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 FB_1 provide information about dose–response effects, relevance of hepatotoxicity during FB_1-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 apparently nongenotoxic mycotoxin. These studies supported the findings of long-term investigations indicating that a cytotoxic/proliferative response is 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).
http://ehpnet1.niehs.nih.gov/docs/2001/suppl-2/291-300gelderblom/abstract.html

Investigations into the toxigenic 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. verticillioides* 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 ducklings 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 possible link between occurrence of this fungus on corn and the development of esophageal cancer has initiated intensive investigations to characterize the toxic and carcinogenic principle(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 fusarin C (*3,4,7*). Chronic feeding studies in male BD IX rats with a freeze-dried corn culture (batch MRC 826B) of *F. verticillioides* at dietary levels ranging from 2 to 4% in a commercial rat feed caused liver cancer in 80% and ductular 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- or 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 9-20) 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 fusarin C, 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 (*9*) 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 fusarin C 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 (*10*) could sensitize rats to develop esophageal cancer when fed low levels of the fungal culture.

We investigated the relative contributions of fusarin C and toxicity to the carcinogenic effects of the fungus in the liver by including a

This article is based on a presentation at the International Conference on the Toxicology of Fumonisin held 28–30 June 1999 in Arlington, Virginia, USA.

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The authors are indebted to W. Moritz for lipid analyses, S.D. Snyman for the preparation of primary rat hepatocytes, N.P.J. Kriek for histologic analyses, and E. Farber for his inspiration and ideas that stimulated the creation of many of the concepts described in this article.

Received 10 April 2000; accepted 21 July 2000.

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 involved in the carcinogenicity of the fungus because culture material *F. verticillioides* strain MRC 1069, containing at least 3 times as much fusarin C and fed at a dietary level 10 times higher than that of MRC 826, produced only a few neoplastic lesions in the liver. The basal cell hyperplastic lesions failed to progress to neoplasia despite the fact that the diet contained marginal levels of lipotropes and some vitamins.

Following these long-term studies with fungal culture material in South Africa, a feeding study in male Fischer 344 rats showed that corn naturally contaminated with *F. verticillioides* and associated with a field outbreak of ELEM in the United States induced nodules, adenofibrosis, and cholangiocarcinomas in the liver after 4 to 6 months (*11*). Although the corn-only diet was deficient in many vitamins and lipotropes, the lesions were very similar to those described by Marasas et al. (*8*), which were induced by *F. verticillioides* strain MRC 826 in male BD IX rats fed a nutritionally balanced diet. The early appearance of the lesions 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_1 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 FB_1, 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 FB_1 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 FB_1 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 FB_1 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 FB_1 (*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 FB_1 caused the hepatotoxic and hepatocarcinogenic effects of the fungal culture material in male BD IX rats. In a subsequent experiment, low dietary levels of FB_1 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 FB_1/kg diet over a period of 24 months. Detailed feed intake profiles were monitored to calculate FB_1 intake profiles during the course of the experiment. No HCC or cholangiofibrotic 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 FB_1 diet [25 mg FB_1/kg (Table 1)], consisted of anisokaryosis (13/17), neoplastic nodules (9/17), oval cell proliferation (2/17), bile duct hyperplasia (3/17), lobular distortion and portal fibrosis (5/17), 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 (GSTP). In the rats fed the 10 mg FB_1/kg diet, fewer lesions

appeared and only mild toxic lesions occurred in the livers of the 1 mg FB_1/kg dietary group. The data indicate that a threshold exists and that a chronic toxic effect is required for FB_1-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 fumonisins in humans (*21*).

Since the discovery of the fumonisins 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 perfomed 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 FB_1 (*19,20*), are summarized in Table 2. Cancer induction by the fumonisins occurs in the presence of adverse hepatotoxicity, including cirrhosis, cholangiofibrosis, and oval cell proliferation (Table 2). An average dietary intake of 0.08 mg FB/100 g body weight (bw)/day induced mild toxic effects with 50% of the rats having neoplastic nodules in the liver; an average intake of 0.16 mg FB/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 FB_1 (*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[a] of AIN 76 diet with the semipurified diet used in fumonisin B_1 feeding studies.

