Fumonisin-Induced Hepatocarcinogenesis: Mechanisms Related to Cancer Initiation and Promotion

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We review the hepatocarcinogenic effects of fungal cultures of Fusarium verticillioides (= Fusarium moniliforme) strain MRC 826 in male BD IX rats. Subsequent chemical analyses of the fumonisin B (FB) mycotoxin content in the culture material used and long-term carcinogenesis studies with purified FBs provided information about dose-response effects, relevance of hepatotoxicity during FB-induced carcinogenesis, and the existence of a no-effect threshold. Fumonisin intake levels of between 0.08 and 0.16 mg FB/100 g body weight (bw)/day over approximately 2 years produce liver cancer in male BD IX rats. Exposure levels < 0.08 mg FB/100 g bw/day fail to induce cancer, although mild toxic and preneoplastic lesions are induced. The nutritional status of the diets used in the long-term experiments was marginally deficient in lipotropes and vitamins and could have played an important modulating role in fumonisin-induced hepatocarcinogenesis. Short-term studies in a cancer initiation/promotion model in rat liver provided important information about the possible mechanisms involved during the initial stages of cancer development by this apparent nongenotoxic mycotoxin. These studies supported the findings of long-term investigations indicating that both cytotoxic/proliferative responses are required for cancer induction and that a no-effect threshold exists for cancer induction. The mechanisms proposed for cancer induction are highlighted and include the possible role of oxidative damage during initiation and the disruption of lipid metabolism, integrity of cellular membranes, and altered growth-regulatory responses as important events during promotion. Key words: fatty acids, fumonisins, Fusarium verticillioides, hepatocarcinogenesis, hypothesis, mechanisms, phospholipids. — Environ Health Perspect 109(suppl 2):291–300 (2001).

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Investigations into the toxicogenic properties of Fusarium verticillioides (= Fusarium moniliforme) have been the focus of many scientific endeavors following the classic finding that the fungus is responsible for natural outbreaks of equine leukoencephalomalacia (ELEM) (1,2). Many isolates of F. verticilloides from different origins in southern Africa were screened on a regular basis at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, Tygerberg, South Africa, in toxicity trials in ducks and rats (3). Comparative toxicity studies of the fungus in different animal species led to the finding that the major target organ differs in each species, whereas certain organs, including the liver and kidneys, appear to be affected consistently to a greater or lesser degree (4). It was proposed that the rat served as the best experimental model to screen toxigenic isolates of F. verticillioides for their potential to induce different lesions in various animal species. Several events following these initial studies have made a major impact on the subsequent research concerning the toxicologic effects of this fungus in animals. One of these was the finding that contamination of corn with F. verticillioides was positively associated with the incidence of human esophageal cancer in the Transkei region of the Eastern Cape Province, South Africa (5,6). Second, toxicity screening of different F. verticillioides isolates obtained from corn cultivated in a high-incidence area of esophageal cancer induced hepato- and cardiotoxic lesions in rats (4). The induction of cirrhosis together with bile duct and nodular hyperplasia was of particular interest with respect to the potential carcinogenic activity of different isolates of the fungus. The possibility that there was a concurrence of occurrence of this fungus in corn and the development of esophageal cancer has initiated intensive investigations to characterize the toxic and carcinogenic potential(s) that occur in corn, the major dietary staple of humans in the Transkei.

Toxicity and Carcinogenicity Studies in Rats

Studies with Cultures of F. verticillioides Strain MRC 826

An isolate of F. verticillioides designated strain MRC 826, obtained from corn grown in a high-incidence area of esophageal cancer in Transkei, induced ELEM in horses and produced the potent mutagenic compound fumaric acid (3,4,7). Chronic feeding studies in male BD IX rats with a freeze-dried material indicated that the hepatocarcinogenicity was related to the toxic effects of the fungal culture material; whether the nontoxic mutagen was related to the toxic effects of the fungal culture material; whether the nontoxic mutagen had toxic effects on rats (5). We have conducted experiments on the development of cholangio- and adenofibrosis, a lesion that appears to develop from the proliferation of hyperplastic epithelial cells, goblet cells, and interstitial cells. The experiment was conducted with both oven-dried (MRC 826, batch B) and freeze-dried (MRC 826, batch B) culture material and identical lesions were induced; however, the degree of the effects was higher with the freeze-dried material. It was suggested that the causative principle(s) was partially destroyed during the oven-drying treatment at 45–50°C. This was of particular interest because the mutagen fumaric acid produced by the strain MRC 826, was highly heat and light labile and not very toxic acutely (7). Three pertinent issues received attention in a subsequent chronic feeding study (6) in rats using the same culture batch of the fungus (MRC 826, batch B): whether the hepatocarcinogenicity was related to the toxic effects of the fungal culture material; whether the nontoxic mutagen fumaric acid could be related to the carcinogenic outcome; and whether the diet that was marginally deficient in certain vitamins and lipotropes known to have protective effects against esophageal cancer (8) could sensitize rats to develop esophageal cancer if fed low levels of the fungal culture.

