels of HGA in the urine and almost no HGD activity in the liver; however, even aged mice show no clinical signs of ochronosis (Montagutelli et al., 1994). It has been hypothesized that this lack of ochronosis is because mice, unlike humans, are able to synthesize ascorbic acid (Montagutelli et al., 1994).

The human and murine HGD genes were recently cloned (Fernández-Cañón et al., 1996; Schmidt et al., 1997); however, the mutation in the Hgd^{aku} mouse model was unknown. Here we report the nature of the mutation in the Hgd^{aku} mouse model, and a non-radioactive genotyping assay for the detection of this mutation.

Results and Discussion

The *Hgd* cDNA was amplified by RT-PCR from the liver of a Hgd^{aku}/Hgd^{aku} mouse and of a wild-type mouse. The wild-type product was approximately 1.4 kb as expected, but PCR on the mutant allele gave two smaller products of approximately 1.1 and 1.3 kb. The deleted regions were then narrowed by restriction mapping and found to be between PCR primers located at positions nt 738 and 1415 (Schmidt et al., 1997) of the cDNA (Fig. 5*A*). These PCR products were sequenced and shown to be missing either one (nt 1052-1178) or two exons (nt 1052-1359). Sequencing at intron/exon boundaries revealed that the Hgd^{aku} mouse mutation is found at the splice donor consensus sequence of the intron following nt 1178 of the cDNA. The mutant allele from a Hgd^{aku} mouse was GA instead of the required GT at the first two bases in the intron (Fig. 5*B*). This mutation caused the Shapiro and Senapathy score (Shapiro and Senapathy, 1987) of the splice donor sequence to be reduced from 80 for the wt to 62 for the mutant sequence. Skipping of either one exon (5' to the mutation), or two exons (5' and 3' to the mutation) creates a frame shift in both cases and predicts severely truncated HGD proteins.

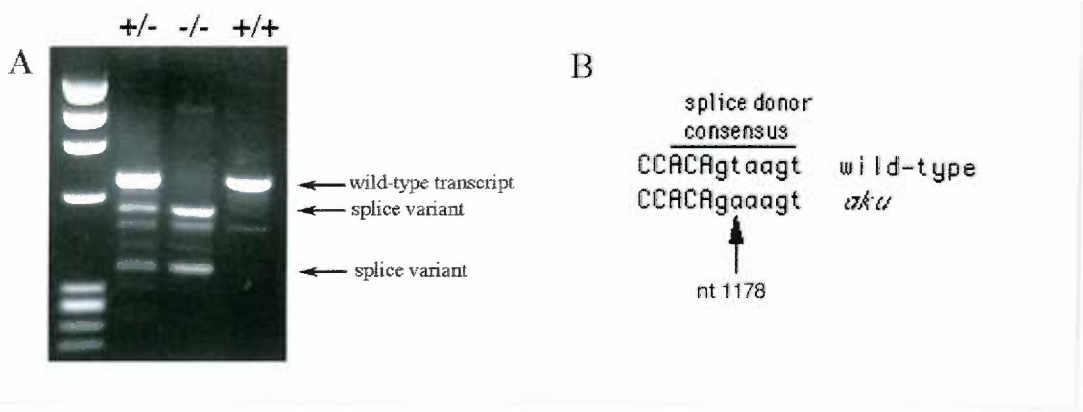

Figure 5. Aku mutation. (*A*) Amplification of Hgd cDNA from wt and Hgd^{aku}/Hgd^{aku} mouse liver. RT-PCR gave two smaller products from the Hgd^{aku}/Hgd^{aku} mouse cDNA, and a band of expected size from the wild-type cDNA. (*B*) The wild-type and Hgd^{aku}/Hgd^{aku} DNA sequence at the intron/exon boundary where the Hgd^{aku}/Hgd^{aku} mouse mutation is located (position 1178 of the cDNA). The mutated base within the splice donor consensus sequence is indicated with an arrow.

The identification of the T to A mutation allowed us to create a genotyping assay for the Hgd^{aku}/Hgd^{aku} mouse using genomic DNA. By using a PCR primer with a one base pair mismatch, a *Rsa* I restriction site was introduced upon amplification of the wild-type sequence, but not in the mutant sequence. A PCR product of 148 bp was amplified from genomic DNA and after cutting with *Rsa* I, the wt product was cut into 126 and 22 bp, whereas the mutant product was not cut. Hgd^{aku}/Hgd^{wt} heterozygotes had 148, 126 and 22 bp bands (data not shown).

Materials and Methods

Primer sequences for the genotyping assay were: 5'-TCCTTCCATTTTCACCGTGC-3' and 5'-TGCTCTGATGAAAGAGACGT-3'. A 20 µl PCR reaction was carried out with GeneAmp 10x buffer and 1.5 mM $MgCl_2$ (Perkin-Elmer). A "touchdown" PCR protocol was used as previously described (Grompe and Al-Dhalimy, 1995). After PCR, *Rsa* I was added directly to the PCR reaction and incubated at 37° for two hours to overnight. Products were then visualized after electrophoresis in a 2.5% agarose gel.

CHAPTER 3

IN VIVO SUPPRESSOR MUTATIONS CORRECT A MURINE MODEL OF HEREDITARY TYROSINEMIA TYPE I

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(Manning et al., 1999)

Abstract

Hereditary tyrosinemia type I (HT1) and alkaptonuria (aku) are disorders of tyrosine catabolism caused by deficiency of fumarylacetoacetate hydrolase (FAH) and homogentisate 1,2-dioxygenase (HGD), respectively. HT1 is a severe childhood disease, but aku is a more benign adult disorder in comparison. Because HGD is upstream of FAH in the tyrosine pathway, mice doubly mutant in both enzymes were found to be protected from the liver and renal damage of HT1 as hypothesized. Mice mutant at the *Fah* locus, but heterozygous for *Hgd* spontaneously developed clonal nodules of functionally normal hepatocytes that were able to rescue the livers of some mice with this genotype. This phenotypic rescue was a result of an inactivating mutation of the wild-type *Hgd,* thus presenting the first example of an *in vivo* suppressor mutation in a mammalian model.

Introduction

Hereditary Tyrosinemia Type I (HT1) is an inborn error of metabolism caused by deficiency of fumarylacetoacetate hydrolase (FAH), the enzyme that carries out the last step of the tyrosine catabolic pathway (see Fig.1)(Lindblad et al., 1977; Mitchell et al., 1999). Of the four genetic diseases known to result from deficiencies in specific enzymes in the pathway, HT1 is the most severe and is characterized by progressive liver dysfunction and renal tubular disease (Mitchell et al., 1999). Moreover, HT1 is unique among the tyrosine catabolic pathway diseases in that patients have a high risk for developing liver cancer (Russo and O'Regan, 1990). Maleylacetoacetate (MAA) and fumarylacetoacetate (FAA), the two compounds that precede FAH in the pathway, and thus accumulate in FAH-deficient hepatocytes, are predicted to be alkylating agents by their chemical structure, and are the primary cause of the liver damage (Jorquera and Tanguay, 1997; Lindblad et al., 1977). Currently, the drug NTBC (2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione), which blocks the pathway upstream of the formation of either MAA or FAA by inhibiting 4-hydroxyphenylpyruvate dioxygenase (HPD) (Fig.1), is used to treat patients with HT1 (Lindstedt et al., 1992). However, NTBC has the disadvantage of markedly elevating blood tyrosine levels and, therefore, needs to be combined with dietary restriction of phenylalanine and tyrosine, because increased tyrosine may lead to corneal ulcers and neurological problems as is seen in patients with hereditary tyrosinemia types II and III (Fig.1) (Mitchell et al., 1999). Additionally, NTBC may not prevent hepatocarcinomas in patients with HT1 (Holme and Lindstedt, 1998; Russo and O'Regan, 1990).

Deficiency of another enzyme upstream of FAH in the pathway, homogentisate 1,2 dioxygenase (HGD), causes alkaptonuria (aku), which has no increase in tyrosine levels (La Du, 1995). Instead, there is an accumulation of the substrate for HGD, homogentisic acid (HGA), which is excreted in the urine and can be oxidized spontaneously to a black pigment, causing darkly colored urine (Zannoni et al., 1969). This pigment also is deposited in many tissues causing ochronosis, arthritis, and connective tissue damage in adulthood (La Du, 1995).

Because elevated blood tyrosine and liver cancer are not found in aku patients, and the toxic compounds MAA and FAA do not accumulate, we hypothesized that inhibition of the pathway at HGD may be a better treatment for HT1 patients than blocking at HPD by using NTBC. This hypothesis could be tested using the mouse models of aku and HT1. An aku mouse model has been described that was created by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis (Montagutelli et al., 1994). Aku mice were found to have a splice mutation, causing the production of two shorter transcripts that predict severely truncated HGD proteins (Manning et al., 1999). These mice have high levels of HGA in the urine; however, older mice show no evidence of ochronosis or joint disease, possibly because mice can synthesize ascorbic acid, unlike humans (Montagutelli et al., 1994). A knock-out mouse model of HT1 also exists ($FAH^{\Delta exon5}$) (Grompe et al., 1993), which is lethal unless kept on NTBC (Grompe et al., 1995). When taken off NTBC, $FAH^{\Delta exon5}$ mice experience liver failure showing necrosis, inflammation, and dysplastic cells histologically. They also exhibit renal tubular disease, and will develop hepatic cancer even while on NTBC (Grompe et al., 1995). By breeding these two mouse models, we tested the hypothesis that a block in the pathway at HGD could rescue FAH knock-out mice from the severe HT1 phenotype.

Results

Cross breeding of aku and HT1 mice

FAH knock-out mice were bred with aku mice to generate mice that are mutant at the *Fah* locus, and wild-type, heterozygous, or mutant at the *Hgd* locus. These mice were kept on NTBC until age 2-4 months. NTBC then was discontinued and mice were sacrificed four weeks later. As predicted, $Fah^{-/-}$, Hgd^{aku}/Hgd^{aku} (double-mutant) mice retained a healthy weight and appearance throughout the 4 weeks off NTBC, whereas $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} (*Hgd* heterozygote) and $Fah^{-/-}$, Hgd^{wt}/Hgd^{wt} (*Hgd* wild-type) mice lost weight and became sick (data not shown). At the time of sacrifice, the liver was pale and jaundiced in all but the double-mutant mice, in which the liver appearance was normal. Liver function tests, plasma amino acid levels, and liver histology were analyzed to confirm these macroscopic observations. Some double-mutant mice were also kept off NTBC for long-term study (12-14 months) of liver and kidney function to compare with those from only 4 weeks off NTBC.

Liver function and amino acid levels

Results of liver function tests of double-mutant mice confirmed that plasma levels of alanine aminotransferase (ALT) (Fig. 6*A*) and conjugated bilirubin (BC) (Fig. 6*B*) were normal in both short-term (4 weeks) and long-term periods off of NTBC. However, both liver function tests of *Hgd* heterozygous and *Hgd* wild-type mice were abnormal. Also, plasma tyrosine levels were in the normal range in double-mutant mice both long and short-term, whereas tyrosine levels in *Hgd* heterozygous and *Hgd* wild-type mice were elevated because of the liver dysfunction in FAH deficiency (Fig.6*C*) (Grompe et al., 1995). Similar results were found for other plasma amino acid levels measured for each mouse genotype (data not shown). Aku mice that are wild-type at the *Fah* locus

also were included as normal controls. Thus, blockage of the tyrosine catabolic pathway at the level of HGD completely protected hepatic function in tyrosinemic mice, but did not increase blood tyrosine levels. Interestingly, liver function tests and amino acid levels were near normal in some of the *Hgd* heterozygous mice, indicating that they were not as severely affected by the liver disease as the *Hgd* wild-type mice.

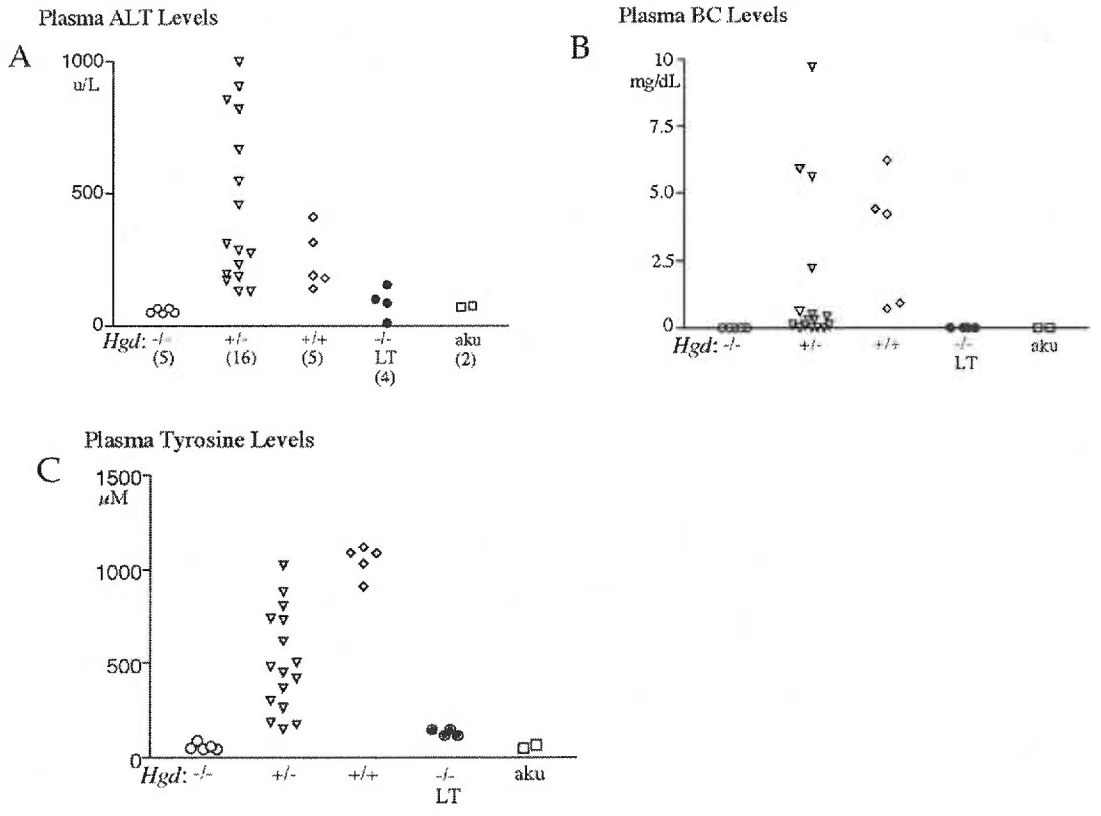

Figure 6. Liver function tests and plasma amino acid levels for *Fah*$^{-/-}$ mice with varying *Hgd* genotypes. (*A*) ALT (alanine aminotrasferase) levels. (*B*) BC (conjugated bilirubin) levels. (*C*) Plasma tyrosine levels. Double-mutant mice (-/-) are shown with both short-term (4-week) and long-term (LT) (12-to 14-month) periods off of NTBC. +/-, *Hgd* heterozygote; +/+, *Hgd* wild-type; aku, alkaptonuria controls (*Fah* wild-type). Numbers of mice from each genotype used throughout this study are given in parentheses.

