Sphingolipid Perturbations as Mechanisms for Fumonisin Carcinogenesis

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There is a great deal of evidence that altered sphingolipid metabolism is associated with fumonisin-induced animal diseases including increased apoptotic and oncotic necrosis, and carcinogenesis in rodent liver and kidney. The biochemical consequences of fumonisin disruption of sphingolipid metabolism most likely to alter cell regulation are increased free sphingoid bases and their 1-phosphates, alterations in complex sphingolipids, and decreased ceramide (CER) biosynthesis. Because free sphingoid bases and CER can induce cell death, the fumonisin inhibition of CER synthase can inhibit cell death induced by CER but promote free sphingoid base-induced cell death. Theoretically, at any time the balance between the intracellular concentration of effectors that protect cells from apoptosis (decreased CER, increased sphingosine 1-phosphate) and those that induce apoptosis (increased CER, free sphingoid bases, altered fatty acids) will determine the cellular response. Because the balance between the rates of apoptosis and proliferation is important in tumorigenesis, cells sensitive to the proliferative effect of decreased CER and increased sphingosine 1-phosphate may be selected to survive and proliferate when free sphingoid base concentration is not growth inhibitory. Conversely, when the increase in free sphingoid bases exceeds a cell's ability to convert sphinganine/sphingosine to dihydroceramide/CER or their sphingoid base 1-phosphate, then free sphingoid bases will accumulate. In this case cells that are sensitive to sphingoid base-induced growth arrest will die and insensitive cells will survive. If the cells selected to die are normal phenotypes and the cells selected to survive are abnormal, then cancer risk will increase. *Key words*: carcinogenesis, ceramide, corn, fumonisin, *Fusarium moniliforme,* glycosphingolipids, sphinganine, sphingolipid, sphingosine, sphingosine 1-phosphate.
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Introduction

The precise mechanism by which fumonisin B_1 (FB_1) induces increased apoptotic and oncotic necrosis and ultimately tumors in rodent liver and kidney is unclear. Several biochemical modes of action have been proposed. Two of these invoke disruption of lipid metabolism as either the initial or a key event in the cascade of molecular changes leading to the diseases associated with exposure to toxic concentrations of fumonisins. Currently a great deal of correlative evidence from *in vivo* studies supports the premise that altered sphingolipid metabolism is closely associated with the liver and kidney toxicity observed in rodents and farm animals:

- Equids: Free sphingoid bases increase in serum, liver, and kidney, and more complex sphingolipids decrease in liver and kidney before indications of hepatotoxicity (*1,2*).
- Pigs: Dose-dependent increase in free sphingoid bases in serum and liver and decreased complex sphingolipids in liver are correlated with hepatotoxicity; the increase in free sphingoid bases in liver, kidney, and lung precedes the onset of hepatotoxicity and pulmonary edema (*3–6*).
- Rats: Free sphingoid base concentration in serum, urine, liver, or kidney and decreased complex sphingolipids in liver and kidney are correlated with the extent and severity of the hepatotoxicity and/or nephrotoxicity or other indicators of cytotoxicity (*7–12*).
- Mice: Free sphingoid base concentration in liver and kidney is correlated with increased apoptosis and oncosis in liver and kidney [(*13–17*) and Figure 1].
- Trout: Free sphingoid base concentration in liver is correlated with promotion of tumors in aflatoxin B_1-initiated trout fed FB_1 (Figure 2).

Numerous studies also hypothesize fumonisin-induced changes in key enzymes involved in cell cycle regulation, differentiation, and/or apoptosis as initial or secondary sites of action:

Alterations in key enzymes or effectors of cell cycle progression and apoptosis:

- Altered expression or activity of protein kinase C, altered phorbol dibutyrate binding (*18,19*)
- Activation of the mitogen-activated protein kinase (*20*)
- Inhibition of serine/threonine phosphatases (*21*)
- Altered expression of cyclins, cyclin-dependent kinases, and dephosphorylation of the retinoblastoma protein (*22,23*)
- Overexpression of transforming growth factor-β1 and c-*myc* in rat liver (*24*)
- Apoptosis inhibitor and protease inhibitor protection from apoptosis (*25*)

Alterations in processes often associated with increased cellular/organ toxicity:

- Increased tumor necrosis factor (TNF)-α secretion in lipopolysaccharide-activated macrophages (*26*)
- Altered calcium homeostasis (*27*)
- Alterations in antioxidants, increased lipid peroxidation, alterations in saturation of fatty acids and other lipid changes (*8,28–33*)
- Stimulation of nitric oxide production (*34*).

Many of these latter studies have been conducted using cultured cells or other *in vitro* systems. In some cases changes in sphingolipids were measured; however, a causal link was not established. This review summarizes the evidence that supports disrupted sphingolipid metabolism as a contributing factor in fumonisin-induced diseases and discusses how disruption of sphingolipid metabolism can alter the rates of cell death and proliferation and thus contribute to the increased cancer risk in the liver and kidney of rodents.