We investigated the relative contributions of fumaric acid and toxicity to the carcinogenic effects of the fungus in the liver by including a dietary levels ranging from 2 to 4% in a commercial rat feed caused liver cancer in 80% and ductal carcinoma in 63% of the surviving rats after 450 days (8). An important finding was that the hepatocellular carcinomas (HCCs) developed in cirrhotic livers showing nodular hyperplasia. Another prominent lesion was the concurrent development of cholangio- and adenofibrosis, a lesion that appears to develop from the proliferation of hyperplastic epithelial cells, goblet cells, and Paneth cells. The experiment was conducted with both oven-dried (MRC 826, batch B) and freeze-dried (MRC 826, batch B) culture material and identical lesions were induced; however, the degree of the effects was higher with the freeze-dried material. It was suggested that the causative principle(s) was partially destroyed during the oven-drying treatment at 45–50°C. This was of particular interest because the mutagen fumaric acid produced by the strain MRC 826, was highly heat and light labile and not very toxic acutely (7). Three pertinent issues received attention in a subsequent chronic feeding study (6) in rats using the same culture batch of the fungus (MRC 826, batch B): whether the hepatocarcinogenicity was related to the toxic effects of the fungal culture material; whether the nontoxic mutagen fumaric acid could be related to the carcinogenic outcome; and whether the diet that was marginally deficient in certain vitamins and lipotropes known to have protective effects against esophageal cancer (8) could sensitize rats to develop esophageal cancer if fed low levels of the fungal culture.

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nontoxic strain of *F. verticillioides*, designated MRC 1069, that produces three times more fusarin C than strain MRC 826 (9). We used a semisynthetic diet marginally deficient in some vitamins and micronutrients to evaluate a possible synergistic effect between nutritional deficiencies and the fungal culture for the induction of esophageal cancer (Table 1). Most lesions found in the rats fed a dietary level of 0.5% of the culture material of MRC 826, batch B, included a high frequency of neoplastic nodules (21/21), ductular hyperplasia (21/21), adenofibrosis (19/21), cholangiocarcinomas (8/21), and HCC (2/21) that metastasized to the lungs. Liver sections of 85% of the animals showed marked increases in the presence of gamma glutamyl transpeptidase positive (GGT+) foci and/or nodules. Unlike results in the previous study (8), very little fibrosis, except in association with adenofibrosis, was noticed in the liver, presumably because of the low dietary levels of the culture material used. Basal cell hyperplasia occurred in 12/21 rats fed culture material of strain MRC 826 (batch B), whereas one rat developed an esophageal papilloma. Very few lesions occurred in the liver of the rats fed culture material of MRC 1069 despite its being fed at a dietary level of 5%. Mild ductular hyperplasia was present (18/22); one rat had a focus of cholangiofibrosis and another presented with a neoplastic nodule. Fatty acid changes occurred in the treated rats of both groups. Hepatocytes in the treated and control rats were prominently loaded with glycogen, presumably caused by the high carbohydrate content of the diet used.

When comparing with the first experiment (8), we must consider several aspects. A clear dose–response effect with respect to hepatotoxicity and carcinogenicity became apparent, suggesting that the hepatotoxicity is related to cancer development by the fungus in the liver of rats. Fusarin C was clearly not related to cancer development by the fungus (garcin C). In the liver of the rats obtained by Wilson et al. (11) seems related to dietary deficiencies, as discussed above. This study further strengthened the hypothesis that the causative toxic principle(s) responsible for ELEM in horses and the hepatotoxicity/carcinogenicity in rats could be identical. Studies of the carcinogenic effects of fusarin C in a short-term cancer initiation/promotion model indicated that culture material of *F. verticillioides* strain MRC 826 exhibited cancer-promoting activity when using diethylnitrosamine (DEN) as a cancer initiator and the induction of GGT-positive foci and/or nodules as end points (12). Subsequently, several other strains of the fungus, isolated from a high-incidence area of esophageal cancer in Transkei, were screened for cancer-promoting activity in a modified version of the resistant hepatocyte rat liver model (13). As described above, DEN was used as a cancer initiator while the culture material of the different strains was fed at a dietary level of 5% for 21 days during promotion with the induction of GGT-positive foci used as end point. Three other strains of *F. verticillioides* in addition to strain MRC 826 exhibited cancer-promoting activity, and a significant correlation was found between toxicity and the cancer-promoting activity. As discussed above, this study also suggested that the compound(s) responsible for the toxic and carcinogenic activity could be identical.

**Studies with Fumonisin B isolated from F. verticillioides Strain MRC 826**

**Long-term studies.** The fumonisin B (FB) mycotoxins were originally isolated (14) using the short-term cancer initiating/promoting model described above and their chemical structures were determined (15). Information about the carcinogenic effects of FB1, the main fumonisin produced by *F. verticillioides*, obtained from a short-term study (14) suggested that this mycotoxin could effect both cancer initiation and promotion, and hence could act as a complete carcinogen. Cancer initiation and promotion were associated with a toxic effect characterized with the proliferation of bile ductules, fibrosis, and nodular regeneration similar to those described for *F. verticillioides* MRC 826 in male BD IX rats (16,17). Dosing of horses proved that FB1 caused the neurotic syndrome ELEM (18). These investigations confirmed the previous hypothesis that the compound responsible for ELEM in horses was also responsible for hepatotoxicity and hepatocarcinogenicity in rats (4). These findings led to carcinogenicity testing of FB1 in male BD IX rats performed with the culture material of strain MRC 826 using a marginally deficient diet, as described by Jaskiewicz et al. (9) and Van Rensburg et al. (10).