Histology results

Figure 7(*A-D*) shows liver H&E staining of double-mutant, *Hgd* heterozygote, and *Hgd* wild-type mice. The latter two genotypes showed evidence of liver disease, with inflammation, necrosis, and dysplasia. The double-mutants, however, had hepatocytes of homogeneous size with few inflammatory cells, in both short- and long-term periods off of NTBC. Interestingly, all *Hgd* heterozygous mice had nodules of healthy-appearing hepatocytes that clearly were shown in the liver sections, and often visible macroscopically at the time of sacrifice. The nodules appeared as distinct regions of normal hepatocytes that were low in glycogen and negative for α-fetoprotein, although both markers were elevated in the surrounding tyrosinemic hepatocytes. The nodules also stained positive for Ki67, a marker for proliferating cells (Fig. 7*E-G*)(Gerdes et al., 1983). These findings confirmed that the nodules consisted of healthy, dividing hepatocytes. The presence of nodules in the livers of *Hgd* heterozygous mice explained why some of the liver function tests and plasma amino acid levels were near normal in these mice, because the nodules created a population of functional hepatocytes in their liver.

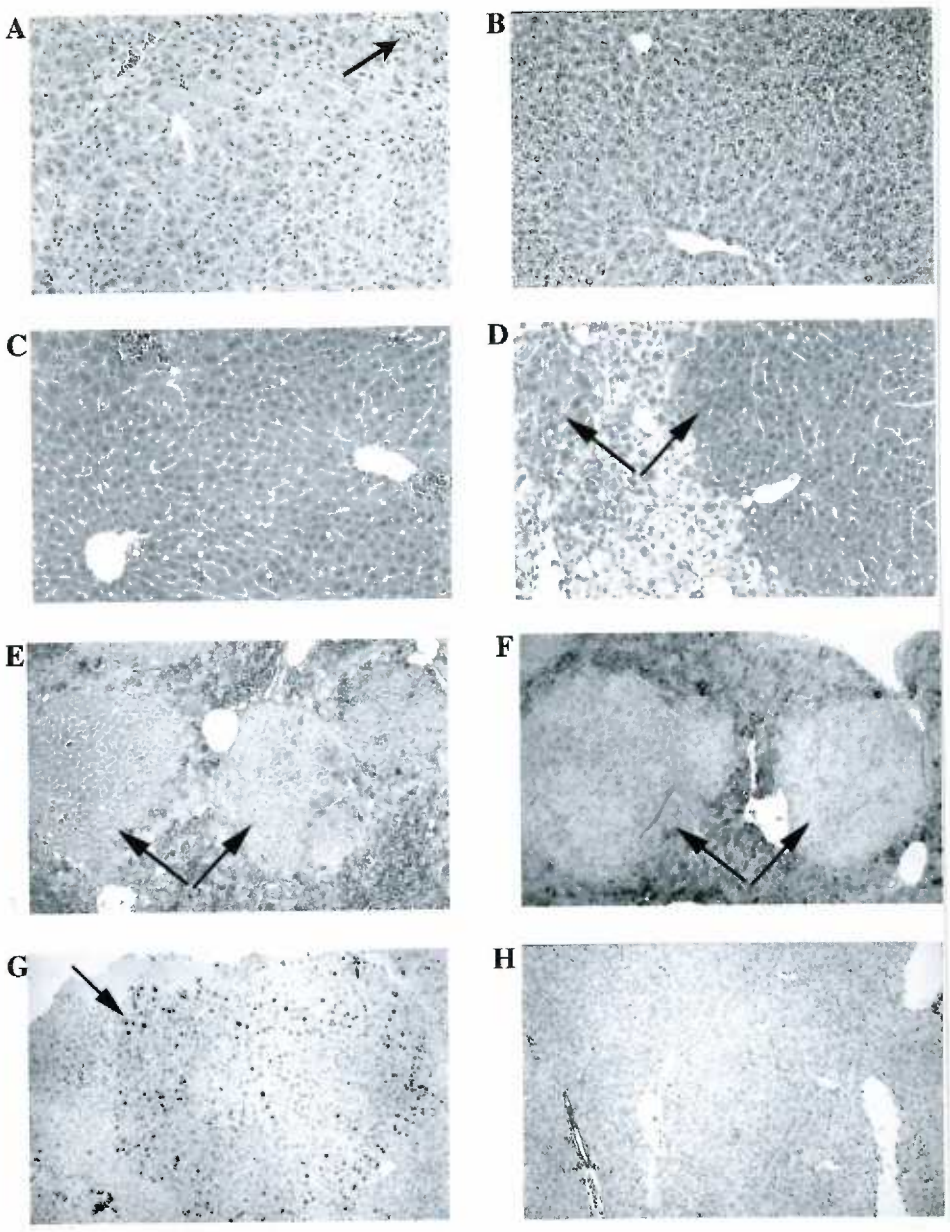

Figure 7. Liver Histology. (*A*) H&E of *Fah*$^{-/-}$, *Hgd* ^{wt}/*Hgd* ^{wt} mouse showing pathology typical of HT1, with many inflammatory cells (black arrow), necrotic cells (white arrow), and disorganized liver architecture, compared with (*B*) H&E for a double-mutant mouse 4 weeks off NTBC showing normal liver cells, with organized, columnar appearance of hepatocytes. (*C*) H&E of double-mutant mouse >1 year off NTBC also demonstrating normal liver histology. (*D*) H&E of *Fah*$^{-/-}$, *Hgd* ^{aku}/*Hgd* ^{wt} , depicting nodules (black

arrows) of healthy hepatocytes against a background of sick cells. Nodules were depleted of glycogen (PAS staining) (*E*), negative for ∝-fetoprotein (*F*), and contained many cells positive for Ki67 (*G*) whereas *Hgd* wild-type cells did not (*H*).

At the time of sacrifice, the kidneys in the double-mutant mice that were off NTBC for more than a year were of normal size and color. Histology results also showed that kidneys of the double-mutant mice were normal, with uniform proximal and distal tubules, whereas mice heterozygous or wild-type at the *Hgd* locus showed the expected tubular dilatation and inflammation seen in an FAH knock-out mouse (Grompe et al., 1995) (data not shown).

Origin of nodules in HGD heterozygotes

Histology examination of the mice sacrificed at 4 weeks off NTBC showed the presence of nodules in all of the *Hgd* heterozygous mice, but not in *Hgd* wild-type mice. Because the experiments described above had shown double-mutant hepatocytes to be metabolically normal, we hypothesized that loss of heterozygosity (LOH) had occurred at the *Hgd* locus in these nodules by an inactivating mutation of the remaining wild-type *Hgd* allele. Because previous work has shown FAA to be mutagenic (Jorquera and Tanguay, 1997), mutations in *Hgd* could be induced by the accumulation of the alkylating substrate of FAH, FAA (and MAA), resulting in a double-mutant cell that is able to proliferate and form a healthy nodule in the liver. To determine whether mutation of the remaining wild-type *Hgd* allele could lead to a complete rescue of the FAH mutant phenotype, a cohort of mice that were $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} were taken off NTBC. Five of 30 animals survived the NTBC withdrawal and recovered their normal weight. Three mice were sacrificed after being off NTBC for 7, 9, or 11 weeks. At the time this paper

was submitted, the other two *Hgd* heterozygotes were still alive off of NTBC and appeared healthy.

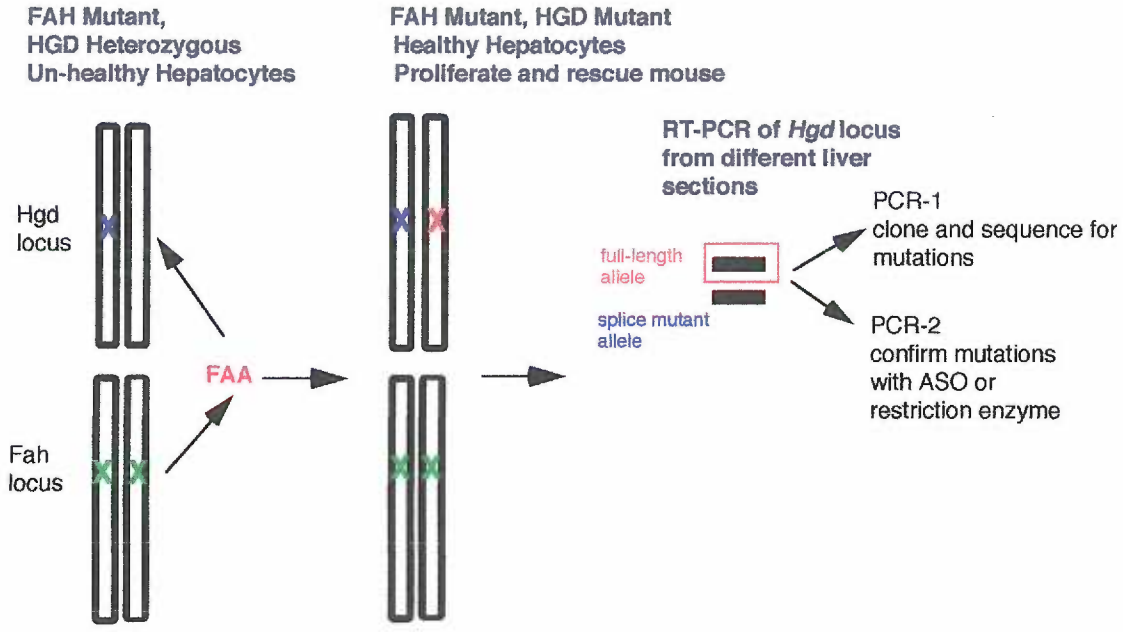

Figure 8. Schematic of LOH hypothesis and experimental design. Details are discussed in the text.

At harvest, the livers of two *Hgd* heterozygous mice (from 9 and 11 weeks off NTBC) appeared normal with no distinct nodules visible macroscopically or in histology sections. Liver function tests also showed that the livers of these two mice were functioning normally (data not shown). Their kidneys, however, were pale and enlarged and showed extensive tubular damage histologically (data not shown). The liver of each animal was dissected into several pieces of about 5 mm in diameter, and RNA was isolated from each aliquot separately. RT-PCR was then performed on the resulting cDNA (PCR-1), using primers to amplify the 3' half of *Hgd*, where the wild-type allele can be separated from the splice variants (see Figs. 8 & 9). The wild-type length PCR

product was isolated from an agarose gel and cloned. Five to 10 of these clones from each separate liver section were sequenced and putative mutations identified. Liver sections from each of the three mice also were tested for HGD enzyme activity and had no HGD enzyme detectable (data not shown).

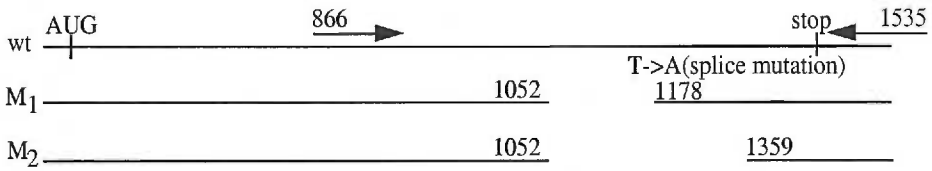

Figure 9. Schematic representation of murine *Hgd*. Wild-type and aku alleles are shown. M_1, splice mutant allele with one exon deleted. M_2, splice mutant allele with two exons deleted. Positions of RT-PCR primers used for mutation analysis of *Hgd* are also shown.

Mutation analysis

A second, independent RT-PCR (PCR-2) was also performed on each cDNA pool to differentiate real mutations from PCR errors. Any mutations identified in PCR-1 that would create obvious disruptions in the HGD protein, such as deletions and insertions, were confirmed in PCR-2 clones. Any sequence changes that caused a change in an amino acid were analyzed further only if they were in a conserved residue between *Aspergillus nidulans* and human HGD proteins (Fernández-Cañón et al., 1996). Table 3 shows the *Hgd* mutations that were confirmed by various methods from 90 clones sequenced in a total of 14 liver sections from the three mice. Eighteen sequence changes from PCR-1 were not present in the 50 independent clones from PCR-2 and then were assumed to be PCR errors or rare mutations that could not be confirmed.