Disruption of Sphingolipid Metabolism

The pathway of *de novo* sphingolipid biosynthesis begins with the condensation of serine with palmitoyl-CoA and proceeds rapidly to the biosynthesis of ceramide (CER) and more complex sphingolipids (Figure 3A). The turnover of more complex sphingolipids results in the production of CER, sphingosine, and sphingosine 1-phosphate (Figure 3A), which are either proven or suspected lipid-signaling molecules [for review see Hannun and Luberto (*35*) and Spiegel (*36*)]. Fumonisins potently

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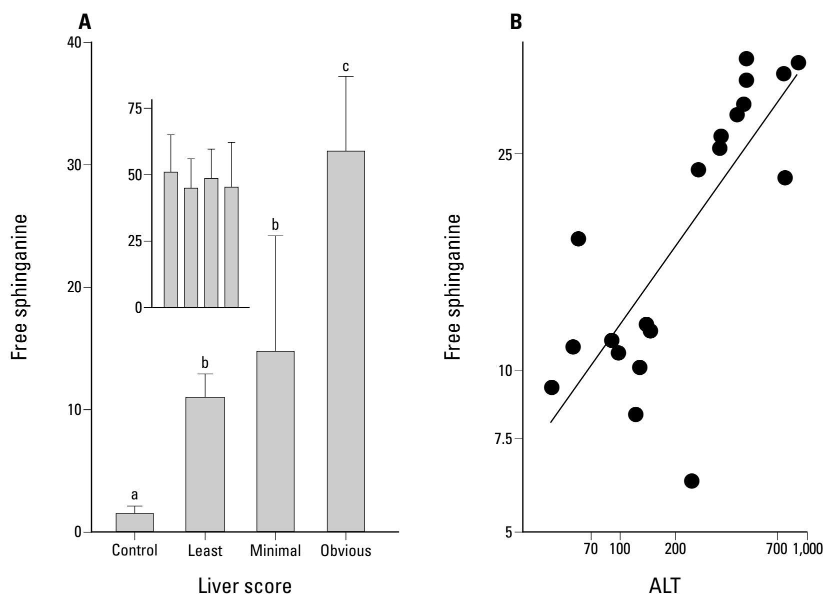

Figure 1. An example of the close correlation between fumonisin-induced disruption of sphingolipid metabolism and onset of toxicity *in vivo* in six strains of mice (*14–17*). The correlation between free sphinganine (nanomoles per gram fresh weight) in liver and (*A*) histopathologic findings (liver scores from hematoxylin- and eosin–stained sections) in livers, and (*B*) liver enzymes (ALT= alanine aminotransferase, units/liter) in serum from six (3–4 of each) different types of male mice dosed subcutaneously with 2.25 mg FB_1/kg body weight/day for 5 days. FVB, FVB mdr1a/b, C57BL/6, C57BL/6NTacfBR-[Tg]TNF mice were obtained from Taconic Farms, Germantown, NY; and C57BL/6J and C57Bl6-*Tnfrsf 1b*^{tm1Mwm} were obtained from Jackson Laboratories, Bar Harbor, ME. Livers from concurrent controls (n = 21) and fumonisin-treated mice (n = 22) were examined without regard to identity and subjectively placed in one of three categories: *a*) normal or least affected—morphologically unremarkable livers containing no or only a few scattered apoptotic hepatocytes ("least" affected were normal-appearing FB_1-treated mice after all readings were complete); and *b*) "minimal" or *c*) "obvious," depending on the amount of apoptosis, mitosis, cytoplasmic vacuolation, cytomegaly, differences in nucleus to cytoplasmic ratio, and necrosis found. All control livers were scored as "least" and are grouped together for comparison to the fumonsin-treated livers. Inset in (*A*) are the total thiobarbituric acid–reactive substances (TBARS) in nanograms malondialdehyde equivalents/20 mg fresh weight for the same liver score groups (control, least, minimal, obvious). The free sphinganine was analyzed as described in Riley et al. (*83*) and TBARS as per Abel and Gelderblom (*33*). Additional details of the dosing regimen, criteria for liver scores, and rationale for selecting mouse strains have been described elsewhere (*14–17*). Care and treatment of animals were approved by the University of Georgia Animal Use Committee.

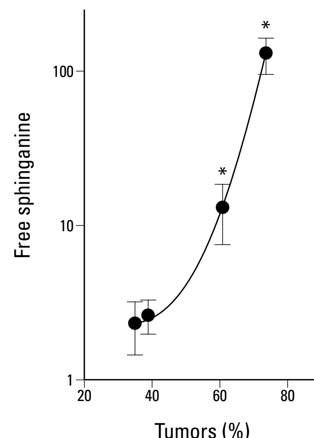

Figure 2. An example of the close correlation between fumonisin-induced disruption of sphingolipid metabolism and cancer promotion *in vivo* in trout (*90*). The correlation between free sphinganine (nanomoles per gram fresh liver weight) tumor incidence in liver from trout fry initiated by immersion in a bath of 100 ng/L of aflatoxin B_1 is presented. After a 1-month outgrowth period fish were fed 0, 5, 25, or 100 ppm FB_1 for 42 weeks before necropsy at 60 weeks. The free sphinganine values are those in livers at 24 weeks (n = 3/dose group) and the tumor incidence at 60 weeks. Methods for the diet formulation, fumonisin analysis, and sphingolipid analysis are given in Meredith et al. (*91*). Care and treatment of animals were approved by the Oregon State University Animal Use Committee.

