CHAPTER 5

Maternal Fumonisin Exposure as a Risk Factor for Neural Tube Defects

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Contents

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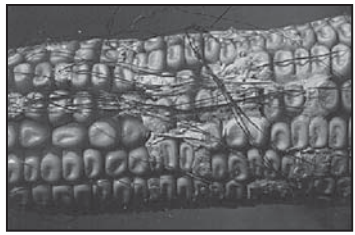
Abstract

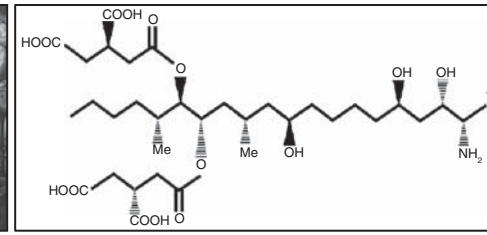
Fumonisins are mycotoxins produced by the fungus *F. verticillioides*, a common contaminant of maize (corn) worldwide. Maternal consumption of fumonisin B_1-contaminated maize during early pregnancy has recently been associated with increased risk for neural tube defects (NTDs) in human populations that rely heavily on maize as a dietary staple. Experimental administration of purified fumonisin to mice early in gestation also results in an increased incidence of NTDs in exposed offspring. Fumonisin inhibits the enzyme ceramide synthase in *de novo* sphingolipid biosynthesis, resulting in an elevation of free sphingoid bases and depletion of downstream glycosphingolipids. Increased sphingoid base metabolites (i.e., *sphinganine-1-phosphate*) may perturb signaling cascades involved in embryonic morphogenesis by functioning as ligands for sphingosine-1-P (S1P) receptors, a family of G-protein-coupled receptors that regulate key biological processes such as cell survival/proliferation, differentiation and migration. Fumonisin-induced depletion of glycosphingolipids impairs expression and function of the GPI-anchored folate receptor (Folr1), which may also contribute to adverse pregnancy outcomes. NTDs appear to be multifactorial in origin, involving complex gene-nutrient-environment interactions. Vitamin supplements containing folic acid have been shown to reduce the occurrence of NTDs, and may help protect the developing fetus from environmental teratogens. Fumonisins appear to be an environmental risk factor for birth defects, although other aspects of maternal nutrition and genetics play interactive roles in determining pregnancy outcome. Minimizing exposures to mycotoxins through enhanced agricultural practices, identifying biomarkers of exposure, characterizing mechanisms of toxicity, and improving maternal nutrition are all important strategies for reducing the NTD burden in susceptible human populations.

I. INTRODUCTION

Fumonisins are mycotoxins produced by the fungus *Fusarium verticillioides*, *F. proliferatum*, and more rarely, other *Fusarium* species (Bolger *et al.*, 2001; Gelderblom *et al.*, 1988) (Fig. 5.1A). They are found in variable amounts in maize (corn) and maize-based foods worldwide



Fusarium verticillioides



Fumonisin

FIGURE 5.1 The photograph on the left shows an ear of maize infected with the *Fusarium verticillioides* fungus. The mold is white to pinkish in color, and produces the fumonisin toxin. Decay often begins with insect-damaged kernels, however, levels of fumonisins capable of causing toxicity in rodents can occur in asymptomatic maize. The chemical structure shown on the right is that of the mycotoxin fumonisin B_1. Fumonisin was first isolated in 1988 by investigators at PROMEC (program on mycotoxins and experimental carcinogenesis) in Tygerberg, South Africa.

(Bolger *et al.*, 2001; Humpf and Voss, 2004) and their presence in other commodities has occasionally been reported (Castoria *et al.*, 2005; da Silva *et al.*, 2000; Kritzinger *et al.*, 2003). Consumption of fumonisin B_1-contaminated maize has been associated with a variety of different diseases in animals, including leukoencephalomalacia in horses (Smith *et al.*, 2002) and pulmonary edema in swine (Constable *et al.*, 2000, 2003; Haschek *et al.*, 2001). Independent studies have also established a causal relationship between fumonisin B_1 exposure and liver and kidney toxicity (Bolger *et al.*, 2001; Voss *et al.*, 2001), and/or liver and kidney carcinogenicity in rodents (Gelderblom *et al.*, 1991; Howard *et al.*, 2001a,b). The International Agency for Research on Cancer has evaluated fumonisin B_1 as a possible human carcinogen (Group 2B) (IARC, 2002). Although the human health effects of fumonisins are not proved, the consumption of foods made from *F. verticillioides*- or fumonisin B_1-contaminated maize has been associated with high rates of esophageal cancer in parts of southern Africa, China, and northeastern Italy (Bolger *et al.*, 2001, Shephard *et al.*, 2007). Elevated levels of fumonisin have also been found in maize from Mazandaran Province in Iran, another region with a high incidence of esophageal cancer (Shephard *et al.*, 2002; Yazdanapanah *et al.*, 2006). However, fumonisin exposures in this and other areas of Iran in which esophageal cancer is less frequent were low ($\leq$ 0.22 μg/kg body weight/day) due to the relatively small amount of maize consumed (Yazdanpanah *et al.*, 2006). In terms of human health risks, maternal consumption of fumonisin B_1-contaminated maize or maize-based food products during early gestation has recently been associated with increased risk for birth defects, specifically, neural tube

defects (NTDs) (Hendricks, 1999; Kromberg and Jenkins, 1982; Marasas *et al.*, 2004; Melnick and Marazita, 1998; Missmer *et al.*, 2006; Moore *et al.*, 1997; Ncayiyana, 1986; Xiao *et al.*, 1990).

II. NEURAL TUBE DEFECTS

A. Neural tube defects: Overview

Approximately 3–4% of all newborns have a significant abnormality in body structure or function. Birth defects (congenital anomalies) are the leading cause of death in children under 1 year of age. NTDs, which occur with a frequency of approximately 1/1000 live births (Nakano, 1973), are among the most common of all human birth defects, yet their etiologic basis and embryology remain poorly understood. NTDs are common congenital malformations that occur when the embryonic neural tube, which ultimately forms the brain and spinal cord, fails to properly close during the first few weeks of development. Anencephaly, which is essentially the absence of the brain, is invariably fatal, and results from failure of the anterior neural folds to properly elevate and fuse along the dorsal midline during early embryogenesis. Spina bifida refers to incomplete development of the posterior neural tube, and failure of fusion of one or more vertebral arches, often accompanied by protrusion of the spinal cord and its associated membranes. Patients with spina bifida can have a variety of associated conditions, the severity of which is often determined by the level of the lesion. Most commonly, patients with spina bifida have difficulty walking and require either braces or a wheelchair, have hydrocephalus requiring shunting, and/or have difficulty with bowel and bladder control. Empirical risk figures, along with numerous clinical and experimental studies, suggest that NTDs are multifactorial in origin, having genetic, environmental, and nutritional components that contribute to their prevalence (Campbell *et al.*, 1986).

B. Environmental risk factors for neural tube defects: Fumonisins

Maternal fumonisin exposure has been proposed as a potential risk factor for human NTDs among populations consuming large amounts of fumonisin-contaminated maize or maize-based products (Marasas *et al.*, 2004). Globally, NTDs present a tremendous burden to human populations in rural areas of the world where maize is a dietary staple, and fumonisin contamination is common (Hendricks, 1999; Kromberg and Jenkins, 1982; Marasas *et al.*, 2004; Melnick and Marazita, 1998; Moore *et al.*, 1997;

Ncayiyana, 1986; Torres *et al.*, 2007; Xiao *et al.*, 1990). The incidence of NTDs in these regions, including Guatemala, northern China, and the Transkei of South Africa, is often 6–10 times higher than the average global neural tube defect rate (Hendricks, 1999; Kromberg and Jenkins, 1982; Marasas *et al.*, 2004; Melnick and Marazita, 1998; Moore *et al.*, 1997; Ncayiyana, 1986; Xiao *et al.*, 1990). Since fumonisins are ubiquitous, and maize-based meals are often the primary food commodity available, human exposures can be significant (Marasas, 1996; Meredith *et al.*, 1999; Miller, 2001; Shephard *et al.*, 1996, 2007). In Guatemala, a recent analysis of fumonisin B_1 in maize samples, coupled with data on daily maize intake, demonstrated that human consumption of maize products could frequently result in fumonisin exposures exceeding the recommended World Health Organization (WHO) provisional maximal tolerable daily intake (Torres *et al.*, 2007). In the United States, the Texas Department of Health reported a neural tube defect cluster among Mexican-American women along the south Texas border in 1990–1991 (Hendricks, 1999). Corn crops in the Lower Rio Grande Valley registered unusually high levels of fumonisin B_1 during this period, and epidemiological studies revealed that Cameron County women who conceived during 1990–1991 had a substantially higher neural tube defect rate (2.7/1000 live births) than those who conceived during the period from 1986 to 1989 (1.5/1000 live births). Mexican-Americans in Texas consume large quantities of maize, primarily in the form of tortillas, and therefore may be exposed to high levels of fumonisins. Results of a follow-up population-based case-control study conducted among the south Texas Mexican-American population suggested that fumonisin exposure increased the risk of NTDs, proportionate to dose, up to a threshold level, at which point fetal death was postulated to be more likely to occur (Missmer *et al.*, 2006).

