Compensatory Regeneration as a Mechanism for Renal Tubule Carcinogenesis of Fumonisins B₁ in the F344/N/Nctr BR Rat

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Fumonisins B₁ (FB₁) and B₂ (FB₂) are fungal metabolites of Fusarium verticillioides (= F. moniliforme), a fungus that grows on many crops worldwide. Previous studies demonstrated that male BD IX rats consuming diets containing 50 ppm fumonisin B₁ developed hepatocellular carcinomas. In our recent studies, diets containing FB₁ at 50 ppm or higher concentrations induced renal tubule carcinomas in male F344/N/Nctr BR rats and hepatocellular carcinomas in female B6C3F₁/Nctr BR mice. The carcinogenicity of FB₁ in rats and mice is not due to DNA damage, as several laboratories have demonstrated that FB₁ is not a genotoxic. FB₁ induces apoptosis in cells in vitro. Including FB₁ in the diets of rats results in increased hepatocellular and renal tubule epithelial cell apoptosis. In studies with F344/N/Nctr BR rats consuming diets containing up to 484 ppm FB₁ for 28 days, female rats demonstrated more sensitivity than male rats in the induction of hepatocellular apoptosis and mitosis. Conversely, induction of renal tubule apoptosis and regeneration were more pronounced in male than in female rats. Induction of renal tubule apoptosis and hyperplasia correlated with the incidence of renal tubule carcinomas that developed in the 2-year feeding study with FB₁ in the F344/N/Nctr BR rats. The data are consistent with the hypothesis that the induction of renal tubule carcinomas in male rats could be partly due to the continuous compensatory regeneration of renal tubule epithelial cells in response to the induction of apoptosis by fumonisins B₁.

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not result in induction of any tumors in male BD IX rats (10). These results indicate that FB1 is a complete carcinogen when included in the diet of male BD IX rats. Additionally, the dietary level of FB1 required to induce hepatic tumors in male BD IX rats is between 25 and 50 ppm.

Including FB1 in the diets of male and female F344/N/Nctr BR rats for 2 years resulted in formation of renal tubule adenomas and carcinomas in male rats (11,12). The doses of FB1 ranged from 5 to 150 ppm in male F344 rats and from 5 to 100 ppm in female F344 rats. The renal tubule adenomas and carcinomas were present in male rats consuming 50 and 150 ppm FB1; there were no dose-related tumors in the female rats. As part of this same study, male and female mice were given diets containing FB1 for 2 years (11,12). The incidence of hepatocellular adenomas and carcinomas was increased in female mice fed 50 and 80 ppm FB1, whereas there were no increases in any tumors in male mice fed diets containing as high as 150 ppm FB1. Therefore, results of studies with F344/N/Nctr BR rats, BD IX rats, and B6C3F1/Nctr BR mice suggest that FB1 is a carcinogenic compound when included in the diet at 50 ppm or higher.

The effects of FB1 in cultures of human cells in vitro were studied in an attempt to understand the mechanism of action of FB1 in cell homeostasis (17). FB1 induced apoptosis in primary human keratinocytes in a dose-dependent manner with a dose as small as 1 µM (17,18). The apoptosis was characterized by morphologic characteristics, the presence of fragmented DNA, histocompatability staining techniques to demonstrate disorganization of the nucleus, and electron microscopic examination of cellular structure (17,18). FB1 also induced apoptosis in normal human fibroblasts, HepG2 human hepatoblastoma cells, and immortalized human esophageal epithelial cells (17). Induction of apoptosis or inhibition of the growth of various cell lines in vitro has also been described for other cultured mammalian cells (19–25).

Administration of FB1 to rats or mice resulted in induction of hepatocellular and renal tubule epithelial cell apoptosis. This was first described as single-cell necrosis in livers of rats and mice treated with FB1 (24,25) and later confirmed as apoptosis (26). FB1-induced hepatocellular and renal tubule apoptosis is reviewed elsewhere in this issue (27).

Because FB1 is a nongenotoxic carcinogen and apoptosis has been suggested as the principal cellular consequence of exposure to FB1, we sought to determine whether the incidence of apoptosis and cell proliferation in F344/N/Nctr BR rats treated for 28 days with diets containing FB1 correlated with the induction of tumors in the 2-year FB1 feeding study.