Deletions (9)	Insertions (3)	Missense (10)
ΔTCG @966 (V) (ASO)	T @1351 (ASO)	TGG->GAC (V->G, A->P)@969 (RE)
ΔG @1287 (ASO)	CG @1363 (ASO)	T->G (W->G) @974 (RE)
ΔG @1292 (ASO)	27bp direct repeat--(1261-1287) (seq.)	A->G (K->E) @998 (RE*)
ΔC @1330 (RE)		G->A (D->N) @1052 (RE)
ΔC @1351 (RE)		A->C (T->P) @1154 (RE*)
ΔAT @1367 (seq.)		C->T (T->I) @1155 (RE*)
ΔCTTC @1380 (ASO)		A->G (R->G) @1160 (RE)
Δ31bp (1253-1283) (seq.)		G->T (E->D) @1195 (ASO)
Δ27bp (1458-1484) (seq.)		C->G (H->D) @1283 (seq.)
		G->A (G->S) @1286 (seq.)

Table 3. Total Confirmed *Hgd* Mutations

Methods of mutation confirmation are noted. Mutations were confirmed in PCR-2 clones by restriction digest (RE) if the mutation altered a restriction site. Otherwise, ASO (allele specific oligonucleotide) hybridization was used when no restriction sites were altered by the mutation. Some mutations also were confirmed by creating a restriction site that recognized either the wild-type or mutant allele, by altering a base in a PCR primer (RE*) (see *Materials and Methods*). Large alterations or mutations from clonal nodules (third mouse; see text) could be confirmed by sequencing alone (seq.). In the missense column, amino acid changes resulting from indicated base changes are referred to in parentheses.

LOH by mitotic recombination or deletion

The liver of the *Hgd* heterozygous mouse sacrificed at 7 weeks off NTBC showed some macroscopic nodularity and was dissected carefully in an attempt to isolate clonal nodules. Ten nodules were dissected and RT-PCR was performed. Interestingly, the wild-type length PCR products could be amplified only in 4 of these 10 nodules (Fig. 10*A*). We hypothesized that this lack of the wild-type length product was due to mitotic recombination or deletion of the wild-type *Hgd* allele in these nodules. To confirm this hypothesis, PCR was performed on genomic DNA from two separate liver sections from each of the three mice. The genotyping assay developed for the aku mice (Manning et al., 1999) was used to separate the wild-type allele from the mutant *Hgd* allele by restriction digest with *Rsa*I. One of the primers was labeled radioactively to allow quantitation of the results (Fig. 10*B*). Indeed, four of the six liver sections tested showed a reduced quantity of the wild-type band (<40%), as expected if gene conversion or deletion of the wild-type *Hgd* allele had occurred. A correction was applied to the ratios for all of the liver sections shown in Fig. 10*B* to include only hepatocyte DNA (see *Materials and Methods*), the only liver cells in which the entire tyrosine catabolic pathway is expressed (Mitchell et al., 1999). One liver section from mouse 2 had only 25% of the signal represented by the wild-type band (Fig.10*B*), thus showing that gene conversion or deletion of the wild-type *Hgd* allele occurred in approximately 50% of the hepatocytes in this liver section.

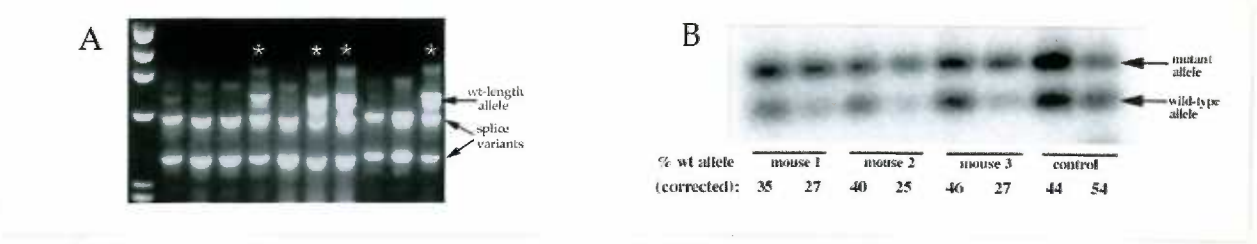

Figure 10. Loss of heterozygosity by mitotic recombination or gene deletion. (*A*) RT-PCR on clonal nodules in a *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mouse. Only 4 of the 10 nodules showed a wild-type length PCR product (*). Mutant PCR products with one or two exons deleted (see Fig. 9) were amplified in each nodule. In those nodules that had an amplifiable wild-type allele, sequencing of cloned RT-PCR products showed the same mutation in all clones, confirming that the dissected sections indeed were clonal nodules. (*B*) PhosphorImager analysis of genotyping assay on liver genomic DNA. Two liver pieces (nonclonal) from three *Hgd* heterozygous mice were analyzed, as well as two heterozygous controls in which the wild-type allele represents approximately 50% of the signal, as expected. The percentage of wild-type allele calculated from the data is corrected to include hepatocyte DNA only (see *Materials and Methods*).

Discussion

An engineered genetic block at HGD protected FAH-deficient mice from the severe liver disease, renal disease, and liver cancer found in the HT1 phenotype. This was shown with mice that were mutant at both *Fah* and *Hgd* loci, in both short-term analysis (4 weeks off NTBC) and in mice off NTBC for more than a year. In addition, double-mutant mice did not have the elevated tyrosine levels seen in mice treated with NTBC, the small molecule inhibitor of HPD (Grompe et al., 1995). Therefore, we conclude that a pharmacologic block at HGD may be a better therapy for HT1 patients than NTBC, because no dietary restrictions for tyrosine and phenylalanine would be required, and liver cancer may be prevented. This approach also is not completely problem-free, because of the joint disease that can be caused by HGA accumulation. Unfortunately, a pharmacological inhibitor of HGD has not yet been reported.

Previously, others have rescued mice deficient in FAH by using a murine model for HPD deficiency (Endo et al., 1997). Endo *et al.* showed that mice mutant at both the *Fah* and *Hpd* loci had normal liver and kidney function, similar to our mice doubly mutant for FAH and HGD. However, the FAH/HPD-deficient mice had markedly elevated blood tyrosine, whereas the FAH/HGD-deficient mice did not. Also, in contrast to our report here, the formation of healthy nodules in the livers of $Fah^{-/-}$, $Hpd^{+/-}$ mice was not reported. Hepatocellular carcinomas were not seen in either double-mutant mouse models, unlike FAH knock-out mice treated with NTBC, which do develop liver cancer. This result suggests that the lack of complete protection by NTBC in FAH knock-out mice must be due to an insufficient amount of drug to completely inhibit HPD *in vivo*.

Here, we have shown that a somatic mutation in a second gene (*Hgd*) in the tyrosine pathway can rescue the lethal liver disease of FAH knock-out mice. Such

suppressor mutations previously have been reported in simple model organisms such as yeast and *Escherichia coli* (Botstein and Maurer, 1982). In fact, a similar finding was discovered in the *Aspergillus* model of FAH deficiency, where suppressor mutations in *Hgd* were also found to prevent toxicity caused by growth of the FAH mutant strain on phenylalanine-containing medium (Fernández-Cañón and Peñalva, 1995). However, here we show an example of a suppressor mutation *in vivo* in a mammal.

Recently, an increasing number of examples of somatic reversion have been documented in regenerating tissues with genetic disease (Hirschhorn et al., 1996; Kvittingen et al., 1993) (Ellis et al., 1995; Jonkman et al., 1997; Waisfisz et al., 1999). The systems involved include the bone marrow, liver, and skin. In the cases of "classical" somatic reversion, one mutant allele is restored to wild-type function. The most common mechanism may be intragenic mitotic recombination (Shao et al., 1999). However, actual removal of point mutations and compensatory frameshifts also have been reported (Kvittingen et al., 1994) (Hirschhorn et al., 1996) (Ariga et al., 1998). We have reported previously that the selective growth advantage of genetically corrected cells can lead to liver repopulation with healthy hepatocytes and complete restoration of liver function (Overturf et al., 1996). Here, we show that second-site mutations in other genes in the same pathway also can result in spontaneous liver repopulation and full phenotypic rescue. This mechanism may be more common than mutation reversion, especially in heterozygous carriers of the suppressor mutation, because any loss of function mutation will result in rescue, whereas the classic somatic reversion requires rare genetic events that restore gene function. *In vivo* suppressor mutations should be considered in cases of spontaneous phenotypic improvement of patients with genetic diseases involving the blood, liver, or skin.

Previous studies on the specific mutagenic effects of FAA and MAA, the two metabolites that accumulate in hepatocytes deficient for FAH, were performed only *in*

vitro (Jorquera and Tanguay, 1997). An advantage of our model is the ability to study the mutagenic effects of FAA *in vivo*. By analyzing mutations that occurred in the wild-type allele of *Hgd* in many different nodules, the spectrum of mutations caused by FAA (and possibly MAA) can be elucidated. As shown in Table 3, a wide variety of mutations were found in *Hgd*. This was surprising because FAA previously was predicted to be an alkylating agent, and this type of mutagen has been shown to cause mostly base substitutions (Vogel and Nivard, 1994). The mutation spectrum also differs from what is seen in the mouse liver because of spontaneous mutagenesis, where greater than 80% of the mutations are missense mutations (de Boer et al., 1997).

In addition to the intragenic mutations found in *Hgd,* possibly up to 50% of the LOH in the nodules was caused by mitotic recombination or deletion of the *Hgd* gene, indicating that these events may be part of the mutation spectrum induced by FAA. However, our current data cannot differentiate between mitotic recombination and complete deletion of the *Hgd* gene. HT1 patients also have been reported to show increased chromosomal breakage (Gilbert-Barness et al., 1990), further supporting the possibility that FAA may cause more complex genetic changes including chromosomal damage or loss. Other mutagens, including the alkylating agent ENU, have not shown this propensity for inducing LOH (Wijnhoven et al., 1998).

Our model could also be used to study the mutation spectrum of FAA more extensively by breeding these mice with one of the transgenic mouse models that have been developed for easy detection of mutations, such as the Muta mouse (Gossen et al., 1994). However, an advantage of our current model is the ability to detect larger deletions, whereas many of the transgenic mouse models cannot because of the limitations of bacteriophage packaging used in these models (Vijg and van Steeg, 1998). Such limitations in other models may explain why larger deletions often have not been found with other alkylating agents.

The high probability for the development of liver cancer in HT1 (Russo and O'Regan, 1990) provides further proof of the potent effects of FAA and MAA. The frequency of nodule formation in the *Hgd* heterozygous mice indicates that the accumulation of these metabolites must be causing many mutations elsewhere in the genome of any FAH-deficient cells, and certainly contributing to the formation of cancer in HT1 patients. A full analysis of the damage that results from the build-up of FAA and MAA in hepatocytes would allow for a better understanding of the pathophysiology of HT1 by using this *in vivo* mouse model.

Materials and Methods

Strains of mice and animal husbandry

Aku mice were obtained from Xavier Montagutelli at the Pasteur Institute (Paris). Aku and *Fah*$^{\Delta exon5}$ mice (Grompe et al., 1993) were bred to create double heterozygotes, which then were crossed to obtain the desired genotypes (without controlling for strain background). Pups were genotyped at the *Fah* locus by using a 3-primer PCR on 200 ng of tail-cut DNA as described (Grompe et al., 1993). Aku mice were genotyped as described by PCR (Manning et al., 1999). All breeders and all mutant animals were treated with NTBC containing drinking water at a concentration of 7.6 mg/L (provided by S. Lindstedt, Gottenburg, Sweden). FAH mutants with varying *Hgd* genotypes were kept on NTBC until an age of at least 2 months, when NTBC was removed. Weights were followed at a weekly basis, and animals were sacrificed at 4 weeks after stopping NTBC, or at a later time point for those that survived NTBC withdrawal for 7 or more weeks. Animal care and experiments were all in accordance with the Guidelines of the Department of Animal Care at Oregon Health Sciences University.

HGD enzyme assay

Liver was homogenized and then sonicated for 2 x 10 sec in 500 µl of 0.25 M sucrose. The liver extract then was clarified by centrifugation (full speed in a microcentrifuge for 15 min at 4°C), and 50-200 µg of protein was added to the reaction mixture containing 100 mM potassium phosphate buffer pH 7/ 2 mM ascorbate/ 50 µM iron sulfate/ 250 µM homogentisate. The production of maleylacetoacetate was measured spectrophotometrically at 330 nm, as described (Edwards and Knox, 1955; Fernández-Cañón and Peñalva, 1995).

Biochemical analysis and quantitative amino acid analysis

Individual plasma samples of 25 µl were analyzed on a Beckman 6300 automated amino acid analyzer (Sturman and Applegarth, 1985). Samples from animals were obtained as follows. Animals were killed by decapitation and blood collected. For anticoagulation, the blood was immediately mixed with 10 µl of sodium-heparin. The red blood cells were removed by a brief centrifugation and the plasma was frozen at -80° C. Twenty microliters of plasma were mixed with 80 µl of a solution of 7% BSA and assayed for alanine aminotransferase, bilirubin and creatinine levels with a Kodak Ektachem 700 chemistry analyzer.

Histology and immune histology

For immunohistology, tissues were fixed in 10% phosphate-buffered formalin, pH 7.4, dehydrated in 100% ethanol and embedded in paraffin wax at 58 °C. Five-micron sections were rehydrated and stained with hematoxylin/eosin (H&E). Endogenous peroxidase activity was blocked with 3% H_2O_2 and methanol. Avidin and biotin pretreatment was used to prevent endogenous staining. Rabbit antibody to mouse AFP was obtained from ICN (cat no. 64-561) and used at a dilution of 1/10,000. Rabbit antibody to mouse ki-67 was obtained from Novocastra (NCL-ki67p) and used at a dilution of 1:300; the antigen-antibody complex was detected with a goat anti-rabbit antibody from Chemicon (AQ 132B), which was used at a dilution of 1:100. Color development was performed with the AEC detection kit from Ventana Medical Systems (Tucson, AZ).