	AIN-76	Semipurified		AIN-76	Semipurified
Protein (g/kg)	214.3	109	Vitamins[b]		
Soy protein		30	Thiamin (mg)	6.4	3.9
Casein	200	10	Riboflavin (mg)	6.1	3.55
Egg albumin		10	Nicotinic acid (mg)	31.1	32
Corn meal		59	Vitamin B6 (mg)	7.1	0.6
Methionine	3	1.91	Folate (mg)	2	0.5
Total CHO (g/kg)	592	659.1	Vitamin B12 (µg)	10	8
Corn starch	216.7	750	Pantothene (mg)	16.8	3
Sucrose	216.7		Biotin (mg)	0.2	0.1
Glucose	216.7	111.25	Vitamin A (IU)	4,005	16,672
Dextrin		54.3	Vitamin D (IU)	1,000	250
			Vitamin E (mg)	79.7	41.33
Total Fat (g/kg)	46.4	47.6	Vitamin K (mg)	5	2.95
Saturated	5.9	6.33	Choline	2	0.7
MUFA	9.0	11.7			
PUFA	29.3	27.0	Minerals[c]		
Sunflower seed oil (g)	50	30	Calcium	5,100	515
			Iron	48	17.3
Energy			Magnesium	604	488
Kcal	3,779	3,448	Phosphorus	4,264	1,133
KJ	15,820	14,426	Potassium	3,925	2,307
			Sodium	1,245	1,028
Fibre (g/kg)	55.4	37.2	Zinc	46	20.8
			Copper	5.7	

Abbreviations: CHO, carbohydrate; KJ, kilojoules. [a]Composition analyses performed using the MRC Food Composition Tables (*80*). [b]mg/kg or units/kg. [c]mg/kg AIN-76 (*23*); semipurified diet (*18*).

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 FB_1 in male BD IX rats.

Reference	Body weight Initial (g)	Body weight Final (g)	Body weight Gain (g)	FCM (% in diet)	FCM intake (mg/100 g bw/day)	FB intake (mg/100 g bw/day)	Duration (days)	Total intake (mg/100 g bw)	Major histologic lesions in the liver
Marasas	63.5 ± 4.7	290.1 ± 46.8	226.6 ± 47.3	4	0.13	0.69	288	198.33	Cirrhosis (20/20)
et al. (*8*)				2	0.06	0.32	606	195.62	Adenofibrosis (19/20)
							Total:	394.95	Ductular carcinoma (10/20)
Control	64.7 ± 4.8	370.8 ± 71.8	306.1 ± 70.2			0.0005	894	0.45	HCC (12/20)
									Basal cell hyperplasia (11/15)
Jaskiewicz	113.9 ± 4.7	359.8 ± 78.0	246.0 ± 77.6	0.25	0.01	0.04	211	9.08	Neoplastic nodules (18/21)
et al. (*9*)				0.50	0.02	0.09	311	26.8	GGT-positive foci (18/21)
				0.75	0.02	0.13	81	10.46	Fatty change (21/21)
				0.50	0.02	0.09	266	22.90	HCC (2/21)
							Total:	70.21	Ductular hyperplasia (21/21)
Control	113.6 ± 5.1	483.6 ± 88.4	370.0 ± 87.2			0.0005	869	0.39	Adenofibrosis (19/21)
									Cholangiocarcinoma (8/21)
Gelderblom	68.6 ± 1.9	416.0 ± 38.2	347.5 ± 38.01		50 mg FB_1/kg diet	0.160	780	124.8	Cirrhosis (15/15)
et al. (*19*)									Regenerative nodules (15/15)
									Cholangiofibrosis (15/15)
Control	68.1 ± 2.7	517 ± 108.8	448.9 ± 107.4						HCC (10/15)
Gelderblom	98.5 ± 7.1	445.1 ± 88.9	346.6 ± 89.9		25 mg FB_1/kg diet[a]	0.080	690	55.20	Anisokaryosis (13/17)
et al. (*20*)	98.4 ± 7.4	474.4 ± 71.8	376.0 ± 71.3		10 mg FB_1/kg diet	0.032	690	22.08	Hyperplastic nodules (9/17)
	101.4 ± 6.3	483.6 ± 114.7	382.2 ± 115.0		1 mg FB_1/kg diet	0.003	690	2.21	Oval cell proliferation (2/17)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2		0.22 g FB_1/kg	0.001	690	0.35	Bile duct hyperplasia (3/17)
									Portal fibrosis (5/17)
									Ground-glass foci (7/17)
									GSTP foci (11/11)

FCM, *Fusarium* culture material. [a]Histologic changes recorded in the high-dose group (*20*).

