A Mouse Model of Renal Tubular Injury of Tyrosinemia Type 1: Development of de Toni Fanconi Syndrome and Apoptosis of Renal Tubular Cells in Fah/Hpd Double Mutant Mice

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Abstract. Hereditary tyrosinemia type 1 (HT1) (McKusick 276700), a severe autosomal recessive disorder of tyrosine metabolism, is caused by mutations in the fumarylacetacetate hydrolase gene Fa/ (EC 3.7.1.2), which encodes the last enzyme in the tyrosine catabolic pathway. HT1 is characterized by severe progressive liver disease and renal tubular dysfunction. Homozygous disruption of the gene encoding Fa/ in mice causes neonatal lethality (e.g., lethal Albino deletion c14CoS mice), an event that limits use of this animal as a model for HT1. A new mouse model was developed with two genetic defects, Fa/ and 4-hydroxyphenylpyruvate dioxygenase (Hpd). The Fa/–/Hpd–/ mice grew normally without evidence of liver and renal disease, and the phenotype is similar to that in Fa/+/Hpd–/ mice. The renal tubular cells of Fa/–/Hpd–/ mice, particularly proximal tubular cells, underwent rapid apoptosis when homogenitase, the intermediate metaboite between HPD and FAH, was administered to the Fa/–/Hpd–/ mice. Simultaneously, renal tubular function was impaired and Fanconi syndrome occurred. Apoptotic death of renal tubular cells, but not renal dysfunction, was prevented by pretreatment of the animals with YVAD, a specific inhibitor of caspases. In the homogenitase-treated Fa/–/Hpd–/ mice, massive amounts of succinylacetone were excreted into the urine, regardless of treatment with inhibitors. It is suggested that apoptotic death of renal tubular cells, as induced by administration of homogenitase to Fa/–/Hpd–/ mice, was caused by an intrinsic process, and that renal apoptosis and tubular dysfunctions in tubular cells occurred through different pathways. These observations shed light on the pathogenesis of renal tubular injury in subjects with FAH deficiency. These Fa/–/Hpd–/ mice can serve as a model in experiments related to renal tubular damage.

Hereditary tyrosinemia type 1 (HT1) (McKusick 276700), a severe autosomal recessive disorder of tyrosine metabolism (1,2), is caused by mutations in the fumarylacetacetate hydrolase gene Fa/ (EC 3.7.1.2) (3–5), encoding the last enzyme in the tyrosine catabolic pathway (1.5). This disorder is characterized by severe progressive liver disease, including liver failure during infancy or in later life, chronic liver damage with a high incidence of hepatoma, and renal tubular dysfunction characterized as de Toni Fanconi syndrome (1,2,6,7). Liver transplantation can save the life of HT1 patients, in some but not all patients, and liver transplantation led to improvement in renal function (7,8). The precise mechanism of renal tubular damage associated with FAH deficiency has required further study.

Microscopical tubular dilation, nephrocalcinosis, involution of epithelial cells, and some degree of glomerulosclerosis were noted in HT1 patients 18 mo to 17 yr of age (2,8). As neonatal Fah-deficient mice died, the kidney from newborn mice with FAH deficiency was investigated, and dilation and vesiculation of the rough endoplasmic reticulum and Golgi apparatus in C14CoS/14CoS newborn mouse kidney were apparent (9). When the Fah-deficient mice were treated with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), their life was spared, and focal degeneration, regeneration of proximal tubular epithelium, and aggregates of cytoplasmic microfilaments were observed (10). Mechanisms for cell injury or cellular dysfunction of the renal tubular cells are not well understood.

The hereditary tyrosinemia type 3 model mice with genetic deficiency of 4-hydroxyphenylpyruvic acid dioxygenase (Hpd; EC 1.13.11.27) on chromosome 5 (Hpd–/– mice) have hypertyrosinemia without liver injury (11–13). Crossing heterozygous c14CoS mice (Fa/ Hpd–/–) with Hpd-deficient mice, we developed a new mutant combination that carried two genetic defects, Fa/–/ and Hpd–/– (14). Although Fah-deficient mice, either c14CoS or target disrupted mice proved to be neonatally lethal, the Fa/–/Hpd–/– mice had a long survival without evidence of hepatic and renal injuries, yet their phenotype is similar to Fa/–/Hpd–/– mice (14).

