NEETESH BHANDARI
Involvement of tumor necrosis factor α and other cytokines in mouse fumonisin B_1 toxicity.
(Under the direction of RAGHUBIR P. SHARMA)

Fumonisin B_1 (FB_1), a toxic metabolite of *Fusarium verticillioides*, is a carcinogen and causative agent of various animal diseases. Our previous studies indicated the involvement of tumor necrosis factor α (TNFα) in FB_1 toxicity. The effect of FB_1 on TNFα and related cytokines with interplay of other downstream signaling molecules was investigated in following studies.

Time course of TNFα and interferon γ (IFNγ) expression after single sc or po dose of 25 mg/kg FB_1 was studied on male BALB/c mice. IFNγ and TNFα induction in liver peaked at 4 and 8 h, respectively after po FB_1 treatment. TNFα induction after po FB_1 treatment, correlated with the increase in liver enzymes. TNFα expression in spleen was unaltered. FB_1 treatment by po route showed greater toxicity as compared to sc route.

Cytokine expression in liver, kidney and spleen was investigated in male B6,129 mice after a multiple subacute dose of 2.25 mg/kg/day FB_1, for five days and sampled after 24 h. FB_1 treatment caused a localized induction of TNFα, IFNγ and interleukin (IL)-12 p40 in liver, with no changes in kidney and spleen. Increased TNFα expression in liver was localized in Kupffer cells. FB_1 toxicity selectively induced T helper 1 cytokines, suggesting a role of liver macrophages and natural killer (NK) cells/$NK1^+$ T cells, in localized cytokine induction.

Male and female BALB/c mice were tested for any gender differences after subacute FB_1 toxicity, using similar five-day repeated treatment model. Female mice

were more sensitive to FB_1 toxicity, showing greater increase in liver enzymes and apoptotic cells. Higher toxicity in females correlated with their higher increase in liver sphingoid bases after FB_1 treatment, and higher basal levels of cytokines.

Gene alterations in cytokine network and apoptosis signaling molecules were measured in mice after acute or subacute FB_1 treatment. FB_1 treatment caused induction of various pro- and anti-inflammatory cytokines in liver. TNFα signaling molecules were induced with no changes in Fas signaling pathway. Induction of IL-1 receptor antagonist and c-Myc oncogene could be responsible for the cancer promoting effects of FB_1.

INDEX WORDS: Fumonisin, Mice, TNFα, Cytokines, Liver, Toxicity, Signaling

INVOLVEMENT OF TUMOR NECROSIS FACTOR α AND OTHER CYTOKINES IN MOUSE FUMONISIN B_1 TOXICITY

by

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DEDICATION

To

My Grandfather
Shri. Pitamber Bhandari

And
Parents
Dr. Mahesh C. Bhandari and Mrs. Bharti Bhandari

Who have been a source of inspiration and encouragement throughout my life.

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Lastly, I wish to thank my family for their unending love and support. For her awareness, understanding and concern a special thank you is owed to my wife, Laxmi. I also owe a great deal to my brother, Deepesh, sister in-law, Ami, and family friends, Ish and Ritu Dhawan for their motivating and unending support.

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CHAPTER 1

INTRODUCTION

The fumonisin B mycotoxins are natural contaminants of corn infected with the fungus *Fusarium verticillioides* (Gelderblom et al., 1988). *F. verticillioides* is prevalent throughout the world and is an important plant pathogen. Ingestion of fumonisin B_1 (FB_1), the major fumonisin produced by the fungus, causes a variety of toxicosis in animals, including equine leukoencephalomalacia and porcine pulmonary edema (Riley et al., 1998). This mycotoxin is carcinogenic in rats and mice (Howard, et al., 2001) and has been implicated as a contributing factor in human esophageal cancer (Sydenham et al., 1990) and primary liver cancer (Ueno et al., 1997). In laboratory animals FB_1 has been shown to be nephrotoxic and hepatotoxic (Voss et al., 2001). Although the liver is affected in the most animal species including horses, pigs, rats (Riley et al., 1998), and primates (Jaskiewicz et al., 1987), other target organs appear to be species specific.

The precise mechanism involved in the pathogenesis of fumonisin-induced toxicosis has not been elucidated to this date. One of the earliest biochemical effects of fumonisin is inhibition of ceramide synthase (sphinganine and sphingosine-N-acyl transferase) leading to accumulation of sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids (Riley et al., 1998). *In vitro* and *vivo*, cells exposed to FB_1 undergo a mixture of necrotic and apoptotic cell death (Tolleson et al., 1996; Voss et al., 1996; Sharma et al., 1997).

In our laboratory tumor necrosis factor α (TNFα) has been shown to be a contributor in FB_1 toxicity, supported by the following findings: (1) macrophages derived from mice treated with fumonisin produce higher amounts of TNFα when stimulated by the mitogen, lipopolysaccharide compared to controls, (2) cells treated with FB_1 *in vitro* produced TNFα, (3) *in vivo* effects of FB_1 are partially reversed by anti-TNFα antibodies

(Dugyala et al., 1998), and (4) mouse strain lacking p75 or p55 tumor necrosis factor receptor also showed tolerance against FB_1 toxicity. Additionally, FB_1 toxicity in CV-1 cells (African green monkey kidney fibroblasts) was prevented by inhibitor of apoptosis protein (IAP), an inhibitor of TNF pathway (Ciacci-Zanella and Jones, 1999).

These observations suggest that the TNFα signaling pathway is involved in fumonisin toxicity. In the case of hepatic injury TNFα induction is one of the earliest events, and can trigger a cascade of other cytokines that cooperate to kill hepatocytes, recruit inflammatory cells, and initiate a wound healing response including fibrogenesis (Diehl, 2000). Liver has its own innate immune system, comprising Kupffer and natural killer cells, which can cause a localized activation of the cytokine network (Seki et al., 2000). Thus a network of cytokines rather than individual cytokine activity may be important in the outcome of any inflammatory response of the liver (Brennan and Feldmann, 2000). TNFα, interleukin (IL) -1β, and interferon γ (IFNγ) have been shown to be involved in different experimental mouse models of hepatotoxicity (Blazka et al., 1995; Ogasawara et al., 1998; Okamoto et al., 1999; Kamimura et al., 1995; Ksontini et al., 1998).

In the current project, the role of TNFα and other cytokines in mediating the toxic effects of FB_1 will be investigated. I therefore propose to test the hypothesis that "**Fumonisin B_1 toxicity involves *in vivo* production of TNFα and other cytokines, with their signaling pathways responsible for the apoptotic and anti-apoptotic responses observed.**" The following specific aims will be attempted to accomplish my objectives.

1. **To demonstrate time related induction of TNFα and IFNγ after acute FB_1 treatment by sc or po route.**
2. **To identify the cells involved in TNFα production, and investigate the expression of various cytokines in liver, kidney and spleen, after subacute FB_1 treatment.**
3. **To examine for any gender differences in FB_1 toxicity in a short-term treatment model.**
4. **To study gene alterations in the cytokine network and apoptosis signaling molecules after acute or subacute FB_1 treatment.**

CHAPTER 2

LITERATURE REVIEW

Fumonisin B_1: A mycotoxin

Fumonisins are produced by *Fusarium verticillioides* (synonymous with *Fusarium moniliforme*) and several other *Fusarium* species and are commonly found on corn worldwide. Fumonisin B_1 (FB_1) was first chemically characterized in culture material of *Fusarium verticillioides* in South Africa (Gelderblom et al., 1988). Since then it has been detected as a natural contaminant of rice, sorghum, and navy beans (Munimbazi and Bullerman, 1996; Patel et al., 1996; Tseng et al., 1995). A total of nine *Fusarium* species that can produce FB_1 under laboratory conditions have been reported. Of these nine, most reports are on FB_1 production by *F. verticillioides*. Additionally FB_1 production was also reported by the fungus *Alternaria alternata* f. sp. *Lycopersici*, a host-specific pathogen of tomato plants (Chen et al., 1992).

Fumonisins: Prevalence

Fumonisins, although mainly restricted to maize, are regarded as a global problem (Dutton et al., 1996). Because *F. verticillioides* is widely distributed in the world, it is not surprising to find global surveys reporting the presence of fungal strains capable of producing Fumonisins in the corn-based animal feeds and foods (Nelson et al., 1991).

In the United States 25 of 26 corn samples were found to be positive for FB_1 in one study (Sydenham et al., 1991) with levels as high as 1.05 ppm. In Iowa levels up to 37.9 ppm were reported by Murphy et al., (1993). The other notable high concentrations reported in corn were 38.5 ppm in Brazil, 50 ppm in France, 334 ppm in Hungary, and 250 ppm in Sardinia (Dutton 1996). In United States in 1989, large number of horses and pigs died from consuming commercial mixed feeds containing fumonisin-contaminated

corn (Harrison et al., 1990; Ross et al., 1991,book). Levels of FB_1 in corn can range from undetectable (less than a few parts per billion) to as high as 150 parts per million (Shephard et al., 1996). In the U.S., processed corn products usually contain less than 2 ppm FB_1 (Pohland, 1996).

Occurrence of FB_1 in corn varies seasonally and geographically. *F. verticillioides* is widespread in both the tropics and humid temperate zones of the world, but is uncommon in cooler temperate zones (Miller, 1994), and hence, corn grown in New Zealand, Canada, and northern Europe is expected to show little fumonisin contamination.

Fumonisins are not significantly destroyed when maize is either dried or thermally processed prior to consumption (Alberts et al., 1990). Fumonisin levels, 50-90%, remain following canning, no significant losses are observed after baking, although roasting (dry heat 220°C for 25 min) results in complete loss of detectable fumonisin B_1 and other fumonisins of the B-series (Scott and Lawrence, 1994). Treatment of corn with calcium hydroxide and heat (nixtamalization) hydrolyses FB_1, forming the corresponding aminopentol, which has been detected in tortilla chips, masa, and canned yellow corn (Hopmans and Murphy, 1993). Studies with laboratory rats have indicated that FB_1 toxicity can remain even after nixtamalization (Hendrich et al., 1993).

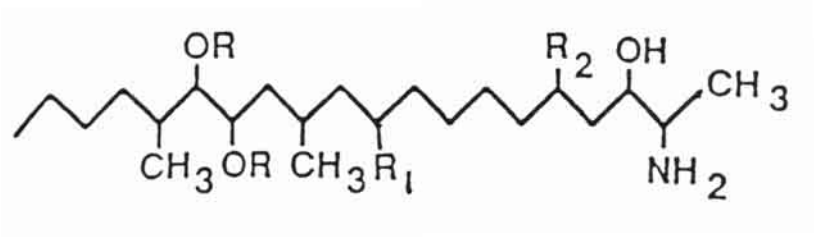
Fumonisins: In corn

F. verticillioides is associated with all stages of maize plant development, infecting the roots, stalk, and kernels, and causing seedling diseases, root rots, stalk rots and ear rots of maize. *F. verticillioides* is an almost constant companion of maize plants

and seed. In many cases, its presence is ignored because it is not causing visible damage. Symptomless infection can exist throughout the plant, and seed-transmitted strains of the fungus can develop systemically to infect the kernels (Munkvold and Desjardins, 1997).

The fungus can produce toxin both in field and presumably storage conditions. The association of the fungus in the corn plant is endophytic, while on corn kernels it is both external and systemic. The systemic seed-borne nature of this fungus suggests that seed treatment is insufficient as a control. The systemic location of this fungus within seed provides favorable conditions to maintain virulence and viability for years (Dungan and Koehler, 1994).

Fumonisin B_1: Structure



R = $COCH_2CH(COOH)CH_2COOH$

FB_1: R_1 = OH, R_2 = OH

FB_2: R_1 = OH, R_2 = H

FB_3: R_1 = H, R_2 = OH

FB_4: R_1 = H, R_2 = H

Figure 2.1. Structure of Fumonisins

Structures of fumonisin are based on a long hydroxylated hydrocarbon chain containing methyl and either amino (i.e. B_1 and B_2) or acetyl amino groups (i.e. A_1 and A_2). In the fumonisins of the "B" series two propane-1,2,3-tricarboxylic acid molecules, are esterified on the hydroxyls at carbons 14 and 15 (Fig. 2.1., Dutton, 1996). Fumonisins of the "B" series are very water-soluble (Norred et al., 1997). FB_1 differs from FB_2 in that it has an extra hydroxyl at position 10. There are at least 14 known fumonisins, although FB_1, FB_2 and FB_3 are the major fumonisins produced in nature. FB_1 is the most abundant of the fumonisins in the naturally contaminated maize, the ratio of FB_1 to FB_2 is approximately 3:1, and of FB_1 to FB_3 is approximately 12:1 (Sydenham et al., 1992).

Fumonisin B_1: Pharmacokinetics

Kinetics of FB_1 involves low gastrointestinal absorption, rapid clearance, biliary excretion of absorbed FB_1, and accumulation of minor amounts of the administered dose in liver and kidneys. FB_1 is rapidly, but incompletely absorbed after oral (po) administration. The T_{max} (time interval for maximum drug concentration) in plasma was 1.02 h and bioavailability 3.5% after po administration of 10 mg/kg FB_1 in rats (Martinez-Larranaga et al., 1999). In a study using ^{14}C-FB_1, up to 96 h after intragastric administration 80% of the radiolabel was recovered in feces, up to 3% in urine and remainder being distributed in liver, kidney and blood, whereas after intravenous (iv) dosing 35% of the iv dose was recovered in feces, 10% eliminated in urine and rest distributed in liver, kidney and blood (Norred et al., 1993). The elimination half-life of FB_1 after po administration was longer (3.15 h) than that obtained after iv administration

(1.03 h; Martinez-Larranaga et al., 1999). Out of all the organs examined, liver contained the greatest portion of the observed dose, reaching a maximum of 0.5% of the dose by 4 h after intragastric administration (Norred et al., 1993). In another comparative study, after intraperitoneal injection of a solution of FB_1, 67% of the dose was recovered in bile over 24 h period, while a similar dose of FB_1 given by gavage resulted in only 0.2% recovery of the toxin in bile (Shephard et al., 1994). Thus, although biliary excretion is the major route of FB_1 elimination from circulation, only small amounts of the toxin appears to be absorbed from the gut.

FB_1 metabolites have not been identified; most of the administered FB_1 can be accounted for as the unmetabolized chemical (NTP Technical Report, 2001). Non-human primate studies (Shephard et al., 1994) produced evidence that gut microflora is capable of removing one or both tricarboxylic acid groups from the molecule.

Fumonisin B_1: Toxicity

Fumonisins produce different types of symptoms in different species although liver is considered to be a target in all animals. These mycotoxins usually produce severe liver and kidney damage (Voss et al., 1989; Riley et al., 1994) that is characterized by a mixture of apoptosis and necrotic cell death (Voss et al., 1996; Sharma et al., 1997).

Horses

Moldy corn disease or equine leukoencephalomalacia (ELEM) was known long before the actual discovery of fumonisin. It was described by Butler in 1902, by producing the symptoms in a test animal with moldy feed. The condition is characterized by hepatopathy and liquefactive necrotic lesions within the cerebral hemispheres. ELEM

has been induced in horses by feeding moldy corn, feed contaminated with *F. monileforme* culture material and pure FB_1. House (1995) reported that at least 38 horses had died of ELEM between October 1994 and February 1995, in Kentucky and Virginia. FB_2 has been shown to be more effective than FB_3 in induction of ELEM and liver injury in ponies (Riley et al., 1997). A maximum fumonisin level of 5 μg/g horse feed has been recommended to avoid the danger of inducing ELEM (Report of Joint Mycotoxin Committee, 1994).

Pigs

In swine the same toxins cause porcine pulmonary edema (PPE), although liver toxicity is observed before and at lower doses than those that induce signs of respiratory distress (Casteel et al., 1993). In general, animals fed high levels of FB_1 die with pulmonary oedema and those surviving lower levels have evidence of subacute hepatotoxicosis. Histologic lesions were only observed in the lung and liver although marked elevations in sphinganine, sphingosine and their ratios were also observed in kidney and pancreas besides lung and liver (Gumprecht et al., 1998). In swine a maximum level of 10 μg/g swine feed has been recommended to avoid PPE (Report of Joint Mycotoxin Committee, 1994). In pigs, cardiovascular effects appear to play a role in pathogenesis of pulmonary edema (Constable et al., 2000).

Laboratory animals

Fumonisin causes liver cancer promotion and subchronic liver and kidney effects in rodents. FB_1 causes apoptosis of hepatocytes and of proximal tubule epithelial cells (Sharma et al., 1997). More advanced lesions in both organs are characterized by simultaneous cell loss (apoptosis and necrosis) and proliferation (Voss et al., 2001). In

rats, feeding with FB_1 causes acute and chronic liver toxicity, bile duct proliferation (hyperplasia), fibrosis progressing to cirrhosis, cholangiofibrosis and often hepatocellular carcinoma and/or cholangiocarcinoma (Voss et al., 1993). FB_1 is a hepatocarcinogen and causes primary hepatocellular carcinoma or cholangiocarcinoma in BD IX rats (Gelderblom et al., 1991). Fumonisins are potent tumor promoters in rat liver after initiation with diethylnitrosoamine (Gelderblom et al., 1996). FB_1 is a non-genotoxic carcinogen and appears to act mainly as a promoter (and possibly weak initiator) of tumors (Gelderblom et al., 1992). The National Toxicology Program in a 28-day and 2-year feeding study of FB_1, showed a clear evidence of renal tubule adenoma in male F344/N rats and hepatocellular adenoma in female $B6C3F_1$ mice. An estimated no observed effect level (NOEL) of FB_1 for renal tumors in rats is between 0.9 and 3.0 mg/kg/day, and for female mouse liver tumors is 2.2-7.5 mg/kg/day based on lifetime exposure (Howard et al., 2001).

Chickens

In day-old broiler chicks and turkey poults 300 mg FB_1/kg body weight for two weeks showed decreased body weight, hepatic necrosis and biliary hyperplasia (Brown et al., 1992).

Ruminants

Calves given feed containing FB_1, up to 148 mg/kg were ineffective in terms of feed intake or weight gain, although certain blood enzymes were elevated along with cholesterol (Osweiler et al., 1993). A study on Angora goats showed that goats can be fed for up to 112 days with diets containing 95 mg FB_1 /kg of diet without any overt signs of toxicosis and also without any effect on weight gain (Gurung et al., 1998). Milk-fed

calves treated with 1 mg/kg FB_1 iv showed increased serum sphinganine concentration with no alteration in serum sphingosine concentration and no observed cardiovascular changes (Mathur et al., 2001).

Humans and Primates

FB_1 has been statistically associated with high incidence of esophageal cancer in certain areas of the Transkei, South Africa (Dutton, 1996) and liver cancer in China (Ueno et al., 1997). On the basis of the toxicological evidence, the International Agency for Research on Cancer has declared *F. moniliforme* toxins as potentially carcinogenic to humans (class 2B carcinogens; Vainio et al., 1993). Inhabitants of Transkei region separate corn into good (not visibly moldy) and moldy corn, the latter being used primarily for beer brewing. Studies on yeast fermentation of corn show that fumonisins are not destroyed during fermentation (Bothast et al., 1992). However, esophageal cancer is a multi-factorial disease, and deficiencies in certain vitamins (Jaskiewicz et al., 1988a) and trace elements (Jaskiewicz et al., 1988b) have been identified in high-risk populations in the Transkei region.

Primate studies were done on baboons and vervet monkeys to support the human epidemiological data, but no species seemed to respond to FB_1 in quite the same way and none developed esophageal cancer. Study on vervet monkeys showed signs of liver damage with increased liver enzymes and cholesterol in serum (Jaskiewicz et al., 1987).

Culture material from *F. verticillioides*, strain MRC 826, significantly enhanced nitrosamine-induced esophageal carcinoma in BD-IX rats (van Rensburg et al., 1985). However, when this rat strain was co-administered purified FB_1 with nitrosamine, there

was no effect of FB_1 on esophageal cancer incidence (Wild et al., 1997), suggesting that FB_1 by itself is not an esophageal carcinogen.

Fumonisin and sphingolipids:

The structural similarity between sphinganine and FB_1 led Wang et al. (1991) to hypothesize that the mechanism of action of this mycotoxin was via disruption of either sphingolipid metabolism or function. Free sphingoid bases are present in low concentrations in all eucaryotic cells. Sphinganine is an intermediate in the *de novo* sphingolipid biosynthetic pathway. The most common sphingoid base in animal cells is sphingosine, which is a component of the ceramide backbone found in all more complex sphingolipids, e.g., glucocerebrosides, gangliosides, sphingomyelins, etc. The presence of free sphingosine in cells is a result of degradation or turnover of more complex sphingolipids. Research on the role of complex sphingolipids, free sphingoid bases, and sphingolipid degradation products in signal transduction pathways, and as mediators of cell growth, differentiation, and cell death has been rapidly expanding (Merrill et al., 1997).

Fumonisins are specific inhibitors of ceramide synthase (sphinganine and sphingosine-*N*-acyl transferase (Wang et al., 1991; Merrill et al., 1993b, Fig. 2.2). Fumonisin-induced disruption of sphingolipid metabolism has been demonstrated in plants, yeast, and all animals that have been studied (Riley et al., 1996). In the *de novo* pathway, sphinganine is converted to dihydroceramide by sphinganine *N*-acyl transferase. Oxidation of the sphinganine backbone of dihydroceramide results in the formation of ceramide. Subsequent modifications at the 1-hydroxyl group of ceramide or

dihydroceramide results in the formation of all more complex sphingolipids i.e., sphingomyelin, and glycosphingolipids. Fumonisin treatment is associated with sphingosine increase, which is probably produced by inhibition of reacylation of sphingosine derived from dietary sources or resulting from turnover of more complex sphingolipids (Fig. 2.2). The degradation of the accumulated sphingosine results in increases in other bioactive lipids such as sphingosine-1-phosphate (sphingosine 1-P) and various phospholipids (Smith and Merrill, 1995). The disruption of sphingolipid metabolism by fumonisins usually precedes and is closely correlated with the incidence and severity of the *in vivo* toxicity (Riley et al., 1996; Tsunoda et al., 1998).