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 marked 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*). 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 by genotoxic carcinogens (*26*). Because FB_1 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 resist the 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*), FB_1, 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 first 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, FB_1 was fed in the diet (1 g FB_1/kg diet) for 26 days followed by partial hepatectomy. Selection occurred 2 weeks later by 2-acetylaminofluorene/carbon tetrachloride (AAF/CCl_4), and the rats were sacrificed after an additional two weeks. The second protocol consisted of partial hepatectomy (PH) followed by single gavage dosages of FB_1 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 FB_1 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 FB_1 nor FB_2 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 FB_1/100 g bw over 7 days did not effect initiation, whereas the same dosage over 21 days did. Initiation by FB_1 also depended on the induction of a hepatotoxic effect together with compensatory or regenerative cell proliferation, a prerequisite for initiation (*34*). FB_1 also appears to be a mitoinhibitor of normal hepatocytes; a dietary treatment of 250 mg FB_1/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 FB_1/100 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 FB_1-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 FB_1-induced cell proliferation is counteracted by its mitoinhibitory effect, producing a much smaller yield of initiated hepatocytes. In addition, a recent study indicated that FB_1 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 FB_1 treatment. The nature of initiation is of particular interest with respect to FB_1 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 FB_1 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 FB_1 reduced the mitotic index and the induction of micronuclei markedly to significantly. These data suggested that FB_1 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 FB_1 (*40*). Because cytotoxic effects and lipid peroxidation occur only at high concentrations of FB_1 (> 75 uM), the induction of chromosomal aberrations at levels of 1.4 and 14 μM need to be investigated further to clarify whether FB_1 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 FB_1 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*), FB_1 increased the rate of oxidation, free radical production, and lipid peroxidation, thereby disrupting membrane

structure and permeability. These effects were noticed at high concentration levels—between 1 and 10 mM FB_1—raising some doubts about inducing similar effects *in vivo*. Cawood et al. (*46*) showed that when radiolabeled FB_1 is used, the compound is tightly associated with plasma and microsomal membranes. However, a specific binding site for the fumonisins has not yet been characterized. Nuclear membrane lipid peroxidation with concomitant DNA strand breaks occurred in isolated rat liver nuclei treated with FB_1 *in vitro* at concentrations ranging from 40 to 300 μM (*44*). The formation of hydroxy and the subsequent formation of peroxyl radicals in the vicinity of nuclear material was proposed to cause DNA strand breaks. It was further postulated that metal ions, specifically iron, endogenously associated with cellular DNA could be important sites for metal-catalyzed oxidative DNA damage. *In vitro* studies in primary hepatocytes showed that lipid peroxidation is effected in a dose-dependent manner closely associated with cytotoxicity induced by FB_1 (*45*). Although α-tocopherol prevented lipid peroxidation by FB_1, cytotoxicty was not completely abolished, indicating that lipid peroxidation is not solely responsible for the cytotoxic effects and could be secondary to cell cytotoxicity. Except for halogenated hydrocarbons such as carbon tetrachloride, oxidative stress and the resultant lipid peroxidation seem to occur as a result of cell injury induced by toxins (*47*). At low levels of exposure (75 μM), FB_1 also enhanced the susceptibility of hepatocytes to undergo lipid peroxidation induced by cumene hydroperoxide, probably via the accumulation of polyunsaturated fatty acids in primary hepatocytes (*40*). In a recent study in rats fed a dietary level of 250 mg FB_1/kg diet, with or without dietary iron loading, FB_1 augmented iron-induced lipid peroxidation in the liver (*48*).

Observations from *in vivo* feeding studies support the results from *in vitro* studies that lipid peroxidation occurs in a dose-dependent manner associated with a hepatoxic effect. Lipid peroxidation appeared to be a secondary effect rather than a causative mechanism of FB_1-induced hepatic injury (*45*). Purification of membranal fractions indicated that FB_1 significantly ($p < 0.05$) increased lipid peroxidation in plasma and microsomal membranes at a dietary level of 250 mg/kg, and it was enhanced (not significantly) in the mitochondrial and nuclear membranal fractions. Oxidative damage could well be an important initial event and in addition to the inhibitory effect on cell proliferation, could explain the slow kinetics of the initiating step. Apoptosis also must be considered because it becomes an important biologic phenomenon at high exposure levels of fumonisins (*36,49*) and because it is known to remove the genetically altered initiated cells in the liver (*50*).