Reverse transcription-PCR (RT-PCR)

At the time of sacrifice, livers from *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mice were dissected into random pieces of ≤5 mm in diameter. In one animal sacrificed at seven weeks off NTBC,

individual nodules 1-2 mm in size were identifiable, and 10 of these were isolated carefully. Each piece was homogenized directly in RNAzol B (Tel-Test, Friendswood, TX), and total cellular RNA was isolated (Chomczynski and Sacchi, 1987). Ten micrograms of total cellular RNA was reverse-transcribed with 0.8 μg oligo(dT) in a volume of 40 μl using a BRL kit (Life technologies, Gaithersburg, MD). Two microliters of the RT reaction was subjected to PCR amplification under the following conditions: 94°C for 5 min., followed by 30 cycles of 90°C x 30 sec, 55°C x 30 sec, and 72°C x 1 min, and then a final extension step of 72°C x 10 min. GeneAmp 10X PCR buffer and 2.5 mM $MgCl_2$ (Perkin-Elmer) were used in a 25 μl reaction with either *Taq* DNA Polymerase (Boehringer Mannheim) or *Pfu* DNA Polymerase (Stratagene). Primer positions are shown in Fig. 9; the reverse primer at position 1535 in the 3'UTR of *Hgd* (Schmidt et al., 1997) had the sequence 5'-TCAATTACAGTAGAGGGCTCCAGTC-3' and the forward primer located at position 866 had the sequence 5'-TAATACGACTCACTATAGGGGCCTGGTATGAAGATCG-3'.

Cloning and sequencing

The PCR products derived from the 3' half of *Hgd* were separated by electrophoresis to isolate the wild-type length product (see Fig. 8). This fragment was isolated using a QIAquick gel extraction kit (Qiagen) and then cloned with either AdvanTAge PCR cloning kit (Clontech) or a PCR-Script kit for *Pfu* products (Stratagene). PCR was performed on the resulting bacterial colonies by using the same PCR protocol as described above, except only 25 cycles were used. Dideoxy sequencing (Sanger et al., 1977) using the same forward primer as listed above, was then performed on the PCR products, after cleaning with a QIAquick PCR purification kit (Qiagen).

Allele-specific oligonucleotide (ASO) hybridization

Fourteen-15 bp oligonucleotides homologous to the wild-type and various mutant sequences were designed (Wu et al., 1989). 10 microliters of the PCR product from PCR-2 clones (from colonies, as described above) was denatured in 100 µl 0.4 M NaOH/ 25 mM EDTA and spotted onto a Hybond-N^+ membrane (Amersham) in a Schleicher & Schuell dot-blot apparatus. Five picomoles of each ASO was end-labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP. The kinase was heat-inactivated and 50 pmol of the opposing, unlabeled ASO was added. The dot blots were hybridized overnight at 25-45°C (depending on the calculated Tm) in 6 x SSPE/ 1% SDS/ 5 x Denhardt's solution. Washes were carried out for 10-30 minutes with either 5 x or 2 x SSC and 0.5% SDS at 25-42°C.

Restriction enzyme mutation analysis

Three mutations were confirmed by altering a base pair in a PCR primer near the mutation to create a restriction site. For mutation, A→G at 998, primer 5'-ATGGAAACTACACACCCTTC-3' created a *Taq* I site recognizing the mutant sequence. For mutation A→C at 1154, primer 5'- GTAATAAGGAGGTCTGAACG-3' created a Psp 14061 (Boehringer Mannheim) site for the wild-type sequence. Lastly, for mutation C→T at 1155, primer 5'-GTGGGGAGTTGCAGATAAGA-3' created a *Bgl* II site for mutant clones only.

Quantitative PCR genotyping

To determine the ratio of wild-type and mutant alleles in genomic DNA, 70 ng of DNA was isolated from separate, 5-mm cubes of *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mouse livers as described (Miller et al., 1988), and used for PCR. 200 ng of the forward primer was end-labeled by T4 polynucleotide kinase and [γ-^{32}P] ATP, and then centrifuged through a

microcon-3 (Amicon) to remove unincorporated nucleotides. Approximately 10 ng of the labeled primer was added to each PCR. PCR and digestion with *Rsa*I were performed as described previously (Manning et al., 1999). Products were run on a 2.5% agarose gel, which then was dried and placed on a phosphoimaging screen for 2 hr. Results were analyzed by using IPLAB GEL software (Scanalytics, Fairfax, VA).

Because only two-thirds of the liver genomic DNA consists of hepatocyte DNA (Rhim et al., 1995), and because the phenotypic reversion occurred only in these cells, the true ratio of mutant/wild-type DNA was calculated by using the following formulas: $X = 1/3 + 2/3\ (m/w)$; $m + w = 1$, where X = measured ratio of mutant (m) over wild-type (w) DNA and m/w = ratio of mutant/wild-type DNA in hepatocytes only. The corrected ratios are given in Figure 10*B*.

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CHAPTER 4

EFFECTS OF HOMOGENTISIC ACID ON THE KIDNEY OF HEREDITARY TYROSINEMIA TYPE I MICE

Abstract

Hereditary tyrosinemia type I is an inborn error of metabolism caused by a defect in the last enzyme of the tyrosine catabolic pathway, fumarylacetoacetate hydrolase (FAH). It is characterized by liver and kidney dysfunction and a high incidence of hepatocarcinoma. The cell damage seen in HT1 is not well understood, but is likely to be caused by the accumulation of the substrate for FAH, fumarylacetoacetate (FAA), in the cell types that express the pathway: renal proximal tubule cells and hepatocytes. Mice that are FAH deficient and were exposed to another metabolite upstream in the pathway, homogentisic acid (HGA), by either exogenous administration or from suppressor mutations in homogentisate 1,2 dioxygenase (HGD) in hepatocytes, showed increased kidney damage over that seen in an untreated HT1 mouse. The combination of metabolites (HGA and FAA) appeared to cause excess damage to the proximal tubule cells, resulting in structural damage to the kidney and continuous regeneration of proximal tubule cells.

Introduction

The tyrosine catabolic pathway is expressed in both hepatocytes and renal proximal tubule cells. The genetic disease hereditary tyrosinemia type I (HT1), resulting from a defect in the last enzyme in the pathway, fumarylacetoacetate hydrolase (FAH), exhibits both liver and kidney dysfunction due to the specific cellular localization of the pathway. The liver dysfunction is more severe and is life threatening. Patients may develop cirrhosis and hepatocellular carcinoma (HCC), and until the recent discovery of NTBC as an inhibitor of the upstream enzyme 4-hydroxyphenylpyruvate dioxygenase (HPD) (Lindstedt et al., 1992), the best treatment was a liver transplant (Mitchell et al., 1999). The kidney manifestations vary and can include renal tubular dysfunction with hypophosphatemic rickets, renal tubular acidosis, and sometimes glomerular dysfunction (Mitchell et al., 1999). The mouse model for HT1 also shows kidney damage including pale and enlarged kidneys and some apparent proximal tubule damage even while on NTBC (Grompe et al., 1995; Grompe et al., 1998). The mice also exhibit generalized aminoaciduria after being taken off NTBC (Grompe et al., 1995). FAH deficient mice transplanted with wild-type hepatocytes, however, have normal kidneys, at least by short-term analysis (Overturf et al., 1996).

Both mice and humans with HT1 accumulate succinylacetone (SA) which is derived from fumarylacetoacetate (FAA) or maleylacetoacetate (MAA) (see Fig. 1) and is not normally found in the circulation. SA is thought to be a possible cause of the kidney damage in the disease because it has been shown to inhibit renal tubular transport in other animal models (Roth et al., 1991). However, since the tyrosine catabolic pathway is expressed in renal proximal tubule cells, the toxic metabolite FAA would also accumulate in these cells with FAH deficiency and could contribute to the kidney damage as it is believed to be the cause of the liver damage in HT1 (Lindblad et al., 1977). This

was also suggested by the work of Endo and colleagues who showed that injection of homogentisic acid (HGA) into mice deficient for both HPD and FAH caused apoptosis of renal proximal tubule cells (Sun et al., 2000).

We have previously shown that mice that are doubly mutant for both FAH and another enzyme in the pathway, homogentisate 1,2 dioxygenase (HGD) which causes the genetic disease alkaptonuria (aku) (La Du, 1995), have normal liver and kidney function (Manning et al., 1999). As discussed in chapter 3, mice that were FAH deficient and heterozygous for HGD were found to lose the wild-type *Hgd* allele by suppressor mutations and formed healthy, double-mutant hepatocytes that could then proliferate and sometimes repopulated the entire liver. If left off NTBC for long-term observation, the kidneys of the $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice became progressively pale and enlarged, with extensive damage to the proximal tubule cells that appeared to be worse than what is seen with FAH deficiency alone (Grompe et al., 1995).

This data led us to further examine the effect of both accumulating metabolites (HGA and FAA) on the kidneys. To test this, we gave FAH knock-out mice on NTBC exogenous HGA, and then examined the effect on the kidneys.

Results and Discussion

$Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mouse kidneys

Kidneys of mice that were FAH mutant and either HGD mutant or HGD heterozygous were examined histologically at the time of sacrifice. As discussed in chapter 3, at one year off NTBC, double-mutant mice had normal appearing kidneys both macroscopically and upon examination of histology (Fig. 11*A*). However, HGD heterozygous mice that were off NTBC for 7 or more weeks had pale and enlarged kidneys with dilatation and focal calcification histologically (Fig. 11*B*). Also, staining with Ki67, a marker for proliferation (Gerdes et al., 1983), showed many positive cells in the tubule epithelium of the *$Fah^{-/-}$, Hgd^{aku}/Hgd^{wt}* mouse (Fig. 11*C*), indicating that there were some regenerating proximal tubule cells.

$Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice can survive off NTBC for over 10 months (I currently have several mice of this genotype off NTBC for over a year). Their livers remain healthy (data not shown), but their kidneys worsen in appearance becoming very pale with many visible cysts. Histologically they show marked cystic dilatation, inflammation, and some areas of regenerating tubule cells (Fig. 11*D*). A 1.5 hour bromodeoxyuridine (BrdU) pulse was given to the same mouse (off NTBC for 10 months) before sacrifice. Increased DNA synthesis was evident in some proximal tubule cells (Fig. 11*E*). Thus, it appears that the *$Fah^{-/-}$, Hgd^{aku}/Hgd^{wt}* mice have enough remaining kidney function to survive, perhaps from constantly regenerating proximal tubule cells, and that the overall kidney damage in these mice is more severe than with FAH deficiency alone (Fig 11*F*).

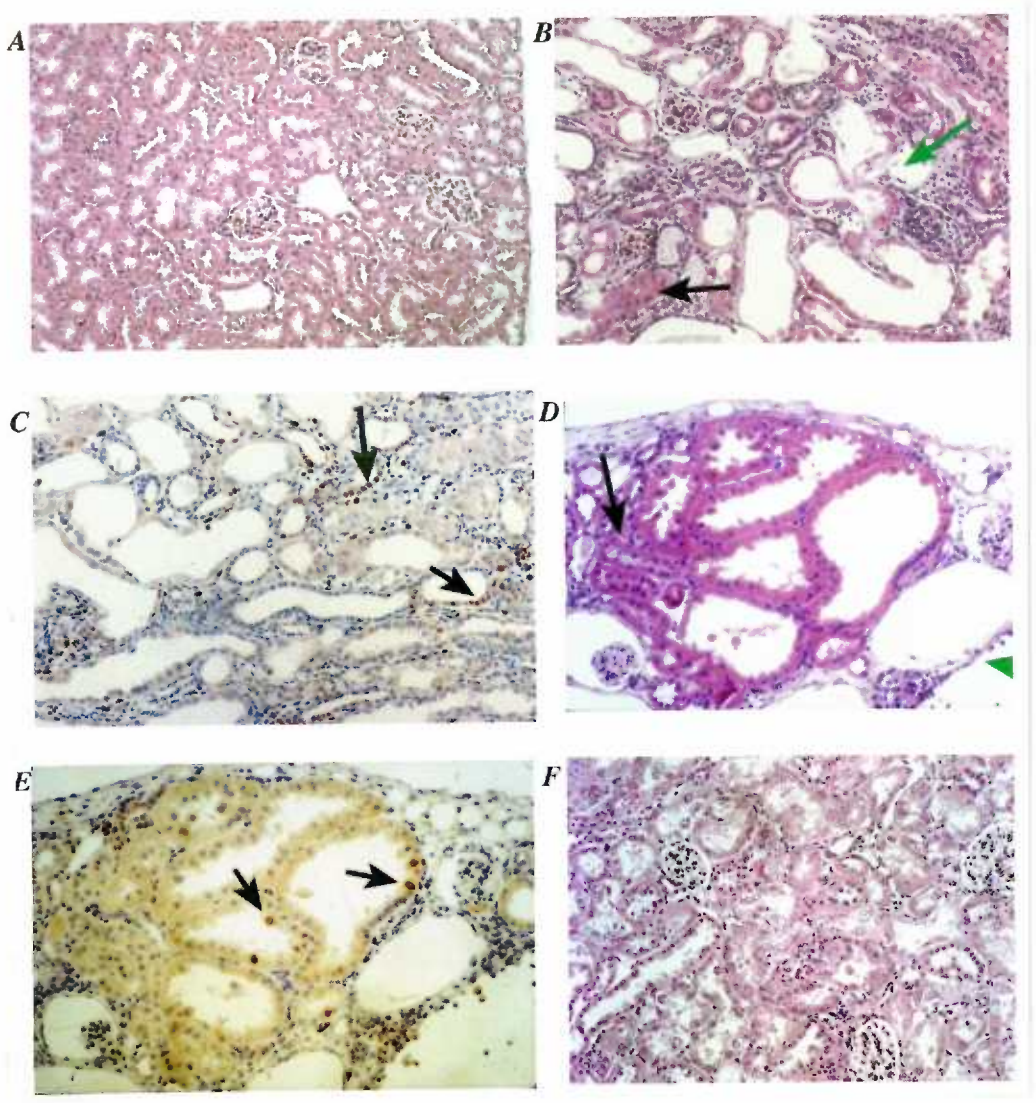

Figure. 11. Kidney histology of *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mice. (*A*) H&E of a double mutant mouse > one year off NTBC showing normal, dense kidney epithelium. (*B*) H&E of a *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mouse kidney off NTBC 7 weeks. Green arrow indicates area of dilatation and damaged epithelium. Black arrow shows regenerating proximal tubule. (*C*) *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mouse off NTBC 7 weeks, showing many cells positive for Ki67 (black arrows). (*D*) H&E of a *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mouse kidney off NTBC for 10 months. Green arrows show area of cystic dilatation. Black arrow shows patch of regenerating tubules. (*E*) BrdU staining of same section from (*D*). Black arrows indicate

examples of BrdU labeled nuclei. (*F*) H&E of an FAH mutant mouse off NTBC for < 6 weeks. Although the tubule epithelium is damaged, it is more structurally intact in comparison to $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mouse kidneys in (*B-E*). All panels represent 200x magnification.