Materials and Methods

Study Material and Feed

Two different preparations of FB1 were used in the studies summarized in this article. FB1 was produced by aqueous cultures of F. proliferatum on corn (P.E. Nelson, Pennsylvania State University, State College, PA, USA). FB1 was extracted from the lyophilized culture material using methanol. The FB1 used in the 28-day feeding study was purified as free acid to a purity of 92.5% using high-performance liquid chromatography (HPLC) (26). The FB1 used in the 2-year feeding study was purified as ammonium salt to a purity of >96% using HPLC. The purity of the FB1 was established using spectroscopic and HPLC techniques (28).

Autoclaved powdered NIH-31 rodent feed (Purina Corp., St. Louis, MO) was the test diet in the study, and FB1 was added as a water-based component using a Patterson-Kelley V-blender (Patterson-Kelley Co., East Stroudsburg, PA, USA). The FB1 control diet was below 0.06 ppm feed.

Animals and Housing

Female and male F344/N/Nctr BR rats were obtained from the breeding colony at the National Center for Toxicological Research at 4 weeks postpartum. Rats were allocated to study dose groups in a random manner to control for weight bias and to reduce sibling allocation to any dose group. Powdered feed was available ad libitum in feeders custom designed for powdered feed, and water was available ad libitum. Cages and water were changed twice weekly.

Study Design

Rats (10/dose/sex) were fed diets containing FB1 for 28 consecutive days. The doses used in this study were 0, 99, 163, 234, and 484 ppm FB1 (26,28). Rats were fasted overnight and euthanized by asphyxiation with carbon dioxide and bled via the orbital sinus before necropsy. Organ weights were determined; tissues were fixed in 10% neutral buffered formalin and processed as described (28). All tissues were examined in rats that received the control diets and the highest FB1 dose (484 ppm); livers and kidneys were examined for all rats in the study.

The design of the 2-year study has been described previously (28). Rats and mice were randomly allocated to dose groups, the study was conducted, and the tissues were analyzed for tumors in accordance with the guidelines of the U.S. National Toxicology Program (29) and U.S. Food and Drug Administration (30).

Determination of Cell Cycle Using Anti-PCNA Immunohistochemical Methods

The proportion of cells in S phase was determined immunohistochemically with minor modification of established methods (31,32). Paraffin-embedded kidney sections that had been fixed for 48 hr in 10% neutral buffered formalin and subjected to heated-citrate antigen retrieval were used. Mouse monoclonal...
Statistical Analysis

Comparison of dosed rats with altered parameters to rats on the control diets was accomplished using either analysis of variance or Fisher’s exact test (SigmaStat, Jandel Scientific, San Rafael, CA, USA). Levels of significance were tested at the 95% confidence level (p < 0.05).

Results

Male and female F344 rats were fed diets containing FB1 for 28 days and their body weights were reduced in all the rats that consumed diets containing FB1. Relative kidney weights of rats fed FB1 were reduced by 19% from the value for rats consuming control diets. Relative kidney weights in female rats fed 484 ppm FB1 were reduced by 11% compared to those of controls (Figure 5).

The most prevalent morphologic changes in the kidneys of the rats were increases in renal tubular epithelial cell apoptosis in the cortico-medullary area. Apoptotic renal tubule epithelial cells were noted by cellular shrinkage from adjacent cells, eosinophilic cytoplasm, and chromatin condensation and margination in the nucleus. Although apoptotic bodies were not detected, clear morphologic markers of apoptosis were present. The morphologic diagnosis of apoptosis was confirmed using the TUNEL assay for detecting fragmented DNA (not shown).

Histopathologic examination was conducted on livers of the rats fed FB1 for 28 days. The most predominant lesions noted were increases in hepatocellular apoptosis and mitosis. Apoptosis was morphologically distinguishable as hepatocytes with decreased cell volume and withdrawal from neighboring hepatocytes. The apoptotic cells were eosinophilic with condensed and marginated nuclei. There was no apparent necrosis in the livers; however, there was disorganization of the sinusoidal structure as a result of the apoptosis. An in situ hybridization method for detection of DNA fragments was used to confirm the identification of some of the apoptotic cells [TUNEL (terminal deoxyribonucleaseI transferase-mediated dUTP nick end labeling) assay (28); data not presented]. The incidence of morphologically distinguishable hepatocellular apoptosis is summarized in Figure 2. The number of female rats with hepatocellular apoptosis increased from 0 at 0 ppm, to 2 of 10 rats at 99 ppm, 9 of 10 rats (90%) at 163 ppm, and all 10 of female rats at 234 and 484 ppm FB1. Male rats evidently were less sensitive to the effects of FB1, because hepatocellular apoptosis was not present in the males fed 0, 99, and 163 ppm FB1 (Figure 2). Hepatocellular apoptosis was present in all male rats fed diets 234 and 484 ppm FB1 (Figure 2). The severity of the hepatocellular apoptosis was graded minimal (1), mild (2), moderate (3), or marked (4). In the female rat livers, the median severity of the apoptosis was 1.2, 1.9, and 2.2 for rats receiving 163, 234, and 484 ppm FB1, respectively. The median severity of apoptosis in the male rat livers was 1.1 and 2.0 for the rats in the 234- and 484-ppm dose groups, respectively.