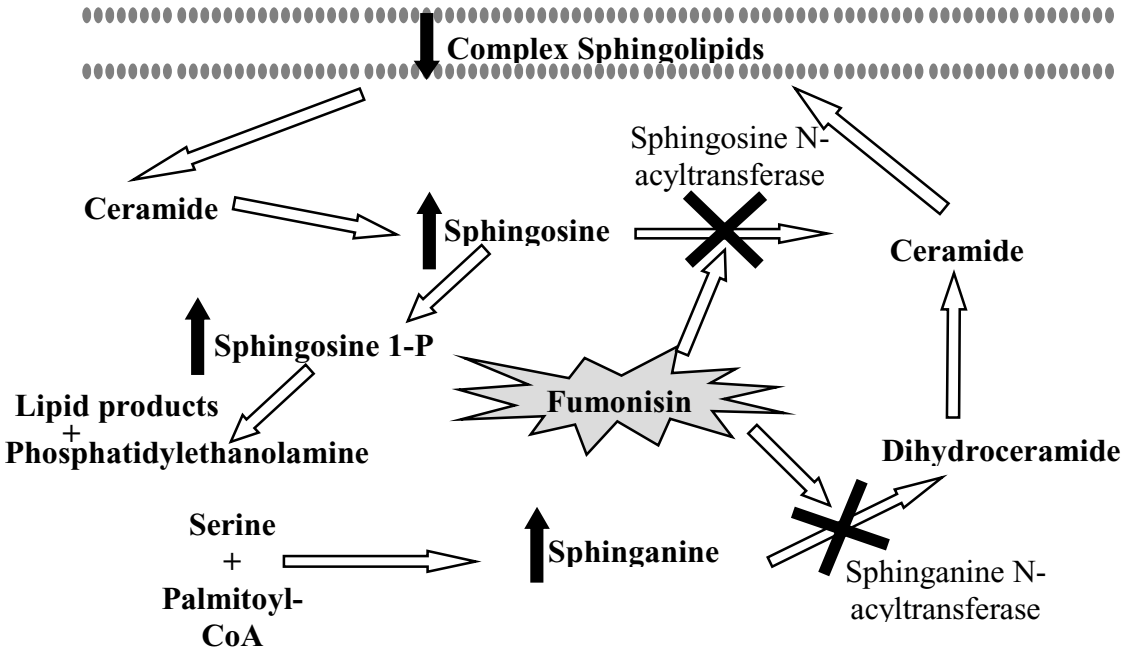


Figure 2.2. Fumonisin-induced alterations in sphingolipid metabolism pathway.

There are many potential molecular sites, which could be affected by fumonisin-induced disruption of sphingolipid metabolism. Because sphingolipids can both

stimulate (Zhang et al., 1991) and inhibit (Merrill, 1993a) cell growth, inhibition of sphinganine *N*-acyltransferase is an attractive hypothesis for the first step in the onset and progression of the diseases associated with consumption of fumonisins. FB_1-induced elevation in free sphingoid bases induces apoptosis, whereas production of sphingosine-1-phosphate or inhibitors of ceramide biosynthesis can prevent apoptosis (Riley et al., 2001). FB_1 elevates endogenous sphinganine and stimulates DNA synthesis in Swiss 3T3 cells (Schroeder et al., 1994). Furthermore, the addition of myriocin, an inhibitor of serine palmitoyltransferase which catalyzes the first, rate-limiting step of *de novo* sphingolipid biosynthesis, resulted in blockage of both fumonisin-induced sphinganine accumulation and apoptosis with reversal of antiproliferative effects (Riley et al., 1999).

Alteration of tissue and serum, sphinganine and sphingosine levels has been established as an early biomarker in FB_1 toxicity, but unable to fully explain its further involvement in toxicity. In Sprague-Dawley and Fischer 344 rats, New Zealand white rabbits, and BALB/c and other mouse strains, disruption of sphingolipid metabolism in liver or kidney occurs at FB_1 doses below those that produce morphologic evidence of injury (Voss et al., 2000). In mouse FB_1-treatment primarily causes liver toxicity without much effect on kidney, but after FB_1-treatment kidney shows a greater change in sphingoid base accumulation as compared to liver (Riley et al., 1998). It has been hypothesized that the threshold for toxicity in liver and kidney is dependent on a corresponding threshold effect on disrupted sphingolipid metabolism (Voss et al., 1998). Increased sphingoid base concentrations have also been found in lung and heart, although apparently in the absence of apoptosis or other indications of toxicity (Smith et al., 1999). While primary rat hepatocytes and liver slices are very sensitive to fumonisin-induced

disruption of sphingolipid metabolism, they were relatively insensitive to the toxic effects of fumonisins (Norred et al., 1997), suggesting that primary rat hepatocytes are not a good model for *in vivo* hepatotoxicity.

Fumonisin B_1 and tumor necrosis factor α:

In various studies tumor necrosis factor α (TNFα) has also been shown to be a contributor in FB_1 toxicity. FB_1 increased TNFα expression in several strains of mice (Sharma et al., 2000, 2001). The *in vivo* hematological effects of FB_1 were partially reversed by anti-TNFα antibodies (Dugyala et al., 1998). Mouse strain lacking p75 or p55 tumor necrosis factor receptor also showed lesser liver toxicity after FB_1 exposure (Sharma et al., 2000; 2001). Macrophages derived from mice treated with FB_1 produced higher amounts of TNFα when stimulated by the mitogen, lipopolysaccharide compared to controls and cells treated with FB_1 *in vitro* also produced TNFα (Dugyala et al., 1998). FB_1 toxicity in CV-1 cells (African green monkey kidney fibroblasts) was prevented by inhibitor of apoptosis protein (IAP), an inhibitor of TNF pathway (Ciacci-Zanella and Jones, 1999). Expression of tumor necrosis factor type 1 receptor associated protein 2 was induced in FB_1-sensitive CV-1 cells but repressed in FB_1-resistant COS-7 cells demonstrating the involvement of TNFα-induced death pathway in FB_1-induced apoptosis (Zhang et al., 2001).

Tumor Necrosis Factor α:

TNFα was isolated nearly 25 years ago, on the basis of its ability to kill tumor cells *in vitro* and to cause hemorrhagic necrosis of transplantable tumors in mice

(Carswell et al., 1975). Since then, TNFα is known to play an important role in immune response, inflammation, autoimmune and pathophysiology of many diseases (Probert et al., 1996). TNFα occurs naturally in two biologically active molecular forms, a 26-kDa type II transmembrane pro-TNF acting locally through cell-to-cell contact, and a 17-kDa secreted mature TNF that is cleaved from pro-TNF by proteolytic enzymes and is capable of acting on distant targets (Kriegler et al., 1988).

TNFα mRNA levels increase strikingly with in 15-30 min of macrophage stimulation with no requirement of *de novo* protein synthesis (Zang and Tracey, 1998). The cell-type-specific regulation of TNFα synthesis is reflected not only by diverse distribution of TNFα receptors, but also by the differential use of regulatory elements on TNFα biosynthesis in different cells.

At the transcriptional level, a number of regulatory sequences are found upstream of the TNFα gene including three nuclear factor-κB (NF-κB) sites (Goldfeld et al., 1993), three nuclear factor-activated T cells (NFAT) binding sites (Tsai et al., 1996), one activating protein-1 (AP-1) and one AP-2 binding site (Leitman et al., 1992). As the factors participating in the regulation of TNFα synthesis pre-exist in unstimulated cells, modification of the transcription factors able to bind to the regulatory elements on the TNFα promoter, such as NFAT, NF-κB and AP-1, has been implicated in the mechanism of TNFα induction. Protein kinases and phosphatases are also involved in the induction of TNFα (Chung et al., 1992; Lee et al., 1994), as well as proteases and phospholipase D (Balboa et al., 1992). Some of these enzymes are able to modify the activity of TNFα induction-related transcription factors such as NF-κB and AP-1. Mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK) kinase (MEKK) can

also activate a few TNFα induction-associated transcription factors such as NF-κB, c-Jun and p38 (Cohen et al., 1996), and thus play a role in TNFα regulation.

At the translational level, TNFα synthesis is inhibited in quiescent cells but highly inducible upon stimulation. It is believed that the enforced inhibition is an important mechanism to protect the host from the harmful effects of TNFα. A key element for translational regulation of TNFα has been identified in the 3' untranslated region (UTR) of TNFα mRNA. It is an AU-rich sequence, UUAUUUAU, and has been shown to decrease mRNA stability (Wilson and Treisman, 1988) or inhibit its translation. The mechanism of translational regulation by the AU-rich sequence has been suggested through physical interaction with the poly(A) tail, resulting in the failure of mRNA to form large polysomes (Grafi et al., 1993). The activation of MAP kinase cascade enhances the translational efficiency of TNFα mRNA (Lee et al., 1994).

TNFα Signaling:

TNFα has been shown to modulate proliferation, differentiation, and apoptotic or necrotic cell death in a number of different cell types (Heller and Kronke, 1994). These disparate responses to TNFα are mediated by TNFα binding to two distinct TNFα receptors, type I (TNFR 55) and type II (TNFR 75, Smith et al., 1994). Both are type I transmembrane glycoproteins and members of the TNF receptor superfamily characterized by the presence of multiple cystine-rich repeats of about 40 amino acids in the extracellular amino-terminal domain. It is suggested by X-ray diffraction study (Banner et al., 1993) that the primary function of TNFα trimers is to mediate receptor aggregation, which activates receptors by inducing receptor trimerization. A silencer of

death domains (SODD) protein has been identified which binds to the TNFR 55 death domain thus preventing its spontaneous signaling (Jiang et al. 1999).

A majority of TNFα activities can be mediated solely by TNFR 55 (Tartaglia et al., 1993). Three functional domains, C-terminal death domain (Tartaglia et al., 1993) and adjacent N-SMase (neutral sphingomyelinase) and A-SMase (acidic sphingomyelinase) activating domains (NSD and ASD) (Schutze et al., 1992; Wiegmann et al., 1994), have been found in the intracellular region of TNFR 55 to be responsible for transferring signals from TNFα to intracellular adaptors.

TNFR 55 death domain mediates both apoptotic and antiapoptotic pathways (Fig. 2.3). TNFR1-associated death domain protein (TRADD, Hsu et al., 1995) is a death domain-containing protein that interacts directly with the death domain of ligand occupied TNFR 55. TRADD is believed to act as an adaptor protein that recruits Fas-associated death domain protein (FADD) and receptor interacting protein (RIP) through the C-terminal death domain, and recruits TNF receptor-associated factor (TRAF2) through N-terminal TRAF-interacting domain (Hsu et al., 1996a,b). FADD's N-terminal death effector domain binds with the death effector domain of caspase-8 (Boldin et al., 1996), whose activation leads to the activation of the protease cascade leading to apoptosis. Another TNFR 55 pathway leading to apoptosis is TRADD-RIP pathway. RIP recruits another adaptor protein, RIP associated Ich-1/CED-3 homologous protein with death domian (RAIDD, Duan and Dixit, 1997). RAIDD binds RIP through its death domain and recruits caspase-2 to RIP to activate the protease cascade.

The antiapoptotic pathway mediated by TNFR 55 involves a serine-threonine kinase or NF-κB-inducing kinase (NIK) that binds specifically to TRAF2 and mediates

TRAF2-dependent activation of NF-κB (Malinin et al., 1997) by stimulating the phosphorylation of I-κB kinase complex (IKK), causing its activation. NF-κB exists in the cytosol of many cell types as an inactive complex of Rel-related factors, bound to a member of IκB. Activation of NF-κB follows phosphorylation of I-κB on serine residues, by IKK complex (Manning and Rao 1997). Phosphorylated IκB is enzymatically degraded by the multicatalytic proteasome complex (Palombella et al., 1994), and liberated NF-κB dimers then translocate to the nucleus and promote transactivation of target genes. NF-κB is required for TNFα-mediated induction of inhibitor of apoptosis protein (IAP), which when over expressed activates NF-κB and suppresses TNFα cytotoxicity through this positive feedback loop (Chu et al., 1997). IAP has been shown to be induced by NF-κB in multiple cell lines (Stehlik et al., 1998). The central mechanisms of IAP apoptotic suppression appear to be through direct caspase and pro-caspase inhibition (primarily caspase 3 and 7) (Roy et al., 1997) and modulation of and by the transcription factor NF-κB. Recently three other anti-apoptosis genes that are induced by NF-κB have been demonstrated: they are A20 (Song et al., 1996), Manganous Superoxide Dismutase (MnSOD, Jones et al., 1997) and an alternate splice product of an immediate early gene, IEX-1L (Wu et al.,1998). In contrast to the IAPs, A20 is a negative regulator of NF-κB activation.

NSD (neutral sphingomyelin domain) mediates a number of TNF responses including inflammatory responses and cell proliferation through MAP kinase ERK and Phospholipase A2 (PLA2). Activated Neutral-sphingomyelinase acts at the outer leaflet of the plasma membrane to trigger the degradation of sphingomyelin into ceramide, which acts as second messenger in inducing apoptosis (Jarvis et al., 1994). Ceramide

generated on membranes can further activate ceramide-activated protein kinase (CAPK) (Winston and Riches, 1995), which then phosphorylates cytoplasmic raf-1. Activated raf-1 can activate MAP kinase cascade followed by the activation of PLA2 (Lin et al., 1993). ASD (acidic sphingomyelin domain) is also suggested to play a role in TNFα-induced apoptosis and NF-κB activation. ASD mediates the activation of acidic-sphingomyelinase via phosphatidyl choline-phospholipase C pathway (Schutze et al., 1992).

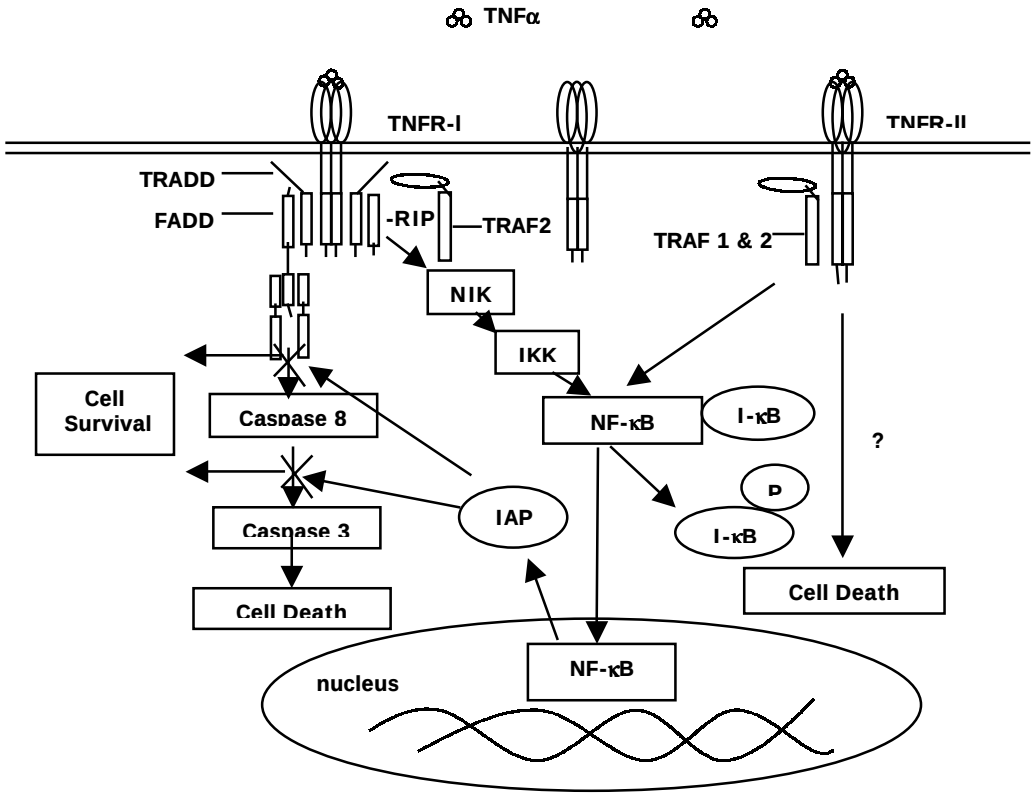


Figure 2.3. TNFα signal transduction pathway

TNFR p75, like TNFR p55, is widely expressed. TNFR p75 binds soluble TNFα poorly, and may only be activated by membrane bound TNFα (Grell et al., 1995). Unlike TNFR p55 it has no cytoplasmic death domain, but it can directly bind TRAF1 and

TRAF2, and in this way give activation signals and/or modulator activities of TNFR p55 (Peschon et al., 1998; Grell et al., 1998). However, TNFR p75 knockout mice have increased resistance to TNFα induced cell death and tissue necrosis (Erickson et al., 1994), raising the possibility that some death signals are mediated also by this receptor. Cytotoxic effects of TNFR p75 can also be mediated by endogenous production of TNFα and autotropic or paratropic activation of TNFR p55 (Grell et al. 1999).

Cytokine network in liver toxicity:

Compared to other mammalian organs, liver is a unique organ in that it has its own innate immune system comprising of Kupffer cells, which are the largest population of fixed macrophages in the body, and the hepatic natural killer cells (Seki et al., 2000). This innate immune system is responsible for the localized activation of cytokine network in liver. Liver is also the major sink for cytokines that circulate in the bloodstream because it harbors the largest number of cells with cytokine receptors and a high density of receptors per cell (Geisterfer et al., 1993). In the case of hepatic injury TNFα induction is one of the earliest events, and can trigger a cascade of other cytokines that cooperate to kill hepatocytes, recruit inflammatory cells, and initiate a wound healing response including fibrogenesis (Diehl et al., 2000).

TNFα induces macrophages to secrete various proinflammatory cytokines like interleukin (IL)-1β and IL-6 (Abbas et al., 1997). TNFα and IL-12 produced by macrophages can also stimulate the natural killer (NK) cells and $NK1^+$ T cells to secrete interferon (IFN) γ, which through a positive amplification loop can further increase TNFα and IFNγ (Kaplan and Schreiber, 1999). On the other hand TNFα, IL-6 and IL-12

up-regulate IL-10 synthesis, which can suppress proinflammatory cytokine synthesis (Pretolani et al., 1999). Thus a network of cytokines rather than individual cytokine activity may be important in the outcome of any immune/inflammatory responses of liver (Brennan and Feldmann, 2000).

Various cytokines are apparently involved in different experimental mouse models of hepatotoxicity. Neutralizing antibodies to TNFα or IL-1 partially prevented liver damage in mice initiated by hepatotoxic doses of acetaminophen (Blazka et al., 1995). TNFα, IFNγ and IL-12 were involved in liver toxicity known as Shwartzman reaction caused by repeated lipopolysaccharide challenge (Ogasawara et al., 1998). IFNγ over-expressed mice also develop chronic active hepatitis (Okamoto et al., 1999). TNFα and IL-6 are involved in alcoholic liver disease (Kamimura and Tsukamoto, 1995). TNFα and IFNγ have also been related to Con A-induced hepatitis (Ksontini et al., 1998).

Cytokines are also the major factors, which determine the differentiation of naive T helper (Th) cells into the Th1 or Th2 phenotypes. The presence of IL-12 during priming is associated with the induction of Th1 cells while IL-4 influences the preferential activation of Th2 cells (Romagnani et al., 2000). The imbalance in Th1/Th2 system has been correlated with different pathophysiological processes (Tanaka et al., 1996).

Apoptosis signaling molecules:

The execution of apoptosis may be initiated by many different signals, either from within or outside the cell involving ligand-receptor interactions, like TNFα/TNFR or Fas/Fas-ligand (FasL), or TGF-β/TGF-receptor, or more potentially by more unspecific

signals such as ceramide or DNA damage. Two most important death receptors involved in liver pathology include TNFα and Fas (Faubion and Gores, 1998). During the modulation phase of apoptosis many different genes such as c-Myc/Mad or Bcl-2/Bad have been shown to able to shift the balance either to cell survival or cell death.

Fas signaling and liver toxicity:

Fas/FasL receptor interactions play an important role in induction of apoptosis in liver. The hepatocytes in the liver appear to be especially susceptible to Fas mediated death, because injection of anti-Fas induces massive injury to liver and not elsewhere (Pinkoski et al., 2000). Hepatocytes constitutively express Fas and may upregulate expression of this receptor in a variety of liver diseases like viral hepatitis or alcohol induced liver disease (Kanzler and Galle, 2000). Fas expression is induced by inflammatory cytokines such as IL-1 (Giordano et al., 1997). At the same time Fas expression is also reduced in particularly aggressive forms of hepatocellular carcinoma (Ito et al., 1998).

Similar to the TNFR pathway, ligation of the FasL to the Fas results in Fas oligomerization, leading to recruitment of the adapter protein, FADD, which further recruits procaspase 8 molecules to the death-inducing signaling complex, resulting in activation of this protease (Pinkoski, et al., 2000). Another important caveat is that the mere expression of Fas does not imply that the cell is susceptible to apoptosis by Fas ligation. Fas-R signaling in many tissues, and likely in the liver, can be inhibited by a variety of processes depending on the cell type, including expression of I-Flice (an endogenous dominant-negative form of caspase 8), Bcl-2 (a cytoprotective molecule),

and X-linked inhibitor of apoptosis (molecules that directly inhibit downstream effector caspases, Faubion and Gores, 1999).

c-Myc/Max/Mad netwok:

The Myc, Max and Mad proteins form a network centered around Max, which forms transactivating complexes when associated with c-Myc but repressive complexes when bound to Mad (Baudino and Cleveland, 2001). Expression of c-Myc is required for entry into the S phase of the cell cycle. Although the transcription factor c-Myc is usually associated with cell proliferation, resting or serum starved cells, in which c-Myc is overexpressed also undergo apoptosis (Henriksson and Luscher, 1996). c-Myc over expression in liver results in increased apoptosis while also inducing a higher incidence of hepatocellular carcinomas (Kao et al., 1996). The ability of overexpressed Myc to facilitate proliferation and inhibit terminal differentiation fits well with the fact that tumors of diverse origins contain genetic rearrangements involving Myc family genes (Grandori et al., 2000).