inhibit the enzyme CER synthase (Figure 3B), which catalyzes the acylation of sphinganine and reacylation of sphingosine (*38–40*). CER synthase recognizes both the amino group (sphingoid-binding domain) and the tricarballylic acid side chains (fatty acyl CoA domain) of fumonisin B_1 (*41,42*). The reduced effectiveness of the hydrolyzed derivatives of the fumonisin B (FB) series (*40,43*) and the inability of pure *N*-acetylated FB_1 to inhibit CER synthase in cultured cells (*43*), support this hypothesis. There is a conflicting report about the ability of *N*-acetylated FB_1 to inhibit CER synthase (*44*). Differing from the results of Norred et al. (*43*), van der Westhuizen et al. (*44*) reported that in primary rat hepatocytes, *N*-acetylated FB_1 was an inhibitor of CER synthase. A possible explanation for this discrepancy is that *N*-acetylated FB_1 can decompose to its *O*-acetylated isomer and possibly to FB_1 during storage (*45*). Interestingly, aminopentol 1, but not FB_1, can be a substrate for CER synthase and the *N*-palmitoyl-aminopentol 1 may also inhibit CER synthase in cultured cells (*46*).

The binding of fumonisin to the catalytic site of CER synthase is the first event in the process referred to as "disruption of sphingolipid metabolism." A common misconception is that inhibition of CER synthase and elevation of free sphingoid bases occur concurrently and are synonymous. The term "fumonisin disruption of sphingolipid metabolism" encompasses all the changes that can occur in the biosynthetic rates and intracellular concentrations of the intermediates and end products within both the *de novo* sphingolipid pathway and branch pathways (such as those leading to changes in biosynthesis of glycerophospholipids and neutral lipids). The biochemical consequences of fumonisin disruption of sphingolipid metabolism (Figure 4) that are most likely to lead to altered cell regulation are: altered CER biosynthesis [for review see Hannun and Luberto (*35*) and Kolsnick and Krönke (*37*)], increased intracellular concentration of free sphingoid bases and their 1-phosphates [for review see Spiegel (*36*) and Merrill et al. (*47*)], and alterations in the cellular concentration of specific glycosphingolipids [for review see Ledeen et al. (*48*)].

Increased Free Sphingoid Bases

The complete inhibition of CER synthase by fumonisins causes the intracellular sphinganine concentration to increase rapidly (*38,39*). However, before this can occur, the capacity of sphingosine kinase to degrade free sphinganine must be exceeded. It is possible that partial inhibition of CER synthase could increase the rate of sphingoid base 1-phosphate biosynthesis without any apparent increase in the free sphinganine concentration. Free sphingosine concentration may also increase through fumonisin inhibition of reacylation of sphingosine derived from sphingolipid turnover or dietary sources/growth medium (Figure 3B). Nonetheless, when toxicity is evident, approximately 95% of the increase in free sphingoid bases in tissues and cultured cells is caused by the increase in free sphinganine (*39*) or sphingoid bases other than sphingosine (*49*).

In cultured cells the increase in free sphinganine and the decreased incorporation of radiolabeled serine or fatty acids into complex sphingolipids can be detected within a few hours after adding fumonisin (*38,39,40*). In mice dosed once subcutaneously with FB_1, the free sphinganine concentration in liver and kidney was significantly increased within 2 hr of dosing and in liver returned to the control concentration after 24 hr (*50*).