When male BD IX rats were fed FB1, at 50 mg/kg diet, regenerative nodules and cholangiofibrosis occurred from 6 months onward (19). The rats that were sacrificed or that died from 18 months until 26 months, when the experiment was terminated, suffered from micro- and macronodular cirrhosis with large expansive nodules of cholangiofibrosis. Histologic changes inside the regenerative nodules varied and included fatty changes, hyaline droplet degeneration, necrosis, and areas with a ground-glass appearance that stained positive for GGT. Of the rats that were killed between 18 and 26 months, 66% developed HCC; in 4 rats this metastasized to the kidney, heart, and lungs. Cholangiofibrosis—manifested as irregular ductlike structures lined with an epithelium consisting of large columnar cells and numerous goblet cells—occurred in 100% of the rats killed between 18 and 26 months. Lesions in the kidneys consisted of diffuse interstitial lymphocytic nephritis and mild membranoproliferative glomerulonephritis and were more pronounced in the rats killed at 26 months. Most of the lesions observed in the liver and kidneys of the rats fed FB1 (19) were also induced by culture material of strain MRC 826, except that the esophageal basal cell hyperplasia and cardiac lesions induced by the culture material (8) were not present. Other compounds present in the fungal culture material may cause these lesions, either separately or synergistically with the fumonisins. However, it was shown that FB1 caused the hepatotoxic and hepatocarcinogenic effects of the fungal culture material in male BD IX rats. In a subsequent experiment, low dietary levels of FB1 were fed to male BD IX rats to establish dose–response effects with respect to cancer development in the liver (20). In short, male BD IX rats were fed a semipurified diet containing 1, 10, and 25 mg FB1/kg diet over a period of 24 months. Detailed feed intake profiles were monitored to calculate FB1 intake profiles during the course of the experiment. No HCC or cholangioblastic lesions were noticed in any of the rats terminated between 18 months and 24 months. The major lesions in the liver of the rats fed the high-dose FB1 diet (25 mg FB1/kg [Table 1], consisted of anisokaryosis (13/17), neoplastic nodules (9/17), oval cell proliferation (2/17), bile duct hyperplasia (2/17), nodular distortions and portal fibrosis (3/17), and ground-glass foci (5/17), whereas the livers of all the rats terminated at 26 months contained positive foci (11/11) of the placental form of gluthathione S-transferase (GSTM). In the rats fed the 10 mg FB1/kg diet, fewer lesions...
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appeared and only mild toxic lesions occurred in the livers of the 1 mg FB1/kg dietary group. The data indicate that a threshold exists and that a chronic toxic effect is required for FB1-induced hepatocarcinogenesis. The results of these toxicity and carcinogenicity studies in rats, together with estimated exposure levels, were used to determine risk-assessment parameters for fumonisons in humans (22).

Since the discovery of the fumonisons in 1988, sensitive analytic detection techniques have been developed (22), which have enabled the retrospective estimation of the FB intake in the initial long-term experiment performed by Marasas et al. (8) and subsequently by Jaskiewicz et al. (9). These calculated data and comparisons with the long-term studies in rats, using purified FB1 (19,20), are summarized in Table 2. Cancer induction by the fumonisons occurs in the presence of adverse hepatotoxicity, including cirrhosis, cholangiofibrosis, and oval cell proliferation (Table 2). An average dietary intake of 0.08 mg FB1/100 g body weight (bw)/day induced mild toxic effects with 50% of the rats having neoplastic nodules in the liver; an average dietary intake of 0.16 mg FB1/100 g bw/day causes liver cancer in 55% of BD IX male rats over a period of approximately 2 years.

**Dietary considerations.** Comparisons of the semipurified diet used in the chronic feeding studies with culture material of MRC 826 (8,9) and FB1 (19) and the synthetic AIN 76 diet (23) used in subsequent experiments (17) are shown in Table 1. The semipurified diet was developed by Van Rensburg et al. (10) to investigate the role of a diet low in micronutrients on the development of esophageal cancer in rats. The rationale behind the study was to evaluate the role of simulated human diets involving corn and wheat, which are invariably used as a main source of food in high-incidence areas for esophageal cancer, on the development of esophageal tumors in rats. The supplementation of marginally deficient corn and wheat diets with various combinations of nicotinic acid, riboflavin, zinc, magnesium, molydenum, and selenium reduced the numbers of esophageal papillomas in rats (10). Indigenous African grains (sorghum and millet) also significantly reduced the incidence of esophageal papillomas compared to the corn-based diet (10). The nutritional composition of the semipurified diet

### Table 1. Comparison of dietary composition of AIN 76 diet with the semipurified diet used in fumonisin B1 feeding studies.

| Reference         | AIN-76 | Semipurified | Protein (g/kg) | BCA (mg/100 g bw/day) | CAF (mg/100 g bw/day) | Total intake (mg/100 g bw) | Major histologic lesions in the liver |
|-------------------|--------|--------------|----------------|-----------------------|-----------------------|---------------------------|-------------------------------------|
| Marasas et al. (8) | 95.0  ± 9.2 | 423.8 ± 107.2 | 348.7 ± 105.2   | 0.22 ± 0.01           | 0.001                 | 800                        | Bile duct hyperplasia (5/17)           |
| Control           | 68.1 ± 2.7  | 416.0 ± 38.2  | 346.6 ± 89.9    | 25 mg FB1/kg diet§    | 0.008                 | 800                        | Cholangiocarcinoma (8/21)               |
| Jaskiewicz et al. (9) | 113.6 ± 5.1 | 483.6 ± 88.4 | 370.0 ± 87.2    | 0.0005                | 0.39                  | 780                        | Cirrhosis (15/15)                      |
| Control           | 68.6 ± 1.9  | 416.0 ± 38.2  | 347.5 ± 38.01   | 50 mg FB1/kg diet      | 0.180                 | 780                        | Cholangiocarcinoma (8/21)               |

**Table 2.** Comparison of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

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**Table 3.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 4.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 5.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 6.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 7.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 8.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 9.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 10.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 11.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 12.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 13.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 14.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 15.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 16.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.