Effects of exogenous HGA on $Fah^{-/-}$ kidneys

$Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice that survive NTBC withdrawal have undergone suppressor mutations in their hepatocytes that created double-mutant cells with a genetic block of HGD. Therefore they accumulate HGA systemically, which is toxic to FAH mutant cells (Sun et al., 2000). To see if exogenous HGA would have the same effect on the kidneys of FAH deficient mice on NTBC (NTBC blocks the pathway upstream of HGA), we determined a dose of HGA (165 mg/kg) that did not result in major harm to the liver when injected intraperitoneally. Following HGA injection, mice were sacrificed at 0, 24, 48, 72, and 96 hours later, after first receiving a one-hour pulse of BrdU. Figure 12 shows that at 0 hours there were very few BrdU positive nuclei (Fig. 12*A*) and the tubule epithelium appeared normal. HGA caused damage specifically to the renal proximal tubules as early as 24 hours after injection (Fig. 12*B*), and many BrdU positive proximal tubule cells were seen by 48 and 72 hours (Fig. 12*C*). By 96 hours, most of the regenerating tubule cells were found in the outer cortex (Fig. 12*D*). After 7 or more days, the kidneys recovered completely both histologically and morphologically (data not shown). Neither this dose, nor higher doses of HGA caused any damage to the kidneys of wild-type mice (data not shown)(Sun et al., 2000).

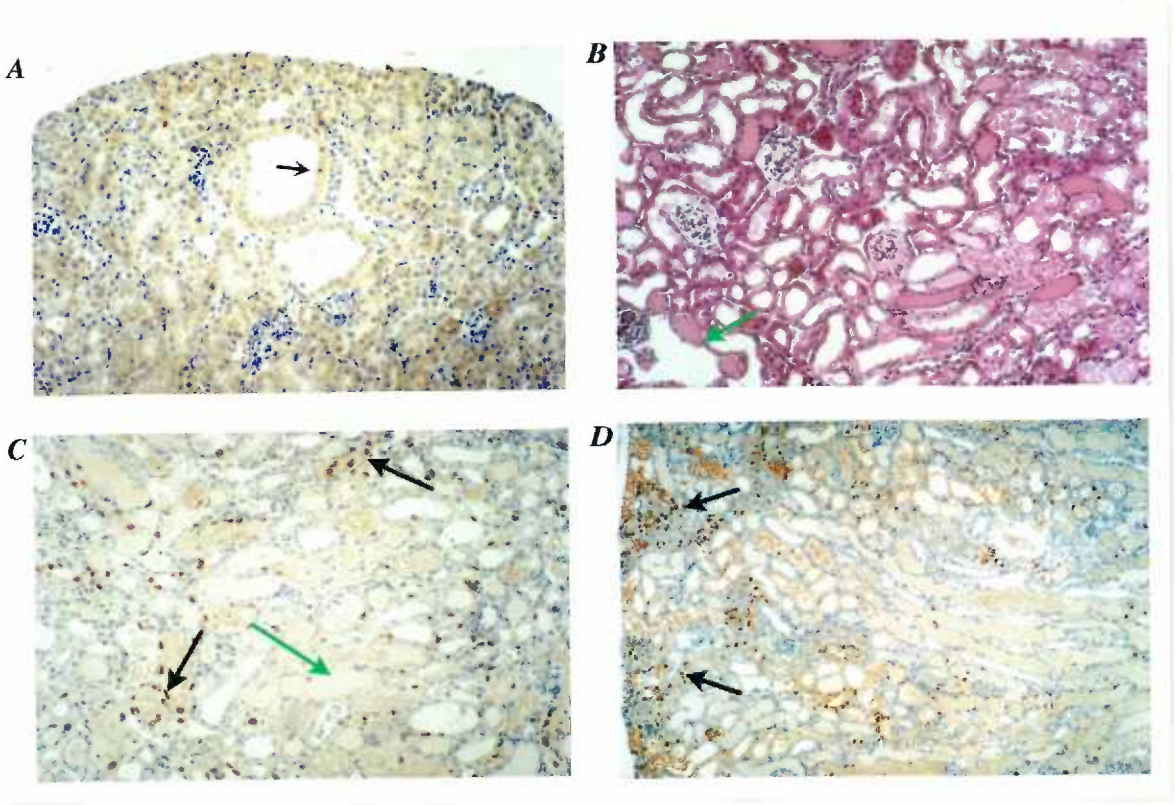

Figure 12. Time course of renal proximal tubule cell regeneration. (*A*) At 0 hours after HGA injection, the tubule epithelium is normal with rare BrdU labeled nuclei (arrow) (200x). (*B*) 24 hours after HGA, H&E shows diffuse tubular dilatation and protein casts (green arrow) (200x). (*C*) BrdU staining at 72 hours after HGA shows proximal tubule necrosis and protein casts (green arrow), with many positive staining nuclei (black arrows) (200x). (*D*) BrdU staining at 96 hours after HGA shows positive nuclei mostly in the outer kidney cortex (100x).

These data show that HGA caused damage specifically to renal proximal tubule cells in FAH deficient mice on NTBC, similar to the kidney damage seen in the $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice. In both models, remaining proximal tubule cells appeared to regenerate and in the case of acute exogenous HGA treatment, restored kidney function

and architecture. Other studies have shown a similar phenomenon of tubule cell regeneration after other types of injury, such as ischemia (Schaudies et al., 1993).

Aku mice have normal liver and kidney function (Montagutelli et al., 1994). Therefore, the kidney damage in the mouse models shown here cannot be attributed to HGA alone, but also requires a background of FAH deficiency. This was also shown in the HPD/FAH double mutant mouse model, where injection with HGA caused apoptosis of renal proximal tubule cells (Sun et al., 2000). This may indicate that the damage is due to an intracellular process in cells that express the tyrosine catabolic pathway. HGA results in increased flux through the pathway, therefore leading to greater amounts of FAA accumulation in the FAH deficient cells and increased cell damage.

Both models shown here, the progressive damage seen in $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice or the acute damage in the HT1 mice given HGA, could be useful for studying renal tubule regeneration and therapy for kidney disease. An *in vivo* selection based therapy may be possible, as has been shown for the liver in the HT1 mouse (Overturf et al., 1998; Overturf et al., 1997; Overturf et al., 1996), by the preferential regeneration of FAH-expressing tubule cells in the kidney. Currently, no such selection models exist for renal tubular disease, and gene therapy for kidney disease is only in the beginning stages of testing in animal models (Imai et al., 1998). Therefore, the use of these mouse models may help develop a better therapy for the renal manifestations in HT1, or for other diseases that affect renal proximal tubule cells.

Materials and Methods

Strains of mice and animal husbandry

Aku mice were obtained from Xavier Montagutelli at the Pasteur Institute (Paris). Aku and $Fah^{\Delta exon5}$ mice (Grompe et al., 1993) were bred to obtain the desired genotypes (without controlling for strain background). Pups were genotyped at the *Fah* locus by using a 3-primer PCR on 200 ng of tail-cut DNA as described (Grompe et al., 1993). Aku mice were genotyped as described by PCR (Manning et al., 1999). All breeders and all mutant animals were treated with NTBC containing drinking water at a concentration of 7.6 mg/L (provided by S. Lindstedt, Gottenburg, Sweden). $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice were kept on NTBC until an age of at least 2 months, when NTBC was removed. Weights were followed at a weekly basis, and animals were sacrificed 7 or more weeks after NTBC removal. Animal care and experiments were all in accordance with the Guidelines of the Department of Animal Care at Oregon Health Sciences University.

BrdU and HGA injections

Homogentisic acid (HGA) (Sigma Chemical Co.) was injected intraperitoneally at 165 mg/kg body weight. HGA stock was made fresh every time at 20 mg/ml in phosphate-buffered saline (PBS), pH 7.4 with 7 µl/ml 10N NaOH and vortexed quickly. For BrdU pulse, mice were injected intraperitoneally with 100 mg/kg 5-Bromo-2-deoxyuridine (Sigma) 1-2 hrs before sacrifice. BrdU was made fresh at 10 mg/ml in PBS with 5 µl/ml 1M NaOH.

Histology and immune histology

For immunohistology, tissues were fixed in 10% phosphate-buffered formalin, pH 7.4, dehydrated in 100% ethanol and embedded in paraffin wax at 58 °C. Five-micron

sections were rehydrated and stained with hematoxylin/eosin (H&E). Endogenous peroxidase activity was blocked with 3% H_2O_2 and methanol. Avidin and biotin pretreatment was used to prevent endogenous staining. Monoclonal antibody for BrdU was used at a dilution of 1:100 (Becton Dickenson, Franklin Lakes, NJ). Rabbit antibody to mouse ki-67 was obtained from Novocastra (NCL-ki67p) and used at a dilution of 1:300; the antigen-antibody complex was detected with a goat anti-rabbit antibody from Chemicon (AQ 132B), which was used at a dilution of 1:100. Color development was performed with the AEC detection kit from Ventana Medical Systems (Tucson, AZ).

CHAPTER 5

ANALYSIS OF MUTATIONS INDUCED BY FUMARYLACETOACETATE AT THE ADENINE PHOSPHORIBOSYLTRANSFERASE LOCUS

Abstract

The genetic disease hereditary tyrosinemia type I (HT1) results from a block in the last step of the tyrosine catabolic pathway, catalyzed by the enzyme fumarylacetoacetate hydrolase (FAH). The substrate for this enzyme, fumarylacetoacetate (FAA) accumulates in FAH deficient hepatocytes and causes cellular damage that can lead to hepatocellular carcinoma (HCC). The mechanism of cell damage has not been shown. We have recently described a mouse model that is FAH deficient and heterozygous for homogentisate 1,2 -dioxygenase (HGD), upstream of FAH in the same metabolic pathway. These mice were found to undergo loss of heterozygosity (LOH) events that caused the loss of the wild-type *Hgd* allele, most likely due to the accumulation of FAA. The mechanism of LOH was shown to be largely due to loss of the entire *Hgd* gene, by mitotic recombination, chromosome loss, or gene deletion. Using *Aprt* heterozygous cells, we have shown that FAA is mutagenic and induced LOH of the wild-type *Aprt* allele mainly by mitotic recombination and chromosome loss, and not by gene deletion or point mutation.

Introduction

The genetic disease hereditary tyrosinemia type I (HT1) is caused by a deficiency of the last enzyme in the tyrosine catabolic pathway, fumarylacetoacetate hydrolase (FAH). Patients accumulate the substrate for FAH, fumarylacetoacetate (FAA), in the cells where the pathway is fully expressed, namely hepatocytes and renal tubular cells. Although it has been suggested that FAA is the main cause of liver damage found in HT1, including progressive liver dysfunction and often cirrhosis and hepatocellular carcinoma (HCC), this has not been formally proven (Mitchell et al., 1999).

FAA is a Michael reaction acceptor and is highly electrophilic (Fig. 3) (Nebert et al., 2000). The method of toxicity has been suggested to be due to the alkylation of cellular molecules by FAA (Lindblad et al., 1977). FAA has also been shown to bind glutathione (GSH) (Edwards and Knox, 1956) and there have been reports of patients with decreased GSH levels (Stoner et al., 1984).

We have previously reported the mutation spectrum resulting from FAA accumulation in mice that were FAH mutant, and heterozygous for HGD (Manning et al., 1999). Homogentisate 1,2 -dioxygenase (HGD) is upstream of FAH in the tyrosine catabolic pathway, and deficiency of this enzyme causes the genetic disease alkaptonuria (AKU), which is a much more benign disorder in comparison to other defects in the pathway (La Du, 1995; Mitchell et al., 1999). The $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice were found to recover from tyrosinemia by undergoing a suppressor mutation in the remaining wild-type copy of *Hgd*, thus resulting in cells that are doubly mutant for HGD and FAH and are functionally normal. These healthy cells were able to divide and rescue the livers of some mice. The most likely cause for the suppressor mutations is damage caused by the accumulation of FAA. The spectrum of mutations was demonstrated by analysis of the inactivated *Hgd* gene. This spectrum consisted of multiple types of point mutations, and

some small deletions and insertions (see Table 3, pg. 42). However, a large proportion of the mutations appeared to be due to the loss of the entire *Hgd* gene, either by large deletions, chromosome loss, mitotic recombination or gene conversion (Fig.13) (Manning et al., 1999).