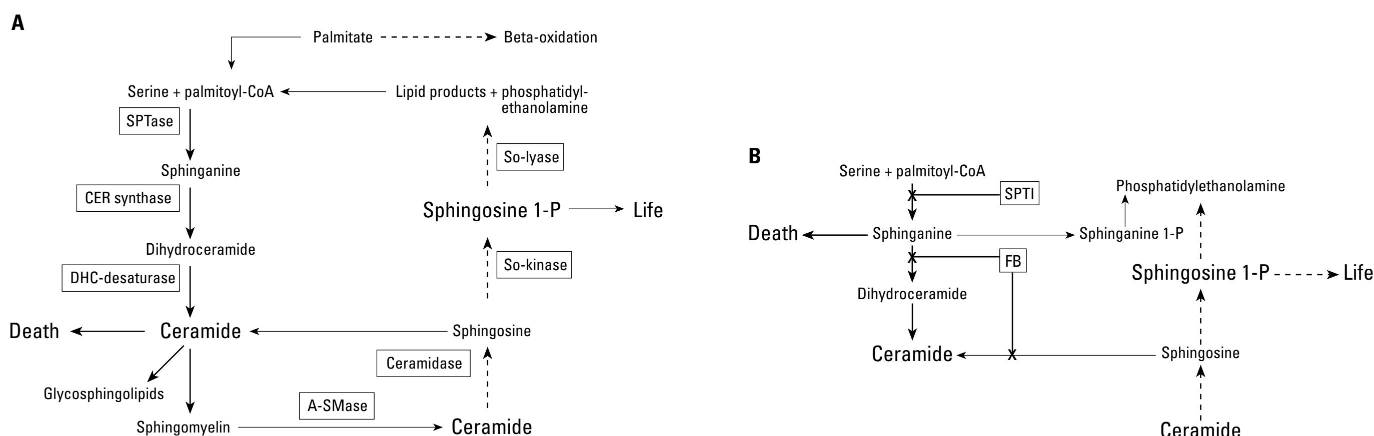

Figure 3. (*A*) The pathway of *de novo* sphingolipid biosynthesis and turnover in a mammalian cell. Abbreviations: A-SMase, acidic sphingomyelinase; DHC-desaturase, dihydroceramide desaturase; FB, ceramide synthase inhibitor; SPTase, serine palmitoyltransferase; SPTI, serine palmitoyltransferase inhibitor. Large solid arrows indicate the enzymatic steps leading to biosynthesis of CER, a known effector of cell death [for review see Hannun and Luberto (*35*)], and large broken arrows show the enzymatic steps leading to the production of SPP, an effector of cell survival or "life" [for review see Spiegel (*36*)]. Free sphinganine is an intermediate and not an end product in the sphingolipid biosynthetic pathway. Thus, in cells that have not been exposed to fumonisin, the free sphinganine concentration is low (< 0.5–3 nmol/g wet weight) and therefore not toxic. Free sphingosine concentration is also low; however, it is not an intermediate in the *de novo* pathway in mammalian cells but is formed as a consequence of sphingolipid turnover. Also shown is the proposed role of mitochondrial perturbations triggering a redirection of palmitate from beta-oxidation into the *de novo* pathway, producing increased biosynthesis of CER under conditions of oxidative stress [for review see Kolesnick and Krönke (*37*)]. (*B*) The sites of action of SPTIs such as ISP-I, and CER synthase inhibitors such as fumonisins (B and C series and their aminopentols). Also shown is the consequence of inhibition of CER synthase under conditions that allow the accumulation of toxic levels (> 12–150 nmol/g wet weight) of free sphinganine (Death). The block on the CER synthase responsible for its reacylation produces an increase in free sphingosine and possibly SPP. However, sphinganine 1-phosphate does not exert a marked cytoprotective effect but binds to and signals via the G protein–coupled receptor encoded by endothelial differentiation gene 1 (*36*). Also shown in (*A*) is the generation of CER by ligand-induced sphingomyelin hydrolysis [for review see Hannun and Luberto (*35*)].

However, in kidney the free sphinganine concentration remained significantly elevated after 48 hr (*50*) but returned to control levels after about 96 hr (*51*).

The half-life of the accumulated free sphinganine inside LLC-PK_1 renal epithelial cells is much longer than the half-life of FB_1 in cells (*49*), which suggests that either the inhibition of sphinganine *N*-acyltransferase is persistent, sphinganine metabolism is slow, and/or sphinganine does not easily diffuse out of cells. When the serine palmitoyltransferase inhibitor produced by *Isaria Sinclairii* (ISP-I =myriocin=thermozymozydin) is added to fumonisin-treated renal cells, the free sphinganine concentration returns rapidly to control concentrations (Figure 5), indicating that the accumulation of free sphingoid bases in renal cells is a consequence of the differences in the kinetics of serine palmitoyltransferase compared to those of the enzymes in the degradative pathway [sphingosine (So-) kinase and So-lyase]. Elevated free sphinganine may also persist in kidney *in vivo*, given that free sphinganine was detected in dead cells collected from rat urine (*7*), free sphinganine in rat urine remained elevated for approximately 10 days after the rats were taken off diets containing FB_1 (*55*), and free sphinganine remained elevated in mouse kidney after it had returned to control levels in the small intestines and liver (*50,51*).

Proliferating renal cells accumulate much higher concentrations of free sphingoid bases than confluent monolayers, and cytotoxicity is

↓ *De novo* sphingosine and ceramide biosynthesis
↑ Free sphingoid base concentration
↓ Glycosphingolipids and *de novo* GSL biosynthesis
↑ GSL biosynthesis via the recycling pathway
↑ Sphingoid base 1-phosphate biosynthesis and concentration
↑ Phosphatidylethanolamine biosynthesis and fatty acid precursors

Figure 4. The biochemical consequences of fumonisin inhibition of CER. GSL, glycosphingolipid. Arrows indicate the direction of the change in either the concentration of the biochemical pool or the rate of biosynthesis of the intermediate or end product.

most readily detected in rapidly proliferating or growing cells (*53,56*). Increased concentration of free sphinganine is also seen in the livers of partially hepatectomized, fumonisin-treated rats relative to the sham-operated fumonisin-treated rat livers (*57*). This probably occurs because the activity of the rate limiting enzyme, serine palmitoyltransferase, is maturationally expressed and is much more active in subconfluent, undifferentiated, metabolically active cells and tissues. Because of the slower turnover rate of more complex sphingolipids, free sphingoid base concentration increases in fumonisin-exposed LLC-PK_1 renal epithelial cells and in liver and kidney *in vivo* before the complex sphingolipid concentration decreases (*3,7,13,53*). However, even though the complex sphingolipid pool size may change slowly, the rate of *de novo* sphingolipid biosynthesis is directly related to the extent of inhibition of CER synthase. Thus, cellular processes dependent on glucosylceramide biosynthesis could be affected with no apparent decrease in glycosphingolipid pool size.