C. Nutritional risk factors for neural tube defects: Folic acid

The etiology of NTDs is largely unknown, but appears to be multifactorial in origin, involving complex gene-nutrient-environment interactions. Consumption of large quantities of fumonisin-contaminated maize or maize-based food products during early gestation may represent an environmental risk factor for having a child with a neural tube defect. However, other aspects of maternal nutrition are also important in ensuring a positive pregnancy outcome, and adequate maternal folate appears to play a critical role in protecting the developing fetus from the harmful effects of environmental teratogens. Diets heavily dependent on maize are likely to be folate deficient since maize contains only low levels of this vitamin (Burton *et al.*, 2008). In Mexico and Central America, maize-based foods are typically prepared using an alkaline process known as

nixtamalization (Palencia *et al.*, 2003) which has been shown to further reduce folate, riboflavin, and other vitamins (Burton *et al.*, 2008; Cárdenas *et al.*, 2001).

Folate is an essential vitamin derived from plant sources that plays an important role in DNA biosynthesis and amino acid metabolism. Multiple transport systems play a role in mediating internalization of folates through the plasma membrane into the cell for utilization in a variety of critical housekeeping functions. The primary mechanisms for folate delivery into the cell are through (1) carrier-mediated (reduced folate carrier; RFC1) or (2) receptor-mediated (folate receptor α, β, δ, γ) processes. These two transport systems are distinguished by their unique patterns of tissue expression, divergent specificities for oxidized vs. reduced folates, and differing protein structures and mechanisms for the transmembrane transport of folates. As mammalian cells are not capable of synthesizing folates *de novo*, these two transport systems play a critical role in mediating folate uptake for the biosynthesis of purines, pyrimidines, and some amino acids that are necessary for cell survival and proliferation.

The murine folate binding protein 1 (*Folr1*) is homologous to the human folate receptor-alpha (FRα), a membrane bound protein with high binding affinity for folic acid. During early development, *Folr1* is highly expressed in the placenta, yolk sac membrane, and dorsal neural tube, and is later observed in the choroid plexus, ependymal cells, and peripheral edge of the retina (Saitsu *et al.*, 2003). The glycosylphosphatidylinositol (GPI)-anchored *Folr1* internalizes folate via endocytosis, and is localized in unique sphingolipid-enriched plasma membrane microdomains known as lipid rafts (Elortza *et al.*, 2003; Foster *et al.*, 2003). Lipid rafts function as specialized platforms for coordinating protein–protein interactions involved in the initiation of signal transduction cascades and intracellular signaling (Brown and London, 1998). Since most embryonic cells do not express *Folr1* (Page *et al.*, 1993), the presence of these receptors in neuroepithelial cells (Barber *et al.*, 1999; Saitsu *et al.*, 2003) suggests a critical role for *Folr1*-mediated folate transport in the normal morphogenetic events involved in neural tube closure (Piedrahita *et al.*, 1999; Spiegelstein *et al.*, 2004).

Human clinical and epidemiological studies have demonstrated that maternal use of folic acid in early pregnancy can significantly reduce both the occurrence (Czeizel and Dudas, 1992), as well as the recurrence (MRC, 1991) of neural tube defect-affected pregnancies. These findings have been further validated by observational studies of women taking daily periconceptional multivitamin supplements containing folic acid (Shaw *et al.*, 1995; Werler *et al.*, 1993). However, while the epidemiologic and experimental data support the hypothesis that this apparent

reduction in neural tube defect risk may be specifically attributable to folic acid, the mechanisms underlying the protective effects of folic acid are not fully understood.

The incidence of NTDs is elevated in regions of the world (Guatemala, South Africa, China) where maize consumption with fumonisin is documented or plausible and where diets are also likely to be deficient in folate (Chu and Li, 1994; Cifuentes, 2002; Marasas, 2001; Marasas *et al.*, 2004; Shannon and Fenichel, 1990; Yoshizawa *et al.*, 1994). The high incidence of NTDs among Mexican-American women living in the U.S.–Mexico border region in Texas is likely related to maternal fumonisin exposure coupled with folate deficiency (Hendricks, 1999; Marasas *et al.*, 2004; Missmer *et al.*, 2006). Interestingly, fumonisin content in Guatemalan and U.S. maize samples has been shown to be similar in some years (Marasas *et al.*, 2004); however, Guatemalans typically consume more maize than Americans, resulting in significantly higher fumonisin exposures. Finally, other studies found a 50% decrease in the incidence of NTDs with folic acid supplementation in Mexico (Martinez de Villarreal *et al.*, 2002), suggesting that folic acid supplementation may offer some protection against the adverse effects of fumonisin exposure.

D. Genetic risk factors for neural tube defects

In addition to environmental and nutritional risk factors, there is clearly a genetic susceptibility component that contributes to the incidence of congenital malformations. Neural tube defect risk has been shown to vary across ethnic groups, suggesting that genetic variation (polymorphisms) may predispose particular individuals, or groups of individuals to NTDs. Several lines of evidence suggest a genetic component to this type of birth defect. First, NTDs are associated with known genetic syndromes, including Meckel syndrome, anterior sacral meningomyelocele, and anal stenosis. Secondly, in NTDs occurring without other syndromes, the recurrence risk for siblings is approximately 2–5%, which is up to a 50-fold increase over that observed in the general population. Khoury *et al.* (1988) have shown that for a recurrence risk to be this high, an environmental teratogen would have to increase the risk at least 100-fold to exhibit the same degree of familial aggregation, making a genetic component essentially required. Additionally, a variety of mutations leading to NTDs in mice have been documented, providing further evidence in support of a genetic factor in humans. To date, more than 80 different murine loci predisposing to NTDs have been identified (Copp *et al.*, 2003; Juriloff and Harris, 2000). The complex, early embryological development of NTDs presents fascinating challenges to the geneticist. One traditional approach to identifying disease genes in families has been

through genomic screening of multiplex pedigrees. Advances in genetic marker availability, including the development of highly polymorphic microsatellite repeat markers (STRPs) and single nucleotide repeat polymorphisms (SNPs), the dense genetic maps of microsatellites (Broman *et al.*, 1998) and of SNPs (Matise *et al.*, 2003), the development of high-throughput genotyping methods (Ben Othmane *et al.*, 1998), and innovations in statistical genetic linkage analysis (Gudbjartsson *et al.*, 2000; Kruglyak *et al.*, 1996; Kong and Cox, 1997; Martin *et al.*, 2000; O'Connell and Weeks, 1995) have paved the way for large-scale investigations of complex human diseases. Recently, a genomic screen of 44 multiplex pedigrees (those with at least two sampled affected individuals) was performed using highly polymorphic microsatellite repeat markers and identified regions of interest on chromosomes 7 and 10 (Rampersaud *et al.*, 2005). Occasionally, large multiplex pedigrees—those with three or more affected individuals—are identified and such families can be invaluable for identifying a gene associated with NTDs (Stamm *et al.*, 2006). Presumably, the affected individuals, since related to one another, all share the same genetic variant. Once a putative causative genetic variant is identified in such a family, its relevance in other families can be quickly assessed.

Another common approach to characterizing the genetic influence of a condition is to assess biologically plausible candidate genes—genes whose involvement in a process is suggested by the known function of a gene. In NTDs, biologically plausible candidate genes might be those known to be involved in neural tube closure in other animal systems, are known to be important generally in early development, or are involved with folate metabolism. Folate metabolism pathway genes have often been examined for association with NTDs due to the well-established ability of folic acid to reduce the risk of this debilitating birth defect by 50–70%. Some studies have shown that the protective effect of folic acid may be decreased in populations of Hispanic descent (Shaw *et al.*, 1995; Suarez *et al.*, 2000). Blood folate levels have a strong genetic component with an estimated heritability of 46% (Morrison *et al.*, 1998), yet maternal folate supplementation can only prevent 50–70% of NTDs (Chatkupt *et al.*, 1994). Folic acid supplementation does not entirely eliminate recurrence risk (Chatkupt *et al.*, 1994; Milunsky *et al.*, 1989), suggesting that additional, genetic factors are responsible for the development of this type of birth defect. These non-folate responsive cases may represent highly genetic cases of NTDs (Scriver, 1985). The ability to identify those women genetically predisposed to having a child with a neural tube defect, and whose risk could be minimized by the use of folic acid supplements, would allow genotype-directed pharmacogenetic interventions.