**Table 17.** Comparisons of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of F. verticillioides and purified FB1 in male BD IX rats.
used in the long-term experiments differs from that of the AIN 76 (23) developed for rats in several respects. These differences included low protein content and marginal to maximal deficiencies in lipotropes (2- to 3-fold lower), vitamins (2- to 10-fold lower), and minerals (2- to 10-fold lower). The caloric contents of the two diets were similar. It is well recognized that diet plays a major role in the induction and spontaneous development of cancer in experimental animals (24). The semipurified diet used in the long-term studies, therefore, could have had an important influence on the outcome of liver and esophageal cancer development in the male BD IX rats—for example, the methionine content of the semipurified diet was marginally lower whereas the folate levels were four times lower. Low levels of the lipotropes, methionine, and choline are involved in cancer development of many organs including the liver (25) and in addition to folic acid, these lipotropes play important metabolic roles in the utilization of methyl groups (24).

**Short-term studies.** Several short-term cancer models, using rat liver, exist to study the underlying mechanisms of cancer development and promotion by genotoxic carcinogens (26). Because FB1 is a complete carcinogen in the liver, subsequent studies were directed to investigate the cancer-initiating and -promoting potential of this apparently nongenotoxic mycotoxin. The cancer-promoting activity of F. verticillioides was first demonstrated in male BD IX rats (13) and, as discussed above, used to purify the different structurally related fumonisin analogues. The basic concepts underlying the processes of initiation and promotion by fumonisins are discussed in detail elsewhere (21,27) and are based on the “resistant hepatocyte” model developed in the liver by Farber (28). In short, there are two basic sequences of which the first is the production or appearance of hepatocytes with a so-called “resistant” phenotype that makes them resistant to growth-inhibitory or toxic effects of many carcinogens. Genotoxic carcinogens rapidly (within several minutes or hours) induce this new phenotype (29), whereas nongenotoxic carcinogens such as clofibrate (30), FB1, and a choline-deficient diet (25) induce a similar phenotype but over a period of several weeks. The induction of this phenotype is complex, and although a mutation-like event is generally considered an important step, this supposition is critically questioned (31). For the ultimate cancer to develop, the altered or initiated cell must be stimulated to grow during promotion or selection. During this process, called differential inhibition, the initiated cell proliferates in an environment created by the promoter that inhibits the growth of the surrounding normal hepatocytes (32).

**Cancer initiation.** Initial studies on the cancer-initiating-potential of the FB mycotoxins were performed in male Fischer rats fed a purified basal diet (16). Two different protocols were used. In the first, FB1 was fed in the diet (1 g FB1/kg diet) for 26 days followed by partial hepatectomy. Selection occurred 2 weeks later by 2-acyethylaminofluorene/carbon tetrachloride (AAF/CCL4), and the rats were sacrificed after an additional two weeks. The second protocol consisted of partial hepatectomy (PH) followed by single gavage dosages of FB1 at various time points before or after PH. The latter regimen is the classic protocol for evaluating the cancer-initiating-potential of genotoxic carcinogens (28). Histologic changes induced by feeding FB1 in the diet for 26 days were similar to those described for BD IX rats and included the generation of early hepatocyte nodules and mild to moderate bile duct proliferation. After the promoting treatment, three to five hepatocyte nodules were visible macroscopically in the liver; the number of GGT positive foci was also significantly increased compared to the controls. In contrast to this finding, neither FB1 nor FB2 exhibited any cancer-initiating activity during the gavage treatment before or after PH (second protocol).

Subsequent studies in male Fischer rats fed the AIN 76 diet focused on dosage studies in relation to the initiating and promoting potential of the mycotoxin (17,33). Initiation depended on both the dosage and the duration of the treatment. A dose of 29.7 mg FB1/100 g bw over 7 days did not effect initiation, whereas the same dosage over 21 days did. Initiation by FB1 also depended on the induction of a hepatotoxic effect together with compensatory or regenerative cell proliferation, a prerequisite for initiation (34). FB1 also appears to be a mitoinhibitor of normal hepatocytes; a dietary treatment of 250 mg FB1/kg bw for 3 weeks (16) or a single gavage dose of 50 mg/kg bw (33) inhibits liver regeneration induced by PH. Thus, a balance seems to exist between the induction and inhibition of hepatocyte regeneration, and the effect on cancer initiation may depend on which of these two processes prevails at a specific time. For example, a total dosage of 29.7 mg FB1/100 g bw over 7 days is likely to create a strong inhibitory effect on cell proliferation and therefore will not support the process of cancer initiation. However, the same dosage administered over 21 days is likely to support regenerative cell proliferation as a result of FB1-induced hepatotoxicity, which then will support cancer initiation (17). The latter concept is not new; initiation by many genotoxic hepatocarcinogens is potentiated either by use of a toxic dosage that stimulates hepatocyte regeneration or by the introduction of PH during the initiating regimen. In combination with PH, which synchronizes the entry of liver cells into the S-phase by approximately 18 hr, cancer initiation by a genotoxic carcinogen could be effected at very low doses when introduced at this stage (35). The same holds true for initiation by the fumonisins, except that the whole process occurs at a far slower rate, probably because the FB1-induced cell proliferation is counteracted by its mitoinhibitory effect, producing a much smaller yield of initiated hepatocytes. In addition, a recent study indicated that FB1 induced apoptosis (36), which has been suggested to reduce the number of initiated cells in the liver (37).