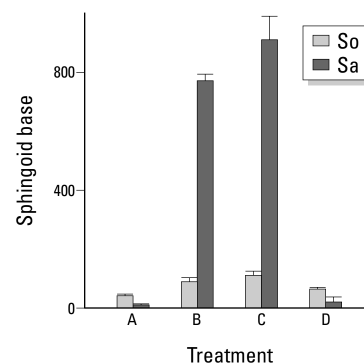

Figure 5. An example of how the activity of enzymes in the *de novo* and turnover pathways can influence the accumulation of sphingoid bases (picomoles per milligram protein) and their metabolism (*52*). Abbreviations: So, sphingosine; Sa, sphinganine. Inhibition of SPTase rapidly returns free sphinganine to control concentrations, indicating that the rate of sphinganine biosynthesis exceeds the rate of sphinganine metabolism in LLC-PK_1 renal epithelial cells. Confluent LLC-PK_1 renal epithelial cells grown in 24-well culture plates (2 cm^2/well) were exposed to fumonisin B_1 (25 µM) for 48 hr. Group A: control-treated (not exposed to fumonisin B_1) at 48 hr. Group B: fumonisin B_1-treated for 48 hr. Group C: fumonisin B_1-treated for 48 hr; then culture medium was replaced with medium without fumonisin B_1 and incubated for an additional 6 hr. Group D: fumonisin B_1-treated for 48 hr; then culture medium was replaced with medium without fumonisin B_1 but with 150 nM ISP-I and incubated for an additional 6 hr. Free sphinganine was analyzed and LLC-PK_1 renal epithelial cells were handled as described in Yoo et al. (*53*). ISP-I was isolated and purified as described in Riley and Plattner (*54*).

Fumonisin exposure also leads to imbalances in phosphoglycerolipid and fatty acid metabolism *in vitro* (*8,38,40*). In fumonisin-treated hepatocytes a portion of the accumulated sphinganine is metabolized to sphinganine 1-phosphate (SPP) and then cleaved into a fatty aldehyde and ethanolamine phosphate (*40*), both of which can be redirected into other biosynthetic pathways. It has been estimated that in fumonisin-treated cells, about one-third of the ethanolamine in phosphatidylethanolamine is derived from long-chain sphingoid base catabolism (*58,59*). The concentration of phosphatidylethanolamine also increases in the liver of rats fed fumonisins (*8*). The ability of cells to rapidly metabolize bioactive sphingoid bases into less bioactive products or into products such as SPP may protect cells from the toxicities associated with either elevated free sphingoid bases or CER (*36*). In mammalian cells, as in yeast, the balance between the endogenous CER and SPP concentration determines whether a cell dies or survives (*36*). This balance is maintained by the relative activity of various key enzymes in the *de novo* and sphingolipid turnover pathways (Figure 3A).

Although *in vivo* studies have found a close correlation between disrupted sphingolipid metabolism and the onset and progression of liver and kidney toxicity, no definitive study *in vivo* has shown that disrupted sphingolipid metabolism is the cause of the increased apoptosis observed in liver and kidney *in vivo*. Several *in vitro* studies with cultured cells have shown that reduction of free sphinganine with ISP-Is completely or partially reverses the effects of FB_1 on cell growth, differentiation, and/or cell death. Free sphingoid bases induce cell death and altered cell growth:

- Sphingoid bases are growth inhibitory, cytotoxic, and induce apoptosis (*53, 60–66*).
- Sphingoid bases or their metabolites can be growth stimulating [for review see Spiegel (*36*)].

Fumonisin effects on cell growth and cell death are reversed by serine palmitoyltransferase inhibitors (SPTI) of sphinganine accumulation:

- SPTI reverses FB inhibition of cell growth and increased cell death and apoptosis in pig renal cells, human colonic cells, primary human keratinocytes (*39,56,65,67*).
- SPTI reverses FB-induced stimulation of $[^3H]$thymidine incorporation in Swiss 3T3 cells (*68*).

Other fumonisin effects closely correlated with elevated free sphingoid bases:

- Endothelial cell permeability (*69*)
- Protein kinase C modulation (*70*).

Other studies demonstrate that supplementation of growth medium with CER or more complex sphingolipids also can completely or partially reverse the effects of FB_1 on cell growth, differentiation, and/or cell death:

- Decreased axonal growth, morphologic changes in fibroblasts, and altered growth factor stimulation of axonal growth are reversed by addition of CER or gangliosides (*71–73*)
- Cell substratum adhesion (*74*)
- Glycosylphophatidylinositol-anchored protein functions such as the folate receptor (*75,76*)
- Assembly and disassembly of cytoskeletal proteins responsible for lipid transport and maintenance of the subcellular architecture (*77*)
- Biosynthesis and retrograde transport of attachment sites for microbial pathogens and toxins and protein transport to the plasma membrane (*78,79*)
- Multidrug resistance in certain cancer cells (*80*)
- Glucosylceramide synthesis is required for axonal growth and chick embryo development (*81,82*).