Induction of hepatocellular apoptosis in the rats was accompanied by increases in morphologically distinguishable hepatocellular mitosis; results are shown in Figure 3 as the percent of rats with increased mitoses. Hepatocellular mitoses were increased in the female rats at 163, 234, and 484 ppm FB1 and were increased in the male rats only at 484 ppm FB1. Induction of both hepatocellular proliferation (Figure 3) and hepatocellular apoptosis (Figure 2) occurred at lower doses in the female rats than in the male rats. Hepatocyte proliferation was also determined using an immunohistochemical method for the detection of PCNA. The percent of hepatocytes in S phase in male rats on the control diet was 0.007%, and increased to 0.18, 0.29, and 1.32% at 163, 234, and 484 ppm FB1, respectively (Figure 4). The percent of hepatocytes in S phase of the cell cycle in female rats was 0.2% in the control group and did not increase until 234 and 484 ppm FB1, whereas the percentage of cells in S phase increased to 0.86 and 0.74%, respectively (Figure 4).

The effect of dietary FB1 on the weights of the kidneys is presented in Figure 5. The data are expressed as weight of the kidneys relative to total body weight. Relative kidney weights were reduced in all the rats that consumed diets containing FB1. Relative weights of kidneys in male rats fed 484 ppm FB1 were reduced by 19% from the value for male rats on control diets, whereas relative kidney weights in female rats fed 484 ppm FB1 were reduced by 11% compared to those of controls (Figure 5).

The most prevalent morphologic changes in the kidneys of the rats were increases in renal tubular epithelial cell apoptosis in the cortico-medullary area. Apoptotic renal tubule epithelial cells were noted by cellular shrinkage from adjacent cells, eosinophilic cytoplasm, and chromatin condensation and margination in the nucleus. Although apoptotic bodies were not detected, clear morphologic markers of apoptosis were present. The morphologic diagnosis of apoptosis was confirmed using the TUNEL assay for detecting fragmented DNA (not shown). Apoptotic renal tubule epithelial cells were present in the kidneys of all male rats treated with 99, 163, 234, and 484 ppm FB1 (Figure 6). The median severity of apoptosis was mild (2.0) in all kidneys of the male rats. Apoptotic renal tubule cells were not detected in kidneys of female rats.
female rats fed 99 ppm FB1 but were present in all kidneys of female rats that received 163, 234, and 484 ppm FB1 (Figure 6). The median severity of the apoptosis in female rats increased from 1.0 at 163 and 234 ppm to 2.4 at 484 ppm. These data demonstrate that renal tubule apoptosis was induced in both sexes; however, male rats were more responsive than female rats to the induction of renal tubule apoptosis by dietary FB1.

Renal tubule cell proliferation at the cortico-medullary junction was determined using immunohistochemical detection of nuclear PCNA (Figure 7). The number of renal tubule epithelial cells in S phase was elevated at all doses of FB1 in male rats and increased in a dose-dependent manner (Figure 7). In female rat kidneys, the percentage of cells in S phase also increased in a dose-dependent manner at all doses of FB1; however, induction of proliferation at 99 and 163 ppm FB1 was less in female than in male rat kidneys (Figure 7).