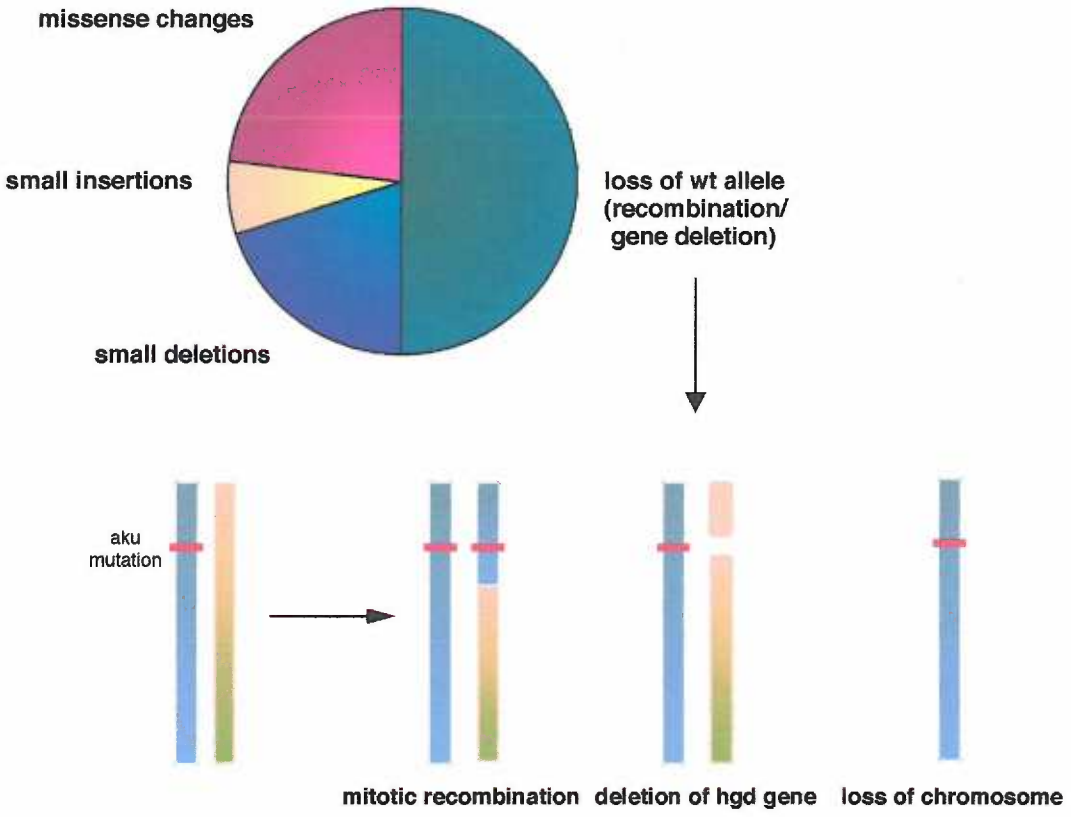

Figure 13. Relative proportions of DNA sequence alterations caused by FAA and possible causes of loss of wt allele

In order to address the potential for FAA to cause these larger, chromosomal loss of heterozygosity (LOH) events, we utilized an *in vitro* system that selects for LOH at the adenine phosphoribosyltransferase (*Aprt*) locus in mouse cells. This system can detect all types of events, including mitotic recombination, by looking at polymorphic markers surrounding *Aprt* on mouse chromosome 8 (Turker et al., 1999).

Previous studies using Chinese hamster V79 cells have shown that FAA is mutagenic at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus. They also showed that the mutagenicity from FAA is increased when the cells are first depleted of GSH with L-buthionine-(S,R)-sulfoximine (BSO), which inhibits GSH synthesis (Griffith, 1981) (Jorquera and Tanguay, 1997). The mutations causing inactivation of HPRT, used as the selection marker, however, were not identified.

Results

APRT LOH Frequencies

LOH by an inactivating mutation at the remaining wild-type (non neo-marked--see *Materials and Methods*) *Aprt* locus is detected by clonal growth in 2,6-diaminopurine (DAP) or 2-fluoroadenine (FA)-containing media. Mutagenicity of FAA was determined by comparing the numbers of APRT deficient clones in FAA-treated cultures to both spontaneous (ø) and BSO-treated (GSH depleted) cultures. Figure 14 shows that treatment of cells with FAA after GSH depletion did cause an increase in mutation frequency, whereas BSO alone did not. The median increase in mutation frequency was statistically significant and was about 2-fold higher for FAA compared to ø (x2.1, P=0.0106, paired t-test) or BSO (x2.4, P=0.0116).

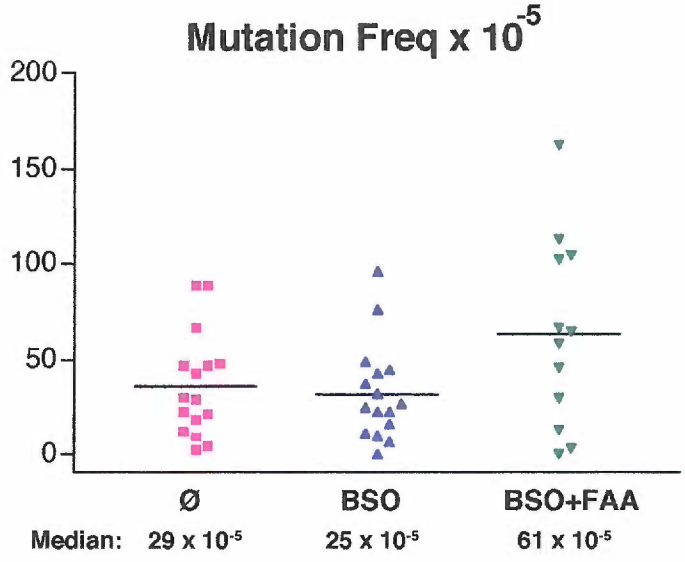

Figure 14. Frequency of APRT- clones in untreated (ø), BSO treated, and BSO + FAA treated cells. Each point represents an individual plate from a total of 4 individual subclones. Each subclone was treated individually and divided into 4 plates for selection. Because of lower viability, some FAA treated subclones were only divided into 2 plates.

Molecular Analysis of APRT Mutant Clones

Two or three clones were picked and expanded for genomic DNA preparation from each plate. An *Aprt* allele-specific PCR was then used to test each clone for LOH at the *Aprt* locus. Clones that remained heterozygous for *Aprt* were presumed to have incurred a point mutation (or epigenetic inactivation) that caused the loss of APRT activity and growth in selection media. Clones that had lost the wild-type *Aprt* were then further analyzed to determine if the LOH event was caused by chromosome loss, deletion, or mitotic recombination. Table 4 shows that there appeared to be an increased amount of LOH at the *Aprt* locus for both BSO and BSO+FAA treated cells, with the FAA treated cells having the highest amount of LOH. If the overall median increase in mutation rate is taken into account, the amount of LOH at the *Aprt* locus in FAA treated cells was increased about 3-fold over spontaneous (5.1×10^{-4} vs. 1.5×10^{-4}) or BSO treated cells (1.8×10^{-4}).

	ø	BSO	FAA+BSO
Aprt +/-	14	8	5
Aprt -/-	16	21	26
% Aprt LOH	53.3	72.4	83.9

Table 4. Aprt LOH

11 polymorphic loci on mouse chromosome 8 were then genotyped to detect deletions or chromosome loss or to identify sites of mitotic recombination. Figure 15 shows an example of two clones analyzed for LOH using the ABI Prism 310 Genetic

Analyzer to detect allele sizes derived from PCR at each marker (see *Materials and Methods*). A mitotic recombination event in one of the clones is demonstrated. Results of this analysis that was completed on all clones that showed LOH at *Aprt* are shown schematically in Figure 16. Only one apparent deletion was found (data not shown) in a BSO clone (region of LOH covering *Aprt*, with surrounding heterozygous regions). The rest of the clones all had either a mitotic recombination event or appeared to have lost the entire chromosome (LOH at all markers). It is interesting to note that only the FAA treated cells showed a recombination event between markers 339 and 100, which have the closest genetic length between them (7.7 cM) as compared to other markers where recombinations took place (from 9.1 to 12 cM). This same event was seen in 3 out of 4 different subclones treated with FAA, so it could not have been a single event.

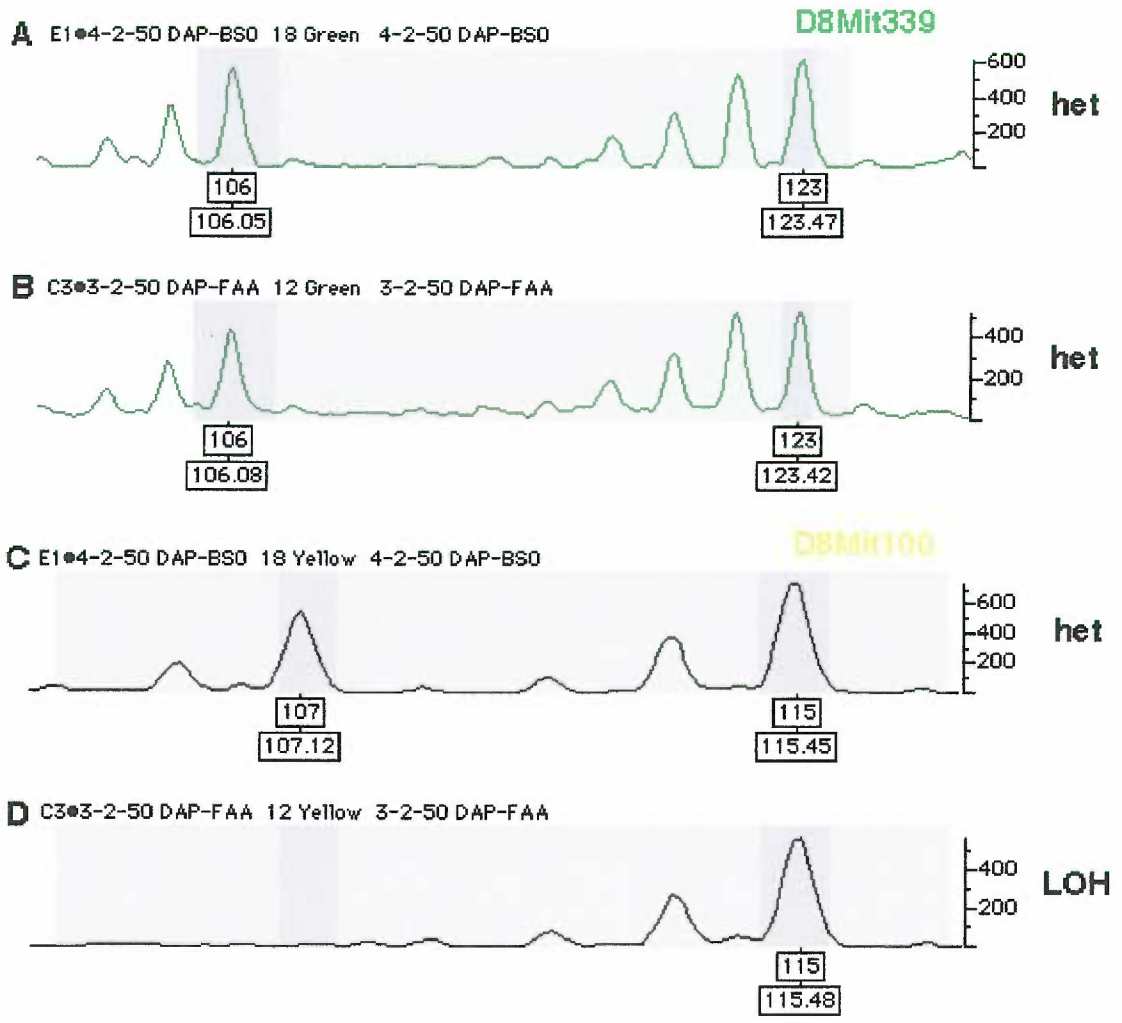

Figure 15. Example of the detection of allele sizes at two marker loci. Two neighboring markers are shown (D8Mit339 and D8Mit100) with the detection of fluorescent-labeled PCR products by the ABI Prism 310 Genetic Analyzer. Results such as these are interpreted using the ABI Prism Genotyper software. A BSO treated clone (*A*, *C*) is shown as an example of maintaining heterozygosity at both markers, and a FAA treated clone (*B*, *D*) that has had a mitotic recombination event between the markers is also shown as heterozygous at D8Mit339, and homozygous at D8Mit100.