With respect to the cancer-initiating potency of FB_1 (*45*), cancer initiation of the choline-deficient diet occurred only after 9 weeks, preceded by lipid peroxidation and a hepatotoxic effect (*25*). The peroxisome proliferator clofibrate (*30*) also causes cancer initiation after prolonged feeding of several weeks. Because FB_1 is considered a weak or slow cancer initiator, the compound may have a strong effect on postinitiation events such as cancer promotion (*17*). Because the fumonisins occur naturally at relatively low dietary levels compared to the levels that initiate cancer in rats, future research should focus on the events related to later phases of cancer development, including promotion and progression.

Cancer promotion. As discussed above, the process of promotion represents an important phase during which the initiated cells are clonally expanded into hepatocyte nodules by a process known as differential inhibition (*28,32*). Although other mechanisms of the clonal expansion of the initiated hepatocyte have been proposed, considerable evidence exists to support a selection process whereby a few resistant hepatocytes proliferate in an environment where the proliferation of normal cells is inhibited (*32*). A compound is called a cancer promoter if it can create such an environment, and studies of fumonisins indicate that a similar hypothesis could be developed for the cancer-promoting property of these compounds. This growth selection of resistant hepatocytes has been recognized as a property of different cultures of *F. verticillioides* (*12,13*) and was successfully used to develop a bioassay for the purification of the fumonisins. Initial studies suggested that hepatotoxicity was associated with the cancer-promoting potential of this mycotoxin (*13*). A recent study showed that cancer promotion, unlike initiation, was effected at relatively low dietary levels (50 mg FB_1/kg diet) in the absence of excessive hepatotoxicity (*33*). The cancer-promoting activity of FB_1 was also associated with an inhibitory effect on hepatocyte proliferation. This suggested an induction of a growth differential whereby the growth of the resistant initiated cells is promoted and that of normal cells inhibited. Inhibition of cell growth occurs in many cell culture systems (*51*) and in rat liver *in vivo* under different experimental conditions involving PH. A dietary level of 250 mg FB_1/kg fed over 21 days significantly inhibited regenerative hepatocyte cell proliferation in hepatectomized male Fischer rats after 24 hr (*17*). Three days after PH the level of DNA synthesis was significantly higher in the FB_1-treated group, whereas at 7 days there was no difference. FB_1 seems to delay hepatocyte regeneration in a reversible manner. Even a single gavage dosage (50 mg/kg bw and higher) 6 hr after PH significantly inhibited DNA synthesis. Thus, the inhibitory effect on cell proliferation is likely to be an important determinant of cancer promotion in rat liver. Many cancer promoters such as 2-acetylaminofluorene, phenobarbital, orotic acid, and ciprofibrate inhibit the epidermal growth factor (EGF)-induced mitogenic response in primary hepatocytes (*33*). This inhibitory effect—also known as mitoinhibition of the EGF response by FB_1—has been used to investigate possible mechanisms involved during cancer promotion (*41*). Binding of EGF to its receptor was not affected in hepatocytes exposed for 12 hr to FB_1. The inhibitory effect was also reversible; maximum inhibition seemed to occur late during the G1-phase of the cell cycle. Pretreatment of hepatocytes with FB_1 only marginally inhibited the EGF response, indicating that there is very little memory after the mycotoxin is removed. *In vivo* and *in vitro* experiments suggest that FB_1 behaves in a manner similar to most cancer promoters in inducing a growth differential that selectively stimulates the outgrowth of initiated cells.

Although a mechanism for cancer initiation has been proposed for FB_1 (see above), the process whereby FB_1 creates a growth differential in the liver that selectively favors the growth of initiated cells still needs to be elucidated. The final part of this review focuses on a mechanism that is likely to create such a "promoting" environment to sustain the process of differential inhibition.

Altered Lipid Biosynthesis as a Possible Mechanism for Cancer Promotion by the Fumonisins: A Hypothesis

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 ω6 and ω3 derived from linoleic acid (C18:2ω6) and α-linolenic acid (C18:3ω3) 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

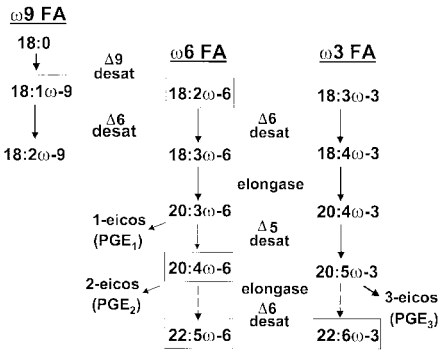

Figure 1. Essential fatty pathways indicating the key role of Δ6 desaturase enzyme and the fatty acids involved in the synthesis of different prostaglandins. The marked fatty acids (boxes) are likely to play a key role in the development of hepatocyte nodules.