The incidence of tumors in male and female F344 rats treated for up to 2 years with FB1 has been reported (28,33). No liver tumors were detected in the male and female F344 rats in the 2-year study; however, renal tubule adenomas and carcinomas were induced in male rats fed diets containing 50 and 150 ppm FB1. Relative weights of the rats and kidneys in that study are shown in Figures 8 and 9. Relative liver weights of the male rats fed FB1 for 2 years were decreased at all doses of FB1 (5, 15, 50, and 150 ppm) compared to relative liver weights in rats receiving control diets (Figure 8). In contrast, relative liver weights in female rats were not affected by inclusion of FB1 in the diets (Figure 8). Relative kidney weights in the male rats were decreased in the groups that received 15, 50, and 150 ppm FB1 for 2 years (Figure 9). This decrease in relative kidney weight plateaued at approximately 21% in male and female rats consuming diets containing 50–150 ppm FB1 (Figure 9).

**Discussion**

Inclusion of FB1 in the diets of F344/N/Nctr BR rats for 2 years resulted in the formation of renal tubule adenomas and carcinomas in male but not in female rats (28,33). We examined kidneys and livers of rats fed diets containing up to 484 ppm FB1 for 28 days to determine whether the presence of apoptosis and proliferation correlated with the incidence of renal tubule epithelial cells by FB1 in the 2-year feeding study.

Consumption of 234 and 484 ppm FB1 for 28 days decreased the total body weight in female rats, and to a lesser extent, in male rats but only at 484 ppm. This toxicity was not reflected in the relative liver weights in male and female rats; a decrease in relative liver weight was detected only in male rats fed 484 ppm FB1 (Figure 1). The presence of hepatocellular apoptosis in female rats was increased by 163, 234, and 484 ppm FB1, whereas higher doses of FB1 were required for induction of hepatocellular apoptosis in male rats (Figure 2). This trend of hepatocytes in the female rat being more sensitive than those in the male rat to the toxicity of FB1 additionally was reflected in the percentage of rats that had morphologically distinguishable hepatocellular mitoses (Figure 3) and hepatocytes in the S phase of the cell cycle (Figure 4).

In the 2-year tumor study where FB1 was fed to male and female F344/N/Nctr BR rats (28,33), relative liver weights in the male rats were reduced in a dose-dependent manner at all FB1 doses, whereas relative liver weights in the female rats were not affected by dietary FB1 (Figure 8). These doses did not induce formation of hepatic tumors in F344/N/Nctr BR rats (28,33). Consequently, a clear role for hepatocellular apoptosis and proliferation in rat liver tumor development cannot be determined from these studies. In contrast, hepatocellular carcinomas have been reported in BD IX rats fed 50 ppm FB1 for up to 26 months (9), and similar doses of FB1 have been shown to induce foci of altered expression of γ-glutamyl transpeptidase and placental glutathione S-transferase activity (11,14,34). Reasons for differences in responsiveness of F344/N/Nctr BR and other rats to the hepatotoxic and hepatocarcinogenic effects of FB1 remain to be determined.

In contrast to liver, toxicity of dietary FB1 in kidneys is more pronounced in male than in female F344/N/Nctr BR rats. Reduction in relative kidney weights was approximately 19% in male rats consuming 484 ppm FB1 compared to relative kidney weights in control rats (Figure 5). In female rats, this reduction in relative kidney weight was only 11% (Figure 5). Reduction in relative kidney weights paralleled the induction of renal tubule epithelial cell apoptosis in rats (Figure 6); the incidence of apoptosis was greater in male rats than in female rats at 99 ppm FB1. Similarly, induction of renal tubule proliferation, most probably a consequence of apoptosis, was increased in male rats at 99 and 163 ppm FB1 compared to that in female rats at the same doses (Figure 7). Induction of renal tubule epithelial cell proliferation was evident in male and female rat kidneys at 234 and 484 ppm FB1 (Figure 7).

Inclusion of FB1 in the diet of rats for 2 years resulted in formation of renal tubule epithelial cell adenomas and carcinomas in 19 and 31% of male rats fed 50 and 150 ppm, respectively (28,33). The FB1-dependent increase in renal tumors was accompanied by an increased incidence of renal tubule hyperplasia in 29 and 17% of male rats fed 50 and 150 ppm FB1, respectively (28). Female rats did not develop renal tubule hyperplasia, adenomas, or carcinomas in response to inclusion of up to 100 ppm FB1 in the diet (28,33). Therefore, it appears that a consistent sex difference exists in the response of rats in the 28-day and 2-year feeding studies, in which male rats are more sensitive than female rats to the toxicity (apoptosis) of FB1.
proteins involved in degradation in the lysosomes (elimination of the embryolethality in homozygous strains of rats. FB1 is an inhibitor of Induction of apoptosis in rat kidneys has plasia in response to FB1-induced apoptosis. pensatory renal tubule epithelial cell hyperplasia in male rat kidneys through induction of com-
difference in response to FB1 is not absolute. male and female rats, suggesting that the sex were decreased in the 28-day (Figure 5) and in these bioactive compounds have been implicated as the mechanism for the induc-
tion of apoptosis in cells in vitro (23,37,40).
It is known that FB1 induces apoptosis in rat kidney renal tubule epithelial cells; therefore, we propose that FB1 induces tumors in male rats through induction of compensatory renal tubule epithelial cell hyperplasia in response to FB1-induced apoptosis.