We found that when homogenitase was administered, the hepatocytes of the Fa/–/Hpd–/– mice undergo rapid apoptosis by retrieval of the tyrosine catabolic pathway (Figure 1) and...
Tyrosine

4-Hydroxyphenylpyruvate

4-Hydroxyphenylpyruvate Dioxygenase (HPD)

Homogentisate

Maleylacetocetate

Fumarylacetocetate

Fumarate + Acetocetate

Fumarylacetocetate Hydrolase (FAH)

Albino lethal

Figure 1. Tyrosine catabolic pathway in a mouse model of hereditary tyrosinemia. The principal metabolites in the pathway are tyrosine, 4-hydroxyphenylpyruvate, homogentisate, maleylacetocetate, and fumarylacetocetate. In hereditary tyrosinemia type III, 4-hydroxyphenylpyruvate dioxygenase (HPD) is deficient, and in hereditary tyrosinemia type I, fumarylacetocetate hydrolase (FAH) is deficient. The Hpd-deficient mice carried a nonsense mutation in the Hpd gene on chromosome 5, and the Fah-deficient mice carried a large deletion on chromosome 7 that disrupted the Fah gene. The mouse model presented here carries deficiency of both Hpd and Fah (Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mouse).

Materials and Methods

Animals

Male Fah<sup>+/+</sup>Hpd<sup>−/−</sup> mice and the male Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice, crossed by heterozygous C<sup>14CoS</sup> mice (Fah<sup>+</sup>/−) and homozygous Hpd<sup>−/−</sup> mice, were used (14). Because the lethal dose of homogentisate is 400 mg/kg body wt for the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mouse (the median lethal dose is 300 mg/kg body wt), the Fah<sup>+/+</sup>Hpd<sup>−/−</sup> mice and Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice were given 100, 200, and 400 mg/kg of neutralized homogentisate intraperitoneally and the kidneys were excised 18 or 48 h after this injection. The control mice were injected with the same volume of physiologic saline. All of the animals were freely fed during the experiments.

To evaluate the protective effects of an apoptosis inhibitor in vivo, the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice were injected intraperitoneally with acetyl-Tyr-Val-Ala-Asp-CHO (YVAD) (200 mg/kg body wt in 500 μl of physiologic saline; n = 4) (Takara, Japan), and 2 h later the mice were given intraperitoneally 200 mg/kg (in 100 μl of physiologic saline) of homogentisate, left for 48 h, and then the kidneys were excised. In contrast, the kidneys of Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice administered the same volume of physiologic saline (n = 2) as controls were excised 50 h after the injection.

Histology

For light microscopic examination, the kidneys fixed in 10% neutralized formalin were dehydrated in 100% ethanol and embedded in paraffin wax at 58°C. The 1.5-μm sections were rehydrated and stained with hematoxylin and eosin and periodic acid-Schiff. Using the one-and-a-half sections, the 3'-OH DNA ends generated by DNA fragmentation were detected using an in situ terminal deoxynucleotidyltransferase-mediated dUTP-digoxigenin nick end labeling assay (TUNEL) (15) kit (ApopTag, Oncor, Gaithersburg, MD), according to instructions from the manufacturer.

For electron microscopic observations, about 1 mm<sup>3</sup> of the kidney were rapidly fixed in 2% glutaraldehyde (Nacalai Tesque, Inc., Kyoto, Japan)-0.1 M cacodylate buffer (pH 7.2) on ice for 3 h. After washing in 0.1 M cacodylate buffer, the sections were post-fixed in 1% osmium (VIII) tetroxide (Nacalai Tesque, Inc.)-0.1 M cacodylate buffer on ice for 2 h, dehydrated in ethanol and propylene oxide, and embedded in epoxy resin. The ultrathin sections were cut on an AO-Reichert Ultracut E microtome (Vienna, Austria), using a glass or diamond knife followed by double staining with 5% uranyl acetate and lead nitrate solution. The sections were examined under a H-300 microscope (Hitachi, Tokyo, Japan). Three mice per intervention and two to four blocks per kidney per mouse were used to observe the pathologic processes.

Biochemical Analysis of Serum and Urine

Blood samples were taken from the heart of the mice at 18 or 48 h after the administration of homogentisate, and the serum were separated and stored at −70°C. Urine collections were made 6 h before administering homogentisate and at 6, 12, 18, 24, 36, and 48 h after this treatment as spot urine samples. Urine was obtained by massaging the lower abdomen of mice directed to the meatus at the indicated time. Serum levels of glucose, phosphate, blood urea nitrogen (BUN), and urinary creatinine, glucose, and phosphate were measured using an automated analyzer. Urinary glucose:creatinine ratio and phosphate:creatinine ratio were calculated. Urinary concentration of succinylacetone (SA) was determined by stable isotope dilution gas chromatography-mass spectrometry assay, as described (16).