Another aspect that could determine the kinetics of the initiating step is the nature of the event(s) leading up to the induction of the initiated hepatocytes as a result of FB1 treatment. The nature of initiation is of particular interest with respect to FB1 because the compound appears not to exhibit any mutagenic or genotoxic effects in different in vivo and in vitro tests (16,38). However, Knasmuller et al. (39) reported that FB1, as well as the mycotoxins moniliformin and deoxynivalenol exhibited clastogenic effects (chromosomal aberrations) at concentrations from 1.4 to 144 µM in primary hepatocyte cultures. At these concentrations FB1 reduced the mitotic index and the induction of micronuclei markedly to significantly. These data suggested that FB1 might exhibit some genotoxic effects. It was postulated that lipid peroxidation could play a role in the chromosomal breakage caused by the accumulation of polyunsaturated fatty acids in primary rat hepatocytes after exposure to FB1 (40). Because cytotoxic effects and lipid peroxidation occur only at high concentrations of FB1 (> 75 µM), the induction of chromosomal aberrations at levels of 1.4 and 14 µM need to be investigated further to clarify whether FB1 is directly or indirectly responsible for chromosomal strand breaks. The disruption of sphingolipid metabolism is effected maximally at these concentrations (41), with the accumulation of sphinganine known to affect cell growth and differentiation (42). However, for the purpose of the present review, the fumonisins will be regarded as not causing direct DNA damage (mutations).

As mentioned above, the role of lipid peroxidation during cancer initiation in rat liver must be considered (17) because relatively high cytotoxic dosages over long time periods are required. Recent investigations indicated that FB1 induces lipid peroxidation in cell membrane preparations (43) and isolated rat liver nuclei (44) and in primary rat hepatocytes and rat liver in vivo (45). When egg yolk phosphatidylcholine (PC) bilayers were used (43), FB1 increased the rate of oxidation, free radical production, and lipid peroxidation, thereby disrupting membrane
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General Introduction

The major constituents of cellular membranes are the phospholipids, which contain fatty acids as important constituents of the typical bilayer structure (52). Essential fatty acids (EFA) are normally linked to the 2 position of the glycerol backbone of the phospholipids and sometimes also to the 1 position. Free cholesterol is closely associated with the fatty acids and hence is an important mechanism in determining membrane fluidity. EFA consists of the o6 and o3 derived from linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3), respectively (Figure 1). A series of alternating desaturations (which add a double bond) and elongations (which add two carbon atoms) are involved in the synthesis of the different long-chained fatty acid metabolites. The desaturation and elongation are not confined to the metabolism of EFA; the saturated fatty acids palmitic and stearic can also be converted to long-chained fatty acids. Apart from the role...
of EFA as structural components of all membranes, they are precursors of the eicosanoids, prostaglandins, leukotrienes, and other oxygenated derivatives.

**In Vitro Studies in Primary Hepatocytes**

Studies in primary hepatocytes indicate that the incorporation of $^{13}$C palmitic acid decreased in the total lipids and the neutral lipids, triacylglycerol (TG), and the cholesterol esters at a nontoxic concentration of 150 μM FB, and at cytotoxic concentrations of 250 μM and higher (40). In contrast, the incorporation of radiolabel into phospholipids increased with a concomitant increase in the concentration level of PC and phosphatidylethanolamine (PE), whereas the total cholesterol decreased. The concentration and labeling of sphingomyelin (SM) decreased, presumably as a result of the inhibitory effect on the ceramide synthase, a key enzyme in the synthesis of different prostaglandins. The marked fatty acids (boxes) are likely to play a key role in the development of hepatocyte nodules.

**In Vitro Studies in Rats**

In vitro studies indicate that FB1 disrupted lipid biosynthesis differently from that in the in vivo studies (56). In contrast to the in vitro studies, the major changes were associated with both the PE and the PC phospholipid fractions, and cholesterol increased in both the serum and liver. In the short-term studies in male Fischer rats, a dietary level of 250 mg FB/kg increased the PE levels in the liver. Fatty acid analyses of the PE and PC fractions of the hepatocytes were heavily affected. This was recognized earlier, as the addition of C20:4 to PE levels in the liver of rats of the long-term study in both the short-term and long-term FB feeding studies confirmed the observations obtained in the liver: an increase in C18:2 and a decrease in C20:4 and C22:5 in the short-term (50 mg FB/kg diet and higher) and long-term (10 mg FB/kg diet and above) experiments. With regard to the total fatty acid parameters, the monounsaturated fatty acids (MUFA) increased in PE, whereas the total n-6 and PUFA decreased in PC. In the long-term experiment, the MUFA increased in PC while the n-3 fatty acids increased in PC and PE, altering the n6/n3 ratio. The effect of FB1 on the n-6 fatty metabolic pathway seems to rely to a greater or lesser extent on the dietary level of FB1, the length of exposure, the specific cellular phospholipid fraction, and differences between in vitro and in vivo experiments.