Bcl-2/Bad network:

Bcl-2 family of genes represents another class of proteins, which are involved in the modulation phase of apoptosis, shifting the balance either to cell survival or cell death. Over expression of Bad, Bax and Bcl-X_S enhance apoptosis process whereas Bcl-2 and Bcl-X_L expression inhibits apoptosis (Korsmeyer, 1999). Bax heterodimerizes with Bcl-2 and homodimerizes with itself. When Bax is overexpressed the cells undergo

apoptosis and whereas when Bcl-2 is overexpressed it heterodimerizes with Bax and represses apoptosis (Kroemer, 1997).

Caspases

The caspases (c-asp-ases) are cysteine proteases that cleave substrate on the carboxyl side of aspartate residues (Alnemri et al. 1996). They are the final executioners of apoptosis. Caspases are thought to participate in apoptosis in a number of ways: (i) auto-activation, (ii) activation of downstream caspases, (iii) activation of DNA fragmentation factor (DFF), which is responsible for internucleosomal DNA cleavage (Liu et al. 1997), and (iv) cleavage of a variety of protein substrates that disable critical cellular processes and break down structural components of cell, e.g. poly (ADP-ribose)polymerase (PARP). They are expressed as inactive proenzymes in the cytosol and are sequentially activated by proteolysis during apoptosis (Wilson, 1998). Initiator caspases (Caspases 8, 9 and 10) activate downstream effector caspases (caspases 3, 6, 7). It has been suggested that pro-caspase-8 may be the apical component of a proteolytic cascade made up of other caspases (Chinnaiyan and Dixit 1997). This is supported by the demonstration that caspase-8 can activate all other known caspases *in vitro* (Srinivasula et al. 1996). Caspase-8 can also cleave death domain kinase RIP which further results in the blockage of TNF-induced NF-κB activation (Lin et al. 1999).

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CHAPTER 3

TEMPORAL EXPRESSION OF FUMONISIN B_1-INDUCED TUMOR NECROSIS FACTOR-α AND INTERFERON γ IN MICE[1]

[1]Bhandari, N., Evaristus N. Enongene, Ronald T. Riley, Filmore I. Meredith, and Raghubir P. Sharma. 2001. Submitted to Comparative Biochemistry and Physiology, 8/8/01

Abstract

Fumonisin B_1 (FB_1), a toxic metabolite of *Fusarium verticillioides*, is a carcinogen and causative agent of various animal diseases. Our previous studies indicated the involvement of tumor necrosis factor-α (TNFα) in FB_1-induced toxic responses. To further investigate the time-course of TNFα production and signaling, mice (4/group) were treated subcutaneously (sc) or orally (po) with either vehicle or 25 mg/kg of FB_1 as a single dose and sacrificed at 0, 2, 4, 8, 12 and 24 h after treatment. The TNFα expression was increased in liver and kidney after both routes of FB_1 exposure without any alterations in spleen. The po route FB_1 treatment caused greater hepatotoxicity as compared to sc route, as depicted by increased alanine aminotransferase and aspartate aminotransferase level in plasma, observed only after po FB_1 treatment. The increase in enzymes at 8 h after po treatment correlated with the highest TNFα expression also noted at 8 h after po treatment, thus further confirming the involvement of TNFα in FB_1 toxicity. The Interferon (IFN) γ expression was increased in liver at 4 h after po FB_1 treatment, suggesting a possible combined role of TNFα and IFNγ in their induction and hepatotoxicity.

Keywords: Fumonisin; IFNγ; Kidney; Liver; Mice; Spleen; TNFα; Toxicity

Introduction

Fumonisin B_1 (FB_1) belongs to a group of naturally occurring mycotoxins produced primarily by *Fusarium verticillioides* (=*F. moniliforme*, Gelderblom et al.,

1988) and related fungi, that are common contaminants of corn and other crops throughout the world (Shephard et al., 1996).

FB_1 has been reported to be carcinogenic in rats (Gelderblom et al., 1991) and mice (National Toxicology Program technical report, 1999), and has been implicated as a contributing factor in human esophageal cancer (Sydenham et al., 1990) and possibly primary liver cancer (Ueno et al., 1997). It has been established as the causative agent in equine leukoencephalomalacia and porcine pulmonary edema (Riley et al., 1993). In laboratory animals FB_1 has been shown to be hepatotoxic and nephrotoxic (Voss et al., 1996). Liver is the common target organ that is affected in most animal species including horses, pigs, rats, mice and primates, with effects on other organs being species specific.

The precise mechanism involved in the pathogenesis of FB_1-induced toxicosis has not been elucidated. Fumonisins bear a remarkable structural and molecular similarity to the backbones of complex sphingolipids, namely sphinganine and sphingosine, and are known inhibitors of ceramide synthase (sphinganine and sphingosine-*N*-acyl transferase), leading to accumulation of free sphingoid bases, sphingoid base metabolites, depletion of more complex sphingolipids and inhibition of ceramide biosynthesis (Riley et al., 1996; Tsunoda et al., 1998). *In vitro* and *in vivo*, cells exposed to FB_1 undergo a mixture of necrotic and apoptotic cell death (Tolleson et al., 1996; Voss et al., 1996; Sharma et al., 1997).

In our laboratory repeated FB_1 treatment has been shown to increase tumor necrosis factor-α (TNFα) expression in several strains of mice (Sharma et al., 2000 a; b; c; d). Multiple FB_1 treatment in male mice caused localized induction of TNFα and interferon (IFN) γ in liver without any alterations in kidney (Bhandari et al., 2000).

TNFα has been shown to be a contributor in FB_1 toxicity where, *in vivo* effects of FB_1 were partially reversed by anti-TNFα antibodies (Dugyala et al., 1998), and mouse strain lacking p75 or p55 tumor necrosis factor receptor showed tolerance against FB_1 toxicity (Sharma et al., 2000a; d). FB_1 toxicity in CV-1 cells was prevented by inhibitor of apoptosis protein (IAP), an inhibitor of TNFα pathway (Ciacci-Zanella and Jones, 1999).

TNFα has been demonstrated as a causal agent in various liver injury and cell death caused by variety of toxins (Jones and Czaja, 1998). Liver damage in mice initiated by hepatotoxic doses of acetaminophen was partially prevented by neutralizing antibodies of TNFα (Blazka et al., 1995). TNFα has been shown to be involved in different mouse models of hepatotoxicity, i.e., alcoholic liver disease and Con A-induced hepatitis (Kamimura et al., 1995; Ksontini et al., 1998). TNFα in conjunction with IFNγ has also been shown to be involved in liver toxicity known as Schwartzman reaction caused by repeated lipopolysaccharide (LPS) challenge (Ogasawara et al., 1998). TNFα and IFNγ induction can generate a positive feed-back loop causing their further increased expression leading to hepatotoxicity (Kaplan and Schreiber, 1999).

The objective of the current study was to investigate the time-related induction of TNFα and IFNγ after a single oral (po) or subcutaneous (sc) dose of FB_1. The two routes of exposure were chosen because of the limited absorption of FB_1 from the gastrointestinal tract and the results were compared for any correlation between the alteration in TNFα and IFNγ expression and hepatotoxicity observed due to FB_1.

2. Materials and Methods

2.1. Animal and housing

Adult male BALB/c mice, 6-8 weeks old (about 20-25 g body weight), were obtained from Harlan Laboratories (Indianapolis, IN). The animals were acclimated in the University of Georgia Animal Resources facility for a week at 23°C and 50% relative humidity with a 12 h light/dark cycle. Pelleted feed and fresh water was provided *ad libitum*. Food and water consumption was recorded daily. The protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee.

2.2. Treatment and sampling

Mice (4/group) were treated subcutaneously (sc) or orally (po) with either physiological buffered saline (PBS) or 25 mg/kg of FB_1 (96% purity, dissolved in PBS), as a single dose and sacrificed at 2, 4, 8, 12 and 24 h after treatment. The 0 h control animals were left untreated. Dose of FB_1 was selected based on previous dose response studies, in which FB_1 produced liver injury without producing any overt toxicity (Sharma et al., 1997). At selected intervals animals were anesthetized (by halothane) and their blood collected via cardiac puncture into a heparinized syringe. The blood was used for blood counts and subsequent isolation of plasma for estimation of liver enzymes. Pieces of liver, kidney and spleen were quickly frozen on dry ice and stored at -85°C until analyzed

2.3. Hematology and estimation of liver enzymes in plasma

Total blood erythrocyte and leukocyte cell counts were determined using an electronic counter (Coulter Electronics, Hialeah, FL). Differential counts were done on

blood smears stained with Giemsa's stain. Levels of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using Hitachi 912 automatic autoanalyzer (Roche Diagnostics Corp. Indianapolis, IN).

2.4. RNA isolation and relative estimation of TNFα and IFNγ mRNA expression

The RNA was isolated from the tissue, converted to cDNA by reverse-transcriptase (RT) and amplified by polymerase chain reaction (PCR) using the protocol as described earlier (Sharma et al., 2000a). For semi-quantitative measurements, the number of cycles within a linear increase in the product was selected for each cytokine. Samples were amplified for 30 cycles for β-actin, 35 cycles for IFNγ and 40 cycles for TNFα in a thermal cycler (Eppendorf Scientific, Westbury, NY). The annealing temperatures were optimized using a gradient. The annealing temperature of 48°C was used for β-actin, and IFNγ; and 54°C for TNFα. The sense and antisense primers for TNFα and β-actin were same as used in a previous study (Sharma *et al*., 2000a). For IFNγ the primers used were 5'- AACGCTACACACTGCATCT-3' (sense) and 5'-AGCTCATTGAATGCTTGG-3' (antisense; chosen by Primer3 program; Whitehead Institute, Cambridge, MA). Amplified products were separated by electrophoresis at 150 V on 2% agarose gels. The gels were photographed using a back lighted UV transilluminator (Ultra Lum, Inc., Carson, CA). The TNFα and IFNγ expression was normalized against β-actin bands and quantified via densitometric imaging using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT).

2.5. RNase protection assay (RPA) for TNFα expression

To confirm the results from RT-PCR, RPA was performed on selected samples using Pharmingen's RiboQuant^{TM} RPA starter kit (San Diego, CA). In brief, the

templates containing mouse TNFα and L32 house keeping gene were used for the T7 RNA polymerase-directed synthesis of high-specific-activity, [α^{32}P]UTP-labeled, anti-sense RNA probes using in vitro transcription kit (Pharmingen, San Diego, CA). Dried probe was dissolved in hybridization buffer to probe concentration of 4×10^5 cpm/μl and 2 μl of this added to the tubes containing 50 μg of sample RNA dissolved in 8 μl hybridization buffer. The samples were overlaid with mineral oil, heated to 90°C, and then incubated at 56°C for 12-16 h. Free probe and other single-stranded RNA molecules were digested with RNase A and RNase T1 at 30°C for 45 min, followed by proteinase K digestion at 37°C for 15 min. The remaining "RNase-protected" probes were purified according to manufacturers protocol and resolved on a denaturing polyacrylamide gel (5% acrylamide, 5 mm wide and 30 cm long) using IBI Base Runner™ 200 (Shelton Scientific, Shelton, CT). The gels were dried onto a filter paper in a pre-heated gel dryer (Labconco, Kansas city, MO) at 80°C for 1 h. Dried gels were placed on XAR film (Kodak, Rochester, NY) with intensifying screens and were developed at -70°C for 48 h. The TNFα expression was normalized against L32 and quantified via densitometric imaging using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT).

2.6. *Statistics*

Data from these studies were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan's Multiple Range Test. The SAS computer program (SAS Institute, Cary, NC) was employed for all calculations. The level of $p < 0.05$ was considered statistically significant for all comparisons. Other levels of significance are indicated with results.

3. Results

Single dose treatment of mice with FB_1 by sc or po routes did not produce any clinical signs of toxicity. Erythrocyte counts were lowered at 4 and 12 h after po FB_1 treatment (Table 3.1). The leukocyte counts were not altered after sc or po FB_1 treatment, although a trend for increase was noted at 8 h after po FB_1 treatment. FB_1 treatment did not alter circulating lymphocytes whereas circulating neutrophils were altered only after po treatment showing an increase at 4 and 8 h after treatment (Fig. 3.1).

A single oral dose of FB_1 at 25 mg/kg caused hepatotoxicity, as indicated by an increase in plasma ALT and AST levels (Fig. 3.2). Both ALT and AST levels increased at 8 h after po FB_1 treatment. No changes in liver enzymes were evident in mice after sc treatment, suggesting that this route of exposure was less hepatotoxic than the oral route.

FB_1 treatment by either sc or po route induced expression of TNFα mRNA in liver. Subcutaneous FB_1 treatment showed increased TNFα expression at 2, 4 and 8 h in liver (Fig. 3.3). FB_1 treatment by po route caused a much greater increase in TNFα expression at 8 h in liver compared to sc route. TNFα expression was also increased in kidney after both sc or po routes of exposure (Fig. 3.4). FB_1 treatment by sc route increased TNFα expression at 2 and 8 h in kidney, whereas the increase was noted at 4 and 8 h after po treatment. The increase in TNFα expression in kidney at 4 and 8 h after po route exposure was significantly greater than the increase noted after sc route. No changes were observed in TNFα expression in spleen after sc or po FB_1 treatment (Table 3.2).

IFNγ expression in liver was increased at 2 h after both sc and po FB_1 treatment (Fig. 3.5). IFNγ expression peaked at 4 h after po FB_1 treatment, whereas no change in IFNγ expression was observed at 4 h after sc FB_1 treatment.

To check the validity of TNFα expression by RT-PCR, RPA was used on a limited number of samples. Measurement of TNFα expression in liver and kidney at 4 and 8 h after po FB_1 treatment by RPA, (Fig. 3.6) also showed increased TNFα expression in liver and kidney at 8 h after FB_1 treatment, thus correlating with the peak of TNFα expression observed at 8 h in liver and kidney measured by RT-PCR.

Control mice treated with PBS after po exposure, at all time points were not different from mice treated after sc exposure (data not shown). For presentation the PBS-treated control mice, sc or po were combined together. The control animals also showed alterations in measured parameters with time, after treatment with PBS. Some of these changes could partly be due to stress-related gene alterations. This emphasizes the reasoning for having individual control groups for each time point after treatment by sc or po route.

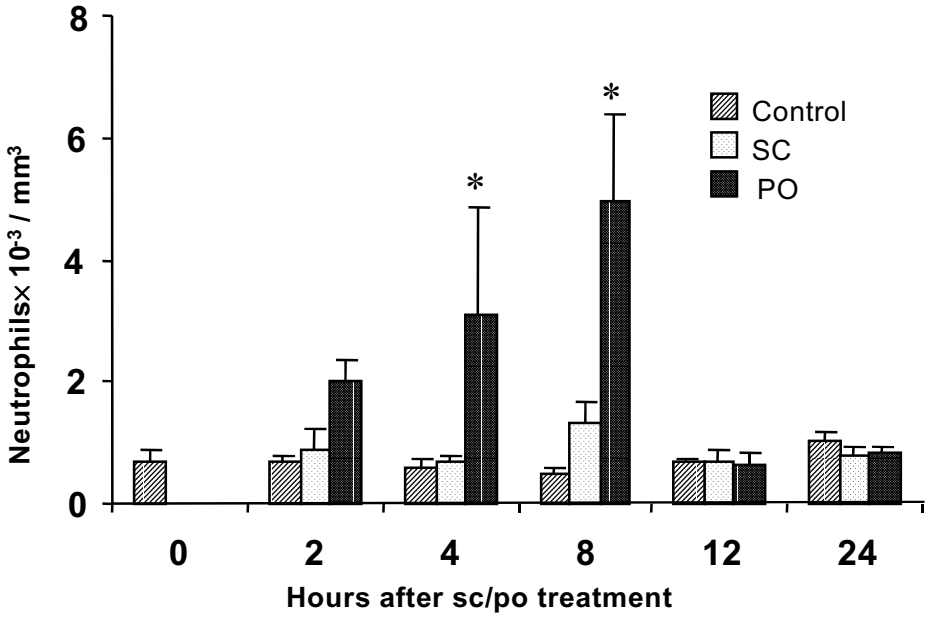

Figure 3.1. Neutrophil counts after sc/po treatment with vehicle (control) and sc or po treatment with FB_1. Results are expressed as mean ± standard error. * Significantly different than the control group at $P < 0.05$.

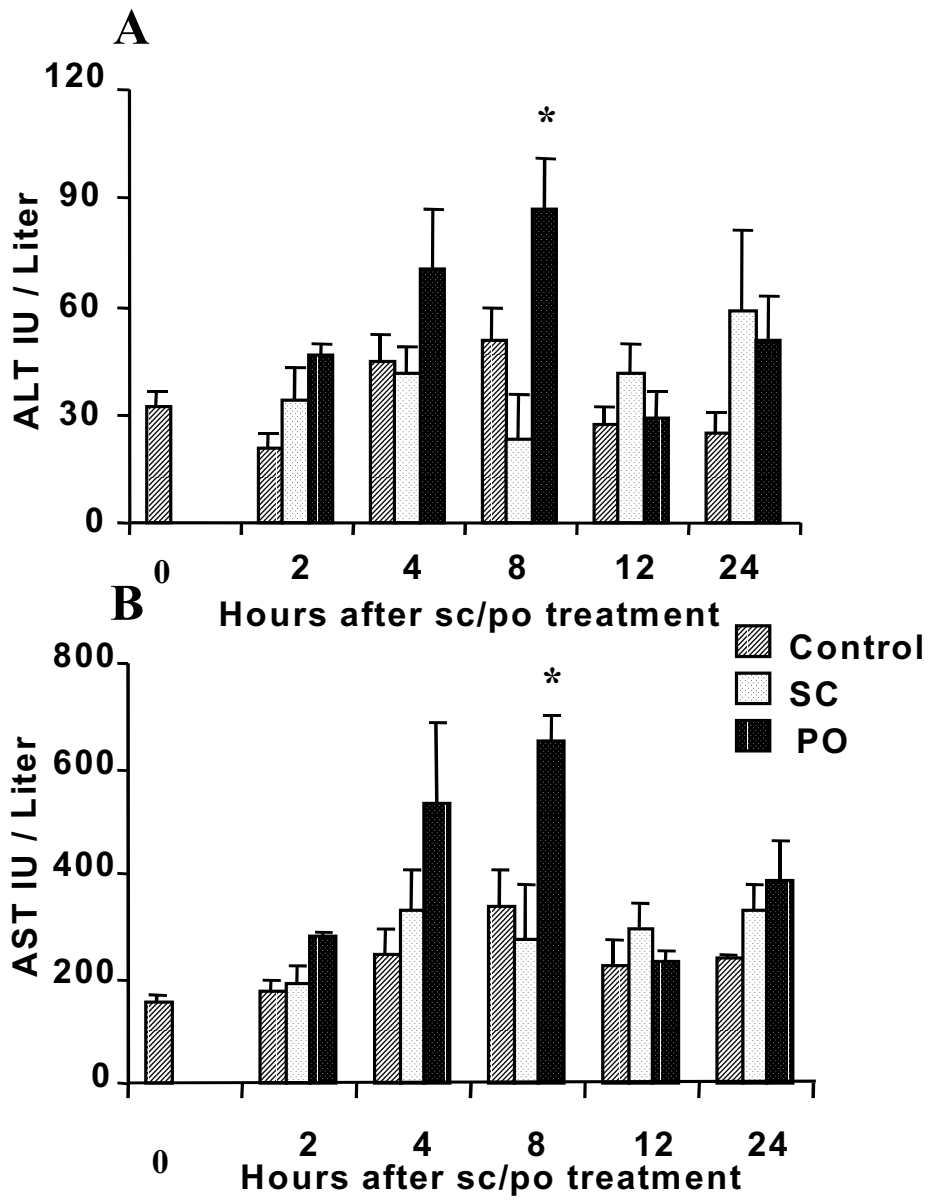

Figure 3.2. Plasma concentrations of liver enzymes (A) ALT and (B) AST after sc/po treatment with vehicle (control) and sc or po treatment with FB_1. Results are expressed as mean ± standard error. * Significantly different than the control group at $P < 0.05$.

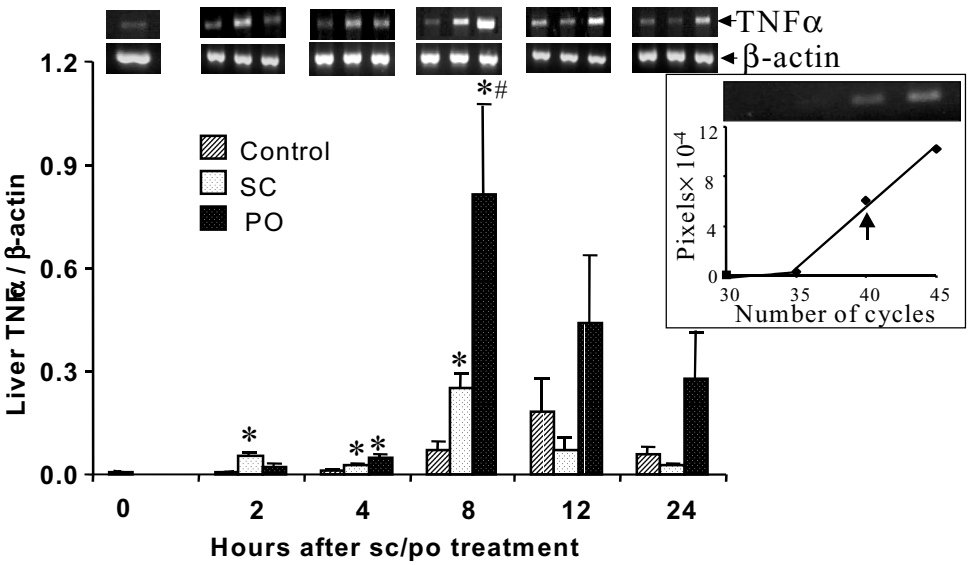

Figure 3.3. TNFα mRNA levels in liver after sc/po treatment with vehicle (control) and sc or po treatment with FB_1. TNFα mRNA levels were quantified by reverse transcriptase polymerase chain reaction (RT-PCR). The TNFα expression was normalized against β-actin. Representative gels are shown on top of respective bars. Results are expressed as mean ± standard error. * Significantly different than the control group at $p < 0.05$ # Significantly different than the corresponding sc FB_1 treatment at $p<0.05$. Graph on right shows the validity of 40 cycles (arrow) for a semi-quantitative measurement of TNFα, within the exponential phase of increase in product size.