The mechanism by which dietary folate supplementation prevents NTDs is not understood (Prevention of Neural Tube Defects: Results of

the Medical Research Council Vitamin Study, MRC Vitamin Study Research Group, 1991). Folic acid derivatives are essential for the synthesis of DNA, as well as for cell division, tissue growth, and DNA methylation (Morrison *et al.*, 1998), and may also protect cells against oxidative damage. DNA methylation enables proper gene expression and chromosome structure maintenance, both of which are critical in the developing embryo (Razin and Kantor, 2005). Low dietary folate levels could directly limit folate availability to cells or indirectly disrupt methionine metabolism, thereby increasing homocysteine in the maternal serum (Rosenquist and Finnell, 2001). Either mechanism implicates folate receptor and methionine–homocysteine regulatory genes. Thus, the study of variation in genes involved in folate metabolism makes good biologic sense. NTDs in humans result from the combined effects of genetic, nutritional, and environmental influences, and as such are a classic example of a multifactorial disorder. Identifying the genetic factors will be critical for characterizing the interactions between genes and the environment, and understanding these interactions will provide the basis for identifying individuals at greatest risk for having a child with a birth defect, as well as facilitating the design of novel preventive strategies.

III. FUMONISIN EXPOSURES

A. Fumonisins and regulatory policy

Based on the adverse health effects observed in animals, the Center for Food Safety and Nutrition, U.S. Food and Drug Administration (FDA) issued industry guidance levels for fumonisins in maize in 2001 (''Guidance for Industry on Fumonisin Levels in Human Foods and Animal Feeds''; http://cfsan.fda.gov/~dms/fumongu2.html) stating that human health risks associated with exposure to fumonisins were possible. Recommended levels for foods range from 2 to 4 ppm total fumonisins (fumonisin B_1 plus fumonisin B_2 plus fumonisin B_3) depending on the intended use of the maize and whether or not the kernels are whole or degermed. Proposed limits (fumonisin B_1 plus fumonisin B_2) in Europe, (effective October 2007), range from 0.2 to 2 ppm depending upon the commodity and the targeted consumer (i.e., adults or infants) (Commission of European Communities, 2005). The International Agency for Research on Cancer (IARC, 2002) classified fumonisin B_1 as ''possibly carcinogenic to humans (class 2B)'' and in 2001 the Joint FAO/WHO Expert Committee of Food Additives (JECFA) recommended a provisional maximum tolerated daily intake (PMTDI) of 2 μg/kg body weight for fumonisin B_1, fumonisin B_2 and fumonisin B_3, ''alone or in

combination'' (Bolger *et al.*, 2001), and suggested that further research was needed to examine the teratogenic potential of this compound.

B. Measurements of fumonisin exposure

One of the difficulties in studying the environmental component to birth defects is that the time of ''injury'' precedes the occurrence of the condition by weeks or months. In humans, neural tube closure is complete by approximately 28 days after conception, frequently before a woman even knows she is pregnant. Prospective studies of exposure would require extensive population screening to identify the occasional affected case and would require years to develop a large enough sample size for the analysis of gene–environment interactions. Thus, use of biomarkers at time points after the event of interest (e.g., pregnancy) may be a useful approach for characterizing environmental influences (Krapels *et al.*, 2004; Leck, 1983; Murray *et al.*, 1997; Prakash *et al.*, 2002; Weis *et al.*, 2005). Fumonisin B_1 is rapidly cleared from the system and can only be detected in human urine and feces for up to 3–5 days after ingestion (Sewram *et al.*, 2003; Riley, *unpublished data*). In the absence of modern technology for prenatal monitoring (i.e., ultrasound), fetal malformations may not be detected until the child is born. At this point, it is too late to accurately determine the level of maternal fumonisin exposure that occurred during the critical gestational window spanning neural tube closure (i.e., 3–4 weeks post-conception). A possible surrogate for direct fumonisin exposure is a rise in the sphinganine/sphingosine ratio, but this measurement is also limited to short-term detection (Sewram *et al.*, 2003) unless exposure is fairly constant. Several studies in rodents have shown that once elevated by a high dose, the levels of sphinganine in either urine or kidney will remain significantly elevated by exposure to a low dose of fumonisin that would not ordinarily elicit an increase in sphinganine (Enongene *et al.*, 2002; Wang *et al.*, 1999). For example, rats were fed diets containing 10 ppm of fumonisin B_1 for 10 days and then changed to either diets containing 0 or 1 ppm fumonisin B_1 (Wang *et al.*, 1999). In the rats consuming the 0 ppm diet, the urinary sphinganine returned to control values ($\sim$0.2 nmol/ml) by day 20, whereas in the animals changed to the 1 ppm fumonisin B1 diet, sphinganine was still significantly elevated on day 20 (2.5 nmol/ml).

Chemical analysis of hair samples may also provide a method for examining chronic mycotoxin exposures. In 2003, Sewram *et al.* (2003) reported that human hair testing could be used to detect fumonisins. After extraction and clean up, high performance liquid chromatography coupled to electrospray ionization-mass spectrometry (HPLC-ESI-MS) was able to detect fumonisin B_1, fumonisin B_2, and fumonisin B_3 from human hair samples (Sewram *et al.*, 2003). However, these were

composite samples taken from barbershops in various regions of South Africa. Thus, hair testing has not yet been employed to measure individual fumonisin exposure level. Hair analysis has many potential advantages. Compared to urine and blood testing, which are only able to detect exposures from ~2 to 4 days past, hair analysis provides a larger window of xenobiotic detection. Exposure detection ranges from weeks to months depending upon the length of the hair (Kintz, 2004) and assessment of the hair in segments can be performed so that the segment most closely reflecting time-at-exposure is selected for study. Nonetheless, the method for measuring fumonisin in hair has not been validated so that levels in hair can be used to predict past or ongoing exposure. More recently, a simple method for measuring fumonisin in urine samples from women in a cohort recruited in Morelos County, Mexico has correlated urinary fumonisin B_1 with tortilla consumption and suggests that urinary fumonisin B_1 could be a valuable tool in investigating the associated health effects of exposure (Gong *et al.*, 2008).

As part of the evaluations of the cluster of NTDs along the Texas–Mexico border in 1990–1991 (Missmer *et al.*, 2006), three different metrics to evaluate fumonisin exposure were considered. These metrics included (1) number of tortillas consumed during the first trimester (collected via questionnaire); (2) post-partum sphinganine/sphingosine ratio to estimate fumonisin exposure from maternal blood; and (3) nanograms of fumonisin ingested per day during the periconceptional period, estimated from grouped 6-month averages for sampled tortillas from homes and markets. The outcome of this study suggested that ingestion of maize-based products in the amounts observed in Mexican-American diets were associated with increased risk for NTDs. All of the experimental findings were robust across exposure metrics, suggesting that the questionnaire data were a realistic measure of fumonisin exposure, particularly when coupled with estimates of seasonal variation in fumonisin levels.