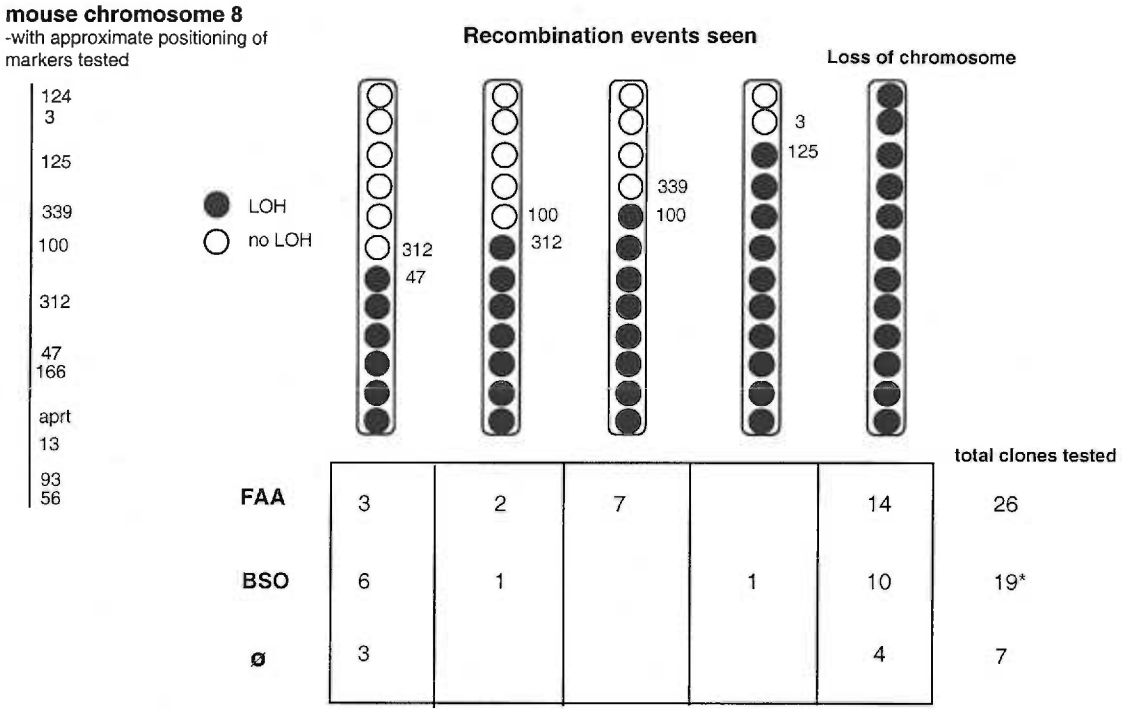

Figure 16. Loss of heterozygosity events and sites of mitotic recombination.

Chromosome 8 is shown with polymorphic markers tested. Markers were located from 4 cM (D8Mit124) to 75 cM (D8Mit56) from the top of the chromosome, along the total genetic length of 84 cM. Mitotic recombination is evidenced as a continuous region of LOH becoming heterozygous at a point centromeric to *Aprt*. Recombination was seen at four different sites as shown. Numbers of clones with the specific event shown for each treatment are listed under the pictured chromosomal event. *One apparent deletion was found in a BSO clone with LOH of markers 339 through *Aprt* only.

Discussion

We have shown that FAA caused an increase in mutation frequency when combined with GSH depletion in *Aprt* heterozygous cells. We have also presented evidence that FAA may induce larger, chromosomal LOH events, in agreement with our previous *in vivo* evidence (Manning et al., 1999). As expected from our previous data, FAA did not cause an increase in the frequency of point mutations or small deletions as compared to spontaneous or BSO-treated cells. However, there was a 3-fold increase in the amount of LOH at the *Aprt* locus due to mitotic recombination or chromosome loss. Also, FAA caused no large intrachromosomal deletions.

Although further experiments will be needed to confirm these results, the apparent shift in position of mitotic recombination in FAA treated clones (between markers D8Mit339/100) may be an interesting effect of its mutagenic action. 100 percent of recombination events identified in untreated and 75 percent of those in BSO treated clones were between markers D8Mit312/47, which have a larger genetic distance between them (10.9 cM) than that between D8Mit339 and D8Mit100 (7.7 cM). Only 25 percent of recombination events in FAA treated clones were between D8Mit312/47. This was similar to that seen in spontaneous clones from other reports, where most recombinations occurred in approximately the same area, close to *Aprt* (Shao et al., 1999; Turker et al., 1999). The overall distribution of chromosome loss and mitotic recombination was also similar to other reports using the mouse kidney cell line (Turker et al., 1999). However, the cell line used in this experiment was chosen because of a general higher percentage of APRT deficient clones caused by point mutation (M. S. Turker, personal communication). Indeed, in the untreated population, only 53% of the cells showed loss of the wild-type *Aprt* allele, where 70-80% was normally seen in other

studies (Shao et al., 2000; Turker et al., 1999). The choice of this cell line made the increase in chromosomal LOH events caused by FAA more evident.

The increase in mutation frequency caused by FAA was modest. Jorquera et al. saw a 10-fold increase in mutation frequency with FAA plus BSO treatment. However, they used a different cell line with HPRT as a selection marker (Jorquera and Tanguay, 1997). *Hprt* is located on the X-chromosome, so mitotic recombination cannot be a method of LOH with that system. *Hprt* takes up 34 kb of genomic DNA (Melton et al., 1984), whereas *Aprt* is only 3 kb (Dush et al., 1985), making *Hprt* a larger target for mutation. Also, since we used a higher dose of FAA (150 μM compared to 100μM), there may have been more cell death at this dose and more mutations may be found at a lower dose. It would be interesting to see the results of a lower FAA dose in the *Aprt* system. Lastly, since we did not test the GSH levels in our cell line, it is possible that the same method of BSO treatment did not cause a depletion of GSH to the same degree as seen in the V79 cells. Jorquera et al. saw only a 3-fold increase in mutation frequency using FAA alone, without prior GSH depletion (Jorquera and Tanguay, 1997).

It is not known whether FAA acts on DNA directly, or acts as a mutagen indirectly through other effects such as the depletion of GSH. Although the depletion of GSH by BSO alone did not cause an increase in mutation frequency as seen in this study and others (Jorquera and Tanguay, 1997), there was an apparent increase in the loss of the wild-type *Aprt* allele in the BSO treated cells compared to untreated cells (Table 4). Thus, depletion of GSH may be part of the general mutagenic effect FAA has on cells.

Overall, our findings support our previous hypothesis from *in vivo* results in the HT1 mouse, that FAA may induce larger chromosomal events as a mechanism of mutagenesis (Manning et al., 1999). There have been reports of chromosomal damage in HT1 patients (Gilbert-Barness et al., 1990), but this has not been looked at extensively in all patients. Further studies using *in vitro* systems such as *Aprt* heterozygous cells in

tissue culture, or *in vivo* studies in $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice, will help to more clearly define the role of FAA in the pathogenesis of HT1 and the development of HCC.

Materials and Methods

Cell culture and APRT-/- clonal selection

APRT heterozygous, mouse kidney cells (cell line 435K (early passage)) were obtained from Mitch Turker's laboratory at OHSU. Cells were derived from *Aprt*$^{+/neo}$ mice with a targeted neo insertion from a 129/Sv, C57BL/6 mixed background (Turker et al., 1999) crossed with DBA/2 mice. Cells were grown in 10% fetal calf serum (FCS) (HyClone, Logan, UT) w/ Dulbecco's minimal essential media (DMEM), 2 mM glutamine and the antibiotics penicillin and streptomycin (Life Technologies, Gaithersburg, MD). Cells were plated at 400 cells/100 mm plate so that individual subclones could be picked (approx.10 days). Five subclones from five different plates were used (1 was later thrown out for low viability). 50,000 or 100,000 cells were plated in a 100 mm dish for no treatment control and put on selection of 80 μg/ml 2,6-diaminopurine (DAP) (Sigma Chemical Co., St. Louis, MO) or 4 μg/ml 2-fluoroadenine (FA) (Turker laboratory) the next day. Cloning efficiency plates were also established.

$1x10^6$ cells were treated with FAA as follows: 6 hrs after seeding, L-buthionine-(S,R)-sulfoximine (BSO) (Sigma) was added directly to media (0.2 mM final concentration). BSO was prepared fresh in HBSS (Life Technologies) with 10 mM Hepes (Life Technologies) and sterile filtered. 18 hrs after the addition of BSO, FAA was added to 150 μM final concentration, or cells were plated for selection as above for BSO-only control. FAA was added with 2x DMEM (45% of total media preparation) and the appropriate amount of water and sterile filtered. 10% FCS was also added. After 24 hrs, cells were washed with HBSS and allowed to recover for 8 days before plating for selection as above.

Media was changed with selection every 6 days. 2-3 clones per plate were picked after 2-3 weeks and expanded for DNA preparation. Plates were stained afterwards with methylene blue (Sigma) and counted.

Genomic DNA preparation

Clones were grown to confluency in a T-25 flask, detached using trypsin EDTA (0.05% w/v) (Life Technologies), washed with PBS, and genomic DNA isolated (Miller et al., 1988).

APRT PCR

Approximately 50 ng of genomic DNA from each clone was subjected to PCR amplification under the following conditions: 94°C for 3 min., followed by 11 touchdown cycles of 94°C x 45 sec, 76°C x 45 sec (Δ-2°C per cycle), and 72°C x 45 sec, and then 25 cycles of 94°C x 45 sec, 54°C x 45 sec, and 72°C x 45 sec with a final extension step of 72°C x 3 min. 10X PCR buffer with $MgCl_2$ (Boehringer Mannheim) was used in a 25 µl reaction with *Taq* DNA Polymerase (Boehringer Mannheim). The reverse primer located between exons 3 and 4 of *Aprt* (Dush et al., 1985) had the sequence 5'-AAGACCCTGCCCTTCCTCTAC-3' and the forward primer located 5' of exon 3 had the sequence 5'-TCCCACAACCTTCCCTCCTTA-3'. A third primer within the knock-out construct (Stambrook et al., 1996) had the sequence 5'-TGCCTGCTTGCCGAATATCATGGT-3'.

Microsatellite analysis

Sequences for PCR primers for markers listed in Figure 16 were obtained from the Whitehead Institute for Biomedical Research/MIT Center for Genome Research. All reverse primers had the sequence 5'-GTTTCTT-3' added to the 5' end of the primer. All

forward primers had 1 of 3 fluorescent tags added to the 5' end: 6-FAM (6-carboxy-fluorescein), Tetrachloro-Fluorescein (TET), or Hexachloro-Fluorescein (HEX) (Integrated DNA Technologies, Inc., Coralville, Iowa). Approximately 40 ng of genomic DNA from each clone was subjected to PCR amplification under the following conditions: 95°C for 12 min., followed by 15 cycles of 94°C x 15 sec, 55°C x 15 sec, and 72°C x 30 sec, and then 30 cycles of 89°C x 15 sec, 55°C x 15 sec, and 72°C x 30 sec with a final extension step of 72°C x 15 min. 10X PCR Gold buffer and 2.5 mM $MgCl_2$ (Perkin-Elmer) were used in a 10 µl reaction with AmpliTaq Gold DNA Polymerase (Perkin-Elmer). Fluorescent-labeled PCR products from all 11 marker sets could be pooled and resolved by capillary electrophoresis in the ABI Prism 310 Genetic Analyzer according to the manufacturer's instructions, and subsequently analyzed using ABI Prism GeneScan and Genotyper software (PE Applied Biosystems, Foster City, CA).

FAA preparation

FAA was prepared by enzymatic conversion from homogentisic acid as has been previously described (Edwards and Knox, 1955).

CHAPTER 6

CONCLUSIONS

HT1 and AKU

This thesis work focuses on the autosomal recessive disease, hereditary tyrosinemia type I (HT1), caused by a defect in the last enzyme in the tyrosine catabolic pathway, fumarylacetoacetate hydrolase (FAH). HT1 patients have liver and renal dysfunction and a high risk for hepatocellular carcinoma (HCC), that are suspected to be caused by the buildup of the substrate for FAH, fumarylacetoacetate (FAA), in hepatocytes and renal proximal tubular cells (Mitchell et al., 1999).

Therapy for HT1 was recently improved by the discovery of 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC) as an inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPD), the second enzyme in the tyrosine catabolic pathway. Blocking the pathway at HPD prevents the formation of FAA and therefore prevents further liver damage (Holme and Lindstedt, 1998; Lindstedt et al., 1992). However, blocking the pathway at this step causes an increase in blood tyrosine which may cause corneal ulcers and mental retardation as seen in patients with tyrosinemia type II and III (see Figure 1) (Cerone et al., 1997; Mitchell et al., 1999). Therefore, one aim of this thesis was to test whether inhibition of the pathway at another step, homogentisate 1,2 -dioxygenase (HGD), would be a better therapy for HT1.

A deficiency of HGD causes the disease alkaptonuria (AKU). AKU patients do not have increased tyrosine, but instead build-up homogentisic acid (HGA) and derivatives that can accumulate in the joints and cause ochronosis and joint disease later in life (Zannoni et al., 1969). There is a mouse model of aku, created by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis. Although the mice do excrete HGA in their urine, they appear healthy with no evidence of joint disease, perhaps because of the ability of mice to synthesize ascorbic acid, unlike humans (Montagutelli et al., 1994).

Our laboratory has created a knockout mouse of FAH ($Fah^{\Delta exon5}$) as a model for HT1. These mice were found to require NTBC to rescue the otherwise lethal phenotype

(Grompe et al., 1995). Taking the mice off of NTBC re-creates both the biochemical and pathological phenotype of HT1, and the mice usually die within two weeks after removal of NTBC. Or, if they are kept on NTBC long-term, they will develop HCC (Grompe et al., 1995).

To test the hypothesis that inhibition of the pathway at HGD would be a better therapy for HT1 than NTBC, we obtained aku mice from Xavier Montagutelli at the Pasteur Institute and bred them with our FAH knockout mice to obtain double-mutant mice. In order to carry out these experiments, we decided to first identify the mutation in the aku mice as discussed in chapter 2, and develop a genotyping assay. The human and mouse *Hgd* genes have been cloned recently (Fernández-Cañón et al., 1996; Schmidt et al., 1997). From the available sequence, we developed primers to amplify the *Hgd* cDNA from both aku and wild-type mice. This revealed that the aku mouse has two truncated gene products. Restriction mapping and sequencing of PCR products showed the mutation to be a splice mutation causing exon skipping and resulting in severely truncated HGD protein. This mutation allowed for the creation of a genotyping assay for genomic DNA by designing a PCR primer with a one base mismatch, creating a restriction site (Manning et al., 1999). This genotyping assay was helpful in maintaining the aku mice as well as for identification of the aku allele for loss of heterozygosity (LOH) studies in chapter 3.