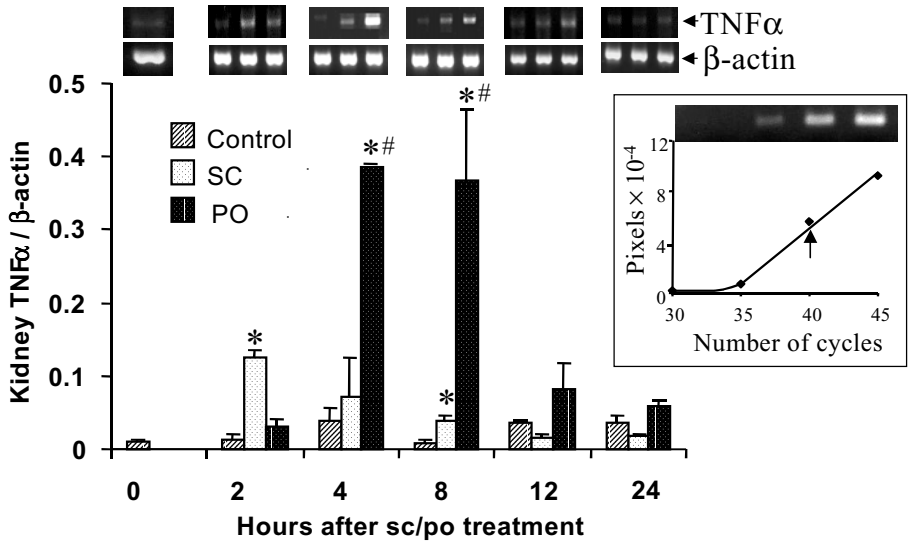

Figure 3.4. TNFα mRNA levels in kidney after sc/po treatment with vehicle (control) and sc or po treatment with FB_1. TNFα mRNA levels were quantified by RT-PCR. The TNFα expression was normalized against β-actin. Representative gels are shown on top of respective bars. Results are expressed as mean ± standard error. * Significantly different than the control group at $p < 0.05$; # Significantly different than the corresponding sc FB_1 treatment at $p<0.05$. Graph on right shows the validity of 40 cycles (arrow) for a semi-quantitative measurement of TNFα, within the exponential phase of increase in product size.

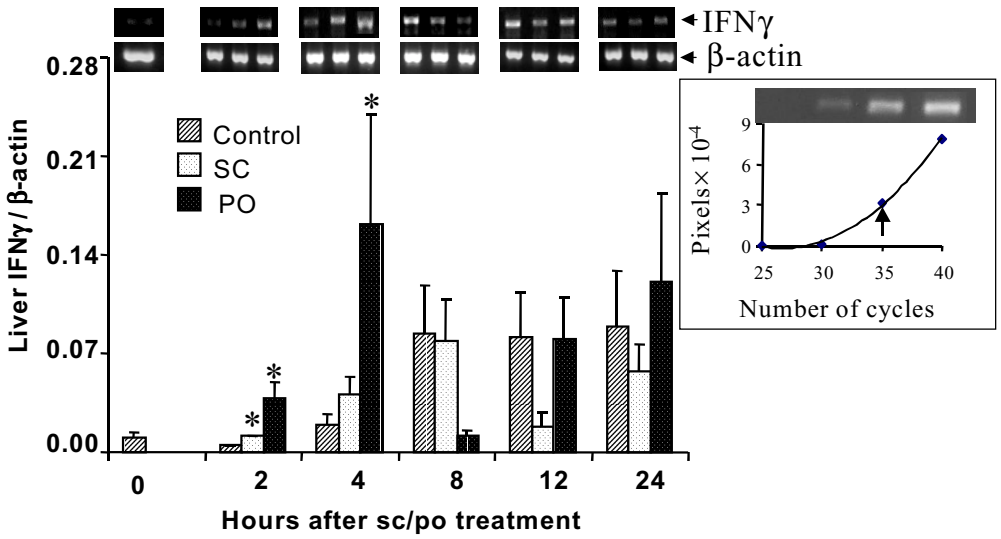

Figure 3.5. IFNγ mRNA levels in liver after sc/po treatment with vehicle (control) and sc or po treatment with FB_1. IFNγ mRNA levels were quantified by RT-PCR. The IFNγ expression was normalized against β-actin. Representative gels are shown on top of respective bars. Results are expressed as mean ± standard error. * Significantly different than the control group at $p < 0.05$. Graph on right shows the validity of 35 cycles (arrow) for a semi-quantitative measurement of IFNγ, within the exponential phase of increase in product size.

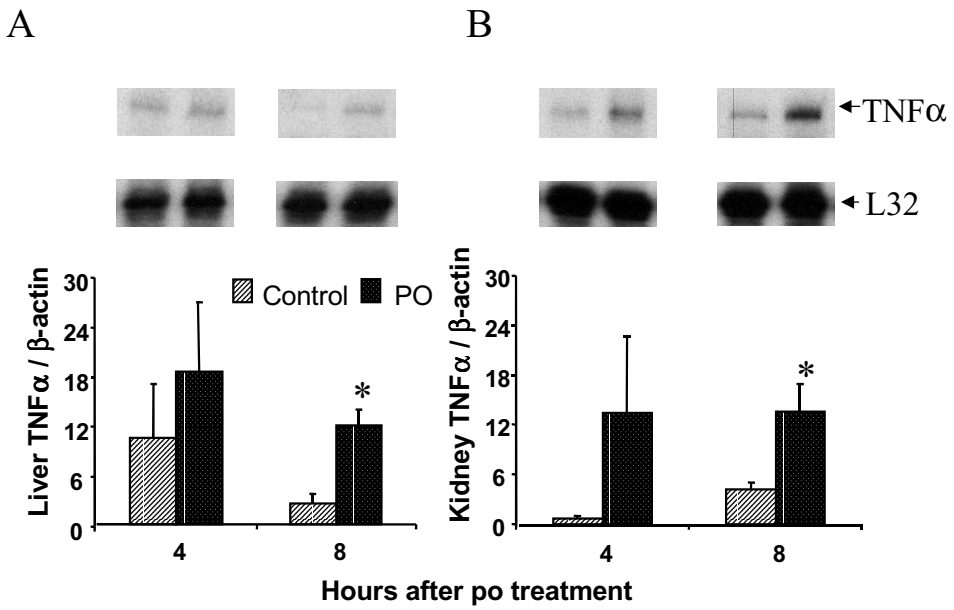

Figure 3.6. TNFα expression in (A) liver and (B) kidney at 4 and 8 h after po FB_1 treatment by RNase protection assay. The TNFα mRNA expression was normalized against L32. Representative gels are shown on top of respective bars. Results are expressed as mean ± standard error. * Significantly different than the control group at $p < 0.05$.

Table 3.1. Erythrocyte and leukocyte counts after single 25 mg/kg FB_1 treatment.

Time after treatment (h)	RBC × 10^{-6} / mm^3			WBC × 10^{-3} / mm^3		
	Saline-treated	SC FB_1-treated	PO FB_1-treated	Saline-treated	SC FB_1-treated	PO FB_1-treated
2	5.19 ± 0.18	5.00 ± 0.17	5.31 ± 0.07	5.05 ± 0.30	3.70 ± 1.11	4.86 ± 0.93
4	5.11 ± 0.10	5.00 ± 0.04	4.77 ± 0.07*	4.61 ± 1.46	3.62 ± 0.34	4.60 ± 1.83
8	4.88 ± 0.11	4.69 ± 0.15	4.73 ± 0.09	4.92 ± 1.00	4.34 ± 0.47	7.90 ± 1.20
12	4.68 ± 0.11	4.51 ± 0.18	4.24 ± 0.06*	3.20 ± 0.12	3.47 ± 0.43	2.98 ± 0.81
24	4.73 ± 0.08	4.66 ± 0.08	4.64 ± 0.07	6.47 ± 1.04	4.10 ± 0.55	3.62 ± 0.38

Results are expressed as mean ± standard error. * Significantly different than the control group at $P < 0.05$.

Table 3.2. Spleen TNFα expression after single 25 mg/kg FB_1 treatment.

Time after treatment (h)	Saline-treated	SC FB_1-treated	PO FB_1-treated
2	0.74 ± 0.18	0.59 ± 0.38	0.87 ± 0.49
4	0.69 ± 0.28	0.12 ± 0.06	0.43 ± 0.21
8	0.05 ±0.04	0.17 ± 0.10	0.45 ± 0.19
12	0.33 ± 0.18	0.05 ± 0.03	0.99 ± 0.48
24	0.21 ± 0.12	0.02 ± 0.01	0.09 ± 0.08

Results are expressed as mean ± standard error.

4. Discussion

FB_1 treatment in male BALB/c mice after two different routes of exposure resulted in differential liver toxicity. Hepatic effects, as demonstrated by increased liver enzymes in circulation, were only observed after po FB_1 treatment, whereas no change in enzyme was observed after sc treatment. The erythrocyte and neutrophil counts were only altered after po FB_1 treatment. The po route FB_1 treatment caused greater hepatotoxicity as compared to sc route.

The disparate responses of FB_1 treatment observed in the two routes of exposure can be supported by the toxicokinetic properties of FB_1. FB_1 is rapidly, but incompletely absorbed after po administration. The T_{max} (time interval for maximum drug concentration) in plasma was 1.02 h and bioavailability 3.5% after po administration of 10 mg/kg FB_1 in rats (Martinez-Larranaga et al., 1999). In a study using ^{14}C-FB_1, up to 96 h after intragastric administration 80% of the radiolabel was recovered in feces, up to 3% in urine and remainder being distributed in liver, kidney and blood, whereas after intravenous (iv) dosing 35% of the iv dose was recovered in feces, 10% eliminated in urine and rest distributed in liver, kidney and blood (Norred et al., 1993). Out of all the organs liver contained the greatest portion of the observed dose, reaching a maximum of 0.5% of the dose by 4 h after intragastric administration (Norred et al., 1993). In another comparative study, after intraperitoneal injection of a solution of FB_1, 67% of the dose was recovered in bile over 24 h period, while similar dose of FB_1 given by gavage resulted in only 0.2% recovery of the toxin in bile (Shephard et al., 1994). Thus, although biliary excretion is the major route of FB_1 elimination from circulation, only small amounts of the toxin appears to be absorbed from the gut. Data presented here

suggests a higher exposure of liver after po administration due to "first-pass" effect, which may not be evident after sc administration of the same dose of FB_1.

While FB_1 is poorly absorbed from gastrointestinal tract, some absorption does occur. FB_1 was more slowly eliminated from liver and kidney tissues than from plasma. The elimination half-life of FB_1 after po administration was longer (3.15 h) than that obtained after iv administration (1.03 h; Martinez-Larranaga et al., 1999). This difference may be the result of continued absorption of FB_1 from the gastrointestinal tract during the elimination phase, thereby prolonging the elimination half-life. In this experiment the sc dose can thus be considered a more acute dose with higher concentration and shorter half-life of elimination as compared to the po dose having transiently lower concentration and longer half-life of elimination. Since in this study the FB_1 treatment by po route caused greater toxicity as compared to sc route, the data suggests lower chronic dose of FB_1 given po could be more toxic than the higher acute dose via sc route.

The increase in expression of TNFα in liver observed as early as 2 h after sc treatment with FB_1 is possible since TNFα mRNA expression and TNF levels are induced within 1-2 h after treatment with LPS (Beutler and Cerami, 1988). We have shown TNFα induction in liver in several strains of mice after repeated treatment of FB_1 (Sharma et al., 2000a; b; c; d). In this study the TNFα mRNA expression in liver was increased after a single sc and po dose of FB_1. The increased TNFα expression at 4 and 8 h after po FB_1 treatment was greater than observed after sc FB_1 treatment. The highest peak of TNFα expression noted at 8 h after po FB_1 treatment correlated with the

increased ALT and AST levels in plasma, thus supporting involvement of TNFα in the FB_1-induced toxicity.

TNFα has been shown to be a contributor in FB_1 toxicity in various experiments. Administration of anti-TNFα antibodies partially reversed the *in vivo* hemopoietic effects in FB_1 treated mice (Dugyala et al., 1998). Transgenic mice lacking p75 or p55 TNFα receptor were also more resistant to FB_1 induced hepatotoxicity as compared to control mice Sharma et al., 200a; d). FB_1 toxicity in CV-1 cells was also prevented by inhibitor of apoptosis protein, an inhibitor of TNFα pathway Ciacci-Zanella and Jones, 1999). Expression of tumor necrosis factor type 1 receptor associated protein 2 was induced in FB_1-sensitive CV-1 cells but repressed in FB_1-resistant COS-7 cells demonstrating the involvement of TNFα-induced death pathway in FB_1-induced apoptosis (Zhang et al., 2001).

TNFα is a mediator of a variety of cellular responses, including apoptotic or necrotic cell lysis as well as cell proliferation (Heller and Kronke, 1994). Besides being cytotoxic to many tumor cells, TNFα has been shown to be mitogenic for a number of normal cells, such as fibroblasts, smooth muscle cells, T cells and B cells (Kahaleh et al., 1988). Low doses of TNFα induced DNA synthesis and enhanced growth, whereas high doses were cytotoxic (Palombella and Vilcek, 1989). The biological effects of TNFα were also strongly dependent on mitotic activity and controlled by P75 TNFR. Mitotically active cells in log growth responded to TNFα by rapidly undergoing apoptosis, whereas TNFα exposure stimulated cellular proliferation in mitotically quiescent cells (Baxter et al., 1999). In PC 60 cells the relative proportion of P75 TNFR

was decisive in predetermining the apoptotic cell death that occurred in response to TNFα (Declerq et al., 1998).

Attempts to correlate the mRNA expression of TNFα in liver tissue with protein measurements using enzyme-linked immunosorbant assay (ELISA) or western blots were not successful (Sharma et al., 2000b). It should be mentioned that changes in intracellular TNFα mRNA level may be an underestimate of the amount of protein secreted; treatment with LPS increased gene transcription 3-fold, intracellular TNFα mRNA 100-fold (post-transcriptional stabilization by acting on the AU-rich 3'-untranslated sequence) and TNFα protein production 1000-fold (Beutler and Cerami, 1988). The relative estimation of mRNA expression by RT-PCR may also underestimate the real increase in cytokine levels produced by the FB_1 treatment. In an earlier study from our laboratory we reported an increase of TNFα after LPS treatment of murine macrophages measured by PCR was 3.5-times less than that estimated by a northern blot (He et al., 2001).

FB_1 treatment by sc or po route caused no increase in TNFα mRNA expression in spleen. FB_1 seems to specifically target the liver and kidney of mice, inducing localized TNFα expression, without any contribution from splenic macrophages. Both secreted and membrane–bound form of TNFα are biologically active (Decoster et al., 1995). The secreted TNFα is capable of acting on distant targets whereas trans-membrane TNFα has a more localized effect, acting through cell to cell contact. The trans-membrane form of TNFα has been shown to be the prime activating ligand of the P75 TNFR (Grell et al., 1995).

IFNγ expression was increased in mice at 2 and 4 h after po FB_1 treatment and at 2 h only after sc FB_1 treatment. IFNγ expression peaked at 4 h and TNFα expression at 8 h after oral FB_1 treatment, suggesting a possible role of IFNγ in the further induction of TNFα. TNFα and IFNγ have shown to be involved in various mouse models of hepatotoxicity (Ogasawara et al., 1998). TNFα secreted by macrophages can activate the natural killer cells and/or Th1 cells to produce IFNγ. This IFNγ through a positive feed back loop can further activate the kupffer cells to produce more TNFα (Kaplan and Schreiber, 1999). IFNγ seems important during the priming phase, whereas TNFα is thought to be the lethal effector molecule acting on target sites already sensitized by IFNγ. IFNγ promotes the inflammatory functions of macrophage-like and endothelial cells, and sensitizes them to environmental stimuli (Ozmen et al., 1994). After a five day repeated dose of FB_1, IFNγ expression was also increased in liver (Bhandari et al., 2001), with out any changes in kidney and spleen (unpublished data).

Alterations in the free sphingoid bases have been measured after sc and po treatment with FB_1 using the same dosing protocol as in the present study (Enongene et al., 2000). After sc treatment a significant time-dependent increase in sphingoid bases occurred in the intestine and liver peaking at 4 to 8 h and declining to control levels by 24 h. In kidney the increase in free sphinganine persisted after 24 h. Similar trends were observed after po treatment with a more delayed decrease in sphingoid bases in the intestine (Enongene et al., 2001). The parallel time course of sphingoid bases and TNFα mRNA induction in liver suggests that inhibition of ceramide synthase and alterations in TNFα expression are related. Recently, in a porcine kidney cell line that is sensitive to FB_1 response, FB_1-induced TNFα expression was shown to be independent of free

sphingoid base accumulation (He et al., 2001). Other aspects of disrupted sphingolipid metabolism, for example sphingosine-1-phosphate or decreased ceramide or the balance may account for the altered TNFα expression.

Possible pathways of TNFα induction involve activation of protein kinase C, or mitogen-activated protein kinases (MAPK), or sphingosine-1-phosphate, which are affected by FB_1 at early time points (Yeung et al., 1996; Pinelli et al., 1999; Riley et al., 2001). MAPKs are also known to increase the translational efficiency of TNFα mRNA (Lee et al., 1994). Other effector molecules which are altered after FB_1 treatment include, increased expression of transforming growth factor-β1 and c-myc in rat liver (Lemmer et al., 1999), inhibition of serine/threonine phosphatase (Fakuda et al., 1996), and altered expression of cyclins and cyclin-dependent kinases (Ciacci-Zanella et al., 1998). Thus, FB_1 toxicity has many secondary sites of action, which could be involved in the final outcome.

In conclusion, the results of the present study showed increased expression of TNFα and IFNγ after single sc or po dose of FB_1. The increased TNFα expression was localized in liver and kidney, without any contribution from splenic macrophages. Increased expression of IFNγ (at 4 h) could act as a potentiating factor for the observed peak of TNFα increase (at 8 h), after po FB_1 treatment. The po route of FB_1 treatment caused greater toxicity as compared to sc route, suggesting importance of enterohepatic circulation in FB_1-induced toxicity.

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CHAPTER 4

FUMONISIN B_1-INDUCED LOCALIZED ACTIVATION OF CYTOKINE NETWORK IN MOUSE LIVER[1]

[1]Bhandari, N., Corrie C. Brown, and Raghubir P. Sharma. 2001. Submitted to Journal of Food and Chemical Toxicology, 8/11/01

Abstract

Fumonisin B_1 (FB_1), a mycotoxin produced primarily by *Fusarium veticillioides* and related fungi, is a carcinogen and causative agent of various animal diseases. Our previous studies indicated the involvement of tumor necrosis factor-α (TNFα) in FB_1-induced hepatotoxicity. Male B6,129 mice (5/group) were injected subcutaneously with vehicle or 2.25 mg/kg/day of FB_1 for 5 days and sampled one day after the last treatment. FB_1 treatment caused an increased expression of TNFα, interferon γ (IFNγ) and interleukin (IL)-12 p40 in liver without any changes in kidney or spleen, suggesting the localized site of their production. IL-1β cytokine expression was increased in liver and kidney after FB_1 exposure. Cells involved in TNFα production after FB_1 treatment in liver were identified as Kupffer cells. FB_1 increased alanine aminotransferase in plasma and increased apoptotic cells in liver. Selective increase in proinflammatory T helper (Th)1-cytokines (IL-12 and IFNγ) and TNFα with no alteration in Th2-cytokines (IL-4, IL-6 and IL-10) suggest the involvement of IL-12, produced by Kupffer cells, in induction of IFNγ production by natural killer (NK) cells and/or $NK1^+$ T cells, which can undergo a positive amplification loop with TNFα produced by macrophages or other hepatic cells to elicit the toxic reaction.

Key words: Fumonisin; Mice; toxicity; cytokine; liver; Kupffer cells; Tumor necrosis factor α; Interferon γ; Interleukin-12

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; FB_1, fumonisin B_1; IFNγ, interferon γ; IL, interleukin; LPS, lipopolysaccharide;

NK, natural killer; RT-PCR, reverse transcriptase-polymerase chain reaction; Th, T helper; TNFα, tumor necrosis factor α; TUNEL, terminal UTP nucleotide transferase end-labeling.

Introduction

Fumonisins are a group of mycotoxins produced by fungus *Fusarium verticillioides (=F. moniliforme*; Gelderblom el al., 1988) and other *Fusarium* species which are common contaminants of corn throughout the world (Shephard et al., 1996). The fungus can produce toxin under field and also presumably under storage conditions. The association of the fungus in the corn plant is endophytic, while on the corn kernels it is both external and systemic. The systemic seed-borne nature of this fungus provides favorable conditions to maintain virulence and viability for years and also suggests that seed treatment is insufficient as a control (Dungan and Koehler, 1994).

Fumonisin B_1 (FB_1), the most abundant of fumonisins, causes the fatal diseases equine leukoencephalomalacia (Marasas et al., 1988) and porcine pulmonary edema (Harrison et al., 1990). Epidemiological data suggests that ingestion of *F. moniliforme*-contaminated corn is linked to human esophageal cancer (Sydenham et al., 1900). In rats kidney is the most sensitive organ whereas in mice liver is the primary site of toxicity (Voss et al., 1996; Sharma et al., 1997). FB_1 has been reported to be a hepatocarcinogen and causes primary hepatocellular carcinoma or cholangiocarcinoma in BD IX rats (Gelderblom et al., 1991). Fumonisins are potent tumor promoters in rat liver after initiation with diethylnitrosoamine (Gelderblom et al., 1996). In rats, feeding with FB_1 causes acute and chronic liver toxicity, bile duct hyperplasia, fibrosis progressing to

cirrhosis and cholangiofibrosis (Gelderblom et al., 1988). The National Toxicology Program in a 28-day and 2-year feeding study of FB_1, showed a clear evidence of hepatocellular neoplasms in female $B6C3F_1$ mice, while no effect was observed in rat liver at similar dietary concentrations (Howard et al., 2001). In FB_1 toxicity, liver is the common target organ that is affected in most animal species; with effects on other organs being species-specific.