IV. REPRODUCTIVE TOXICOLOGY OF FUMONISINS

A. Animal studies: Overview

Previous reproductive toxicology studies in laboratory animals examining the effects of prenatal exposure to fumonisin demonstrated a potential risk to the developing fetus. Studies using an aqueous extract of contaminated maize-culture material of *F. verticillioides* reported that fumonisin was developmentally toxic in hamsters (Floss *et al.*, 1994; Penner *et al.*, 1998). In addition, purified fumonisin B_1 was shown experimentally to cause fetal toxicity in rats and mice (Collins *et al.*, 1998; Reddy *et al.*, 1996). In another study, pregnant CD1 mice treated with a semipurified extract

of *F. verticillioides* culture material exhibited a dose-dependent increase in the number of resorptions and fetal abnormalities (Gross *et al.*, 1994). However, in several of the studies, developmental abnormalities also occurred in the presence of maternal toxicity. In animal model systems, cultured rat embryos exhibited an increase in the incidence of NTDs (Flynn *et al.*, 1997) following exposure to the hydrolyzed fumonisin B_1, also known as aminopentol 1 (AP_1), which is produced during the nixtamalization process used to make maize flour for masa and tortillas (Dombrink-Kurtzman *et al.*, 2000). Sadler *et al.* (2002) reported an association between fumonisin B_1 exposure and NTDs in murine embryo culture and showed that folinic acid supplementation was able to ameliorate the teratogenic effects of fumonisin. Early gestational exposure to the mycotoxin also induces a high incidence of NTDs in offspring of LM/Bc mice (Gelineau-van Waes *et al.*, 2005), and a somewhat lower, but biologically significant incidence of NTDs in embryos of CD1 mice following maternal intraperitoneal injections of purified fumonisin B_1 (Voss *et al.*, 2006). In the inbred LM/Bc mouse strain, maternal administration of 20 mg/kg/day fumonisin B_1 on gestational days 7.5 and 8.5 (intraperitoneal injection) resulted in NTDs (exencephaly, which is the mouse equivalent of anencephaly in humans) in all litters examined ($n = 10$ litters), and in 79% of the exposed embryos. Similar to the results obtained in murine embryo culture (Sadler *et al.*, 2002), maternal supplementation with folic acid was able to reduce the *in vivo* incidence of fumonisin-induced NTDs in LM/Bc mice by approximately 50% (Gelineau-van Waes *et al.*, 2005). Preliminary results using the LM/Bc mouse model ($n = 3$ l) indicate that the oral route of fumonisin exposure (20 mg/kg of fumonisin B_1 on E7.5 and E8.5; equivalent to 80–120 mg of fumonisin B_1/kg diet) also results in NTDs (20%) in exposed embryos.

B. Mouse models of fumonisin-induced neural tube defects

1. Fumonisin B_1-induced neural tube defects in cultured mouse embryos

Sadler *et al.* (2002) assessed the effect of fumonisin B_1 treatment on development of neurulating mouse embryos in culture. Fumonisin B_1 treatment resulted in a dose-dependent increase in the percentage of embryos exhibiting NTDs (Sadler *et al.*, 2002). Concurrent alterations in levels of sphingoid bases were also found. Supplementation of the embryos with high levels of folate during the fumonisin B_1 treatment reduced the incidence of NTDs without correcting the sphingolipid abnormalities, suggesting that the mycotoxin influenced neural tube maturation by affecting the function of the folate receptor. Cumulatively, these findings provided a conceptual framework to account for how

exposure to fumonisin B_1 could increase the risk of NTDs by disrupting lipid rafts through sphingolipid depletion and, consequently, impaired folate receptor function.

2. Maternal fumonisin exposure and neural tube defects

Further evidence of a causal relationship between gestational fumonisin exposure and altered embryonic morphogenesis was provided by the establishment of an *in vivo* mouse model (Gelineau-van Waes *et al.*, 2005). Early gestational administration of the purified fumonisin B_1 mycotoxin to pregnant mice of the inbred SWV and LM/Bc mouse strains resulted in an increased incidence of fetal resorptions and/or malformations (Gelineau-van Waes *et al.*, 2005). Although reproductive toxicology studies in which purified fumonisin B1 was administered to hamsters (Penner *et al.*, 1998) or fumonisin-contaminated culture material was administered to mink (Powell *et al.*, 1996) reported a decrease in birth weight and/or an increase in fetal death as the primary finding(s), very few LM/Bc embryos/fetuses were resorbed, even at the highest dose of fumonisin administered. Rather, a significant proportion of fumonisin-exposed LM/Bc embryos failed to complete neurulation. The number of affected fetuses per litter increased as the dose of fumonisin was increased, and at the highest dose administered (20 mg/kg/day on E7.5 and E8.5), the litters of all treated dams were positive for NTDs, and 79% of the exposed LM/Bc fetuses were exencephalic (Gelineau-van Waes *et al.*, 2005) (Fig. 5.2).

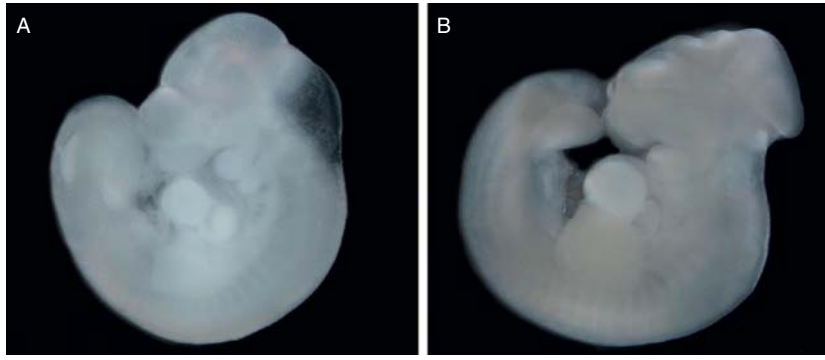


FIGURE 5.2 (A) Image of a normal (vehicle control) E10.5 embryo; (B) image of an E10.5 exencephalic embryo collected from an LM/Bc dam that was treated with purified fumonisin B1 early in gestation (20 mg/kg i.p. on E7.5 and E8.5).

The same fumonisin treatment regime performed in mice from the highly inbred SWV strain, however, yielded very different results. At the highest fumonisin B_1 dose administered (20 mg/kg/day on E7.5 and E8.5), approximately 15% of the implants were resorbed, and only one exencephalic fetus was observed in the 10 SWV litters examined. Sphinganine levels were elevated in the placentas and embryos of both strains, although to a much greater extent in the LM/Bc mice, suggesting that genetics plays a role in the observed differences in sphingolipid metabolites and pregnancy outcome after fumonisin exposure. Observations from preliminary feeding studies (summarized in Voss *et al.*, 2006) are consistent with the concept of strain-dependent differences. In these studies, LM/Bc and CD1 mice were fed fumonisin-contaminated diets beginning 5 weeks before mating and continuing throughout gestation. At a dietary fumonisin B_1 concentration of 150 ppm, late fetal deaths occurred in two of the nine (22%) CD1 litters, and the incidence of late fetal deaths in affected litters ranged from 40 to 64%. No NTDs were found in the CD1 mice. In contrast, no late fetal deaths were found in the five LM/Bc litters, but one LM/Bc litter was positive for NTDs. The results from the studies in LM/Bc and SWV mice, in conjunction with the preliminary results of the feeding studies comparing the LM/Bc and CD1 strains clearly point to an as yet unknown genetic susceptibility component with respect to risk for NTDs following maternal fumonisin B_1 exposure.

In the LM/Bc mouse strain, maternal fumonisin exposure resulted in decreased expression of folate receptor (Folr1) protein in the yolk sac membrane and neuroepithelial cells of developing embryos. In addition, reduced levels of ^{3}H-folate uptake by neurulating embryos demonstrated that fumonisin depletion of glycosphingolipids (gangliosides) had an impact on both expression and function of the GPI-anchored folate receptor. Moreover, similar to the results observed in the embryo culture model of fumonisin exposure, supplementing pregnant dams with high levels of folic acid (50 mg/kg/day on E0.5–E9.5) afforded some protection against fumonisin teratogenicity, and reduced the incidence of NTDs from 79 to 50% in exposed LM/Bc fetuses (Gelineau-van Waes *et al.*, 2005).

V. MECHANISMS OF FUMONISIN TOXICITY

A. Structural considerations

The chemical structure of fumonisin (Fig. 5.1B) is remarkably similar to that of the sphingoid bases sphinganine (Sa) and sphingosine (So), and fumonisin has been shown to inhibit the enzyme ceramide synthase in *de novo* sphingolipid metabolism (Wang *et al.*, 1991). Ceramide synthase

catalyzes the formation of ceramide from the condensation of fatty-acyl CoA and sphinganine (Sa) or sphingosine (So). Inhibition of ceramide synthase by fumonisins is competitive (Merrill *et al.*, 2001; Riley and Voss, 2006; Riley *et al.*, 2001, 2006) and likely involves the tricaballylic acid and amine groups, which occupy the enzyme's respective fatty-acyl CoA and the sphingoid base binding sites (Merrill *et al.*, 2001). Experimental evidence indicates that the primary amine is essential for the fumonisin molecule's ceramide synthase inhibitory activity (Lemke *et al.*, 2001; Norred *et al.*, 2001), which is believed to be the mechanistic ''trigger'' for toxicity. Inhibition of ceramide synthase results in an accumulation of upstream sphingoid bases and sphingoid base-1-phosphates, and a depletion of downstream complex glycosphingolipids (Fig. 5.3). To date, at least 28 analogues of fumonisin have been identified (Reeder *et al.*, 2002). Fumonisin B_1 (FB_1) is the most common and, from a toxicological standpoint, the most thoroughly studied. Structures of the less common