Supporting the notion that fumonisin-induced alterations in sphingolipid metabolism can lead to increased apoptosis and altered cell proliferation is the fact that sphingolipids and their metabolites are known regulators of cell growth, differentiation, apoptosis, necrosis, and immune response:

Sphingoid bases and their metabolites [for review see Spiegel (*36*) and Merrill et al. (*47,84*)]:

- Inhibition of protein kinase C, activation of proteases
- Activation of phospholipase D/inhibition of phosphatidic acid phosphatase
- Activation of the epidermal growth factor receptor kinase (probably via mitogen-activated protein kinase)
- Control of intracellular calcium (seemingly via SPP)
- Control of plasma membrane potassium permeability in myocytes
- Inhibition of DNA primase and increases in transcription factor activator protein-1
- Ligand (SPP) for the endothelial differentiation gene 1 receptor.

CER [for review see Hannun and Luberto (*35*) and Kolesnick and Krönke (*37*):

- Second messenger in cytokine signal transduction
- Activity of protein kinases, phosphatases, and mitogen-activated protein kinases
- Activity of phospholipase D, cytosolic phospholipase A2
- Transcription factor nuclear factor kappa B
- CPP32-like caspases.

More complex sphingolipids [for review see Futerman (*85*), Yates and Rampersaud (*86*), and Radin (*87*)]:

- Binding of cytoskeletal proteins
- Participation in cell–cell communication and cell–substratum interactions
- Receptor-mediated transport and sorting by lipid rafts
- Modulation of growth factor receptors.

Also, mutants defective in sphingolipid biosynthesis are growth suppressed (*88*). Because of the large number of regulatory processes known to be affected by sphingolipids and the crosstalk between sphingolipid and glycerophospholipid signaling pathways, unraveling the downstream mechanisms by which fumonisin-induced sphingolipid alterations cause tissue damage will be extremely challenging.

In Sprague-Dawley and Fischer 344 rats, New Zealand white rabbits, and BALB/c and other mouse strains, disruption of sphingolipid metabolism (as evidenced by increased free sphinganine concentration) occurs at fumonisin dosages that do not cause morphologic evidence of injury (*7,8,10–13,89*). Where liver pathology is observed, there is a close correlation between the incidence and severity of the pathology and the increase in free sphinganine indicative of disrupted sphingolipid metabolism (for example, see Figures 1 and 2). In mice, this occurs with no apparent increase in lipid peroxidation (Figure 1A, inset). In rats (*92*) and rabbits (*93*), the kidney is extremely sensitive to fumonisin-induced nephrotoxicity. Voss et al. (*92*) found the dietary no-observed-effect level (NOEL) for nephrosis in male Fischer 344 rats was 3 parts per million (ppm), whereas increased renal free sphinganine has been found in male Sprague-Dawley rats fed AIN-76 diets containing 1 ppm FB_1 (*55*). In male RIVM:WU rats, liver free sphinganine was significantly elevated at > 0.19 < 0.75 mg FB_1/kg body weight (equivalent to 1.9 and 7.5 ppm dietary FB_1). The increase occurred in the absence of any evidence of hepatosis (*11*). The low-observed-effect level for tubular cell death and significant increases in kidney free sphinganine was < 0.19 mg FB_1/kg body weight, which was equivalent to < 1.9 ppm in feed (*11*).

In Sprague-Dawley rat urine, free sphinganine accumulation in dead cells closely reflected the changes in sphingolipids and nephrotoxicity that occurred in the kidney (*7*). The results of the recently completed U.S. Food and Drug Administration long-term feeding study in rats confirms these findings (*12*). Feeding studies with pure FB_1 in American Institute of Nutrition-76 diets indicate that the NOEL for elevation of urinary free sphinganine in Sprague-Dawley rats is 1 ppm (*55*). Once elevated by feeding an apparently nephrotoxic concentration of FB_1, an apparently non-nephrotoxic concentration (1 ppm) will keep the free sphinganine

concentration at a level approaching that of the nephrotoxic fumonisin dosage (10 ppm) (*55*). Nonetheless, the elevation in free sphingoid bases and the associated fumonisin-induced toxicities are reversible (*94*).

Alterations in Complex Sphingolipids

Inhibition of sphinganine (sphingosine) *N*-acyltransferase (CER synthase) in cells also leads to a concentration-dependent reduction in more complex sphingolipids (*41,53*), with sphingomyelin biosynthesis being inhibited earlier and at lower fumonisin concentrations than glycosphingolipid biosynthesis (*41*). There is no doubt that the loss of complex sphingolipids also plays a role in the abnormal behavior and altered morphology of fumonisin-treated cells. For example, in the LLC-PK_1 renal cells, the morphologic changes such as decreased cell–cell contact and increased fibroblast-like appearance are not reversed using ISP-Is, suggesting that they are due to depletion of more complex sphingolipids (*67*) or other factors.