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 ^{14}C palmitic acid decreased in the total lipids and the neutral lipids, triacylglycerol (TG), and the cholesterol esters at a noncytotoxic concentration of 150 μM FB_1 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 sphingolipid biosynthetic pathway (*53*). Fatty acid changes were restricted mainly to PC and TG with a decrease in the relative levels of C16:0 and the C16:1ω7 fatty acids and C18:1ω9. Changes in polyunsaturated fatty acids (PUFA) were restricted mainly to an increase in C18:2ω6 in TG and PC; C20:4ω6 also increased in both PC and TG. These changes were observed in cytotoxic and noncytotoxic dosage levels. As a result, the PUFA increased in both PC and TG. The increases in PC, PE, and PUFA are likely to counteract the shift to a more rigid membrane structure caused by the decrease in total cholesterol. The increase of C18:2ω6 could be related to the impairment of the Δ6 desaturase enzyme, the rate-limiting enzyme in fatty acid metabolism (Figure 1), and the increase in C20:4ω6 could be caused by the disruption of prostaglandin biosynthesis by inhibiting the cyclooxygenase enzyme. This was supported by the finding that the inhibition of monooxygenase by ibuprofen also inhibits the EGF mitogenic response (*54*). At lower concentrations of FB_1, the relative levels of C20:4ω6 were unaffected, whereas C18:2ω6 still increased, indicating that, depending on the dosage, different effects could be induced in primary hepatocytes. This implies that the disruption of the Δ6 desaturase could be an important early event that occurs in hepatocytes exposed to low concentrations of FB_1. The accumulation of C20:4ω6 was effected at dosages that significantly inhibit the EGF response (*41*). The inhibition of C20:4ω6 metabolism by FB_1 was further strengthened by the following: First, the addition of C20:4ω6 and C20:5ω3 to primary hepatocytes in the presence of EGF respectively stimulates and inhibits the mitogenic response; second, the mitoinhibitory effect of FB_1 was counteracted by the addition of prostaglandin E_2 (*54*). Changes to the fatty acid profiles of hepatocytes membranes and specifically C20:4ω6 also effected the mitogenic response. This was shown by the addition of C20:5ω3, which inhibits the EGF response presumably by replacing C20:4ω6 from the membrane phospholipids. The resulting formation of the prostaglandin E_3 series has fewer, even opposite, properties from those of the prostaglandin E_2 series that are produced from C20:4ω6 (*51*). These results suggest that C20:4ω6 is central to the regulation of the EGF response in primary hepatocytes. This was recognized earlier, as the disruption of C20:4ω6 metabolism affects the mitogenic response of EGF and hepatocyte growth factor (HGF) in primary hepatocytes (*55*).

In Vivo Studies in Rats

In vivo studies indicate that FB_1 disrupted lipid biosynthesis differently from that in the *in vitro* 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_1/kg increased the PE level, whereas SM decreased. No effect was noticed in the rats that received the 50 and 100 mg FB_1/kg diets. However, in long-term studies using BD IX rats, dietary levels of 1, 10, and 25 mg/kg increased the PE levels in the liver. Fatty acid analyses of the PE and PC fractions of the liver indicated that, as was shown in the *in vitro* study, the relative level of C18:2ω6 increased in PE and markedly in the PC fraction of the rats from the short-term study. The level of C20:4ω6 was not altered, although there was a marked decrease in the PC fraction using the 250 mg/kg dietary level. C22:5ω6, the end product in the n-6 metabolic pathway, was reduced in PE in the liver of the rats that received the 100 and 250 mg/kg diets. A similar pattern was noticed in the liver of rats of the long-term study in both the PC and PE fractions, but because of the small numbers of rats (3 per group) used for analyses, these changes were not significant. However, analysis of the total fatty acid profiles of the liver, using a larger number of rats per group (4 to 6 animals/group), indicated that the relative levels of C18:2 increased. Fatty acid analyses of serum PC of both the short-term and long-term FB_1 feeding studies confirm the observations obtained in the liver: an increase in C18:2ω6 but a decrease in C20:4ω6 and C22:5ω6 in the short-term (50 mg FB_1/kg diet and higher) and long-term (10 mg/FB_1/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 FB_1 on the n-6 fatty metabolic pathway seems to rely to a greater or lesser extent on the dietary level of FB_1, 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_1/kg diet) of FB_1 fed for 21 days were evaluated on lipid metabolism in rat liver microsomal membranes (*57*). These dietary levels of FB_1 were used to investigate the cancer-promoting potential of the fumonisins in 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 C18:1ω9, increased significantly in the treated groups (100 mg FB_1/kg diet and higher) in all the phospholipids except phosphatidyl serine (PS). The relative (%) and absolute (μg) values of C18:2ω6 increased in the PC, PI, PS, and PE phospholipid fractions. C20:4ω6 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 C20:4ω6 in PE increased, presumably because of the prominent increase (> 2×) in the level of PE compared to that of the other phospholipids. A similar effect was noted with C22:4ω6 and C22:5ω6 in PC and PE. The relative values of the n-3 fatty acid, C22:6ω3, 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