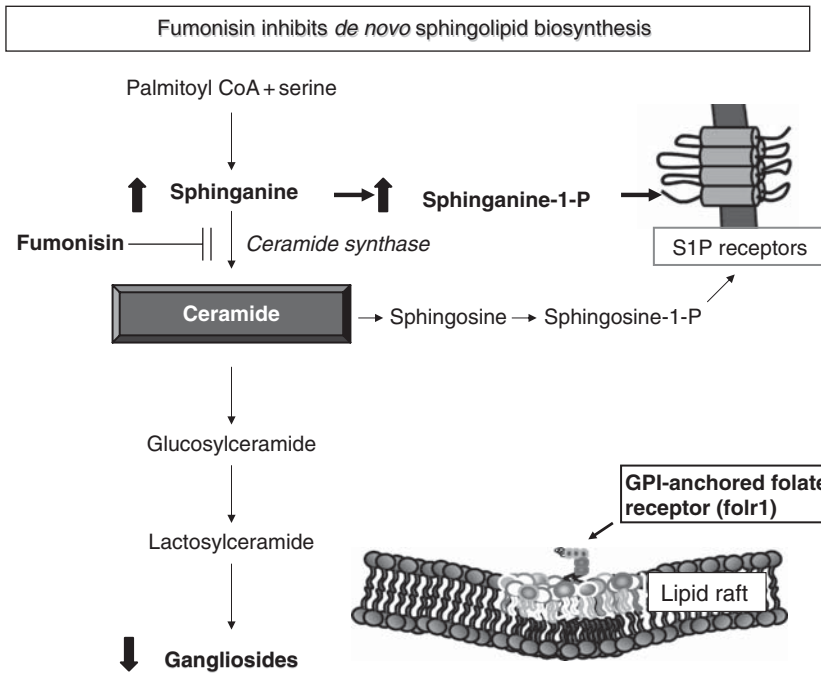


FIGURE 5.3 Simplified schematic of the *de novo* sphingolipid biosynthetic pathway. Fumonisin inhibits the enzyme ceramide synthase, resulting in (1) an accumulation of sphinganine, which is subsequently phosphorylated to form bioactive sphinganine-1-phosphate, a ligand for the G protein-coupled S1P receptors; and (2) a depletion of downstream glycosphingolipids (gangliosides), important components of "lipid rafts" in which GPI-anchored receptors such as the folate receptor (*Folr1*) are found.

fumonisin B_2, and fumonisin B_3, differ from that of fumonisin B_1 by the number and position of hydroxyl groups on the backbone.

Base hydrolysis removes the tricarballylic acid groups yielding the corresponding hydrolyzed fumonisins (HFB_1, HFB_2) (Dombrink-Kurtzman *et al.*, 2000; Merrill *et al.*, 2001). Hydrolyzed fumonisins form during the traditional corn cooking (in alkaline water) method known as nixtamalization that is used to make masa for tortillas (Dombrink-Kurtzman *et al.*, 2000; Palencia *et al.*, 2003). Hydrolyzed fumonisin B_1 inhibits ceramide synthase less effectively *in vitro* (Merrill *et al.*, 2001) and, in contrast to fumonisin B_1, was not toxic when fed to female mice for 28 days (Howard *et al.*, 2002). Likewise, *N*-(acetyl)-fumonisin B_1 and *N*-(carboxymethyl)-fumonisin B_1 which, like *N*-(1-deoxy-D-fructose-1-yl) fumonisin B_1, lack a free primary amine group, were not toxic and did affect sphingolipid metabolism when fed to female mice (Howard *et al.*, 2002). Heating fumonisin B_1 with reducing sugars such as glucose yields browning reaction products such as *N*-(1-deoxy-D-fructose-1-yl) fumonisin B_1 and *N*-(carboxymethyl)-fumonisin B_1 (Lu *et al.*, 2002). Fumonisin B_1 also forms conjugates when heated with model starch or protein compounds (Humpf and Voss, 2004). In this case, however, binding likely occurs through the tricarballylic group. The extent to which fumonisin binds to food matrices and the bioavailability of matrix-bound fumonisins is unknown. However, the presence of matrix bound (or ''hidden'') fumonisins in maize-based cereal (Kim *et al.*, 2003), and alkali-processed food (Park *et al.*, 2004) has been demonstrated.

B. Role of sphingolipids in fumonisin toxicity

Several biochemical modes of action have been postulated to explain fumonisin-induced toxicity and carcinogenicity in animals, but the primary hypothesis involves perturbation of sphingolipid metabolism (Bolger *et al.*, 2001; IARC, 2002; Wang *et al.*, 1991; WHO, 2000). Sphingolipids are important structural components of the plasma membrane and also function as intra- and intercellular signaling molecules (Chalfant and Spiegel, 2005; Merrill *et al.*, 2001). The toxic effects of fumonisin appear to depend on disruption of various aspects of lipid metabolism, membrane structure, and signal transduction pathways mediated by lipid second messengers. The demonstrated effects include altered rates of cell proliferation vs. apoptosis, altered cell–cell communication and cell adhesion, oxidative stress, and modulation of gene expression (Abel and Gelderblom, 1998; Bhandari *et al.*, 2002; Seefelder *et al.*, 2003). The biomolecular events linking ceramide synthase inhibition, disruption of sphingolipid metabolism, and toxicity have not been elucidated and mechanistic roles for oxidative damage (Abel and Gelderblom, 1998; Kouadio *et al.*, 2005; Lemmer *et al.*, 1999; Sahu *et al.*, 1998), critical changes

in cell fatty acid composition (Gelderblom *et al.*, 2001), up- or down-regulation of transcription or post-transcriptional stabilization of cytokines and other signaling molecules (Bhandari *et al.*, 2002; Bondy *et al.*, 2000; Gelderblom *et al.*, 2001; Merrill *et al.*, 2001; Sharma *et al.*, 2003) have also been proposed. The extent to which such events occur independently or secondary to sphingolipid metabolism disruption is unknown. Aside from influencing apoptosis and mitosis (Dragan *et al.*, 2001; Howard *et al.*, 2001a), fumonisin interferes with cell–cell or cell–matrix adhesion and membrane permeability, perhaps through sphingolipid dependent mechanisms (Gon *et al.*, 2005; Pelagalli *et al.*, 1999; Ramasamy *et al.*, 1995). It is noteworthy that detachment and sloughing of tubule epithelial cells is a feature of fumonisin-induced renal disease (Hard *et al.*, 2001). In addition, complex sphingolipids are important for the uptake and trafficking of folate (Stevens and Tang, 1997) via the GPI-anchored folate receptor.

Apoptosis and mitosis in liver (hepatocytes) and kidney (proximal tubule epithelium of the outer medulla) are early consequences of fumonisin exposure (Bolger *et al.*, 2001; Dragan *et al.*, 2001; Howard *et al.*, 2001a; Voss *et al.*, 2001). They occur simultaneously and it is proposed that an imbalance between cell death and increased regenerative pressure through continuous mitosis is a critical component of fumonisin's carcinogenic mode of action, especially in the kidney (Dragan *et al.*, 2001; Howard *et al.*, 2001a). In this regard, the survival and replication of damaged cells escaping apoptosis is likely critical for renal carcinogenesis. Sphingoid bases and other sphingolipids are signaling molecules for apoptosis and mitosis (Merrill *et al.*, 2001; Taha *et al.*, 2006). Specifically, sphingosine and ceramide are pro-apoptotic, growth inhibitory and cytotoxic whereas sphingosine-1-phosphate (S1P) exerts pro-growth and anti-apoptotic effects. Sphinganine causes apoptosis in LLC PK_1 kidney cells *in vitro* (Kim *et al.*, 2001; Riley *et al.*, 1999; Yu *et al.*, 2001), possibly through a calmodulin-dependent mechanism (Kim *et al.*, 2001). The importance of elevated levels of free sphinganine in fumonisin-induced apoptosis has been demonstrated using myriocin, a potent inhibitor of serine palmitoyltransferase that abolishes the fumonisin-induced increase in free sphinganine and the increased apoptosis (Riley *et al.*, 1999) Addition of exogenous sphinganine to the incubation medium also enhanced the toxicity of fumonisin to Chinese hamster ovary (CHO) cells, which are relatively resistant to the sphingolipid and pro-apoptotic effects of fumonisin (Yu *et al.*, 2001). Thus, the overall metabolic balance of the various sphingolipids is likely a critical determinant of whether cells undergo apoptosis, remain quiescent, or replicate and, in this regard, the ratio of S1P to ceramide is likely to be important (Spiegel and Milstien, 2002).