Glycosphingolipid changes also modulate apoptosis. For example, fumonisin inhibition of glycosphingolipid biosynthesis will protect cells from the death induced by Shiga-like toxins (SLT) in combination with butyric acid, which sensitizes cells to SLT-induced apoptosis (*78*). SLT (B subunit) ligation of globoside Gb3 has been shown to induce apoptosis (*95*). Recent studies have shown that endotoxin and cytokines can increase serine palmitoyltransferase activity (*96*), a process that can sensitize cells to *de novo* CER- or sphingoid base-induced cell death. In LLC-PK_1 renal epithelial cells, treatment with SLT and endotoxin-containing bacterial lysates had little effect on cell viability in the absence of the proinflammatory cytokine, TNF-α (Figure 6). However, cell death was increased markedly in cultures treated with the bacterial lysates when followed by TNF-α and this increased cell death is prevented by FB_1 and/or ISP-I (Figure 6). Thus, fumonisin inhibition of *de novo* sphingolipids biosynthesis *in vivo* could alter the proapoptotic signals mediated by as yet unidentified endogenous ligands for sphingolipid receptors. Fumonisins could also protect cells from the apoptosis associated with microbial toxins and the sensitization induced by cytokines or other factors that stimulate *de novo* sphingolipid biosynthesis.

Decreased CER Biosynthesis

The inhibition of CER biosynthesis necessarily precedes any change in the intracellular concentration of intermediates or end products in the sphingolipid biosynthetic process. Because increased CER concentration is an important signal in the induction of cell death, the inhibition of CER synthase by fumonisins can inhibit cell death induced by CER in short-term experiments, whereas prolonged inhibition will promote free sphingoid base-induced cell death if free sphingoid bases accumulate to toxic concentrations. Fumonisin inhibition of CER synthesis may also regulate numerous other critical cell regulatory functions that lead to altered proliferation and cell death. For example, FB_1 protects cells from stress-induced apoptosis that is mediated by increased *de novo* CER biosynthesis:

- Sphingosine-induced germinal vesicle breakdown and oocyte maturation (*98*)
- Apoptosis induced by pharmacologic agents (*99–103*)
- Carnitine palmitoyltransferase inhibition-induced apoptosis (*104*)
- Lipopolysaccharide/platelet-activating factor-induced arachidonic acid release (*105*)
- Chemical hypoxia-induced cell death (*106*)
- CD95 antigen-transduced, caspase-dependent T-cell proliferation (*107*)
- Serum-stimulated retinoblastoma protein dephosphorylation and cell cycle progression (*108*)
- Multidrug resistance modulator-dependent cytotoxicity (*109*)
- TNF-α/cycloheximide-induced endothelial cell death (*110*)
- 12-*O*-tetradecanoylphorbol-13-acetate-induced apoptosis (*111*)
- Fatty acid-induced nitric oxide synthase-dependent apoptosis (*112*)
- Ionizing radiation-induced DNA damage and cell death (*113*).

The ability of fumonisin inhibition of CER biosynthesis to protect cells is of considerable interest because primary rat hepatocyte necrotic cell death is mediated by CER (but not dihydroceramide) mitochondrial dysfunction (*114*), and dihydroceramide desaturase activity depends on the redox state of the cell (*115*). The ability of fumonisins to protect oxidant-damaged cells from CER-mediated apoptosis could also result in an accumulation of cells with damaged DNA.

Many of the processes that stimulate apoptosis via increased *de novo* CER biosynthesis appear to be caused by increased serine palmitoyltransferase activity. Ideally, specific inhibitors of serine palmitoyltransferase and inhibitors of CER synthase should be used in combination to test the hypothesis that *de novo* CER biosynthesis modulates cell function (*54*). The reason for this is that fumonisin inhibition of CER synthase causes changes in many lipid metabolites (Figure 4) that are known modulators of cellular regulation, including both inducers of cell death and promoters of cell survival. Use of serine palmitoyltransferase inhibitors in combination with CER synthase inhibitors will block all *de novo* fatty acyl CoA-dependent sphingolipid biosynthesis and CER biosynthesis that use free sphingoid bases derived from sphingolipid turnover or dietary sources. Nonetheless, use of fumonisins alone has revealed a great deal about the processes that are controlled by *de novo* sphingolipid biosynthetic pathways and thus reveals the potential of fumonisin to perturb cellular regulation. Specific inhibitors of serine palmitoyltransferase (myriocin) are commercially available, as are inhibitors of CER synthase (fumonisins).

Added to these sphingolipid-mediated effects is the likelihood that fumonisins target other processes that can also contribute to the observed cellular deregulation and increased cancer risk in rodent liver and kidney. In addition, other factors independent of fumonisins (diet, immune status, infectious agents, other toxins) can contribute to increased cancer risk. Although the *in vivo* evidence for most of these other proposed targets is currently only weakly supported, the evidence for fumonisin-induced disruption of sphingolipid metabolism in target tissues has been demonstrated repeatedly in many independent studies.