Subsequently, the effects of different dietary dosages (10, 50, 100, and 250 mg FB/kg diet) of FB1 fed for 21 days were evaluated on lipid metabolism in rat liver microsomal membranes (57). These dietary levels of FB1 were used to investigate the cancer-promoting potential of the fumonisins in the DEN-initiated rats (29). The major changes associated with the microsomes were increased levels of PC, phosphatidyl inositol (PI), PE, and cholesterol. The levels of the saturated fatty acids and MUFA, especially C18:199, increased significantly in the treated groups (100 mg FB/kg diet and higher) in all the phospholipids except phosphatidyl serine (PS). The relative (%) and absolute (μg) values of C18:2 were increased in the PC, PI, PS, and PE phospholipid fractions. C20:4 showed a decrease in the relative values, whereas the absolute values remained constant in PC, PI, and PS despite the fact that the concentration of the phospholipids increased in the high-dosage groups. In PE, however, the relative value was not altered. However, the absolute value of C20:4 in PE increased, presumably because of the prominent increase (>2x) in the level of PE compared to that of the other phospholipids. A similar effect was noted with C22:6 and C22:5 in PC and PE. The relative values of the n3 fatty acid, C22:6, also decreased in a dose-responsive manner in all the phospholipids; the absolute values remained the same except PE, where they significantly increased. The relative levels of total PUFA were not altered; the absolute levels increased (PC, PE, and PI) because of...
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The polyunsaturated/saturated (P/S) fatty acid ratio also decreased in the PC phospholipid fraction because of alterations in the PUFA and saturated fatty acid levels. Changes to membrane environment of FB1 also expanded and included the plasma, mitochondrial, and nuclear membrane fractions of rat livers exposed to 250 mg FB1/kg diet for 21 days. Some differences exist in the lipid profiles of the different membrane fractions with respect to the effect of FB1 on the levels of cholest erol and PC and PE. In the plasma membrane and nucleus, only PE significantly increased; PC, SM, and cholest erol were unchanged. The mitochondrial membrane structure was also altered differently from the plasma membrane. The level of PE increased, PC and SM decreased, and cholesterol remained unchanged. The fatty acid patterns were similar, with minor differences between PC and PE—e.g., PUFA decreased in PC (both the relative and absolute values), whereas in PE the relative value decreased and the absolute value increased. The absolute values of the saturated fatty acids and MUFA increased, causing a decrease in the P/S ratio of PC and PE and suggesting a less fluid mitochondrial membrane.

It can be argued that some of these changes can be related to the hepatotoxic effects induced by FB1 in the liver. Apart from cancer promotion, the toxic effects are closely related to cancer initiation by the fumonisins, making it difficult to associate specific changes in lipid metabolism with cancer induction at this stage. However, a characteristic fatty acid pattern seems to emerge in the livers of rats exposed to dietary levels of FB1 that effect both cancer initiation and promotion. These include the following: First, an increase in saturated fatty acids and MUFA (C18:1ω9) fractions was observed in both PC and PE. Second, the relative level of C18:2ω6 increased in PC, whereas the absolute value was enhanced in PC and PE. Third, the relative and absolute values of C20:4ω6 tended to decrease in PC and increase in PE. Fourth, the relative and absolute values of C22:4ω6 and C22:5ω6 decreased in PC, whereas only the relative value of C22:5ω6 decreased in PE. The n-3 fatty acid, C22:6ω3, also decreased in PC but tended to increase in PE. Fifth, both the relative and absolute total PUFA values decreased in PC but only the relative levels decreased in PE. And, sixth, the P/S ratio decreased in both PC and PE, suggesting a less fluid plasma membrane structure.

Mechanistic Implications with Respect to Cancer Promotion

Apart from the role of the PUFA in regulating many processes in the cells via their production of different classes of prostaglandins, their role in determining the structure and function of cellular membranes also must be considered. A change in saturation could determine the responsiveness of cells to transformation or the expression of specific phenotypes supporting differential growth that produces clonal expansion of certain cell types associated with neoplastic development. With respect to cancer promotion, the disruption of growth-stimulatory responses in primary hepatocytes and regenerating liver could be important in establishing the growth differential.

Altered lipid parameters associated with the growth of hepatocyte nodules. Abel et al. (58) recently investigated the role of different lipid parameters in the development and/or progression of hepatocyte nodules at different time intervals (1, 3, 6, and 9 months). The concentration of the phospholipid PE increased, whereas the total cholesterol increased in the 1- and 9-month nodules. Despite the fact that PC increased in the 1-month nodules, the increased level of PE caused a decrease in the PC/PE ratio in hepatocyte nodules. Fatty acid analyses indicated that C18:1ω9 and C18:2ω6 increased in PE and PC, while C20:4ω6 decreased in PC but increased quantitatively in PE. The end products of the n-6 and n-3 pathways, C22:5ω6 and C22:6ω3, decreased both qualitatively and quantitatively in PC, causing a decrease in PUFA. The lipid profiles of the surrounding tissue reflect those of the control tissue. In regenerative liver (over 7 days after partial hepatectomy), used as a control for cell proliferation, the fatty acid profiles of PE and PC are very similar to those of hepatocyte nodules except that C18:1ω9 decreased in PC. Other differences were the increased membrane fluidity and the tendency of PC to decrease in regenerating liver. Apart from a few differences, the lipid parameters associated with increased cell proliferation in the hepatocyte nodules closely mimic those of normal regeneration in the liver. However, one major difference is that the lipid changes in the nodules are persistent whereas they are reversed in regenerating liver. In the hepatocyte nodules the altered lipid metabolic pattern, specifically the fatty acid profiles, could be important in regulating growth in these lesions. In this regard, the increased levels of PE and C20:4ω6 are of interest because the fatty acid regulates many processes related to cell growth, such as proliferation and apoptosis (58). With respect to cell proliferation in hepatocyte nodules, the role of C20:4ω6 and its cyclooxygenase prostaglandin E2 series products in the activation of protein kinase C and mitogen activation protein kinases should be considered (59). Tang et al. (60) suggested that the metabolism of C20:4ω6 is involved in the evolution of preneoplastic foci into nodules and hepatocellular carcinomas in rat liver. C20:4ω6 has also been linked to the action of transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α (62), which together with the deregulation of c-my expression could be important determinants during FB1-induced apoptosis in the liver of rats.