Disruption of sphingolipid metabolism, altered fatty acid metabolism, and induction of tumor necrosis factor-α (TNFα) are the some of the known biochemical alterations of FB_1 *in vivo*. FB_1 inhibits ceramide synthase (sphinganine or sphingosine-*N*-acyl transferase), a critical enzyme in the metabolism of sphingolipids (Riley et al., 1996). FB_1 exposure *in vivo* causes accumulation of free sphingoid bases, sphingoid base metabolites, depletion of more complex sphingolipids and inhibition of ceramide biosynthesis (Riley et al., 1996; Tsunoda et al., 1998). FB_1 also increases TNFα expression in several strains of mice (Sharma et al., 2000a; 2000b; 2000c; 2000d). The acute *in vivo* hemopoietic effects of FB_1 were partially reversed by anti-TNFα antibodies (Dugyala et al., 1998), and a mouse strain lacking p75 or p55 TNFα receptor showed reduced hepatotoxicity after FB_1 treatment (Sharma et al., 2000a; 2000d). FB_1 caused an increased expression of TNFα in liver without the induction of TNFα in kidney and spleen (Bhandari et al., 2000).

Liver has its own innate immune system comprised of Kupffer cells, which are the largest population of fixed macrophages in the body, and the hepatic natural killer cells (Seki et al., 2000). This innate immune system is responsible for the localized action involving the cytokine network in liver. Liver is also the major sink for cytokines

that circulate in the bloodstream because it harbors the largest number of cells with cytokine receptors and a high density of receptors per cell (Geisterfer et al., 1993). In the case of hepatic injury TNFα induction is one of the earliest events, and can trigger a cascade of other cytokines that cooperate to kill hepatocytes, recruit inflammatory cells, and initiate a wound healing response including fibrogenesis (Diehl et al., 2000).

TNFα induces macrophages to secrete various proinflammatory cytokines like interleukin (IL)-1β and IL-6 (Abbas et al., 1997). TNFα and IL-12 produced by macrophages can also stimulate the natural killer (NK) cells and $NK1^+$ T cells to secrete interferon (IFN) γ, which through a positive amplification loop can further increase TNFα and IFNγ (Kaplan and Schreiber, 1999). On the other hand TNFα, IL-6 and IL-12 up-regulate IL-10 synthesis, which can suppress proinflammatory cytokine synthesis (Pretolani et al., 1999). Thus a network of cytokines rather than individual cytokine activity may be important in the outcome of any immune/inflammatory responses of liver (Brennan and Feldmann, 2000). TNFα, IL-1β and IFNγ are involved in different experimental mouse models of hepatotoxicity (Blazka et al., 1995; Ogasawara et al., 1998; Okamoto et al., 1999; Kamimura et al., 1995; Ksontini et al., 1998).

Since TNFα induction in response to FB_1 has been shown earlier in liver, the current study was undertaken to investigate the effect of FB_1 on the modulation of other cytokines in liver, kidney and spleen of male B6,129 mice, and to identify if the response of liver to FB_1 was unique or also shared by other organs such as kidney or spleen. In addition, the cellular location of TNFα production in liver was also investigated. Male mice were employed using a protocol that has resulted in consistent hepatic toxicity in a short-term treatment model in our earlier studies.

2. Material and methods

2.1. Animals and housing

Adult male B6,129 mice, 7-8 weeks old (about 18-22 g body weight), were obtained from Jackson Laboratories (Bar Harbor, ME). The animals were acclimated in the University of Georgia Animal Resources facility for a week at 23°C and 50% relative humidity with a 12 h light/dark cycle. Pelleted feed (fumonisin free) and fresh water was provided *ad libitum*. Food and water consumptions were recorded daily. The protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee.

2.2. Treatment and sampling

Mice (5/group) were treated with five daily subcutaneous injections (in a volume of 1 ml/100 g body wt.) of endotoxin-free FB_1 (99.6% purity; Promec, Tygerberg, South Africa) in physiological buffered saline; the dose per mouse being 0 (saline-treated controls) or 2.25 mg FB_1/kg/day. The protocol for treatment was based on our previous dose response studies in which FB_1 produced liver injury characterized by apoptosis in male mice (Sharma et al., 1997; 2000a). One day after the final injection, animals were killed with halothane and their blood collected via cardiac puncture into a heparinized syringe. The plasma was isolated by centrifugation and used for estimation of liver enzymes. Parts of liver and kidney tissues were fixed in 10% neutral buffered formalin for 12 h. Remaining pieces of liver and kidney tissues were quickly frozen in aliquots on dry ice and stored at -85°C until analyzed. An additional mouse was treated with single dose of lipopolysaccharide (LPS, Sigma, St. Louis, MO) 5 mg/kg body weight intra-

peritoneally in physiological buffered saline or another with vehicle alone. The animals were killed after 2 h of LPS treatment. Parts of liver were fixed in 10% neutral buffered formalin for 12 h.

2.3. RNA isolation and semi-quantitative estimation of cytokine expression

The RNA was isolated from the tissue, converted to cDNA by reverse transcriptase (RT) and amplified by polymerase chain reaction (PCR) using the protocol described earlier (Sharma et al., 2000), in a thermal cycler (Eppendorf Scientific, Westbury, NY). For semi-quantitative measurements, the number of cycles within an exponential increase of the product was optimized for each cytokine. The desirable annealing temperatures were determined using a gradient program in the cycler. The primer sequences, annealing temperature, and numbers of cycles used for each cytokine are indicated in Table 4.1. Each amplified product was separated by electrophoresis at 150 V on 2% agarose gels. The gels were photographed using a back-lighted UV trans-illuminator (Ultra Lum, Inc., Carson, CA). The cytokine expression was normalized against β-actin and quantified via densitometric imaging using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT).

2.4. Immunohistochemistry

Paraffin-embedded, formalin-fixed liver sections were treated with 0.01% trypsin for 20 min at 37°C for antigen retrieval. Primary rat anti-mouse Mac-3 antibody (BD Pharmingen, San Diego, CA), diluted as 1:200, was used at 37°C for 2 h followed by biotinylated secondary anti-rat antibody at 37°C for 1 h. Mac-3 antibody reacts with the glycoprotein Mac-3 antigen expressed on mouse mononuclear phagocytes. The slides

were incubated with avidin-biotin peroxidase complex for 1 h followed by 3,3' diaminobenzidine substrate.

2.5. In-situ hybridization

Digoxigenin-labeled antisense mouse TNFα riboprobes were synthesized and partially hydrolyzed with 0.1 M NaOH at 4°C for 40 min to generate 300 bp fragments and then adjusted to pH 6.0 by 0.2 M sodium acetate and 0.1 N HCl (Cox et al., 1984). *In situ* hybridization was performed on paraffin-embedded formalin-fixed liver sections. Briefly, sections were de-paraffinized, then digested with 100 μg/ml proteinase K at 37°C for 15 min, followed by overnight hybridization at 42°C with 25 ng probe/slide. The following day, sections were subjected to stringent washes and then incubated with 1:300 dilution of anti-dig-AP at 37°C for 2 h. Substrate development was with 4-nitroblue tetrazolium chloride (NBT) + 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and progressed for 12 h. The slides were counterstained with hematoxylin and covered with permount and a glass cover slip for a permanent record.

2.6. Estimation of liver enzymes in plasma

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using Hitachi 912 automatic autoanalyzer (Roche Diagnostics, Indianapolis, IN).

2.7. In situ staining of the apoptotic bodies

To identify positive apoptotic cells in liver and kidney *in situ*, formalin-fixed tissues were embedded in paraffin; 5 μm sections were made and used for apoptosis analysis. Terminal UTP nucleotide transferase end-labeling (TUNEL) of apoptotic cells was performed using a peroxidase-based ApopTag® Plus kit (Intergen, Purchase, NY).

Digoxigenin-UTP labeled DNA was detected with anti-digoxigenin-peroxidase antibody followed by peroxidase detection. Tissues were counterstained with hematoxylin for 2 seconds. The number of TUNEL-positive cells were counted and normalized to the total area of each section.

2.8. Statistics

Data from these studies were analyzed by one-way analysis of variance (ANOVA). The SAS computer program (SAS Institute, Cary, NC) was employed for all calculations. The level of $P < 0.05$ was considered statistically significant for all comparisons.

3. Results

Treatment of mice with FB_1 for 5 days produced no obvious gross clinical or behavioral effects. The FB_1 treatment had no influence on the body weight or food or water consumption (data not presented). The 5-day repeated daily treatment of FB_1 caused induction of several (but not all) cytokines in liver. The expression of TNFα in liver was increased five-fold that of control as shown in Fig. 4.1. Kidney and spleen did not show a similar induction of TNFα. Figure 4.2 illustrates the increase of IL-12 p40 (32-fold of control) again in liver only. The increase of IFNγ (>5-fold of control) in liver was observed, without significant changes in kidney and spleen (Fig. 4.3). IL-1β expression was increased in liver and kidney after FB_1 exposure but no changes were observed in spleen (Fig. 4.4). Changes in IL-1β expression in liver were greater in magnitude than those observed in kidney (3.2-fold vs. 1.8-fold, respectively). Other

cytokines evaluated in this study, namely IL-2, IL-4, IL-6 and IL-10, remained unaltered in liver, kidney and spleen after FB_1 treatment (Table 4.2).

In order to determine the types of cells involved in the production of TNFα in liver, immunohistochemistry using an anti-macrophage antibody was employed. The localization of Kupffer cells by Mac-3 antibody was in the lining of sinusoidal spaces of liver as shown in Fig. 4.5A. Kupffer cells were identified throughout the sinusoidal linings in the liver sections. For localization of cells that produced TNFα, *in-situ* hybridization was employed. LPS-treated mice liver was used as a positive control for increased TNFα expression by Kupffer cells as was evident by *in situ* hybridization. Cells lining the sinusoidal spaces showed the presence of TNFα, although the distribution of positive cells was less than all the Kupffer cells detected by immunohistochemistry (Fig. 4.5B). No TNFα was noticed in liver sections of vehicle-treated mice after *in situ* hybridization (Fig. 4.5C). After FB_1 treatment the pattern of TNFα expression correlated with the location of Kupffer cells in liver, although the number of cells producing this cytokine was much lower than those observed after LPS treatment (Fig. 4.5D).

FB_1 treatment caused hepatotoxicity as indicated by increased level of ALT in plasma (Table 4.3). Nearly 10-fold increase in circulating ALT was observed after a sub-acute treatment of mice with FB_1. The presence of apoptotic cells in liver was also increased after FB_1 treatment (Fig. 4.6). Very few apoptotic foci were observed in liver of vehicle-treated mice, whereas a large number of TUNEL-positive cells were apparent in liver sections from FB_1-treated mice. In kidney apoptotic changes were not significantly altered after FB_1 treatment. The number of apoptotic cells/cm^2 in kidney were 4±3 (Mean±SE) in control vs. 25±10 in FB_1-treated animals ($P = 0.09$).

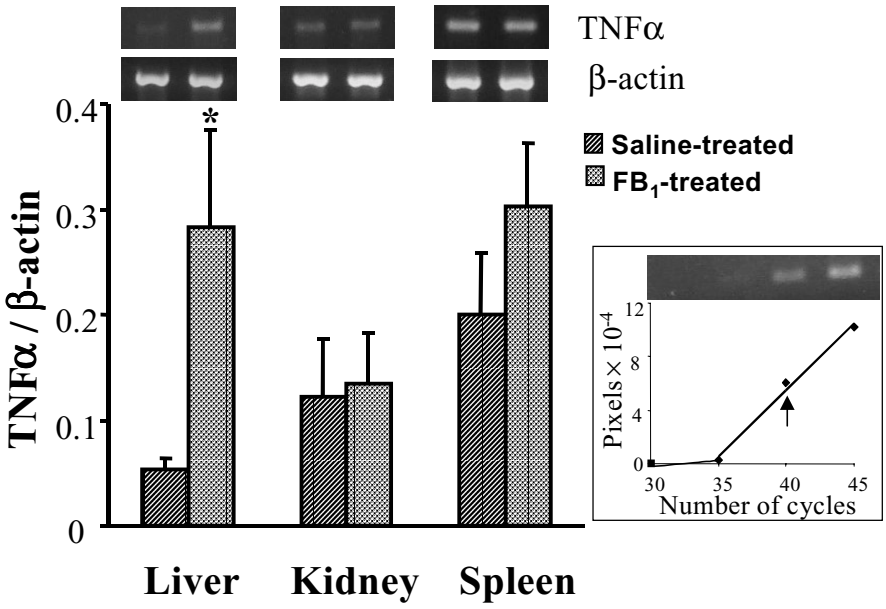

Figure 4.1. Tumor necrosis factor α (TNFα) expression in mouse tissues after fumonisin B_1 (FB_1) treatment. The mRNA levels were quantified by reverse transcriptase-polymerase chain reaction and normalized against β-actin. Representative gels are shown on top of respective bars. Results in graph are expressed as mean ± SE, n=5. * Significantly different from saline-treated control at $P < 0.05$. Inset shows use of 40 cycles (arrow) for a semi-quantitative measurement of TNFα, lying within the exponential phase of increase in product size.

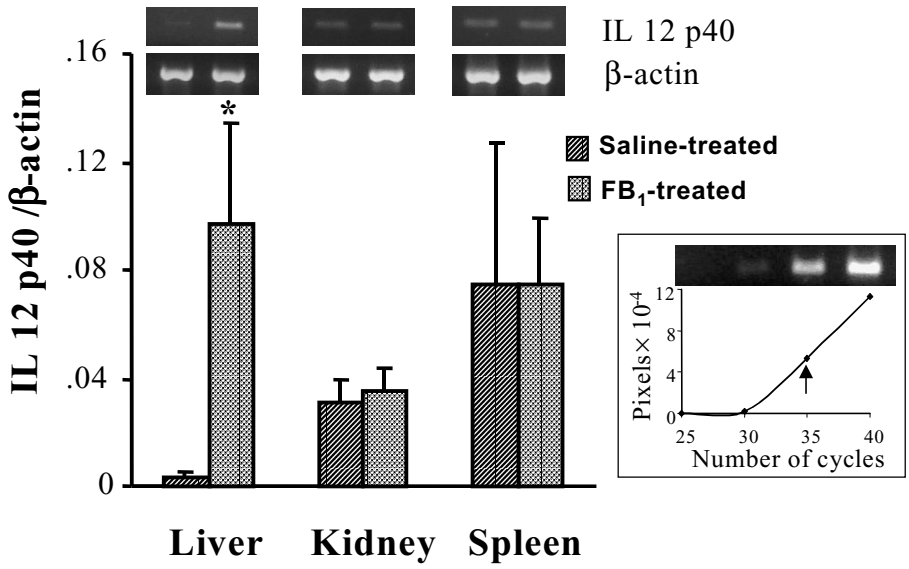

Figure 4.2. Interleukin (IL)-12 p40 expression in mouse tissues after fumonisin B_1 (FB_1) treatment. The mRNA levels were quantified by reverse transcriptase-polymerase chain reaction and normalized against β-actin. Representative gels are shown on top of respective bars. Results in graph are expressed as mean ± SE, n=5. * Significantly different from saline-treated control at $P < 0.05$. Inset shows use of 35 cycles (arrow) for a semi-quantitative measurement of IL 12p40, lying within the exponential phase of increase in product size.

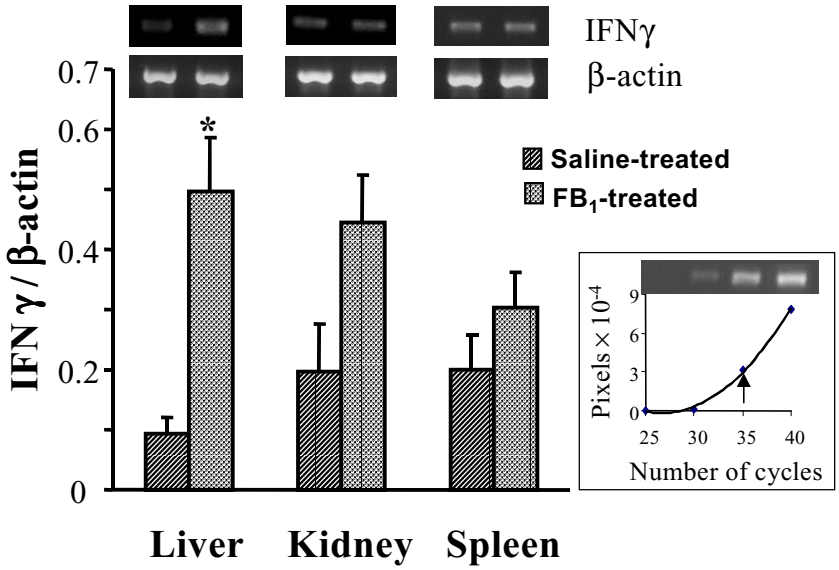

Figure 4.3. Interferon γ (IFNγ) expression in mouse tissues after fumonisin B_1 (FB_1) treatment. The mRNA levels were quantified by reverse transcriptase-polymerase chain reaction and normalized against β-actin. Representative gels are shown on top of respective bars. Results in graph are expressed as mean ± SE, n=5. * Significantly different from saline-treated control at $P < 0.05$. Inset shows use of 35 cycles (arrow) for a semi-quantitative measurement of IFNγ, lying within the exponential phase of increase in product size.

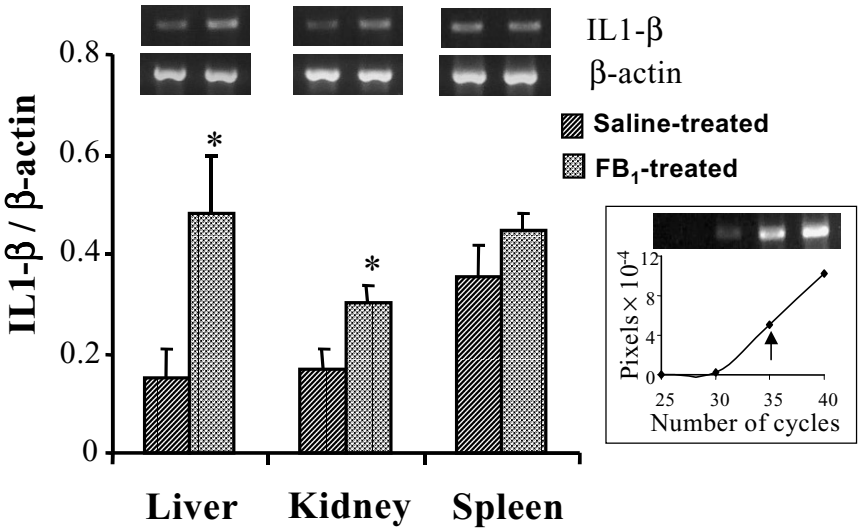

Figure 4.4. Interleukin (IL)-1β expression in mouse tissues after fumonisin B_1 (FB_1) treatment. The mRNA levels were quantified by reverse transcriptase-polymerase chain reaction and normalized against β-actin. Representative gels are shown on top of respective bars. Results in graph are expressed as mean ± SE, n=5. * Significantly different from saline-treated control at $P < 0.05$. Inset shows use of 35 cycles (arrow) for a semi-quantitative measurement of IL1-β, lying within the exponential phase of increase in product size.

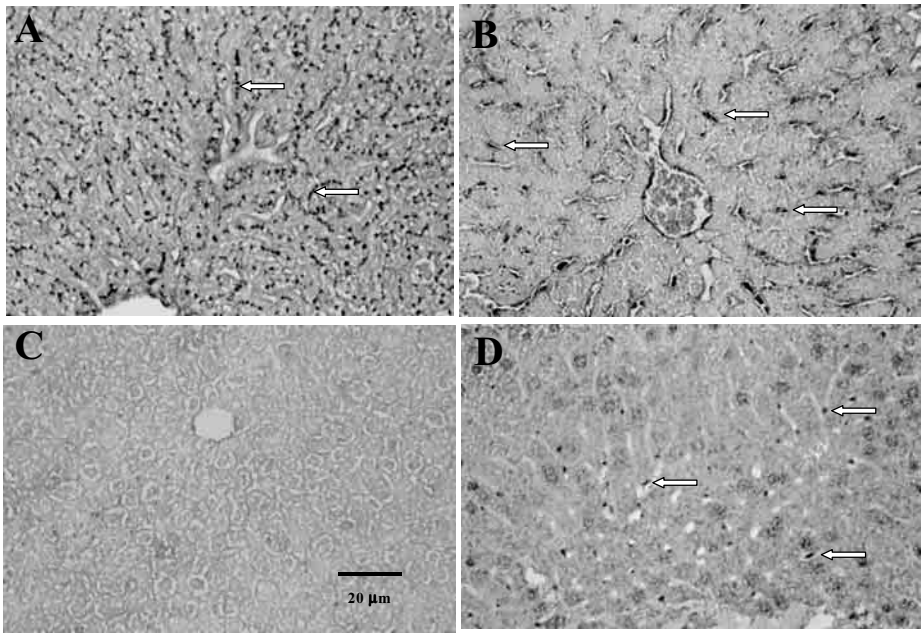

Figure 4.5. Kupffer cells involved in fumonisin B_1 (FB_1)-induced tumor necrosis factor α (TNFα) expression. Micrograph of liver section showing (A) spatial distribution of Kupffer cells (arrow) in a normal mouse liver after immunohistochemistry using Mac-3 antibody, which specifically binds with Mac-3 surface antigen on macrophages. (B) Liver from mouse treated with lipopolysaccharide, used as a positive control, showing TNFα expression (arrow) localized in the Kupffer cells after *in-situ* hybridization using mouse anti-sense TNFα riboprobe. (C) Saline-treated mouse liver after *in situ* hybridization indicating no noticeable TNFα production in liver. (D) Liver from FB_1-treated mouse after *in situ* hybridization suggesting involvement of Kupffer cells in TNFα expression (arrow).