Statistical Analyses

For statistical analyses, we used unpaired t tests and one-way factorial ANOVA and multiple comparison tests.

Results

Biochemical Data on Blood and Urine

To evaluate renal tubular functions in the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice after the administration of homogentisate, serum levels of glucose, phosphate, and BUN were measured after treatment at appropriate intervals (Figure 2). Fah<sup>+/+</sup>Hpd<sup>−/−</sup> mice served as the control animals. A significant reduction in serum glucose levels was seen in the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice treated with 200 and 400 mg/kg homogentisate (Figure 2, A and B). In contrast, there was a significant increase in serum BUN after treatment with 200 and 400 mg/kg homogentisate (Figure 2, A and B) and in serum phosphate after treatment with 400 mg/kg homogentisate to the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice (Figure 2B). However,
the levels of serum glucose, BUN, and phosphate in the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice were not affected by 100 mg/kg homogentisate (data not shown). Similarly, the level of serum phosphate in the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice was not affected in case of 200 mg/kg homogentisate (Figure 2A). In the control mice, the same treatment did not significantly change any of these parameters (Figure 2).

In Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice and the control mice, urinary levels of creatinine, glucose, and phosphate were measured before and after treatment with homogentisate. Figure 3, A and B, shows time courses of changes in urinary glucose and phosphate, respectively. The urinary glucose:creatinine ratio was markedly increased in the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice after the administration of 100 to 400 mg/kg homogentisate. In contrast, the urinary glucose:creatinine ratio was essentially unchanged in the control (Figure 3A). Similarly, the urinary phosphate:creatinine ratio was significantly increased in the
treated Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice 18 to 48 h after the administration (Figure 3B). These results suggest that in the Fah<sup>−/−</sup>Hpd<sup>−/−</sup>mice, administration of homogentisate resulted in reduced reabsorption of glucose and phosphate by the renal tubules and that the degree of renal dysfunction in the treated Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice depended on the dose of homogentisate administered.

**Histologic Investigations**

Renal sections from Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice treated with various doses of homogentisate showed pathologic features of bleeding in vast areas, accumulation of mononuclear cells at the interstitium, proximal tubular dilation, and cytoplasmic vacuolation. A representative view of the kidney sections obtained from the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice given 400 mg/kg homogentisate is shown in Figure 4D. At the proximal tubule, the nucleus of the epithelial cell was sometimes abnormal (Figure 4D). The appearance of glomeruli, interstitial cells, distal tubular cells, and blood vessels was normal in all sections. The proximal tubular epithelial cells in the untreated Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice, and in the untreated or homogentisate-treated Fah<sup>+/+</sup>Hpd<sup>−/−</sup> mice appeared unchanged (Figure 4, A through C).

Representative electron microscopic views of proximal tubular cells from homogentisate-treated Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice are shown in Figure 5, C through F. In the cytoplasm, droplets of fat (Figure 5D) and large lysosomes were present and many mitochondria were swollen (Figure 5, C and D). The breakage and vacuolization were present at the brush border. In some cells, compaction and degradation of chromatin were present in association with convolution of the nuclear profile (Figure 5C). Thus, severe cellular damage of proximal tubular cells in the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice occurred after treatment with 400 mg/kg homogentisate. Similar changes, albeit to a lesser extent, including the characteristic nucleus of apoptosis, were seen in some epithelial cells of the proximal tubules from Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice after injection of 200 mg/kg homogentisate (Figure 5E). However, in the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice treated with 100 mg/kg homogentisate, there were no extensive ultrastructural abnormalities of renal proximal tubular epithelial cells (Figure 5F). These features were similar to findings in control Fah<sup>+/+</sup>Hpd<sup>−/−</sup> mice treated with various doses of homogentisate or untreated Fah<sup>+/+</sup>Hpd<sup>−/−</sup> mice (Figure 5). The most distal tubular and glomerular cells were normal in treated Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice (data not shown). Some pathologic changes, including swollen mitochondria, vacuolization,
Figure 5. Electron microscopic views of renal proximal tubular cells. Sections from an untreated $Fah^{+/+}Hpd^{+/+}$ mouse (A), an untreated $Fah^{-/-}Hpd^{-/-}$ mouse (B), and treated $Fah^{-/-}Hpd^{-/-}$ mice with 100 mg/kg (F), 200 mg/kg (E), and 400 mg/kg (C and D) of homogentisate are shown, respectively. Note the compaction, segregation, and homogenization of chromatin at the nuclear periphery (arrowhead; C, D, and E) and the broken nuclear envelope (C). At the cytoplasm, numerous mitochondria are swollen, cristae are curved and broken (C and D), and fatty droplets are numerous (D). Magnification: $\times3520$ in A; $\times3200$ in B; $\times3500$ in C; $\times3000$ in D; $\times3600$ in E; and $\times4380$ in F.
brush border loss, and large lysosomes, seem to be secondary damage in apoptotic cells. It may be that the apoptotic cells were inaccessible to phagocytes following extrusion into the lumen of the tubule or the extent of apoptotic cell death exceeded the phagocytic capacity.