Possible Mechanism of Cancer Induction

Theoretically, at any specific time the balance between the intracellular concentration of

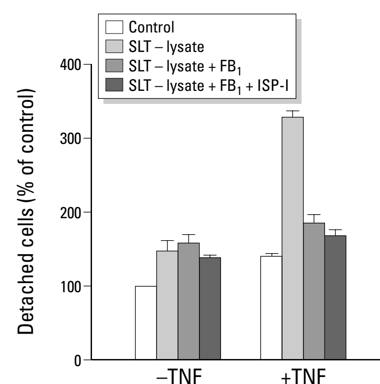

Figure 6. An example of how FB_1 can protect cells from cell death induced by treatments that require *de novo* sphingolipid biosynthesis as a critical step in the onset of toxicity (*97*). Confluent (24 well plates, 2 cm^2/well) LLC-PK_1 renal epithelial cells treated with phosphate-buffered saline vehicle (control), 20 ng/mL of a lysate of Shiga toxin–producing bacteria (SLT-lysate; Toxin Technologies, Inc., Sarasota, FL), SLT-lysate plus 150 μM FB_1 (+FB_1), or SLT-lysate plus FB_1 and ISP-I (450 nM) for 20 hr followed by addition of 10 ng/mL of TNF-α (+TNF) and additional incubation for 72 hr, and similar treatments without addition of TNF-α (-TNF). Neither the FB_1 or ISP-I alone caused any detachment of cells greater than the vehicle controls. Cell detachment was determined as described in Riley et al. (*67*). Values are means ± SD, *n* = 2 from one representative experiment. ISP-I alone also prevents the cytotoxicity of SLT-lysates plus TNF (data not shown).

sphingolipid effectors that protect cells from apoptosis (decreased CER, increased SPP) and the concentration of effectors that induce apoptosis (increased CER, free sphingoid bases, fatty acids, or specific glycosphingolipids) will determine the observed cellular response (Figure 3). *In vitro*, the response of different cell lines to fumonisin-induced disruption of sphingolipid metabolism is difficult to predict. Some cell lines responded to inhibition of CER synthase with increased apoptosis and decreased proliferation (*23,39, 53,56,65*), whereas in other cell lines inhibition of CER synthase increases proliferation (*68*) or has no effect on cell proliferation (*23*). In addition, CER synthase inhibition prevents the apoptotic effects of certain treatments. Thus, the effects of disrupted sphingolipid metabolism are cell type and treatment dependent. It has been proposed that for nongenotoxic (not DNA reactive) carcinogens, the balance between the rates of apoptosis and proliferation are critical determinants in tumorigenesis (*116,117*). Thus, in affected tissues cells sensitive to the proliferative effect of decreased CER and increased SPP (*36*) should be selected to survive and proliferate when the conditions under which the cells are exposed to fumonisins are such that increased free sphingoid base concentration does not inhibit growth. Conversely, when the rate of increase in free sphingoid bases exceeds a cell's ability to convert sphinganine/sphingosine to dihydroceramide/CER or their sphingoid base 1-phosphate, then free sphingoid bases will accumulate. In this latter case, cells sensitive to sphingoid base-induced growth arrest will cease growing, and insensitive cells will survive. In either case, if the cells selected to die are not DNA-damaged cells and the cells selected to survive are DNA damaged, then the relative abundance of the DNA-damaged cells will increase. The cancer risk will also increase because the probability of a transformed cell's surviving will have increased. In this theory, fumonisin acts to increase the population of cells that have preexisting DNA defects, and fumonisin is not itself DNA reactive. How these cells become DNA damaged is irrelevant if the end result is to increase their chance of survival. Another condition that could alter the balance between cell death and survival would be if the block on CER synthase were reduced or CER synthase expression were increased while free sphinganine levels were still quite high. In such a case, CER levels might increase rapidly to toxic levels. Similar results could occur with induction of serine palmitoyltransferase (increasing free sphinganine, as seen in rapidly dividing cells) or inhibition of enzymes in the degradative pathway. Several studies have shown that fumonisin inhibits *de novo* CER production and apoptosis resulting from treatments that induce stress sufficient to cause DNA damage (*104, 106,113*). Again, if DNA-damaged cells survive, one can imagine an increase in the population of DNA-damaged cells and an increased cancer risk.

In conclusion, we hypothesize that FB_1-induced alterations in sphingolipid signaling pathways will lead to altered rates of cell death and regeneration. In certain situations, CER synthase-inhibited cells that are DNA damaged may survive under conditions that would normally lead to their death from CER generated *de novo*. Conversely, CER synthase inhibition can lead to the accumulation of sphingoid bases and their metabolites, which would also alter rates of cell death and proliferation. Given the current lack of evidence for the DNA reactivity of fumonisins, the carcinogenic risk from fumonisin may *a*) be related to its ability to increase the chance of survival of cells that have been DNA damaged by other means; *b*) stimulate cell division directly (via SPP); *c*) increase regeneration in response to increased cell death (via sphingoid bases or depletion of more complex sphingolipids); or *d*) increase the chances of survival of preexisting DNA-damaged cells through an insensitivity to the apoptotic effects of disrupted sphingolipid metabolism.

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