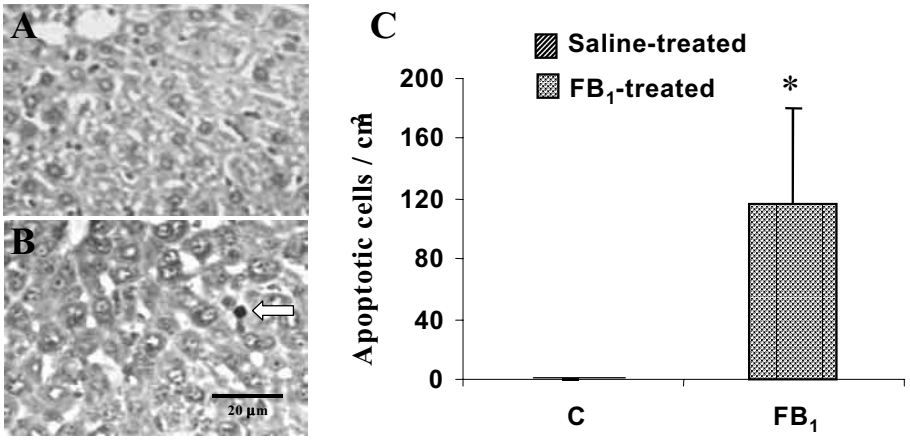

Figure 4.6. Fumonisin B_1 (FB_1)-induced apoptosis in liver by *in situ* terminal-UTP nucleotide transferase end labeling (TUNEL) staining. (A) Apoptotic cells were absent in control liver. (B) Showing apoptotic TUNEL-positive cells (arrow) in FB_1-treated mice. Tissues were counterstained with hematoxylin. TUNEL-positive cells stained dark brown. The magnification is shown by a bar equivalent to 20 µm. (C) Quantitative estimation of number of TUNEL-positive apoptotic cells/cm^2 tissue section. Results are expressed as mean ± SE, n=5. * Significantly different from saline-treated control at $P < 0.05$.

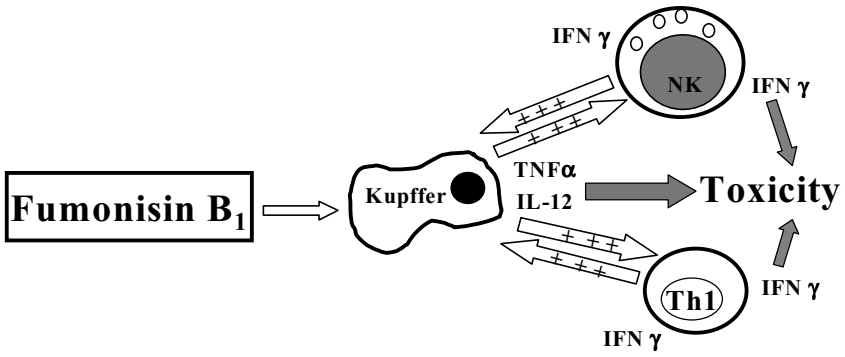

Figure 4.7. Schematic diagram of cytokines involved in fumonisin B_1 (FB_1)-induced hepatotoxic response. Liver shown with resident Kupffer cells, natural killer cells (NK) and T helper-1 cells (Th1), inducing a Th1 dominant cytokine response when challenged by FB_1. FB_1 may induce Kupffer cells to produce tumor necrosis factor α (TNFα) and interleukin (IL)-12. TNFα and IL-12 can act on lymphocytes to produce interferon γ (IFNγ), which through a positive feedback loop (+++) will further activate Kupffer cells to produce more TNFα and IL-12 and eventually more IFNγ, leading to liver toxicity (based on Diehl, 2000; Kaplan and Schreiber, 1999).

Table 4.1.

Primer sequences of various cytokines and their optimized annealing temperatures and the number of amplification cycles employed in the PCR amplification.

Cytokine	Primer sequences[a]	Annealing temperature	Amplification cycles
TNFα	5'-CTCTTCAAGGGACAAGGCTG-3' (sense) 5'-CGGACTCCGCAAAGTCTAAG-3' (antisense)	54°C	40
IL-1β	5'-GCAACTGTTCCTGAACTCA-3' (sense) 5'-CTCGGAGCCTGTAGTGCAG-3' (antisense)	55°C	35
IL-2	5'-CTCGCATCCTGTGTCACATT-3' (sense) 5'-ATCCTGGGGAGTTTCAGGTT-3' (antisense)	48°C	40
IL-4	5'-TCAACCCCCAGCTAGTTGTC-3' (sense) 5'-CGAGCTCACTCTCTGTGGTG-3' (antisense)	48°C	35
IL-6	5'-TTCCATCCAGTTGCCTTCTT-3' (sense) 5'-CAGAATTGCCATTGCACAAC-3' (antisense)	48°C	40
IL-10	5'-CCAAGCCTTATCGGAAATGA-3' (sense) 5'-TTTTCACAGGGGAGAAATCG-3' (antisense)	55°C	35
IL-12 p40	5'-AGGTGCGTTCCTCGTAGAGA-3' (sense) 5'-AAAGCCAACCAAGCAGAAGA-3' (antisense)	55°C	35
IFN γ	5'-AACGCTACACACTGCATCT-3' (sense) 5'-AGCTCATTGAATGCTTGG-3' (antisense)	48°C	35
β-actin	5'-ATGGATGACGATATCGCT-3' (sense) 5'-ATGAGGTAGTCTGTCAGGT-3' (antisense)	48°C	30

[a]All primer sequences were selected from data in GenBank and using the Primer3

Table 4.2.

Expression of IL-2, IL-4, IL-6 and IL-10 in liver, kidney and spleen by reverse transcriptase-polymerase chain reaction after FB_1 treatment.[a]

Cytokine	Liver		Kidney		Spleen	
	Saline-treated	FB_1-treated	Saline-treated	FB_1-treated	Saline-treated	FB_1-treated
IL-2	0.06±0.01	0.04±0.00	0.09±0.02	0.09±0.03	0.10±0.02	0.12±0.01
IL-4	0.35±0.08	0.51±0.11	0.06±0.01	0.05±0.01	0.21±0.06	0.22±0.51
IL-6	0.13±0.05	0.17±0.03	0.12±0.03	0.17±0.05	0.11±0.04	0.15±0.04
IL-10	0.29±0.04	0.34±0.04	0.56±0.12	0.39±0.07	0.26±0.05	0.39±0.04

[a] Mean ± SE (n=5).

Table 4.3.

Plasma concentration of liver enzymes in saline and FB_1-treated mice.[a]

Group	ALT (IU/liter)	AST (IU/liter)
Saline-treated	44 ± 12	641 ± 292
FB_1-treated	411 ± 118*	949 ± 118

[a] Mean ± SE (n=5). * Significantly different from the saline treated control group at $P < 0.05$.

4. Discussion

Results from the current study indicate that hepatotoxic response to FB_1 in mice was accompanied by modulation of several pro-inflammatory cytokines in liver. The induction of cytokines appeared to be a localized phenomenon in the liver, as the changes in spleen or kidney were much less pronounced or not observed. For TNFα, the cytokine that has been reported to be the first inflammatory factor in inducing hepatotoxicity, the cells involved in its production were found lining the sinusoidal spaces.

Various cytokines are apparently involved in different experimental mouse models of hepatotoxicity. Neutralizing antibodies to TNFα or IL-1 partially prevented liver damage in mice initiated by hepatotoxic doses of acetaminophen (Blazka et al., 1995). TNFα, IFNγ and IL-12 were involved in liver toxicity known as Shwartzman reaction caused by repeated LPS challenge (Ogasawara et al., 1998). IFNγ over-expressed mice also develop chronic active hepatitis (Okamoto et al., 1999). TNFα and IL-6 are involved in alcoholic liver disease (Kamimura and Tsukamoto, 1995). TNFα and IFNγ have also been related to Con A-induced hepatitis (Ksontini et al., 1998).

In our laboratory TNFα has been shown to be involved in FB_1-induced toxicity in mice (Dugyala et al., 1998; Sharma et al., 2000a; 2000d). In the current study FB_1 treatment caused an increased expression of TNFα, IL-12 p40 and IFNγ only in liver. This localized cytokine induction in liver suggests the involvement of liver innate immune system comprising of Kupffer cells and NK cells. Similar to TNFα, the primary cellular source of IL-12 production is also macrophages, i.e., Kupffer cells in liver. IL-12 is a disulphide linked heterodimeric cytokine consisting of p35 and p40 kd subunits. Out

of the two subunits, p40 is mostly regulated whereas p35 is expressed in an almost constitutive fashion (Chizzonite et al., 1998).

It is worthwhile to indicate here that the relative estimation of mRNA expression by RT-PCR may underestimate the real increase in cytokine levels produced by the FB_1 treatment. In an earlier study from our laboratory we reported an increase of TNFα after LPS treatment of murine macrophages measured by PCR was 3.5-times less than that estimated by a northern blot in same murine macrophages (200- vs. 700-fold, respectively; He et al., 2001). In another study using male BALB/c mice and an identical treatment with FB_1 we found that increases in liver TNFα, IFNγ and IL-1β expression measured by ribonuclease protection assay were 33-, 12-, and 4-fold, respectively (Bhandari, N., Sharma, R.P., unpublished data).

Cytokines are also the major factors, which determine the differentiation of naive T helper (Th) cells into the Th1 or Th2 phenotypes. The presence of IL-12 during priming is associated with the induction of Th1 cells while IL-4 influences the preferential activation of Th2 cells (Romagnani et al., 2000). The imbalance in Th1/Th2 system has been correlated with different pathophysiological processes (Tanaka et al., 1996). In this study FB_1 treatment selectively induced Th1 cytokines (IL-12 and IFNγ) without any alterations in Th2 cytokines (IL-4, IL-6, and IL-10).

TNFα, IL-12 p40 and IFNγ have shown to be involved in a positive feed back loop causing their induction and leading to hepatotoxicity (Kaplan and Schreiber, 1999). Figure 4.7 illustrates the cytokine network between TNFα, IL-12, and IFNγ in liver as it could be involved in the observed FB_1 hepatotoxicity in mice. TNFα and IL-12 produced by the Kupffer cells in liver can act on NK cells and/or Th1 cells to produce IFNγ. This

IFNγ can then further back-activate the Kupffer cells through a positive feedback loop to produce more TNFα and IL-12 and eventually producing more IFNγ from the NK cells and/or Th1 cells. This elevated TNFα and IFNγ has been shown to be involved in various models of liver toxicity and thus could also play an important role in the FB_1-induced liver toxicity (Kaplan and Schreiber, 1999; Ogasawara et al., 1998).

The mice repeated FB_1 treatment resulted in nearly 5-fold increase in the expression of TNFα. TNFα is a pleiotropic cytokine primarily produced by activated macrophages and in smaller amounts by several other cell types. In the liver TNFα is primarily produced by Kupffer cells and cholangiocytes (Kanzler and Galle, 2000). Kupffer cells have been demonstrated to play a causal role in hepatotoxicity of CCl_4 and alcoholic liver disease (Edwards et al., 1993; Adachi et al., 1994). In alcoholic liver disease Kupffer cells have been shown to be involved in increased TNFα production (Kamimura and Tsukamoto, 1995). Other cell types, including biliary epithelial cells and venous endothelial cells have also been shown to be involved in TNFα induction after partial hepatectomy (Loffreda et al., 1997).

In this study Kupffer cells were shown to be the source of induced TNFα production in both LPS (positive control) and FB_1-treated mice. Kupffer cells are located lining the endothelial cells of the sinusoids. The TNFα mRNA distribution depicted by *in-situ* hybridization closely paralleled the localization of mac-3 expressing Kupffer cells.

The toxicity of FB_1 in male B6,129 mice was similar to that observed in C57BL/6J and BALB/c mice employing an identical treatment protocol (Sharma et al., 2000a; 2000d). In the current study the hepatic effects of FB_1 treatment were demonstrated by increased liver enzyme, ALT, in circulation (Table 4.3) and presence of

TUNEL positive apoptotic cells in liver (Fig. 4.6). AST levels were also increased after FB_1 treatment but not significantly. Elevation of ALT activity is a more specific indicator of hepatic disease than AST (Burt and James, 1994). Apoptotic cells were not detected in control mice livers. It has been reported that under physiological conditions only 1-5 per 10,000 cells will be detected as apoptotic in liver given the low cell turnover of both hepatocytes and cholangiocytes (Schulte-Hermann et al., 1995).

TNFα has been shown to be a contributor in FB_1 toxicity in various experiments. Administration of anti-TNFα antibodies partially reversed the *in vivo* hemopoietic effects in FB_1 treated mice (Dugyala et al., 1998). Transgenic mice lacking p75 or p55 TNFα receptor were also more resistant to FB_1 induced hepatotoxicity as compared to control mice (Sharma et al., 2000a; 2000d). FB_1 toxicity in CV-1 cells was also prevented by inhibitor of apoptosis protein, an inhibitor of TNFα pathway (Ciacci-Zanella and Jones, 1999). In our additional investigations the expression of *c-myc*, which is associated with proliferative, inflammatory, and apoptotic changes, was increased 4.3 times (measured by RT-PCR) in liver after FB_1 treatment of mice (Bhandari, N., Sharma R.P., unpublished data).

Another biochemical mode of action of FB_1 toxicity involves inhibition of ceramide synthase enzyme, leading to accumulation of free sphingoid bases, sphingoid base metabolites and depletion of more complex sphingolipids. There is a good correlation between the increase in sphingoid bases and the toxicity observed in different rodents and farm animals, but this fails to explain the observed tissue and species specificity. Increase in sphinganine after FB_1 treatment has been observed in all target and non-target organs in various experimental models. In mouse FB_1-treatment primarily

causes liver toxicity without much effect on kidney, but after FB_1-treatment kidney shows a greater change in sphingoid base accumulation as compared to liver (Tsunoda et al., 1998). Thus free sphingoid bases are not sufficient to account for the presence and variations noticed in toxicity. In a porcine kidney cell line that is sensitive to FB_1 response, the FB_1-induced TNFα expression was recently shown to be independent of free sphingoid base accumulation (He et al., 2001).

Other important pathways which can be involved in TNFα induction in response to FB_1 include PKC translocation from cytosol to membrane in rat cerebrocortical slices via direct interaction with diacylglycerol binding site in rat cerebrocortical slices (Yeung et al., 1996), and/or activation of the mitogen-activated protein kinase (MAPK) in swiss 3T3 cells and human bronchial epithelial cells (Wattenberg et al., 1996; Pinelli et al., 1999). The relationship of these events with cytokine-mediated effects of FB_1 is not understood at present.

In conclusion the results of the present study identify the involvement of Kupffer cells in increased TNFα expression in liver of mice after FB_1 exposure. FB_1 treatment also caused localized expression of TNFα, IL-12 p40 and IFNγ in liver, suggestive of a Th1 dominant response involving macrophages, NK cells and/or $NK1^+$ T cells. Further studies are required to understand the importance of individual cytokines in toxicity caused by FB_1.

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CHAPTER 5

GENDER-RELATED DIFFERENCES IN SUBACUTE FUMONISIN B_1 HEPATOTOXICITY IN BALB/C MICE[1]

[1]Bhandari, N., Quanren He, and Raghubir P. Sharma. 2001. Toxicology. 165:195-204.

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Abstract

Fumonisin B_1 (FB_1), a potent mycotoxin prevalent in corn, is a carcinogen and causative agent of various animal diseases. Species and sex variations to chronic FB_1 toxicity have been reported. Free sphingoid bases and cytokine levels are the two major biochemical alterations of FB_1 *in vivo* and may explain any sex differences in FB_1 toxicity. Male and female BALB/c mice (5/group) were injected subcutaneously with either saline vehicle or 2.25 mg/kg/day of FB_1 for 5 days. One day after the last injection females showed a greater increase in circulating alanine aminotransferase and greater number of apoptotic cells in liver after FB_1 treatment than males, indicating greater hepatotoxicity. Peripheral leukocytic counts, including neutrophils, were increased in females only after FB_1 treatment. The increased toxicity in females correlated with a greater increase of sphinganine and sphingosine levels in liver after FB_1 treatment compared to males. No sex differences in kidney sphinganine or sphingosine levels were observed after FB_1 treatment. Previously we have shown the induction of tumor necrosis factor α (TNFα) in FB_1-induced hepatotoxicity. While in males FB_1 treatment caused increased expression of TNFα, interleukin (IL)-12 p40, interferon γ (IFNγ), IL-1β, IL-6 and IL-10, females showed an increased expression of IL-6 only, and a downward modulation of IFNγ, indicating gender differences in cytokine pathways in liver activated by FB_1. The basal expression of TNFα, IL-12 p40, IL-1β and IFNγ in liver of females was higher compared to males. Gender differences in alterations of free sphingoid bases and cytokine modulation after FB_1 treatment suggest their possible involvement in sex-dependent differential hepatotoxicity in mice.

Keywords: Fumonisin; gender; hepatotoxicity; cytokines; mice; sphingoid bases

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; FB_1, fumonisin B_1; IFNγ, interferon γ; IL, interleukin; LPS, lipopolysaccharide; NK, natural killer; PCR, polymerase chain reaction; TNFα, tumor necrosis factor α; TUNEL, terminal UTP nucleotide transferase end-labeling.

1. Introduction

Fumonisins are natural contaminants of corn and other crops infected with the fungus *Fusarium verticillioides (=F. moniliforme*, Gelderblom et al., 1988). The occurrence of these mycotoxins has been reported worldwide (Shephard *et al*., 1996). Ingestion of fumonisin B_1 (FB_1), the major fumonisin produced by the fungus, causes a variety of toxicosis in animals, including equine leukoencephalomalacia (Marasas et al., 1988) and porcine pulmonary edema (Harrison et al., 1990). This mycotoxin has been reported to be carcinogenic in rats (Gelderblom et al., 1991) and has been implicated as a contributing factor in human esophageal cancer (Sydenham et al., 1990) and possibly primary liver cancer (Ueno et al., 1997). The mouse liver is the primary site of FB_1 toxicity whereas the kidney is the most sensitive organ in rats (Voss et al., 1996; Sharma et al., 1997).

The National Toxicology Program (1999) in a 28-day and 2-year feeding study in $B6C3F_1$ mice reported females to be more sensitive to FB_1 hepatotoxicity than males. Male mice showed no evidence of toxicity when exposed to 5, 15, 80 or 150 ppm FB_1 for 2 years, whereas females showed clear evidence of hepatocellular neoplasm after

exposure to 50 or 80 ppm. The present study was performed to investigate gender differences in response of female and male BALB/c mice to effects of FB_1 in a short-term treatment model.

Species, gender and tissue-specific toxicity of FB_1 may involve complex mechanisms with interplay between several molecular sites. One well-known biochemical effect of FB_1 is inhibition of ceramide synthase (sphinganine and sphingosine-*N*-acyl transferase) leading to accumulation of free sphingoid bases, sphingoid base metabolites, and depletion of complex sphingolipids (Riley et al., 1996; Tsunoda et al., 1998). Fumonisin B_1 was reported to induce tumor necrosis factor-α (TNFα) expression in mice (Sharma et al., 2000 a; b; c; 2001). The *in vivo* hemopoietic effects of FB_1 were partially reversed by anti-TNFα antibodies (Dugyala et al., 1998), and the hepatotoxic response was decreased in mice strains lacking either p75 or p55 TNFα receptor (Sharma et al., 2000a; 2001). FB_1 caused an increased expression of TNFα in liver without the induction of TNFα in kidney or spleen (Bhandari et al., 2000).

TNFα causes apoptosis in cells and its induction is one of the earliest events in hepatic injury, leading to modulation of a cascade of other cytokines (Diehl et al., 2000). In a lipopolysaccharide (LPS)-induced model TNFα caused macrophages to release increased amounts of interleukin (IL)-1β, which in turn acted on macrophages and vascular endothelial cells to release more IL-6 and IL-8 (Abbas et al., 1997). TNFα, along with IL-12 produced by macrophages in response to antigenic challenge, can stimulate natural killer (NK) cells and NK 1^+ T cells to secrete interferon γ (IFNγ), which cause T cell-dependent liver injury (Tanaka et al., 1996). Thus a network of cytokines

rather than individual cytokine expression may be important in the overall outcome of a hepatotoxic response (Brennan and Feldmann, 2000).

Accumulation of free sphingoid bases and altered cytokine levels are two major biochemical responses to FB_1 *in vivo*. In order to explain the observed sex differences in FB_1 toxicity in mice we evaluated the gender-related changes in free sphingoid bases and cytokine expression. A greater increase in free sphinganine in liver of female mice and a differential cytokine modulation after FB_1 treatment may help explain the greater hepatotoxicity in females than in males.

2. Methods

2.1. Animals and housing

Adult male and female BALB/c mice, 7-8 weeks old (about 16-22 g body weight), were obtained from Harlan Laboratories (Indianapolis, IN). The animals were acclimated in the University of Georgia Animal Resources facility for a week at 23°C and 50% relative humidity with a 12 h light/dark cycle. Pelleted feed (fumonisin free) and fresh water were provided *ad libitum*. Food and water consumptions were recorded daily. The protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee.

2.2. Treatment and sampling

Male and female mice (5/group) were treated with five daily subcutaneous injections (in a volume of 1 ml/100 g body wt.) of endotoxin-free FB_1 (99.6% purity; Promec, Tygerberg, South Africa) in physiological buffered saline (PBS), the dose per mouse

being 0 (PBS-treated controls) or 2.25 mg FB_1/kg/day. This treatment protocol was based on our previous studies in which FB_1 produced consistent dose-related liver injury characterized by apoptosis in male mice (Sharma et al., 1997; Tsunoda et al., 1998; Dugyala et al., 1998). One day after the final injection, animals were killed with halothane after overnight fasting and their blood collected via cardiac puncture into a heparinized syringe. The blood was used for blood counts and subsequent isolation of plasma for estimation of liver enzymes. Each kidney was cut into two pieces along its transverse axis. Parts of liver and kidney tissues were fixed in 10% neutral buffered formalin for 4 h. Remaining pieces of liver and kidney tissues were quickly frozen in aliquots on dry ice and stored at -85°C until analyzed for sphingolipids and cytokines.