**In Situ Detection of Apoptosis in Kidney Sections**

Apoptosis was identified using the modified TUNEL assay. In the renal sections obtained from Fah−/− Hpd−/− mice after treatment with various doses of homogentisate, many nuclei showed evidence of apoptosis (Figure 6). Most of the apoptotic nuclei were located in the proximal tubules (Figure 6, C, D, and F). Counting of signal-positive cells in the kidney sections from the homogentisate-treated Fah−/− Hpd−/− mice revealed that administration of the 400 mg/kg homogentisate resulted in apoptosis of approximately 80% of proximal tubular cells, as shown in Table 1. When low doses of homogentisate were administered, the ratio of positive cells decreased (Table 1). The appearance of apoptotic cells in the proximal renal tubules from the homogentisate-treated Fah−/− Hpd−/− mice depended on the dose of homogentisate administered. Few positive signals were present in distal tubular nuclei, however, essentially no positive nucleus was found in glomeruli, interstitial cells, or collecting duct cells (Figure 6). Findings in the untreated Fah−/− Hpd−/− mice were similar to those seen in Fah−/− Hpd−/− mice (Figure 6, A, B, and E).

**Effects of a Caspase Inhibitor on Renal Tubular Cells**

To evaluate in vivo effects of a caspase inhibitor on the kidney, the Fah−/− Hpd−/− mice were preinjected intraperitoneally with YVAD, then 2 h later, 200 mg/kg homogentisate was administered. All mice survived for 48 h after the injection of homogentisate, then were killed and the kidney was investigated. Renal sections from the Fah−/− Hpd−/− mice pretreated with YVAD showed few apoptotic nuclei, as determined using TUNEL assay (Figure 6G, Table 1). In addition, histology of renal epithelial cells remained unchanged, as seen in the hematoxylin and eosin and periodic acid-Schiff staining when YVAD was administered before the administration of homogentisate (data not shown). These results suggest that pretreatment with the caspase inhibitor can effectively prevent cellular damage induced by homogentisate in the Fah−/− Hpd−/− mice.

We next investigated the effects of a caspase inhibitor on renal tubular functions in the Fah−/− Hpd−/− mice. We preadministered YVAD, a potent, selective, cell-permeating inhibitor of caspase-1 (17–19) to the Fah−/− Hpd−/− mice, then the mice were treated with homogentisate. However, preadministration of YVAD did not alter the urinary levels of glucose and phosphate (Figure 7). This evidence suggested that the impaired function of renal tubules was not overcome by pretreatment of the homogentisate-treated Fah−/− Hpd−/− mice with the caspase inhibitor. It is notable that the serum BUN level remained high in the Fah−/− Hpd−/− mice pretreated by YVAD and treated by homogentisate (130.0 ± 8.49 mg/dl, n = 4). Thus, elevation of serum BUN seen in homogentisate-treated Fah−/− Hpd−/− mice is not directly related to the massive apoptosis of tubular epithelial cells. SA is derived from fumarylacetoacetate (FAA) and is excreted into the urine of HT1 patients and in the homogentisate-treated Fah−/− Hpd−/− mice (14). We confirmed that SA levels were markedly increased in Fah−/− Hpd−/− mice after the injection of homogentisate (204700 ± 3360.67 nmol/mmol creatinine, n = 4), whereas the untreated Fah−/− Hpd−/− mice showed low values (150 ± 14.75 nmol/mmol creatinine, n = 2). Therefore, the caspase inhibitor YVAD did not alter the urinary excretion of SA in the homogentisate-treated Fah−/− Hpd−/− mice.