The decrease in PUFA and the increase in C18:1ω9 in hepatocyte nodules have been suggested to play important roles in the lower levels of lipid peroxidation normally seen in cancerous lesions (62). Cancer cells have low levels of PUFA and the degree of depletion in vitro can be an accurate predictor of its malignancy in vivo.

Disruption of growth control by FB1. The effects of FB1 on phospholipid and fatty acid metabolism closely mimic those seen in hepatocyte nodules, although there are some differences, as described for nodules and regenerating liver (see above). The decrease in fatty acid saturation, induced by FB1, implies a more rigid membrane structure such as found in hepatocyte nodules. The n-6 fatty acid pathway is markedly affected with an accumulation of C18:2ω6 and a decrease in C20:4ω6 as well as in the subsequent products C22:4ω6 and C22:5ω6. This specific altered fatty acid pattern (Figure 1) likely caused an impaired Δ6 desaturase enzyme. This hypothesis was further strengthened by the fact that another substrate for the enzyme, C18:1ω9, increased and an n-3 fatty acid product of the enzyme, C22:6ω3, decreased. However, the modulating effect of FB1 on this rate-limiting enzyme in fatty acid metabolism still needs to be elucidated. The decrease in PUFA, in addition to the disruption of fatty acid metabolism, could also result from lipid peroxidation induced by FB1 at high-dosage levels (45). In this regard the accumulation of C18:1ω9 is of interest because C18:1ω9 exhibits potent antioxidant activity (62) that, in the case of FB1–induced hepatotoxicity, could provide a specific survival mechanism to hepatocytes under conditions of stress.

The concentration of PE is markedly increased in the membrane fractions of the hepatocyte, increasing the absolute values of C20:4ω6 within the cell. The latter state—together with the increased level of C18:1ω9, which implies a lower oxidative status—is likely to favor cell proliferation, especially in the initiated hepatocyte cell population (60,62). This becomes evident with a similar fatty acid pattern found in hepatocyte nodules (58), presumably sustaining cell proliferation, whereas in the surrounding and normal liver it appears to inhibit growth, thereby creating an environment for the differential inhibition of growth. This is also true for FB1, which effects this altered lipid profile in the liver and can inhibit cell proliferation in
regenerating liver (16,17,33). However, in such an environment the initiated hepatocytes would proliferate into hepatocyte nodules, and some of these might develop into tumors after continued exposure to FB1. Very little is known about the nodules induced by FB1 in the liver, but because hepatocyte nodules appear to be very similar with respect to the resistant phenotype regardless of the initiator or promoter used (26), the differential created by FB1 is likely to promote their growth. Changes to membrane structure and fluidity appear to have important implications with respect to membranal processes related to normal growth and differentiation. In early persistent and late preneoplastic nodules, the binding of EGF, lipoproteins, and desialylated glycoproteins is markedly reduced (63). A decreased ligand binding might play a role in the altered responses to external growth inhibitory and stimulatory factors that regulate cell proliferation and other physiologic factors in the liver.

Modulation of growth regulatory molecules in the liver. The molecular mechanisms underlying FB1-induced hepatotoxicity and carcinogenesis have not been examined in depth. A recent study employing Northern blot (mRNA) analysis showed increased hepatic expression of HGF, TGF-α, and especially TGF-β1 and c-myc during short-term feeding of FB1 (36). Immunostaining with LC1(1–30) antibody for mature TGF-β1 showed that zone 1 and 2 hepatocytes were responsible for the increased expression of TGF-β1. Overexpression of TGF-β1 may be responsible for the prominent proapoptotic effects of FB1 in the liver. The proto-oncogene c-myc is a positive regulator of cell proliferation that is involved in tumor progression (64,65) and has also been implicated in TGF-β1 signaling (66). Increased expression of c-myc oncogene and TGF-β1 may cooperate in the promotion of liver tumors during feeding FB1, possibly by providing an environment that selects for the growth of TGF-β1-resistant transformed liver cells. Oncogenesis due to overexpression of both c-myc and TGF-α appears to involve disruption of the Rb/E2F pathway and deregulation of cell cycle control. Both c-myc and TGF-α contribute to induction of cyclin D1 expression and resultant inactivation of the retinoblastoma tumor suppressor protein, and c-myc may directly induce E2F (67). With respect to apoptosis, overexpression of c-myc together with the depletion of growth factors and/or disruption of growth signaling pathways could cause imbalances of cell cycle progression and hence induction apoptosis (68). In this regard, FB1 overexpressed c-myc in rat liver (36), whereas it disrupted growth-related responses in different cell types such as primary hepatocytes (41) and in the liver in vivo (33).