2.3. Hematology and estimation of liver enzymes in plasma

Total blood erythrocyte and leukocyte cell counts were determined using an electronic counter (Coulter Electronics, Hialeah, FL). Differential counts were done visually on blood smears stained with Giemsa's stain and then converted to absolute cell counts. Levels of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using Hitachi 912 automatic autoanalyzer (Roche Diagnostics Corp. Indianapolis, IN).

2.4. In situ staining of apoptotic bodies

Formalin-fixed tissues were embedded in paraffin; 5 μm sections were made and used for apoptosis analysis using a terminal UTP nucleotide transferase end-labeling (TUNEL) of apoptotic cells with a peroxidase-based ApopTag® Plus kit (Intergen, Purchase, NY). Tissues were counterstained with hematoxylin for 10 seconds. The

number of TUNEL-positive cells were counted and normalized to the total area of each section.

2.5. Analyses of sphingolipids

Free sphinganine and sphingosine were determined in base-treated lipid extracts from liver and kidney by high performance liquid chromatography (HPLC) utilizing a modification of the extraction methods originally described by Merrill et al. (1988). Sphingoid bases were quantitated based on the recovery of a C_{20}-sphinganine internal standard (Matreya Inc., Pleasant Gap, PA). The complete description of the extraction, derivatization procedure, and HPLC apparatus is similar to one described earlier (Riley et al., 1994).

2.6. RNA isolation and semiquantitative estimation of cytokine expression

The RNA was isolated from the tissue, converted to cDNA by reverse-transcription and amplified by polymerase chain reaction (PCR) using the protocol described earlier (Sharma *et al*., 2000a). The number of cycles within an exponential increase of the product was optimized for each cytokine. Samples were amplified for 30 cycles for β-actin and IL-1β, 35 cycles for IL-12 p40, IFNγ, and IL-10, and 40 cycles for TNFα and IL-6, in a thermal cycler (Eppendorf Scientific, Westbury, NY). The optimized annealing temperatures were 48°C for β-actin, IL-6 and IFNγ; 54°C for TNFα; and 55°C for IL-1β, IL-10 and IL-12 p40. The sense and antisense primers for TNFα and β-actin were same as used in a previous study (Sharma *et al*., 2000a). For other cytokines, the primers were 5'-GCAACTGTTCCTGAACTCA-3' (sense) and 5'-CTCGGAGCCTGTAGTGCAG-3' (antisense) for IL-1β; 5'-TTCCATCCAGTTGCCTTCTT-3' (sense) and 5'-CAGAATTGCCATTGCACAAC-3' (antisense) for IL-6; 5'-

AGGTGCGTTCCTCGTAGAGA-3' (sense) and 5'-AAAGCCAACCAAGCAGAAGA-3' (antisense) for IL-12 p40; 5'-AACGCTACACACTGCATCT-3' (sense) and 5'-AGCTCATTGAATGCTTGG-3' (antisense) for IFNγ; and 5'-CCAAGCCTTATCGGAAATGA-3' (sense) and 5'-TTTTCACAGGGGAGAAATCG-3' (antisense) for IL-10 (chosen by Primer3 program; Whitehead Institute, Cambridge, MA). Amplified products were separated by electrophoresis on 2% agarose gels, and gels were photographed using a backlighted UV transilluminator (Ultra Lum, Inc., Carson, CA). The cytokine expression was quantified by densitometric imaging using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT), and normalized against β-actin amplified from the same sample.

2.7. Statistics

Data from these studies were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan's Multiple Range test using the SAS computer program (SAS Institute, Cary, NC). The level of $P < 0.05$ was considered statistically significant for all comparisons.

3. Results

Treatment of either male or female mice with FB_1 for 5 days did not produce any apparent signs of behavioral or gross toxicity. The FB_1 treatment caused a significant decrease in body weights of females only (Table 5.1). The mean liver weight relative to the body weight was increased by FB_1 treatment in both sexes of mice; however, the increase in the relative liver weight of females (34%) was greater than the corresponding

increase in males (18%). The kidney weight relative to the body weight increased in females only after FB_1 treatment.

The hematological parameters after FB_1 treatment also showed some gender differences (Table 5.2). The erythrocyte counts were increased in males where as females showed increased total leukocyte counts after FB_1 treatment. The increase in leukocyte counts was primarily because of increased number of circulating neutrophils. The number of lymphocytes remained unaltered after FB_1 treatment.

The concentration of liver enzymes (ALT and AST) in circulation was increased after FB_1 treatment in both sexes (Fig. 5.1). However, in the case of ALT the increase was relatively greater in FB_1-treated females than in corresponding males.

The changes in liver enzymes corresponded well with the enumeration of apoptotic bodies using *in situ* TUNEL staining (Fig. 5.2). A 5-day treatment of FB_1 induced apoptosis in the liver of both males and females. The number of TUNEL-positive cells/cm^2 in FB_1-treated female liver was six times greater than that in FB_1-treated males. The number of apoptotic cells/cm^2 in kidney were 4 ± 3 (Mean ± SEM) in control males vs. 25 ± 10 in FB_1-treated males, and 8 ± 3 in control females vs. 40 ± 29 in FB_1-treated females. Apoptotic changes in kidney after treatment were not statistically significant due to a high variability in FB_1-treated mice of both sexes.

Exposure of both male and female mice to FB_1 caused an accumulation of free sphingoid bases, sphingosine and sphinganine, in liver and kidney. Both PBS- and FB_1-treated females showed a higher level of liver sphinganine and sphingosine compared to the corresponding group of males (Fig. 5.3). The increase in free sphinganine in liver of females after FB_1 treatment was 30% higher compared to that in males. Kidneys of both

male and female mice showed a similar increase of free sphingoid bases after FB_1 treatment.

FB_1 treatment increased the expression of TNFα, IL-1β, IL-6, IFNγ, IL-12 p40 and IL-10 in liver of male mice (Fig. 5.4). Female mice had a relatively higher expression of basal TNFα, IL-1β, IFNγ and IL-12 p40 in liver as compared to control males. FB_1 treatment in females caused a higher expression of IL-6 than in males and a reduced expression of IFNγ in liver. No changes in any of the cytokines were observed in male or female kidney after FB_1 treatment (data not presented), except that FB_1 treatment in females caused a decrease in IFNγ expression.

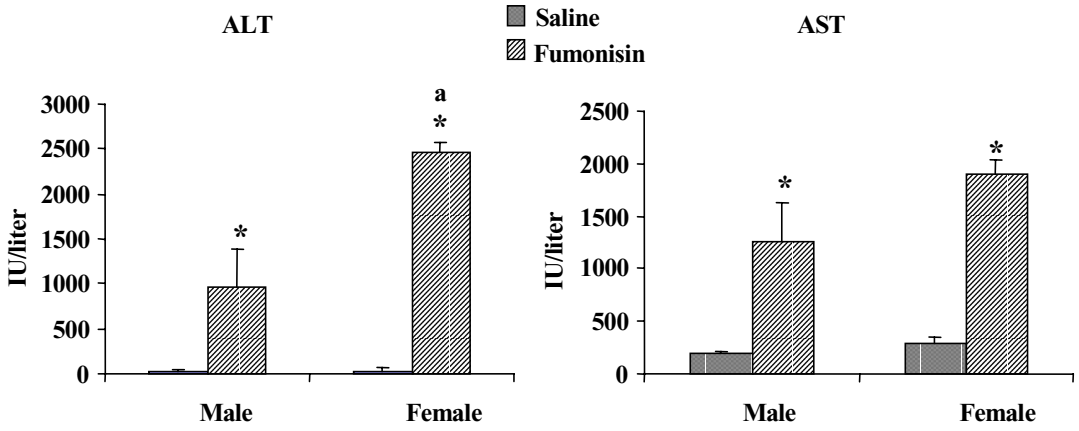

Figure 5.1. Concentrations of liver enzymes, ALT and AST, in PBS- and FB_1-treated male and female mice. Results are expressed as mean ± SEM, n=5. * significantly different from the respective saline-treated control at $P < 0.05$, [a] significantly different from the corresponding male group at $P < 0.05$.

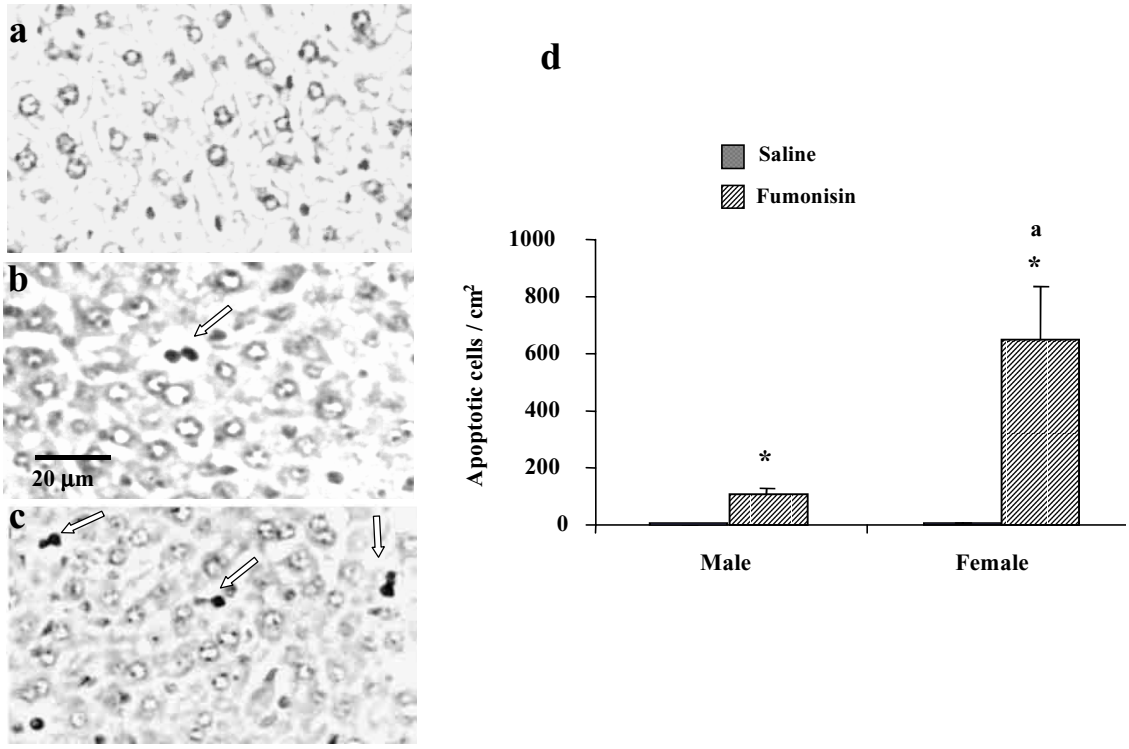

Figure 5.2. Micrograph of liver section after *in situ* TUNEL staining. a, Apoptotic cells were absent in control liver. b, Showing apoptotic TUNEL-positive cells (arrow) in FB_1 treated male and c, female. Tissues were counterstained with hematoxylin. TUNEL-positive cells stained dark brown. The magnification is shown by a bar equivalent to 20 µm. d, Quantitative estimation of number of TUNEL-positive apoptotic cells/cm^2 tissue section. Results are expressed as mean ± SE, n=5. *significantly different from the respective saline-treated control at $P < 0.05$, [a] significantly different from the corresponding male group at $P < 0.05$.

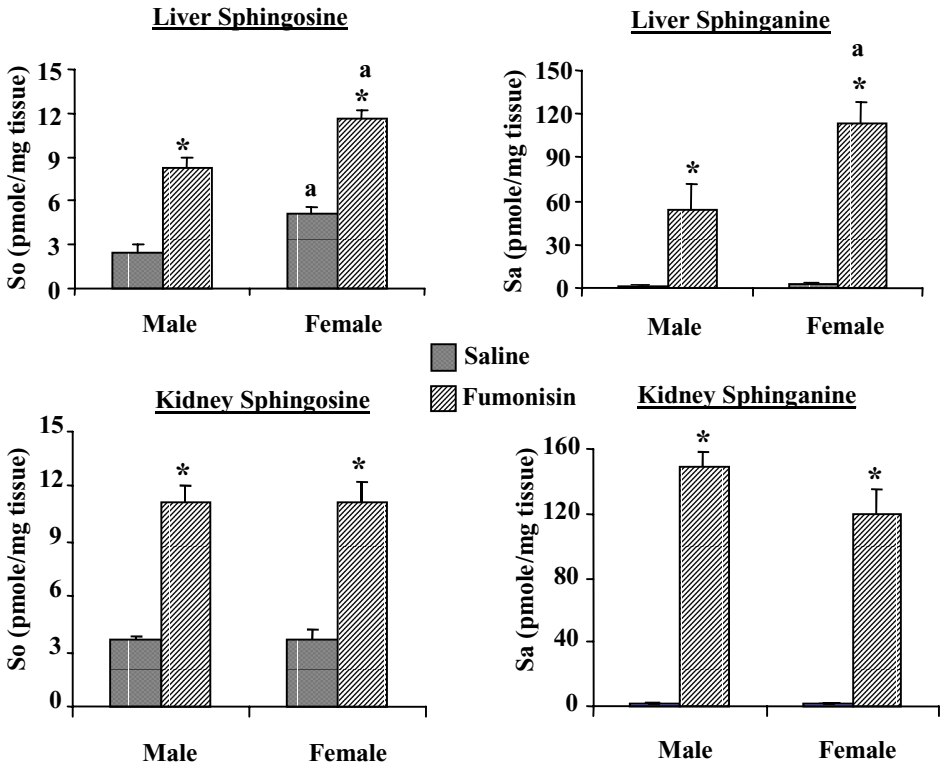

Figure 5.3. Concentration of sphingoid bases sphingosine (So) and sphinganine (Sa) in liver and kidney of male and female mice after saline or FB_1 exposure. Results are expressed as mean ± SE, n=5. *significantly different from the respective saline-treated control at $P < 0.05$, [a] significantly different from the corresponding male group at $P < 0.05$

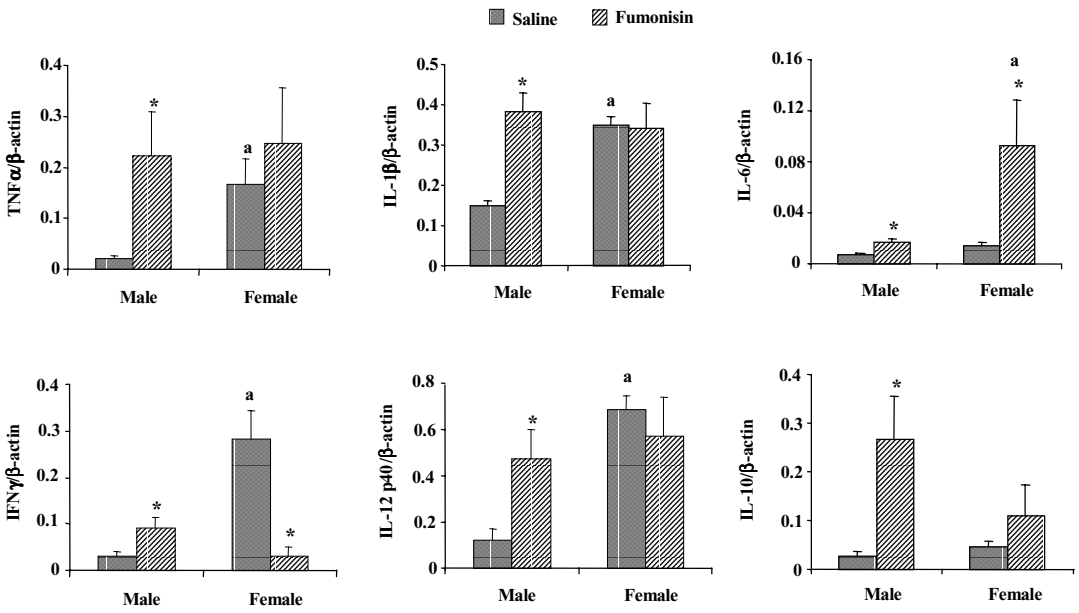

Figure 5.4. Expression of TNFα, IL-1β, IL-6, IL-12 p40, IFNγ and IL-10 mRNA levels in liver after treatment with FB_1. The mRNA levels were quantified by reverse transcriptase-PCR and normalized against β-actin. Results are expressed as mean ± SE, n=5. * significantly different from the respective saline-treated control at $P < 0.05$, [a] significantly different from the corresponding male group at $P < 0.05$

Table 5.1

Body and organ weights of mice treated with vehicle or FB_1 [a]

Group	Initial body wt., g	Body wt. at last treatment, g (% change)	Liver wt., g [b] (%)	Kidney wt., g [b] (%)
Male, saline-treated	21.6±0.7	21.5±0.6 (0.6 ±1.6)	0.92±0.03 (4.47±0.05)	0.30±0.01 (1.46±0.02)
Male, FB_1-treated	22.4±0.9	21.2±0.7 (-5.2±1.0)	1.05±0.04* (5.28±0.18)*	0.27±0.01 (1.35±0.06)
Female, saline-treated	16.3±0.4	17.7±0.4 (9.2±0.8)	0.76±0.03 (4.38±0.11)	0.21±0.01 (1.25±0.05)
Female, FB_1-treated	19.0±0.8	16.2±0.8 [c] (-14.8±1.2)	0.90±0.03* (5.88±0.10)*	0.22±0.01 (1.45±0.03)*

[a] Mean ± SE (n=5). [b] The relative organ weights are as % of the final body weight after overnight fasting. [c] Significantly different from the initial body weight ($P < 0.05$). * Significantly different from respective saline-treated control group at $P < 0.05$

Table 5.2

Hematological parameters of mice treated with vehicle or FB_1 [a]

Group	Red blood cells ($10^{-6}/mm^3$)	Leukocytes ($10^{-3}/mm^3$)	Lymphocytes ($10^{-3}/mm^3$)	Neutrophils ($10^{-3}/mm^3$)
Male, saline treated	5.34±0.11	3.13±0.33	1.96±0.20	0.70±0.15
Male, FB_1 treated	5.94±0.08*	4.06±0.80	2.33±0.51	0.97±0.10
Female, saline treated	4.73±0.17	3.16±0.12	2.53±0.05	0.46±0.07
Female, FB_1 treated	5.12±0.13	4.76±0.47*	2.46±0.08	1.59±0.31*

[a] Mean ± SE (n=5). * Significantly different from its respective saline-treated control group at $P < 0.05$

4. Discussion

A 5-day treatment of male and female BALB/c mice to FB_1 resulted in gender related differences in hepatotoxicity. Over the course of the short-term treatment there were no significant body weight changes in control groups of either sex; however, females after FB_1 exposure showed a decrease in body weight. The increase in liver weights after FB_1 treatment was higher in females than in male mice. Liver is the target organ in mice for FB_1 toxicity and the increased liver weight could be due to compensatory hypertrophy after FB_1 treatment (Voss et al., 1996). Circulating neutrophils were increased only in females after FB_1 treatment. In the present study, no inflammatory changes were observed in the liver sections of mice in either sex after FB_1 treatment.

In females, greater hepatotoxicity was demonstrated compared to males after FB_1 treatment by increased ALT level and *in situ* TUNEL staining. As shown by histopathology in our previous study using the same treatment protocol, apoptosis was the only lesion observed in liver and kidney after FB_1 treatment, without noticeable necrosis (Sharma et al., 1997). The only other change in liver was eosinophilic appearance of periportal hepatocytes suggesting bridging between portal triads.

Gender differences after chronic exposure to FB_1 toxicity have been reported earlier. The National Toxicology Program (1999) reported that in a 2-year feeding study female $B6C3F_1$ mice were more sensitive to FB_1 toxicity than males. In a 13-week feeding study of mice given 81 ppm FB_1, only females showed liver lesions consisting of individual hepatocyte necrosis, hepatocellular cytomegaly and increased number of mitotic figures (Voss et al., 1995). In another study with male and female $B6C3F_1$ mice

Bondy et al. (1997) indicated no consistent sex-related changes after a 2 week daily gavage with FB_1; however, at daily doses of 15 and 35 mg/kg FB_1 females had increased serum ALT levels whereas no enzyme alterations were observed in males.

The extent of accumulation of free sphingoid bases, sphingosine and sphinganine, was greater in female liver than in males after FB_1 treatment. The control females also showed higher basal levels of sphinganine and sphingosine than their respective male counterparts, thus suggesting a possible contribution of sphingoid bases in increased sensitivity to FB_1 in females. No sex-related changes in kidney sphingoid base levels were observed after FB_1 treatment although the increases in this organ were similar to those in liver.

FB_1 is a known inducer of TNFα, which may be involved in its toxicity (Sharma et al., 2000a; 2001). TNFα can trigger a cascade of other cytokines, such as IL-1β, IL-6 and IL-10, which can modify various immune processes and cause cytotoxicity. TNFα induces macrophages to secrete other toxic cytokines like IL-1β and IL-6 (Abbas et al., 1997). On the other hand TNFα, IL-6 and IL-12 up-regulate IL-10 synthesis, which suppresses the synthesis of IL-1β and IL-6 (Pretolani et al., 1999). TNFα and IL-12 produced by macrophages can also stimulate the NK cells and $NK1^+$ T cells to secrete IFNγ, which through a positive amplification loop can further increase TNFα and IFNγ (Kaplan and Schreiber, 1999). FB_1-induced TGF-β1 expression in hepatocytes has also been suggested to play a role in liver apoptosis and fibrosis (Lemmer et al., 1999).

FB_1 treatment in males increased the expression of all liver cytokines evaluated in this study (TNFα, IL-1β, IL-6, IL-10, IL-12 p40 and IFNγ), suggesting an amplification of the cytokine network and its possible involvement in FB_1 hepatotoxicity. However,

the levels of various proinflammatory cytokines (TNFα, IL-1β, and IFNγ) in control (PBS-treated) females were already close to those in FB_1-treated males. Higher basal expression levels of TNFα, IL-1β, IL-12 p40 and IFNγ in liver of females as compared to males may explain a lack of further increase after FB_1 treatment.