**Discussion**

In the present study, we investigated kidneys of a recently developed mouse model of human HT1 with both FAH and HPD deficiency. As expected from previous studies in the liver (14), kidneys from the Fah−/− Hpd−/− mice are phenotypically normal in terms of morphology and function. This indicated that the introduction of a null mutation of Hpd prevented any pathology of the kidney associated with Fah deficiency. Our previous study revealed that hepatocytes which lack Fah and Hpd undergo rapid apoptosis when the tyrosine catabolic pathway was reopened at the step of HPD (14), and FAA is at least one of the chemicals that reacts with mitochondria and releases cytochrome c from the mitochondria (20). Recent advances in the understanding of mechanisms related to apoptosis indicated that activation of caspases is one of the main processes leading to cell death (17–19). In the present study, apoptotic death of renal tubular epithelial cells was almost completely prevented by preadministration of the caspase inhibitor YVAD (Figure 6G, Table 1). This means that apoptosis of the epithelial cells resulted from activation of caspases in the cells.

Previous mouse models for HT1, either albino-lethal c14COS or targeted disrupted FAH deficiency, were neonatally lethal (21–27), or required treatment with NTBC (10), an effective inhibitor for HPD activity. Although the neonatal c14COS mice showed no apparent kidney injury, abnormalities were characterized by changes in membranous structures (9,10) and were limited to proximal epithelial cells of tubules (9,23), an area where FAH is physiologically expressed (23,25). In the NTBC-treated Fah-deficient mice, renal proximal tubular cells of the kidney showed abnormalities in nuclei and lysosomes (10). In addition, the caspase inhibitor YVAD effectively prevented the appearance of these changes in cellular structures of the Fah-deficient mice (Figure 6G). These results suggest that abnormalities in the structure of the cells noted in the earlier studies are signs of ensuing apoptotic death. Accordingly, the death signal was specific in triggering cell death processes.

Previously, we analyzed hepatocellular apoptosis induced by homogentisate in Fah−/− Hpd−/− mice, and we suggested that cell death of hepatocytes that carried FAH deficiency was due to intracellular events (14,20). For renal tubular cells, however, there are at least two possibilities for the origin of cell injury: The cell death is due entirely to an intracellular process, or is initiated by chemicals derived from the extracellular metabo-
Figure 6. Homogentisate-induced apoptosis of kidney cells in the Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice and effects of a caspase inhibitor. The renal sections were obtained from variously treated mice and stained for DNA fragmentation, as described in Materials and Methods. The controls are untreated Fah<sup>++/+</sup>Hpd<sup>−/−</sup> mice (A), untreated Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice (B), and Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice treated with 400 mg/kg homogentisate (E). In the experimental animals, Fah<sup>−/−</sup>Hpd<sup>−/−</sup> mice were treated with 100 mg/kg homogentisate (C), 200 mg/kg homogentisate (D), or 400 mg/kg homogentisate (F). A section from acetyl-Tyr-Val-Ala-Asp-CHO (YVAD)-pretreated Fah<sup>−/−</sup>Hpd<sup>−/−</sup> and homogentisate-treated (200 mg/kg body wt) mice is shown in G. The arrowheads indicate apoptotic cells. Magnification: ×318 in A through G.
syndrome seen in the homogentisate-treated cellular changes in morphology. It seems likely that Fanconi reversible inhibitor for cellular metabolism, but does not cause no specific changes in morphology, even with high concentra-
tion of SA on renal tubules in rats (29 –32). SA inhibited uptake of glucose and phosphate was impaired. Interestingly, treatment of the Fah⁄⁄ Hpd⁄⁄ mice with the caspase inhibitor YVAD did not prevent the development of Fanconi syndrome (Figure 3), which means that the cell death pathway and the metabolic process to Fanconi syndrome can be separated in the nephropathy associated with FAH deficiency. If the pathway for cell death and the biochemical process leading to Fanconi syndrome are completely separable, one possible explanation is that Fanconi syndrome is caused by chemicals derived from glomerular filtrates. In this context, SA was present in the urine of HT1 patients (6 –8,28) and in the urine of Fah-deficient mice (10,14,20). However, SA did not cause cellular damage (29,30). Other chemicals such as FAA or MAA were not detected in urine from FAH-deficient patients (6) or mice (25). In addition, apoptotic death is confined to proximal renal tubular cells where Fah is likely to be expressed. We speculate that intracellular metabolite(s) such as FAA may explain the cell death in proximal tubules.