Recent evidence shows that FB1 stabilizes cyclin D1, causing accumulation of the protein in the nucleus of altered hepatocytes in foci, nodules, adenomas, and carcinomas (69) in the livers of rats (male BD IX) fed FB1 over a period of two years (19). In male Fischer rats fed FB1 over a period of 21 days (17), cyclin D1 protein levels in liver also increased up to 5-fold in a dose-responsive manner, with no simultaneous increase in mRNA. The increase in FB1-treated samples of cyclin-dependent kinase (Cdk4) complexes with cyclin D1 and consequently elevated Cdk4 activity were confirmed by an increased phosphorylation of the retinoblastoma protein. Levels of cyclin E and Cdk2 did not differ between controls and FB1-treated livers (short term) except for one sample in which a decrease in both proteins was detected. Alterations in cyclin D1 were specific to the livers, and all other tissues were negative for cyclin D1 overexpression except the kidney. Kidney showed some positive nuclear staining in the proximal tubules in both untreated and treated rats. This finding must be interpreted with caution in view of the tendency of proximal tubules often to stain nonspecifically in immunohistochemistry. Because chronic interstitial nephritis was present in the kidneys and FB1 can have toxic effects in rat kidneys (70), this may also reflect a role of cyclin D1 in this pathology. To test whether the overexpression of cyclin D1 was a common property of rat HHC, liver sections from paraffin-embedded rat HCC caused by nitroglycerin or diethylnitrosamine/pheno- barbital (DEN/PB) (71,72) were compared to those induced by FB1. The cyclin D1 overexpression, characteristic of FB1-induced preneoplastic lesions and HCC, was not changed in HCC caused by DEN/PB or nitroglycerin. However, HCCs induced by nitroglycerin or DEN/PB showed proliferating cell nuclear antigen staining rates similar to those in the FB1-induced tumors. These findings suggest that altered cyclin D1 and Cdk4, as major cell cycle oncogenes, may be role players in the carcinogenic effects of FB1. Presently, we are in the process of determining which signaling molecules, known to participate in the regulation of cyclin D1 stability/degradation, could be affected by FB1. The modulating effects of FB1 on both sphingolipids and phospholipids could play a major role in the molecular events involving cyclin D1 protein stability (69).

Conclusions

The toxicity induced by FB1 in the liver appears to play an important role during the cancer initiation, and the induction of oxidative damage and lipid peroxidation could be important initial events. Selective inhibition seems to be the likely mechanism during cancer promotion. Changes in the balance of the different cell regulatory molecules discussed above are likely to be involved in the induction of a growth differential that selectively stimulates the growth of initiated cells. This was shown with the peroxisome proliferator Wy-14,643, which decreased the level of HGF in the liver (73). In vitro studies (74) indicate that HGF stimulates the growth of normal hepatocytes while inhibiting the growth of preneoplastic or neoplastic cells. The reduction of HGF therefore could play an important role in the promotion of preneoplastic cell growth. Apoptosis is also very important during the growth of hepatocyte nodules because cancer promoters such as phenobarbital are known to decrease the rate of apoptosis in these lesions (75). FB1 can induce apoptosis in the liver (36,49) and the disruption of sphingolipid metabolism has been implicated because it disrupts sphingolipid metabolism and therefore ceramide synthesis (53), an important signaling molecule for apoptosis. However, fatty acids, especially C20:4ω6, have been found to be important second messengers during TNF-α–induced apoptosis by the release of ceramide via the stimulation of sphingomyelinase (61). In rat hepatoma cells, C20:4 metabolites were also shown to be involved in apoptotic cell death elicited by TGF-β1 (76). In a recent study in an esophageal cancer cell line, C20:4ω6 and its cyclooxygenase products prostaglandin E2 and prostaglandin A2 induced apoptosis, a process that was inhibited by FB1 (77). The effect of FB1 can be explained either by the reduction of ceramide or the regulation of C20:4ω6 levels, as discussed above. In contrast, a recent study indicated that FB1 induced apoptosis in esophageal epithelial cells and neonatal human keratinocytes (51). It would appear that, depending on the cell type, the extent to which different pathways are interrupted could determine whether the cell would undergo apoptosis (78). A unique pathway has been proposed whereby the glycerophospholipids and the sphingolipid cycle interact to control a variety of cellular processes including apoptosis, with C20:4ω6 and ceramide as the key role players (61). A similar interactive pathway is likely to exist for the fumonisins in the liver to regulate processes related to cell proliferation and apoptosis (Figure 2). FB1 effects a similar phospholipid metabolism and hence fatty acid pattern in the liver, as was noted in hepatocyte nodules. However, subsequent effects on sphingolipid and/or prostaglandin production seems to inhibit the growth of normal hepatocytes, which together with the overexpression of TGF-β1 and c-myc could effect apoptosis. Oxidative damage and the resultant lipid peroxidative products could also further enhance apoptosis.
in the liver (79); on the other hand, the increased C18:1 and C20:4 fatty acids and the decrease in PUFAs are critical with respect to cancer promotion, especially at low dietary levels of FB1, where cancer promotion is effected in the absence of apoptosis and the disruption of the sphingolipid metabolic pathway. Future studies will focus on the role of C20:4 as a second messenger molecule, including the regulation of its release by phospholipase A2 and the subsequent modulating effects on cell proliferation and apoptosis that could potentially cause development of the cancer phenotype in the liver.

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Figure 2. Diagram illustrating the role of FB1 on lipid, sphingolipid, and fatty acid metabolism as a model for the enhanced proliferation in hepatocyte nodules (bold arrows). Abbreviations: PLA2, phospholipase A2; ω-6 fatty acid metabolic pathway, involv-
ing the Δ6 desaturase enzyme and PGE2. Subsequent effects regarding the growth regulation [inhibition] in normal cells included the disruption of growth factor responses with the induction of apoptosis, involving p-

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