increased phospholipid concentrations. The polyunsatured/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 membranal environment of FB_1 also expanded and included the plasma, mitochondrial, and nuclear membrane fractions of rat livers exposed to 250 mg FB_1/kg diet for 21 days. Some differences exist in the lipid profiles of the different membrane fractions with respect to the effect of FB_1 on the levels of cholesterol and PC and PE. In the plasma membrane and nucleus, only PE significantly increased; PC, SM, and cholesterol 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 FB_1 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 FB_1 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 tend 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 E_2 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)-α and tumor necrosis factor (TNF)-α (*61*), which together with the deregulation of c-*myc* expression could be important determinants during FB_1-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 FB_1. The effects of FB_1 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 FB_1, 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 FB_1 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 FB_1 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 FB_1-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 FB_1, 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 FB_1. Very little is known about the nodules induced by FB_1 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 FB_1 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 FB_1-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 FB_1 (*36*). Immunostaining with LC(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 FB_1 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 FB_1, 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, FB_1 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 FB_1 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 FB_1 over a period of two years (*19*). In male Fischer rats fed FB_1 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 FB_1-treated samples of cyclin-dependent kinase (Cdk)4 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 FB_1-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 FB_1 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/phenobarbital (DEN/PB) (*71,72*) were compared to those induced by FB_1. The cyclin D1 overexpression, characteristic of FB_1-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 FB_1–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 FB_1. 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 FB_1. The modulating effects of FB_1 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 FB_1 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*). FB_1 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 apototic cell death elicited by TGF-β1 (*76*). In a recent study in an esophageal cancer cell line, C20:4ω6 and its cyclooxygenase products prostaglandin E_2 and prostaglandin A_2 induced apoptosis, a process that was inhibited by FB_1 (*77*). The effect of FB_1 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 FB_1 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). FB_1 effects a similar phospholipid 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 TGFβ-1 and c-*myc* could effect apoptosis. Oxidative damage and the resultant lipid peroxidative products could also further enhance apoptosis

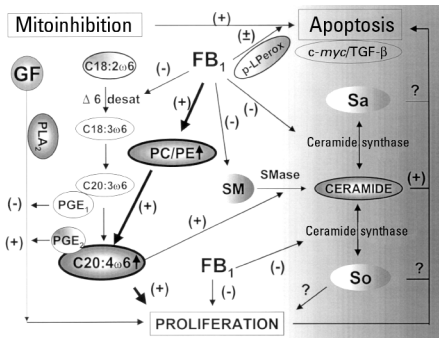

Figure 2. Diagram illustrating the role of FB_1 on lipid, sphingolipid, and fatty acid metabolism as a model for the enhanced proliferation in hepatocyte nodules (bold arrows). Abbreviations: PLA_2, phospholipase A_2; p-LPerox, products of lipid peroxidation. Inhibition of growth of normal cells seems to be related to the disruption of the ω6 fatty acid metabolic pathway, involving the Δ6 desaturase enzyme and PGE_2. Subsequent effects regarding the growth regulation (inhibition) in normal cells included the disruption of growth factor responses with the induction of apoptosis, involving p-LPerox, overexpression of c-*myc*/TGF-β, and the disruption of sphingolipid metabolism via the inhibition of ceramide synthase by FB_1.

in the liver (*79*); on the other hand, the increased C18:1ω9 and C20:4ω6 fatty acids and the decrease in PUFA are critical parameters likely to favor cell proliferation (*60,62*), especially in the initiated cell population.

The disruption of the phospholipid and n-6 fatty acid metabolic pathway, producing changes in the level of C20:4ω6, appears to be critical with respect to cancer promotion, especially at low dietary levels of FB_1, 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 C20:4ω6 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 eventually cause development of the cancer phenotype in the liver.

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