Increased cell proliferation in the induc-
tion of kidney cancer may play a role in the development of spontaneous and chemical-induced renal tumors in the Eker rat. Formation of renal cell carcinomas in the Eker rat was first described as an autosomal domi-

nent trait (41) and involves a mutation in the Tsc2 gene (42,43). The presence of homoy-
gous mutant Tsc2 genes is embryothal (44); however, animals bearing heterozygous Tsc2 genes develop spontaneous renal cell carcinomas by 1 year of age (44). The Tsc2 gene codes for tuberin (45), which regulates the GTPase activity for Rap-1 and rap-5, two proteins involved in ras signal transduction (46,47). Inhibition of tuberin expression in cultured cells resulted in a decreased G1 transit time, and an induced proliferation of G0-

arrested cells (48). Transgenic insertion of wild-type Tsc2 into Eker rats resulted in the elimination of the embryol lethality in homozygous animals and reduced the ability of N-ethyl-N-nitrosourea to induce renal tumors in heterozygotics (49). Therefore, loss of control at critical cell cycle checkpoints through loss of tuberin expression, and the sustained hyperplasia results in eventual renal tubule tumor formation in the Eker rat.

Sustained cellular hyperplasia has been proposed as a model for the induction of renal cell tumors in rats by chlorofom and by compounds that induce α2µ-globulin accumu-
lation in renal tubule cell lysosomes of male rats. In the latter case, chemicals that bind to α2µ-globulin (e.g., d-limonene) apparently interfere with α2µ-globulin protein degradation in the lysosomes (50–52). The modified α2µ-globulin accumulates in the lysosomes, resulting in cell death (hyaline droplet nephropathy). This loss of tubule cells is followed by compensatory cell prolif-
eration in the tubules (53) and eventual for-
mation of renal tumors. In support of the role of this mechanism of tumor formation, NCI Black Reiter rats do not produce α2µ-globulin (54). Compounds that induce renal tumors in conventional rats through α2µ-globulin accumulation and hyaline droplet nephropathy do not induce nephropathy or tumors in the NCI Black Reiter rats (54). The role of α2µ-globulin nephropathy and sustained pro-
flleration in the induction of renal cell tumors was substantiated in the mouse where trans-
genic expression of α2µ-globulin in mice resulted in development of hyaline droplet nephropathy (55).

Continuous cell proliferation has also been associated with increased risk for tumor development in other tissues. Several comp-
ounds have been shown to induce urinary bladder regenerative hyperplasia and eventu-
cally cancer in rodents (56). The apparent mechanism of action is the formation of insoluble calculi, and the physical trauma of the interaction of the calculi with the urinary bladder epithelium results in cell death, sus-
tained hyperplasia, and eventual tumor for-
mation (56,57). This is the mechanism in the induction of urinary bladder tumors in male rats by high doses of sodium saccharin due to the high concentrations of protein and alka-
linity of the urine (56,58,59).

In conclusion, we believe there is suffi-
cient evidence to support the hypothesis that sustained renal tubule regeneration in response to FB1-induced renal tubule epithe-
lial cell apoptosis participates in the develop-
ment of renal tubule tumors in male F344/N/Nctr BR rats fed FB1. However, the increased hyperplasia may not be solely responsible for the induction of the tumors. The decrease in relative kidney weights (Figure 5) and the induction of renal tubule proliferation (Figure 7) in male and female F344/N/Nctr BR rats and the induction of renal tubule apoptosis and hyperplasia in male but not female rats fed FB1 for 2 years support the hypothesis. It is anticipated that ongoing studies concerning the role played by signal transduction pathways in the induction of apoptosis will shed additional light on the molecular mechanism of FB1-induced renal tubule apoptosis and tumor formation in male rat kidneys.

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