Kidney pathology associated with HT1 includes glomerulosclerosis and interstitial fibrosis, and there frequently is a loss of the brush border and cytoplasmic vacuolation in ultrastruc-
tures of the tubular cells (2,8). Such pathology in the renal tubular cells is likely to be related to occurrence of cell death signal in the cells. Because apoptotic cells are rapidly removed from the tubules, it may be difficult to detect such cells in kidney sections from patients. Long-term investigations of Fah⁄⁄ Hpd⁄⁄ mice given low doses of homogentisate may elucidate the precise process of renal damage seen in the HT1 patients.

Fanconi syndrome in HT1 patients (2,6) was reproduced in treated Fah⁄⁄ Hpd⁄⁄ mice (Figure 3), as reabsorption of glucose and phosphate was impaired. Interestingly, treatment of the Fah⁄⁄ Hpd⁄⁄ mice with the caspase inhibitor YVAD did not prevent the development of Fanconi syndrome (Figure 7), which means that the cell death pathway and the metabolic process to Fanconi syndrome can be separated in the nephropathy associated with FAH deficiency. If the pathway for cell death and the biochemical process leading to Fanconi syndrome are completely separable, one possible explanation is that Fanconi syndrome is caused by chemicals derived from filtrates of glomerulus in this mouse model, and in HT1 pa-
tients. Roth and colleagues extensively investigated the effects of SA on renal tubules in rats (29 –32). SA inhibited uptake of methyl α-D-glucose, but electron microscopic analysis revealed no specific changes in morphology, even with high concentra-
tions of SA (29,30). Thus, it is likely that SA can act as a reversible inhibitor for cellular metabolism, but does not cause cellular changes in morphology. It seems likely that Fanconi syndrome seen in the homogentisate-treated Fah⁄⁄ Hpd⁄⁄ mice is, at least partly, relevant to SA.

The possibility that abnormalities in intracellular metabol-
ism, for example chemicals derived from glomerular filtrates.

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*The percentages of signal-positive cells after TUNEL staining were calculated based on counting in 20 random fields of one slice from each kidney. Ranges are given in parentheses. The caspase inhibitor YVAD was injected 2 h before the administration of homogentisate.*

Table 1. Percentages of apoptotic cells in proximal renal tubules of Fah⁄⁄ Hpd⁄⁄ mice after the administration of homogentisate

There are observations that apoptosis of kidney cells under-
lies the pathogenesis of kidney diseases, conditions that in-
clude polycystic kidney disease (34), glomerulonephritis (35,36), exposure to toxic agents (37–39), and some cases of kidney transplantation (40). However, clear relationships between Fanconi syndrome and apoptosis have not been demonstrated (41). We found that renal tubular damage in HT1 involves cell death due to apoptosis. As discussed above, apoptosis associated with Fah deficiency is caused by interaction between FAA and mitochondria. Changes in morphology of proximal cells after injection of homogentisate are character-
ized by abnormalities in mitochondria, disruption of the nuclear membrane, and fragmentation of nuclei. These changes are obviously relevant to apoptosis and are completely pre-
vented by administration of a caspase inhibitor before the administration of homogentisate. We suggest that the apoptosis of renal tubular epithelial cells observed in our study is likely due to the accumulation of FAA in epithelial cells.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, the Ministry of Health and Welfare (Japan), and a Grant-in-Aid from
Figure 7. Effects of the caspase inhibitor YVAD on the urinary excretion of glucose and phosphate. Urinary levels of glucose and phosphate were monitored after the administration of homogentisate (200 mg/kg body wt) in Fah\(^{−/−}\)Hpd\(^{−/−}\) mice after pretreatment with YVAD.

The Fah\(^{−/−}\)Hpd\(^{−/−}\) mice were given 200 mg/kg YVAD intraperitoneally (n = 4) (■), then 2 h later the mice were given 200 mg/kg homogentisate intraperitoneally. The urinary excretion of glucose (A) and phosphate (B) in YVAD-pretreated Fah\(^{−/−}\)Hpd\(^{−/−}\) mice was similar to findings in the Fah\(^{−/−}\)Hpd\(^{−/−}\) mice treated with 200 mg/kg homogentisate without YVAD (▲, n = 5). Data from the YVAD-treated or untreated Fah\(^{−/−}\)Hpd\(^{−/−}\) mice were compared with data from the Fah\(^{+/−}\)Hpd\(^{−/−}\) mice (△, n = 6). *P < 0.05; **P < 0.01; ***P < 0.001. The arrow indicates the point of injection of homogentisate.

IBM Japan. We are grateful to T. Kubo for technical assistance with the histology and to M. Ohara for assistance with the translation.

References