Sphingolipid metabolism is also affected by cytokines and other signaling molecules. Tumor necrosis factor α (TNFα) is a cytokine that

exerts both pro- or anti-apoptotic effects and has been shown to modify the extent of fumonisin-induced liver injury in mice (Sharma *et al.*, 2002), perhaps through a mechanism that involves NF-κB (Gopee and Sharma, 2004). Enhancement of liver apoptosis (compared to wild-type mice) in TNFα knockout mice has been observed and was likely affected through over-expression of Fas ligand, indicating that Fas-dependent pathways also mediate fumonisin-induced apoptosis (Sharma *et al.*, 2003). Activation of neutral sphingomyelinase by TNFα could also enhance apoptosis by increasing cell ceramide or sphingosine levels or, alternatively, promote cell survival in the event that significant amounts of sphingosine and, ultimately, S1P were formed from the sphingomyelinase-generated ceramide pool. Like TNFα and possibly Fas ligand, other cell surface receptor agonists influence cell survival and replication by activating sphingomyelinase, ceramidase or sphingosine kinases, thereby increasing the production of ceramide, sphingosine, and S1P, respectively (Merrill *et al.*, 2001; Spiegel and Milstien, 2002).

C. Fumonisin inhibition of *de novo* sphingolipid metabolism

Fumonisin exposure has been shown to result in subsequent alterations in the major pools of sphingolipids, including increased concentrations of free sphingoid bases and their 1-phosphate metabolites, and decreased biosynthesis of ceramide and downstream glycosphingolipids (Merrill *et al.*, 2001; Riley *et al.*, 1996) (Fig. 5.3). Sphinganine levels accumulate rapidly following inhibition of ceramide synthase, providing a biomarker for fumonisin exposure that has been validated *in vitro*, as well as in tissue, serum, and urine (Norred *et al.*, 1997; Riley *et al.*, 1993; Wang *et al.*, 1991). The significantly elevated levels of sphinganine observed in LM/Bc maternal and fetal tissues following fumonisin exposure are indicative of fumonisin inhibition of the enzyme ceramide synthase. The marked increase in sphinganine in embryonic tissue following early gestational exposure to fumonisin (E7.5 and E8.5) suggests that the toxin crosses the placenta and that there is an effect at the level of the embryo that cannot simply be attributed to maternal toxicity. At this early time point, the placenta is not well developed, leaving the embryo potentially vulnerable to teratogenic insult.

Fumonisin inhibition of ceramide synthase leads to the accumulation of free sphinganine and sphingosine in tissues, serum and urine in a wide range of species including catfish, trout, poultry cattle, horses, rabbits, and mice (Bolger *et al.*, 2001; Merrill *et al.*, 2001; Riley *et al.*, 2001). Free sphingosine or sphinganine are subsequently phosphorylated by *sphingosine kinase* to form the corresponding sphingoid base-1-phosphates (S1P or sphinganine-1-P), which may then be dephosphorylated by either *sphingosine phosphate phosphatases (Sgpp1, Sgpp2)* or *lipid phosphate*

phosphatases (*Ppap2a, Ppap2b*), or alternatively, irreversibly degraded by *sphingosine phosphate lyase (Sgpl)* to form phosphoethanolamine and hexadecenal (Fig. 5.4). Phosphoethanolamine is a precursor for the biosynthesis of phosphatidylethanolamine, a compound that has also been shown to accumulate following fumonisin exposure both *in vitro* and *in vivo*. Accumulation of bioactive S1P or sphinganine-1-P in serum or tissues of horse (Constable *et al.*, 2005), swine (Piva *et al.*, 2005), rats (Riley and Voss, 2006), adult mice (Suzuki *et al.*, 2007), ducks (Tardieu *et al.*, 2006), and livers of mouse embryos (Riley *et al.*, 2006) has been reported after fumonisin exposure.

The *in vivo* sphingolipid effects of fumonisins are reversible (Merrill *et al.*, 2001). Within 3 weeks after replacing fumonisin-contaminated diet with control feed, the elevated tissue sphinganine (Sa) and sphingosine (So) concentrations and Sa/So ratios (used as a biomarker of fumonisin exposure) of rats decreased markedly, almost reaching pretest values, and the histological appearance of the liver and kidney returned (loss of fumonisin-linked lesions) to normal (Voss *et al.*, 1998). In ponies, serum sphingoid base concentrations and serum chemistry indicators of hepatic injury (alanine and aspartate transaminase activities) rose and fell together as the fumonisin contaminated feed was given to and withdrawn from the animals (Wang *et al.*, 1992).

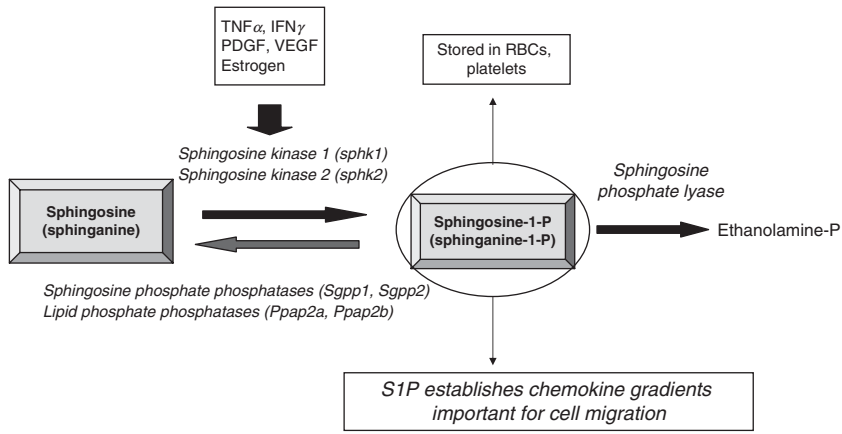


FIGURE 5.4 Biochemical pathway illustrating (1) phosphorylation of sphingosine or sphinganine by the enzymes sphingosine kinase 1 (*sphk1*) and/or sphingosine kinase 2 (*sphk2*); (2) dephosphorylation of sphingosine-1-phosphate (S1P) or sphinganine-1-phosphate by sphingosine phosphate phosphatases (*sgpp1, sgpp2*) and/or lipid phosphate phosphatases (*ppap2a, ppap2b*); and (3) irreversible degradation of S1P or sphinganine-1-phosphate by sphingosine phosphate lyase (*sgpl*) to form phosphoethanolamine. Both S1P and sphinganine-1-phosphate are stored in red blood cells and platelets, and function as ligands for the G protein-coupled S1P receptors.

D. Fumonisin and accumulation of bioactive sphingoid base-1-phosphates

Although sphingolipids are important structural components of the exoplasmic leaflet of the plasma membrane, their role in mediating cell signaling processes has also recently been recognized. Sphingoid base metabolites, such as *sphingosine-1-phosphate (S1P)* are involved in signal transduction cascades that regulate key biological processes (Futerman and Hannun, 2004; Spiegel and Milstien, 2002, 2003; Stunff *et al.*, 2004; Watterson *et al.*, 2003). S1P turnover is mediated either by reversible dephosphorylation to sphingosine or by irreversible cleavage to ethanolamine phosphate and fatty aldehyde (Zhou and Saba, 1998). The dynamic balance between S1P (pro-survival) and ceramide/sphingosine (pro-apoptosis) has been proposed to form a ''cellular rheostat'' that determines cell survival vs. cell death (Spiegel and Milstien, 2002). S1P has both extracellular and intracellular signaling functions, and functions as a ligand on cell membrane S1P receptors, a family of five G-protein-coupled receptors ($S1P_{1–5}$; previously endothelial differentiation gene (Edg) receptors) that play important roles in such diverse functions as cell survival/proliferation, differentiation and migration, as well as angiogenesis and endothelial barrier integrity (Brinkmann, 2007; Dev *et al.*, 2008; Takabe *et al.*, 2008) (Fig. 5.5). SIP gradients and S1P receptors also play an important role in mediating immune cell migration to sites of inflammation (Brinkmann, 2007; Cyster, 2005). The phenotypes observed in S1P receptor knockout mice indicate a certain degree of functional redundancy between the various receptor isoforms, but also establish a critical role for S1P receptors in angiogenesis and neurogenesis during embryonic development (Kono *et al.*, 2004). Inactivation of $S1P_1$ in mouse models results in embryolethality, and the offspring die between E12.5 and E13.5 due to severe hemorrhage. $S1P_{1,2,3}$ are expressed in neural tissue (especially the telencephalon), $S1P_5$ is expressed in CNS white matter (oligodendrocytes), and both sphingosine kinases (*Sphk1*, *Sphk2*) are expressed in the developing brain and spinal cord (Dev *et al.*, 2008; Kono *et al.*, 2004; Mizugishi *et al.*, 2005), suggesting a role for S1P receptor-mediated signaling in neural tube closure. Although there is no obvious phenotype when either *Sphk1* or *Sphk2* alone is inactivated in a knockout mouse model, *Sphk1/Sphk2* double knockout embryos die by E12.5 with severe vascular and neural abnormalities, including failure of neural tube closure (Mizugishi *et al.*, 2005). Interestingly, *Sphk1-/-/Sphk2* +/− mutant mice are infertile, and follow-up experiments suggest a critical role for early gestational activation of *de novo* sphingolipid metabolism in the maintenance of pregnancy (Mizugishi *et al.*, 2007).