Inhibition of HGD

A pharmacological inhibitor of HGD does not currently exist. However, the creation of double-mutant mice ($Fah^{-/-}$, Hgd^{aku}/Hgd^{aku}) could also demonstrate the effect of a block in the tyrosine catabolic pathway at HGD in mice that were deficient for FAH. As shown in chapter 3, these double-mutant mice were analyzed for liver function and shown to be normal as hypothesized. Liver function tests, plasma amino acid levels

including tyrosine, and liver and kidney histology were all in the normal range for double-mutant mice that had been off NTBC for over a year (Manning et al., 1999). These results show that the creation of an inhibitor for HGD would be an effective therapy for HT1. Since there was no increase in tyrosine as shown in our mice, this therapy may be a better alternative than NTBC because some HT1 patients on NTBC therapy still require a diet low in tyrosine and phenylalanine to reduce complications of high tyrosine (Holme and Lindstedt, 1998).

New mechanism of somatic reversion in mammals

In breeding the aku and FAH knockout mice to generate double-mutants, $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} and $Fah^{-/-}$, Hgd^{wt}/Hgd^{wt} were also created and used in the study for comparison. Both genotypes lost weight and showed macroscopic changes characteristic of the HT1 mouse after they were taken off NTBC. However, on closer look of the $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice after four weeks off NTBC, nodules of healthy appearing tissue were visible on the otherwise pale and sick liver (Fig. 16). These nodules were found in all $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice on examination of histology sections, and were shown to consist of healthy hepatocytes. We then hypothesized that these nodules were due to loss of heterozygosity (LOH) of the wild-type *Hgd* allele, and that this LOH was caused by accumulation of FAA in the FAH mutant hepatocytes. More mice of this genotype were taken off NTBC for longer periods and some were able to survive for several months to a year or more. Examination of mice off NTBC for 9 or more weeks showed normal appearing livers with no distinct nodules. We further hypothesized that the hepatocytes that had undergone a LOH event were able to divide and rescue the liver, as has been shown with transplanted hepatocytes in this mouse model (Overturf et al., 1996).

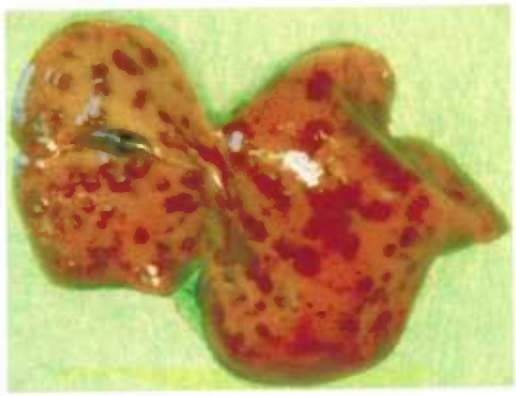

Figure 17. Liver from a *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mouse with clear nodules of red, healthy tissue.

As shown in chapter 3, we proved this hypothesis by finding mutations in the wild-type length *Hgd* allele. It was helpful that the aku mutation had created shortened mRNA products, so that the wild-type length *Hgd* allele could be easily isolated. This finding of spontaneous LOH of the wild-type *Hgd* allele is important to the field of genetics overall because it is, to our knowledge, the first example of an *in vivo* suppressor mutation in a mammal. This phenomenon had previously been reported in lower organisms, including in a fungal model of HT1, where a suppressor mutation in *Hgd* was also found that allowed growth of the FAH mutant strain on phenylalanine medium (Fernández-Cañón and Peñalva, 1995).

This model in the mouse represents a new mechanism of somatic reversion (see Table 2). Although there are now several examples of reversion events seen in genetic disease, they have only been demonstrated to occur within the same locus that was originally mutant. A second-site mutation causing inactivation of another gene in the pathway may be a more common event because any inactivating mutation in the second gene could result in phenotypic reversion. Corrections of frameshift (Darling et al., 1999; Waisfisz et al., 1999) (Ariga et al., 1998; Thanh et al., 1995) or back mutation of the

original point mutation (Hirschhorn et al., 1996; Kvittingen et al., 1994; Stephan et al., 1996) both require more specific events that must be extremely rare.

LOH of wild-type Hgd allele

One *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mouse sacrificed after 7 weeks off NTBC for *Hgd* mutation analysis had some areas in the liver where nodules could be distinguished and were then carefully isolated. RT-PCR on these nodules showed that the wild-type length *Hgd* allele could be amplified in only 4 out of the 10 nodules tested (Fig. 10*A*). This finding suggested that some LOH events may involve the loss of the entire wild-type *Hgd* allele, by chromosome loss, mitotic recombination, gene conversion, or large deletion. Using the aku genotyping assay (Manning et al., 1999) on genomic DNA from the livers of all three mice used in these studies confirmed that loss of the wild-type *Hgd* allele occurred in all mice, and in some liver sections accounted for as much as 50% of the mutation events (Fig. 10*B*).

Some types of mutagens such as the bulky-adduct inducing 7,12-dimethyl-1,2-benz[a]anthracene (DMBA) can cause larger, chromosomal events rather than point mutations or small deletions and insertions seen with many alkylating agents (Bremner et al., 1994; Wijnhoven et al., 1998). FAA may also cause these larger events as seen in the above studies. Recently, hydrogen peroxide was shown to cause increased mitotic recombination as a mechanism of LOH at the adenine phosphoribosyltransferase (*Aprt*) locus in murine kidney epithelial cells (Turker et al., 1999). We used the same system with *Aprt* as a marker locus to determine the mechanism of LOH induced by FAA. It has been shown that FAA is mutagenic in an *in vitro* system, and that the mutagenicity was enhanced by first depleting cells of glutathione (GSH) (Jorquera and Tanguay, 1997). However, this was shown using hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) as a selection marker. *Hprt* is hemizygous and therefore cannot utilize mitotic

recombination as a mechanism of LOH. We have confirmed that FAA increased the mutation frequency in a different cell system, and also have demonstrated the mechanism of the LOH events caused by FAA at the *Aprt* locus, which has not been shown before.

As discussed in chapter 5, FAA caused about a 2-fold increase in mutation frequency in *Aprt* heterozygous cells. This increase was mainly due to an increase in loss of the wild-type *Aprt* allele (see Table 4) similar to what was seen in the $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice with loss of the wild-type *Hgd* allele. This system can detect mitotic recombination events by looking at polymorphic markers along mouse chromosome 8, surrounding the *Aprt* locus. Marker analysis showed that LOH induced by FAA was due to both chromosome loss and mitotic recombination, and not to deletions. A shift in the site of recombination was also seen in clones resulting from FAA treatment, compared to recombination events seen in clones from spontaneous or BSO treatment (Fig. 15). These data reflect what was seen in the $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice *in vivo* and show that larger, chromosomal events may play an important role in the cell damage caused by FAA.

Chromosomal events such as mitotic recombination have only recently been shown to occur in normal somatic cells (De Sepulveda et al., 1995). These events could contribute to the somatic mosaicism and phenotypic variance within families that have been found with increasing frequency in genetic diseases (Hirschhorn, 1995). HT1 is included among the genetic diseases that show phenotypic variance, and this variance in disease severity is believed to be caused by revertant nodules that contain some FAH activity (Mitchell et al., 1999). Such nodules may arise by mitotic recombination in a compound heterozygote, and could be caused by FAA. Other diseases involving DNA damage such as Bloom syndrome and Fanconi anemia (D'Andrea and Grompe, 1997; Ellis et al., 1995) have also shown somatic reversion events in patients that were caused by intragenic mitotic recombination. Recombination events in some cases could be

induced during the repair process (Tischfield, 1997) and contribute to somatic mosaicism in the genetic diseases mentioned, or in other diseases.

Mechanism of FAA toxicity

Although FAA has been thought to cause the cell damage found in HT1 since the suggestion by Lindblad in 1977 (Lindblad et al., 1977), it has not been shown to be directly mutagenic until recently (Jorquera and Tanguay, 1997). This thesis work has contributed to the data supporting FAA as the disease-causing agent in HT1. We have demonstrated for the first time the spectrum of mutations caused by FAA, and have shown that this spectrum includes chromosomal events such as mitotic recombination. FAA is highly electrophilic and predicted to be an alkylating agent by its chemical structure (Mitchell et al., 1999). Interestingly, the spectrum of mutations found did not reflect what is usually seen with other alkylating agents (Vogel and Nivard, 1994). Instead, FAA produced a variety of mutations including larger, chromosomal events that may be an important part of the damage caused to hepatocytes and renal proximal tubule cells in HT1.

HCC is a frequent outcome in HT1 (Russo and O'Regan, 1990). Other genetic diseases with high rates of cancer, such as Fanconi anemia and Bloom syndrome (D'Andrea and Grompe, 1997) have chromosomal aberrations due to DNA damage. HT1 has also shown evidence of chromosomal damage in one patient (Gilbert-Barness et al., 1990). However, the DNA from liver tumors found in HT1 patients has not been examined extensively for any characteristic DNA lesions.

FAA was shown to bind GSH *in vitro* (Edwards and Knox, 1956) and was also evidenced *in vivo* in HT1 patients where GSH levels were low (Stoner et al., 1984). GSH is the most abundant nonprotein thiol and is important in a variety of cellular processes including maintaining reduced sulfhydrl groups and defense against oxidative damage

(Lu, 1999). As shown in *in vitro* studies, prior depletion of cellular GSH enhanced the mutagenicity of FAA (Jorquera and Tanguay, 1999; Jorquera and Tanguay, 1997). Depletion of GSH may be an important part of FAA damage by disrupting essential functions such as DNA repair or any cellular process involving a thiol-requiring protein.

The involvement of oxidative damage in HT1 is also suggested to be a mechanism of toxicity. This could also be a result of GSH depletion since GSH is an important antioxidant (Nebert et al., 2000). The finding of increased 8-hydroxy-2'-deoxyguanine (8-OH-dG) levels in HT1 mice after NTBC removal (M. Grompe, unpublished data) supports the role of oxidative damage in the HT1 disease process.

Induction of kidney damage by HGA in FAH deficiency

As discussed in chapter 4, kidneys of $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice showed progressive damage with increased proliferation of renal proximal tubule cells. This extensive damage may result from increased stress on the tyrosine catabolic pathway from HGA accumulation in the liver and its circulation to the kidneys. Similarly, HGA injected into FAH deficient mice on NTBC also showed damage specifically to renal proximal tubule cells. These observations suggest that build-up of FAA contributes to both the liver and the kidney damage in HT1.

The extensive renal damage seen in these mice also provides a model for testing therapies of renal proximal tubule disease. $Fah^{-/-}$, Hgd^{aku}/Hgd^{wt} mice with corrected livers from LOH events, or HT1 mice on NTBC given exogenous HGA, both represent models for specific damage to renal proximal tubule cells. This separation of liver and kidney damage in HT1 has not been shown before. The strong selection for FAH-positive cells seen in liver repopulation in the HT1 mouse model (Overturf et al., 1996) may also occur in the kidney. We have demonstrated the time course of renal proximal tubule cell

regeneration after HGA injection that may be useful as a first step in determining a FAH positive cell selection strategy in HT1 kidneys.

Future studies

The *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mouse model described in this thesis work could be used as a tool for identifying the specific mechanism of liver and kidney toxicity induced by FAA. *In vivo* suppressor mutations in *Hgd* allow the study of FAA induced DNA alterations in a chronic setting, which more accurately reflects the disease process in HT1. Other models such as the *Hpd/Fah* double-mutant mice given HGA (Endo et al., 1997) can only study acute effects due to the sudden re-opening of the pathway after HGA injection. The *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mice could be used for testing therapies that would protect against FAA induced mutations because of the reproducible appearance of nodules in the livers of these mice after removal of NTBC that could function as a read-out of mutation accumulation. Therapies that protected the FAH mutant hepatocytes from mutations caused by FAA would show a reduced number of nodules compared to untreated controls. Testing antioxidants such as Vitamin E using this model may be useful for HT1 therapy as Vitamin E has been shown to decrease the liver cancer incidence in another mouse model (Factor et al., 2000).

The extreme kidney damage seen in the *Fah*$^{-/-}$, *Hgd*^{aku}/*Hgd*^{wt} mice is another potential use for therapy development. Preferential renal proximal tubule cell regeneration due to *in vivo* selection has not been shown before in an animal model. This could be happening to some degree in this mouse model, and the use of an HGD antibody (already developed by our laboratory) may show areas of renal proximal tubule cells that have no HGD due to LOH events as seen in the liver. Kidney gene therapy using vectors already developed in this laboratory (Overturf et al., 1997; Overturf et al., 1996) could be tested in this mouse model or in FAH mutant mice on NTBC given HGA. For the latter,

the regeneration time-course identified after HGA injection as discussed in chapter 4 will be useful for such experiments.

Further examination of the mechanism of FAA induced LOH would also help in the understanding of FAA toxicity and HCC development in HT1. Using the *Aprt* system discussed in chapter 5, it may be beneficial to test lower concentrations of FAA, or possibly different mechanisms of GSH depletion to see if the mutation frequency can be increased to an even higher level over untreated cells. The subsequent analysis of clones may reveal a difference in FAA treated clones as to the site of recombination as shown in Fig. 15, or perhaps the analysis of more clones will show higher levels of mitotic recombination like that seen after treatment with hydrogen peroxide (Turker et al., 1999).

This thesis work has described a mouse model that can be rescued from liver failure by *in vivo* suppressor mutations as a mechanism of reversion that has not been demonstrated before in a mammal. This mouse model also revealed the spectrum of mutations and other chromosomal alterations caused by FAA that has also not been previously described. We have also suggested the possible involvement of mitotic recombination as a major mechanism of LOH both *in vivo* in the mouse model and *in vitro* in *Aprt* heterozygous cells. This work has significance to the field of genetics and has contributed to the understanding of HT1 pathophysiology and HCC development in this genetic liver disease.

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