Sphinganine-1-P also functions as a ligand for S1P receptors, although the binding affinity for the different S1P receptor isoforms differs from

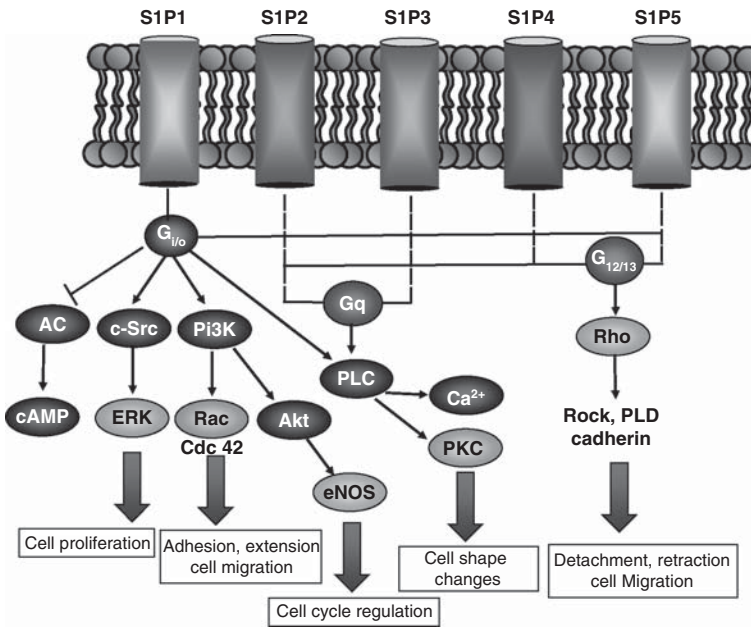


FIGURE 5.5 There are five isoforms of S1P receptors ($S1P_{1-5}$), a family of G protein-coupled membrane receptors previously known as "Edg" receptors (endothelial differentiation gene). Ligand binding and activation of S1P receptors initiates multiple intracellular signaling cascades involved in cellular proliferation, differentiation, survival and migration.

that of S1P (Im *et al.*, 2001). The high levels of sphinganine and sphinganine-1-P that accumulate after fumonisin exposure may therefore elicit different downstream signaling cascades than S1P due to differential binding and activation of the various S1P receptor isoforms. Moreover, high concentrations of ligand have been shown to cause internalization and downregulation of S1P cell surface receptors, thereby acting as a ''functional antagonist'' rather than as an agonist (Brinkmann, 2007). Further differences in downstream signaling effects include a role for S1P in intracellular signaling, which apparently is not observed for sphinganine-1-phosphate (Taha *et al.*, 2006).

The activation of S1P receptors reduces renal and mesenteric blood flow in rats (Bischoff *et al.*, 2001), and it can be speculated that decreased blood flow and hypoxia contribute to the extreme sensitivity of rat kidneys to fumonisins. Fumonisin exposure has also been shown to decrease cardiac output, heart rate, and mean arterial pressure as well as increase pulmonary arterial pressure and pulmonary resistance in pigs (Constable *et al.*, 2000, 2003), findings which suggest that left-sided cardiac insufficiency underlies porcine pulmonary edema. Plasma sphingoid base

concentrations (Constable *et al.*, 2003) and serum S1P (and sphinganine-1-P) (Piva *et al.*, 2005) increase in fumonisin-exposed pigs. Sphingosine blocks L-type calcium channels and ryanodine receptors in rabbits (Sabbadini *et al.*, 1992). Therefore, one possible mechanism for fumonisin-induced porcine pulmonary edema involves interference with calcium ion transport, which in turn is critical for cardiac function and vascular tone (Constable *et al.*, 2003). Both sphinganine and sphingosine relaxed phenylephrin-contracted and uncontracted thoracic aortic and pulmonary arterial rings *in vitro* (Hsiao *et al.*, 2005) and, while S1P had little effect on the aortic rings, it increased the tension of uncontracted pulmonary arterial rings. From these observations, it can be further speculated that plasma S1P also contributes to pulmonary edema by increasing pulmonary arterial tension, likely through a mechanism involving S1P receptors. $S1P_{1,2,3}$ receptors are known to mediate endothelial barrier integrity and vascular permeability (Brinkmann, 2007; Gon *et al.*, 2005; Sanchez *et al.*, 2007; Singleton *et al.*, 2005), while agonism of $S1P_3$ receptors results in sinus bradycardia (Sanna *et al.*, 2004).

The process of neural tube closure requires the integration of multiple signaling cascades involved in cell proliferation, migration, and differentiation. It is therefore likely that fumonisin inhibition of ceramide synthase, resulting in elevated levels of sphinganine-1-phosphate (as opposed to tightly regulated levels of S1P) may alter the dynamics of S1P receptor-mediated signaling pathways involved in coordinating these events, thereby contributing to the failure of neural tube closure.

E. Fumonisin depletion of glycosphingolipids and disruption of folate transport

In addition to an accumulation of upstream sphingoid bases and sphingoid base-1-phosphates, depletion of the downstream, complex sphingolipid metabolites of ceramide (i.e., gangliosides) has also been shown to occur after fumonisin exposure (Fig. 5.3). Stevens and Tang (1997) reported in an *in vitro* study that fumonisin-induced depletion of glycosphingolipids inhibited vitamin uptake via the GPI-anchored folate receptor and suggested that dietary exposure to fumonisin could therefore adversely affect folate uptake, and potentially compromise cellular processes dependent on this vitamin. The high-affinity folate receptor (murine *Folr1*; human FRα) is a GPI-anchored protein found associated with detergent-insoluble membrane microdomains rich in cholesterol and sphingolipids known as ''lipid rafts'' (Elortza *et al.*, 2003). These rafts create domains of reduced fluidity in membranes and arise from the shared biophysical properties of sphingolipids and cholesterol (Brown and London, 2000; Rietveld and Simons, 1998). They are hypothesized to function in membrane and endocytic sorting of proteins in polarized

epithelial cells, as foci for recruiting signaling molecules to the plasma membrane, and in cell adhesion (Ilangumaran *et al.*, 1999; Lin *et al.*, 1998; Simons and Ikonen, 1997). A role for membrane rafts in regulating the folate receptor was suggested by studies in which the depletion of cellular cholesterol was found to impair the uptake of 5-methyltetrahydrofolate into cells (Chang *et al.*, 1992).

Gangliosides stabilize the association of GPI-anchored proteins with the outer leaflet of cell membranes (Watanabe *et al.*, 2002) and are important components of specialized membrane microdomains, or ''lipid rafts'' in which GPI-anchored proteins cluster. Ganglioside GM1 has been shown to be highly expressed in the developing brain and spinal cord (Silani *et al.*, 1993), and decreased levels of ganglioside GM1a and GD1a have previously been reported in anencephalic human fetal brain (Cacic, 1995). In the LM/Bc *in vivo* mouse model of fumonisin exposure, maternal supplementation with ganglioside GM1 was more effective than folate in preventing NTDs. Expression of ganglioside GM1 in plasma membrane microdomains of the developing neuroepithelium may be necessary for coordinating/orchestrating protein–lipid interactions and signal transduction events involved in normal neural tube closure. GPI-anchored proteins such as the folate receptor associate with gangliosides in the trans-golgi network, prior to sorting and transport to the plasma membrane. Association of Folr1 with ganglioside GM1 may be important for delivery of the GPI-anchored folate receptor to the apical plasma membrane in neuroepithelial cells. In addition, co-localization of Folr1 with GM1 in lipid rafts appears to facilitate receptor function, and promote normal neural tube closure. Different gangliosides associate with different proteins in ''lipid rafts'' in a cell-type specific manner, and the rafts function as specialized platforms for the initiation of signal transduction (Brown and London, 1998; Hoessli *et al.*, 2000). Although the specific interactions between ganglioside GM1 and Folr1 and their precise role in neural tube closure are unknown at this time, several hypotheses are currently under investigation. Since the GPI-anchored folate receptor has previously been isolated from lipid raft microdomains enriched in signaling molecules, including kinases/phosphatases, heterotrimeric G proteins, and small GTP-binding proteins (Elortza *et al.*, 2003; Foster *et al.*, 2003; Miotti *et al.*, 2000), it seems reasonable to hypothesize that Folr1 may play a critical role in signal transduction pathways necessary for cell survival, proliferation, and activation of the actin cytoskeleton necessary for normal neural tube closure.

Fumonisin inhibition of *de novo* sphingolipid biosynthesis (Merrill *et al.*, 2001; Riley *et al.*, 1996), resulting in depletion of complex glycosphingolipids, disruption of lipid rafts, and compromised folate transport via Folr1, which may have significant implications with respect to developmental anomalies such as NTDs (Hansen *et al.*, 2003; Piedrahita

et al., 1999; Rothenberg *et al.*, 2004; Saitsu *et al.*, 2003). The inhibition of folate uptake by cells is expected to have the same consequences as a dietary deficiency in this vitamin. Therefore, the possibility that inhibition of folate receptor-mediated vitamin uptake contributes to the development of NTDs has been investigated. Mice in which expression of the gene encoding the folate receptor (*Folr1*) was knocked out were found to have defects in neural tube development and die before birth (Piedrahita *et al.*, 1999). This phenotype could be reversed by supplementing the pregnant knockout mice with excess folate, suggesting that the loss of vitamin uptake mediated by the folate receptor was responsible for the developmental defects (Piedrahita *et al.*, 1999). Reduction of folate receptor expression in mouse embryos with antisense technology (Hansen *et al.*, 2003) or blocking Folr1 activity in pregnant rats with antibodies to this transporter (da Costa *et al.*, 2003) both resulted in impaired embryogenesis that could be reversed with excess folate supplementation. Rothenberg *et al.* (2004) found autoantibodies against this transporter in serum from a subset of women with neural tube defect-affected pregnancies, suggesting that antibody-induced inactivation of the folate receptor may be responsible for some NTDs in humans. Cumulatively, these findings indicate that impairment of folate receptor function could result in NTDs.

Fumonisin B_1-induced depletion of the major sphingolipids to 40–65% of their normal levels in Caco-2 cells (which express high levels of the folate receptor) was found to significantly impair 5-methyltetrahydrofolate uptake (Stevens and Tang, 1997). While this result demonstrates that sphingolipid depletion inhibits vitamin uptake, it does not indicate a specific effect on the folate receptor because 5-methyltetrahydrofolate can be taken up by both the RFC1 and the GPI-anchored folate receptor (Folr1) (Corona *et al.*, 1998; Miotti *et al.*, 1997). Therefore, to specifically determine the effects of fumonisin B_1 treatment on the GPI-anchored folate receptor, uptake was measured with folic acid. This synthetic folate is bound by the folate receptor (Folr1) with an affinity that is approximately 100,000 times greater than that of the RFC1, and folic acid is transported almost exclusively by the former (Goldman, 1971; Kamen and Capdevila, 1986). Folate receptor function was found to be inhibited by 20–35% in cells derived from a human placental choriocarcinoma (JAR), monkey kidney epithelium (MA-104), and a human adenocarcinoma (Caco-2) that had been depleted of roughly 40% of their sphingolipids by fumonisin B_1 treatment. Other uptake processes, including receptor-mediated and facilitative transport, were not affected by this treatment (Stevens and Tang, 1997). Thus, depletion of cellular sphingolipids specifically inhibits folate receptor-mediated vitamin uptake.

Folate receptor function can be altered by changing the level of this protein, its activity, or both. To determine if folate receptor levels were

affected by sphingolipid depletion, the amount of this transporter was measured in fumonisin B_1-treated cells. Total cellular folate receptor was measured by quantifying specific folic acid binding in detergent solubilized cells while the amount of receptor active in endocytosis and available for folate uptake was measured in intact cells under conditions where endocytosis was maintained. Fumonisin B_1-induced sphingolipid depletion did not change total cellular levels of folate receptor significantly in Caco-2, JAR and MA-104 cells. However, this treatment did decrease the amount of receptor active in endocytosis by between 25 and 40%. This decrease in the amount of folate receptor available for vitamin uptake could contribute to the fumonisin B_1-induced inhibition of this transporter.

The effect of fumonisin B_1 treatment on folate receptor function was investigated by determining if sphingolipid depletion altered the endocytic kinetics of this transporter. These experiments were done using FRαTb-1 cells, which were generated by stably transfecting CHO cells with the GPI-anchored folate receptor and the transferrin receptor (Mayor *et al.*, 1998). The movement of the folate receptor through the endocytic pathway was followed by using a fluorescent folic acid analog that remained tightly bound to this protein. Fumonisin B_1-treatment did not significantly alter the rate at which the folate receptor was internalized. However, the recycling of this receptor to the cell surface was accelerated approximately threefold by sphingolipid depletion (Chatterjee *et al.*, 2001). The recycling rate was also faster in cells depleted of cholesterol through treatment with the HMG-CoA reductase inhibitor compactin (Chatterjee *et al.*, 2001). The fact that reduction of either of the major lipid species found in membrane rafts specifically perturbed the recycling of the folate receptor suggests that association with these domains regulates the intracellular sorting and trafficking of this GPI-anchored protein. Thus, the inhibition of folate receptor-mediated vitamin uptake caused by fumonisin B_1-induced sphingolipid depletion appears to result from a combination of altered endocytic trafficking and reduced levels of this receptor available for folate uptake. The molecular mechanisms by which sphingolipid depletion causes these changes are unknown.

In summary, fumonisin mycotoxins exert a wide range of diverse and species-specific toxicological effects. The weight of evidence suggests that inhibition of ceramide synthase and disruption of sphingolipid metabolism is the initial mechanistic event. The ensuing disruption of the metabolism of the sphingoid bases, sphingoid base 1-phosphates, and complex glycosphingolipids and the physiological processes modulated by these molecules are critical for fumonisin toxicity.

VI. CONCLUSIONS

NTDs present a tremendous burden to human populations in rural areas of the world where maize is a dietary staple (Hendricks, 1999; Kromberg and Jenkins, 1982; Marasas *et al.*, 2004; Melnick and Marazita, 1998; Moore *et al.*, 1997; Ncayiyana, 1986; Xiao *et al.*, 1990). The observed association between consumption of fumonisin-contaminated maize and increased incidence of NTDs in several world communities, coupled with the existing experimental evidence in animal models strongly supports the need for further investigation into the teratogenic potential of this compound. Epidemiologic data on dietary fumonisin exposures, including levels in foods, and daily food consumption, coupled with measurements of reliable biomarkers of exposure are needed. These exposure assessments, along with information on maternal genetics and nutrition (i.e., maternal folate status) as they relate to pregnancy outcome will help to establish a comprehensive understanding of the impact of fumonisin as a human health hazard, and risk factor for birth defects. Animal feeding trials incorporating dietary administration of the mycotoxin will help to establish relevant ''no observable adverse effect levels'' (NOAEL), and mechanistic studies in animal models will help to identify and validate biomarkers of exposure, and establish the complex biochemical, genetic, immune, and nutritional factors that contribute to increased susceptibility to fumonisin-induced NTDs. Establishing the basic mechanisms underlying fumonisin toxicity and identifying polymorphisms in candidate genes that contribute to susceptibility will facilitate our understanding of neural tube defect risk in women exposed to this mycotoxin during early pregnancy. Collectively, this information will also help in developing strategies for prevention. The experimental evidence suggests that maternal folate supplementation is effective in ameliorating the teratogenic effects of fumonisin. Maternal diet and nutritional status play a key role in fetal development, and strategies such as folate supplementation or folate fortification of foods may prove effective in reducing the incidence of NTDs in communities likely to be consuming high levels of fumonisin-contaminated maize. Fumonisins are present at low levels in maize throughout the world, but high levels of the toxin may occur, depending on the environmental conditions and genetics of the host plant. Strategies for reducing the risk of fumonisin contamination in maize supplied to the market include improved crop management practices, and improved breeding strategies, as well as transgenic approaches for increased ear-mold resistance (Betz *et al.*, 2000; Duvick, 2001). Minimizing exposures to mycotoxins through enhanced agricultural practices and biotechnology, characterizing potential health risks and mechanisms of toxicity, and improving maternal nutrition are all important strategies or considerations for reducing the neural tube defect burden in high risk human populations.

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