The expression of IFNγ was increased in males and decreased in females after FB_1 treatment. The significance of this opposite effect in two sexes is not apparent at present; however, we have confirmed this finding in other experiments employing an identical experimental protocol (unpublished data). Females showed a greater induction of IL-6 whereas males showed a greater induction of IL-10 after FB_1 treatment. IL-6 is a proinflammatory cytokine and its induction can be related to increased hepatotoxicity in females, whereas a higher increase in IL-10, a protective cytokine (Meager, 1998), could be responsible for the decreased response of males to FB_1 treatment.

Hormonal influences on cytokine modulation have been suggested in numerous earlier studies. Male rat peritoneal macrophages exposed to estradiol released greater amounts of TNFα as compared to untreated macrophages (Chao et al., 1995). Peritoneal adherent cells from adult female rats secreted higher amounts of IL-1β spontaneously than those from age-matched male rats (Hu et al., 1988). Periodic surges of estrogen and prolactin in sexually mature female mice can also stimulate IFNγ, since the IFNγ promoter is regulated by estrogen and exposure to 17β-estradiol has been reported to increase IFNγ mRNA expression in concanavalin A-stimulated mice (Fox et al., 1991).

In various studies, females have been shown to be more sensitive to toxic responses against a variety of chemicals, with interplay of different factors (Morgan et al., 1995; Kari et al., 1992). Toxicokinetic differences as a cause of gender specificity for

FB_1 can be ruled out since this mycotoxin is largely excreted unmetabolized (Shephard et al., 1992). It is possible that either the influence of androgens reduces the toxicity to FB_1 in males or the effect of reproductive hormones in female mice makes them more sensitive. Studies are currently underway in our laboratory to determine the role of gonadal hormones in FB_1-induced toxicity.

In summary the results of this study showed female mice to be more sensitive to hepatotoxic effects of the 5-day repeated treatment of FB_1 as compared to their male counterparts. Gender-related changes to fumonisin treatment were reflected by differences in accumulation of free sphingolipid bases and differences in cytokine modulation in liver, suggesting that these factors may be involved in the hepatotoxic responses to FB_1 in mice.

Acknowledgements

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CHAPTER 6

MODULATION OF THE EXPRESSION OF SELECTED CELL SIGNALING GENES IN MOUSE LIVER BY FUMONISIN B_1[1]

[1]Bhandari, N., and Raghubir P. Sharma. 2001. To be submitted to Journal of Pharmacology and Experimental Therapeutics.

ABSTRACT

Fumonisin B_1 (FB_1) is a naturally occurring mycotoxin produced primarily by *Fusarium verticillioides* and related fungi, which are common contaminants of corn throughout the world. FB_1 is a carcinogen and causative agent of several lethal animal diseases. Liver is the primary target organ in mice. Our previous studies showed altered expression of cytokines in mouse liver after FB_1 treatment. To further investigate the genes involved in the cytokine network and apoptosis signaling, male and female BALB/c mice (5/group) were injected subcutaneously with either saline or 2.25 mg/kg/day of FB_1 for 5 days. FB_1 treatment caused increased expression of tumor necrosis factor α (TNFα), interleukin (IL)-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-6, IL-10, IL-12 p40, IL-18 and interferon γ (IFNγ) in male liver, with a similar increase in females except that IL-1β and IL-18 were unaltered. Control females showed higher basal levels of IL-1α, IL-1Ra, IL-10, IL-12 p40 and IFNγ as compared with males. Expression of TNF receptor 55 and TNF receptor associated death domain (TRADD) was increased, with no changes in Fas signaling molecules, Fas, Fas ligand, Fas associated death domain (FADD) and Fas-associated protein factor (FAF). Expression of c-Myc, B-Myc, Max and Mad oncogenic transcription factors and apoptotic genes, namely Bcl-2, Bax and Bad, was also increased after FB_1 treatment. FB_1 caused an activation of cytokine network in liver along with TNFα signaling pathways. Induction of IL-1Ra and oncogenes could be responsible for the cancer promoting properties of FB_1.

ABBREVIATIONS: FADD, Fas-associated death domain; FAF, Fas-associated protein factor; FAP, Fas-associated phosphatase; FasL, Fas ligand; FB_1, fumonisin B_1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFNγ, interferon γ; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; MIF, macrophage migration inhibitory factor; NK, natural killer; RIP, receptor-interacting protein; RPA, ribonuclease protection assay; RT-PCR, reverse transcriptase-polymerase chain reaction; Th, T helper; TNFα, tumor necrosis factor α; TNFR, tumor necrosis factor receptor; TRADD, TNF receptor-associated death domain; TRAIL, TNF-related apoptosis-inducing ligand.

Fumonisins are mycotoxins produced by *Fusarium verticillioides* (= *F. moniliforme*, Gelderblom et al., 1988) and other *Fusarium* species, which commonly contaminate corn. Of all the fumonisins, fumonisin B_1 (FB_1) is the most prevalent. Consumption of corn-based feed contaminated with high levels of fumonisins or pure FB_1 have been shown to cause the farm animal diseases, equine leukoencephalomalacia and porcine pulmonary edema (Riley et al., 1998). FB_1 is carcinogenic in rats and mice (Howard et al., 2001) and has been implicated as a contributing factor in human esophageal cancer (Sydenham et al., 1990) and possibly primary liver cancer (Ueno et al., 1997). In laboratory animals FB_1 has been shown to be hepatotoxic and nephrotoxic (Riley et al., 1998).

The mechanism of FB_1 toxicity is complex; it is species-, gender-, and tissue-specific. For example, the primary target organ of FB_1 toxicity is brain in horse, lungs in pig, kidney in rat and liver in the mouse (Riley et al., 1998). In a two-year feeding study

conducted by the National Toxicology Program, the carcinogenic lesions were found in male rat kidney and female mouse liver (Howard et al., 2001).

Biochemical alterations induced by FB_1 include inhibition of ceramide synthase (sphinganine and sphingosine-*N*-acyl transferase) leading to accumulation of sphingoid bases, sphingoid base metabolites, depletion of more complex sphingolipids and inhibition of ceramide biosynthesis (Riley et al., 1998). FB_1 increased the expression of tumor necrosis factor-α (TNFα) in mouse liver (Sharma et al., 2000a, 2001). In addition, the *in vivo* hematological effects of FB_1 were partially reversed by anti-TNFα antibodies (Dugyala et al., 1998), and mouse strain lacking p75 or p55 TNFα receptor (TNFR) showed reduced hepatotoxicity after FB_1 treatment (Sharma et al., 2000a, 2001). FB_1 treatment caused an increased expression of TNFα in liver with relatively less effect in kidney (Bhandari et al., 2001).

In vivo studies in mice and rats have demonstrated that FB_1 induces apoptosis in liver and kidney (Sharma et al., 1997, Riley et al., 1998). FB_1 treatment caused apoptosis in several different human cells (Tollenson et al., 1996). Since apoptosis can be involved both in cancer development and disease process, the ability of FB_1 to induce apoptosis appears to be important with respect to its toxicological effects. TNFα causes apoptosis in cells and its induction is one of the earlier events in hepatic inflammation, which triggers a cascade of other cytokines (Diehl, 2000).

The present study was undertaken to investigate the expression of various genes involved in cytokine network and signaling molecules that could play a role in the FB_1-induced apoptosis. In a recent study we showed that TNFα, interleukin (IL)-1β, IL-6, IL-10, IL-12 p40 and interferon γ (IFNγ), measured by reverse transcriptase-polymerase

chain reaction (RT-PCR) in the liver of male mice, were induced after FB_1 treatment (Bhandari et al., 2001). Female mice were more sensitive to FB_1 toxicity and showed higher basal levels of TNFα, IL-1β, IL-12 p40 and IFNγ compared with males. Suggesting that higher basal levels of certain cytokines may predispose female mouse liver to FB_1 toxicity. To further evaluate the activation of cytokine network in liver using a quantitative method, expression of various cytokines was measured by ribonuclease protection assay (RPA) in mouse liver after FB_1 treatment of animals. In addition, expression of signaling molecules involved in TNFα and Fas apoptotic pathways were also investigated. Two most important death receptors involved in liver pathology include TNFα and Fas (Faubion and Gores, 1998). The FB_1-induced c-Myc expression has also been suggested in cancer promoting effects of FB_1 (Lemmer et al., 1999); therefore, expression of Myc/Max/Mad network oncogenes was measured after FB_1 treatment. Other intracellular molecules involved in regulation of apoptosis, like caspases (caspase 3, caspase 6 and caspase 8) and Bcl-2 family pro-apoptotic (Bax and Bad) and anti-apoptotic (Bcl-2) molecules were also measured.

Materials and Methods

Animal and Housing. Adult male and female BALB/c mice, 7 weeks old (about 18 g body weight), were obtained from Harlan Laboratories (Indianapolis, IN). The animals were acclimated in the University of Georgia Animal Resources facility for one week at 23°C and 50% relative humidity, with a 12 h light/dark cycle. Pelleted feed (fumonisin-free) and fresh water were provided *ad libitum*. Food and water consumptions were recorded daily. The protocols for animal use followed the Public

Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Treatment and Sampling. Mice (5/group/sex) were treated with five daily subcutaneous injections (in a volume of 1 ml/100 g body wt.) of endotoxin-free FB_1 (99.6% purity; Promec, Tygerberg, South Africa) in physiological buffered saline; the dose per mouse being 0 (saline-treated controls) or 2.25 mg FB_1/kg/day. The protocol for treatment was based on our previous studies in which FB_1 produced consistent liver injury characterized by apoptosis in both male and female mice, and females showing greater hepatotoxixicty (Sharma et al., 1997; Bhandari et al., 2001). One day after the last injection, animals were euthanized with halothane, and pieces of liver tissue were obtained, quickly frozen in aliquots on dry ice and stored at -85°C until further analysis.

RNA Isolation and RNase Protection Assay (RPA). RNA was isolated from frozen livers (each 70-80 mg) with TRI® reagent (Molecular Research Center, Cincinnati, OH), using a protocol described earlier (Sharma et al., 2000a). Frozen tissues were grounded in liquid nitrogen in a mortar and then TRI reagent added to the mortar and tissues further homogenized. RPA was performed on samples using RiboQuant^{TM} RPA starter kit (Pharmingen, San Diego, CA). In brief, three Pharmingen template sets, one for cytokines (mCK-2b), the second for TNFα and Fas signaling molecules (mAPO-3), and a third mouse template set (containing TNFα/Myc/Max/Mad/Bcl-2/Bax/Bad/Caspases) were used. The synthesis of high-specific-activity, [α^{32}P]UTP-labeled, T7 RNA polymerase-directed, anti-sense RNA probes was done using the *in vitro* transcription kit and manufacturer's directions (Pharmingen, San Diego, CA). 3000 Ci/mmol and 10 mCi/ml [α^{32}P]UTP (ICN Biomedicals, Costa Mesa, CA) was used for

probe synthesis. The dried probe was dissolved in hybridization buffer to a concentration of 4×10^5 cpm/µl; 2 µl of this was added to the tubes containing 50 µg of sample RNA dissolved in 8 µl hybridization buffer. A mouse control RNA (Pharmingen) was used as a positive control for RNA integrity, whereas yeast tRNA used as a negative background control. The samples were overlaid with mineral oil, heated to 90°C, and incubated at 56°C for 12-16 h. Free probe and other single-stranded RNA molecules were digested with RNase A and RNase T1 at 30°C for 45 min, followed by proteinase K digestion at 37°C for 15 min. The remaining RNase-protected probes were purified according to manufacturer's protocol and resolved on a denaturing polyacrylamide gel (5% acrylamide, 8 *M* urea, 5 mm × 30 cm) using IBI Base Runner^{TM} 200 (Shelton Scientific, Shelton, CT). The non-hybridized probe was run to indicate the integrity and labeling efficiency and as a size marker. The gels were dried onto a filter paper in a pre-heated gel dryer (Labconco, Kansas city, MO) at 80°C for 1 h. Dried gels were placed on XAR film (Kodak, Rochester, NY) with intensifying screens and were developed at -70°C for 48 h. Using undigested probes as markers, a standard curve of migration distance versus log nucleotide length was plotted on semi-log graph paper and used to establish the identity of RNase-protected bands. Because the probe contains polylinker sequences it migrates less than the protected species in the gels. The relative gene expression was quantified via densitometric imaging using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT) and normalized against ribosomal protein L32 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) house keeping genes.

Statistics. Data from these studies were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan's Multiple Range test. The SAS computer

program (SAS Institute, Cary, NC) was employed for all calculations. The level of $P < 0.05$ was considered statistically significant for all comparisons.

Results

Multiple dose treatment of either male or female mice with FB_1 for 5 days produced no obvious gross clinical or behavioral effects. Gene alterations in mice liver after FB_1 treatment were measured by RPA. Fig. 6.1 shows the representative gels obtained from the three RPA templates. The data reported have been normalized to ribosomal protein L32 (accession # K02060). Identical results were obtained with GAPDH as the normalizing gene (accession # M32599, data not shown). Genes from the three template sets have been grouped into different figures based on their relevance and the range of their relative expression values.

Gene alterations in cytokine network were observed in both males and females after FB_1 treatment. FB_1 treatment in males caused increased expression of TNFα, IL-1α, IL-1β, IL-1Ra, IL-6, IL-10, IL-18 and IFNγ in liver (Fig. 6.2). In males maximum increase was noted in TNFα (33-fold) followed by IL-1Ra (18-fold), IFNγ (12-fold), and IL-6 (11-fold). Table 6.1 displays calculated fold-increase in expression of cytokine genes, which were significantly altered after FB_1 treatment either in males or females.

FB_1 treatment in females caused increased expression of TNFα, IL-1α, IL-1Ra, IL-6, IL-10 and IFNγ (Fig. 6.2). In females, maximum increase was noted in IL-6 followed by TNFα and IFNγ. In general, control females showed higher basal levels of most cytokines compared with control males, of which expression of IL-1α, IL-1Ra, IL-10, and IFNγ was significantly greater in females than males (Fig. 6.2). Expression of

IL-12 p40 was increased in males but unaltered in females after FB_1 treatment (Fig. 6.3). Females also showed higher basal levels of IL-12 p40 compared with males. IL-12 p35 (Fig. 6.3) and macrophage migration inhibitory factor (MIF, data not shown) expression was unaltered in both males and females after FB_1 treatment.

Signaling molecules involved in TNFα apoptotic pathway were increased in both males and females after FB_1 treatment. Expression of TNFR 55 increased 40% over control after FB_1 treatment (Fig. 6.4). No alterations were observed in expression of receptor interacting protein (RIP) with FB_1 (Fig. 6.4). FB_1 treatment increased TNF receptor associated death domain (TRADD) gene expression 70% over control (Fig. 6.5). Observed increases in expression of apoptosis and signaling genes, which were significantly altered after FB_1 treatment, are shown in Table 6.2.

FB_1 treatment caused no alteration in Fas signaling molecules, namely Fas-associated protein factor (FAF, Fig. 6.4), Fas and Fas-associated death domain (FADD, Fig. 6.5). Expression of Fas ligand (FasL) and Fas-associated phosphatase (FAP) could not be detected in any of the liver samples (Fig. 6.1). No alterations were observed in the expression of TNF-related apoptosis-inducing ligand (TRAIL) after FB_1 treatment (Fig. 6.5).

Gene expression of c-Myc, B-Myc, Max and Mad, involved in the Myc/Max/Mad network were all activated after FB_1 treatment, without any gender differences (Fig. 6.6). Among these four genes the induction of c-Myc was highest, followed by B-Myc, Mad and Max. FB_1 treatment caused induction of pro-apoptotic Bcl-2 family genes, Bax (Fig. 6.4) and Bad (Fig. 6.6). Expression of anti-apoptotic gene Bcl-2 was induced only in

females after FB_1 treatment (Fig. 6.6). In males, the Bcl-2 expression did increase 2-fold after FB_1 treatment, but was not significant because of variation within animals.

FB_1 treatment caused increased expression of caspase 3 in both males and females (Fig. 6.5). Caspase 6 (Fig. 6.4) and caspase 8 (Fig. 6.5) expression was unaltered after FB_1 treatment (Fig. 6.4). This assay was capable of detecting less than 50% increase in gene expression (for TNFR 55). Only very few genes with fold increase greater than 1.5 were shown unaltered.

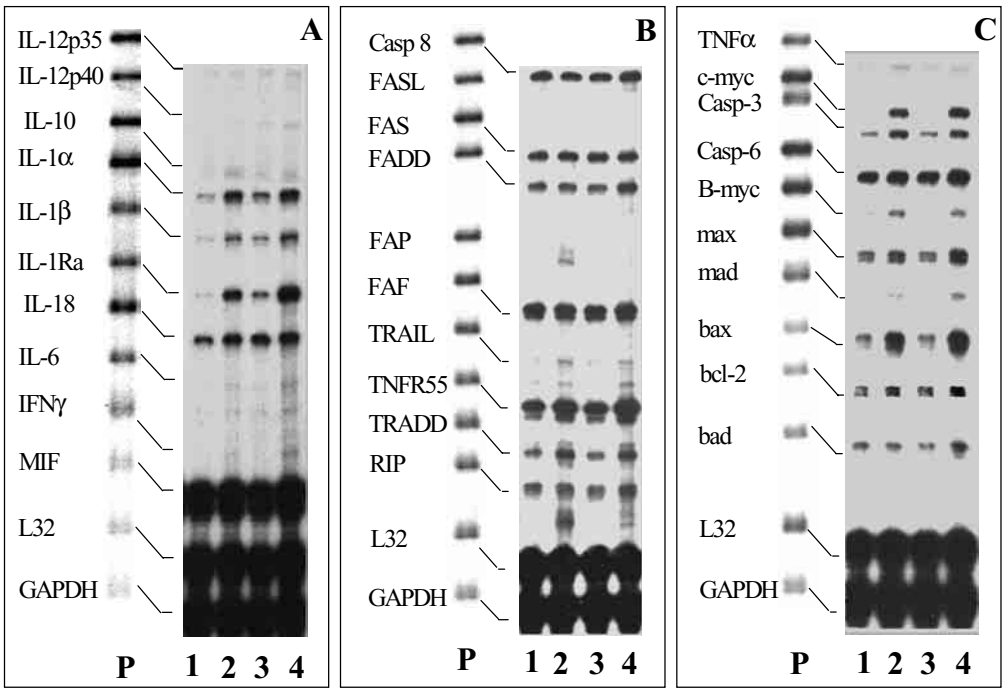

Figure 6.1. Representative gels of RNase protection assay. Three template sets were used (A) for cytokines (mCK-2b), (B) for TNFα and Fas signaling molecules (mAPO-3), and (C) for apoptosis signaling molecules (custom template). A non-hybridized probe set (P) was run as a size marker. The gene names are listed on the left side of each probe set. Treatment groups are indicated as (1) male, saline-treated, (2) male, FB_1-treated, (3) female, saline-treated and (4) female, FB_1-treated.

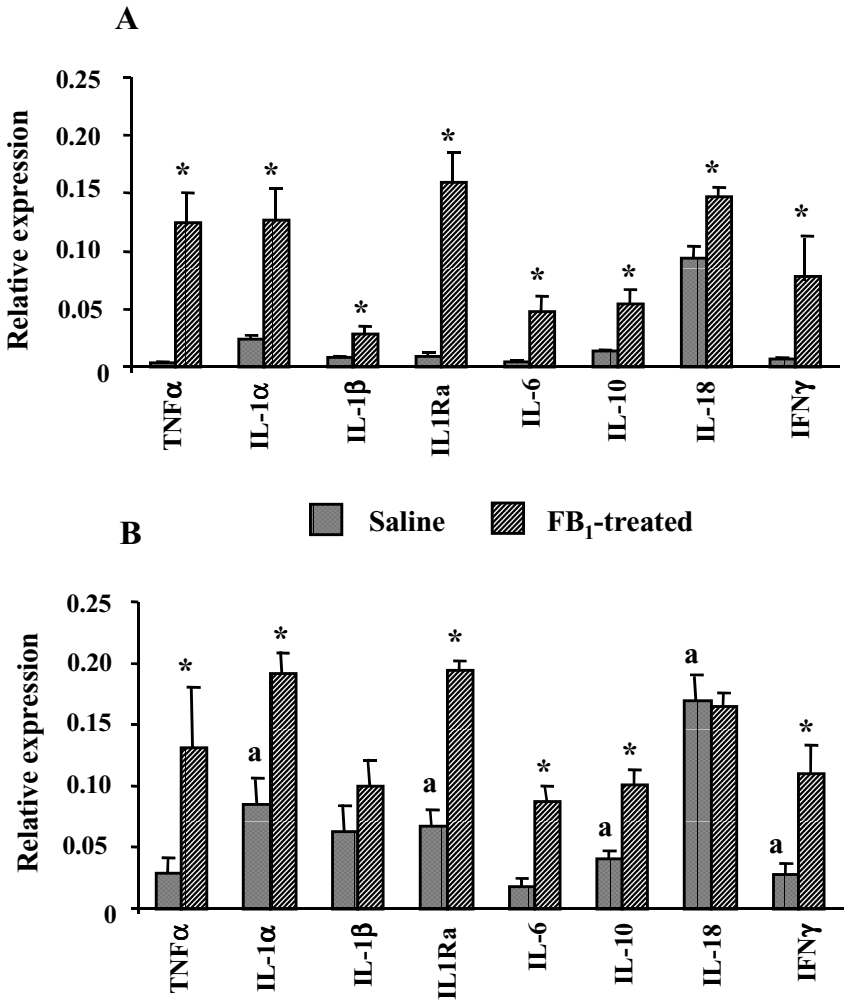

Figure 6.2. Alterations in cytokine expression in male (A) and female (B) liver after FB_1 treatment. The relative mRNA expression is indicated and normalized against L32. Results are expressed as mean ± standard error. (n=5) * Indicates significantly different from the control group at $P < 0.05$ and [a] indicates significantly different from the corresponding male group at $P < 0.05$.

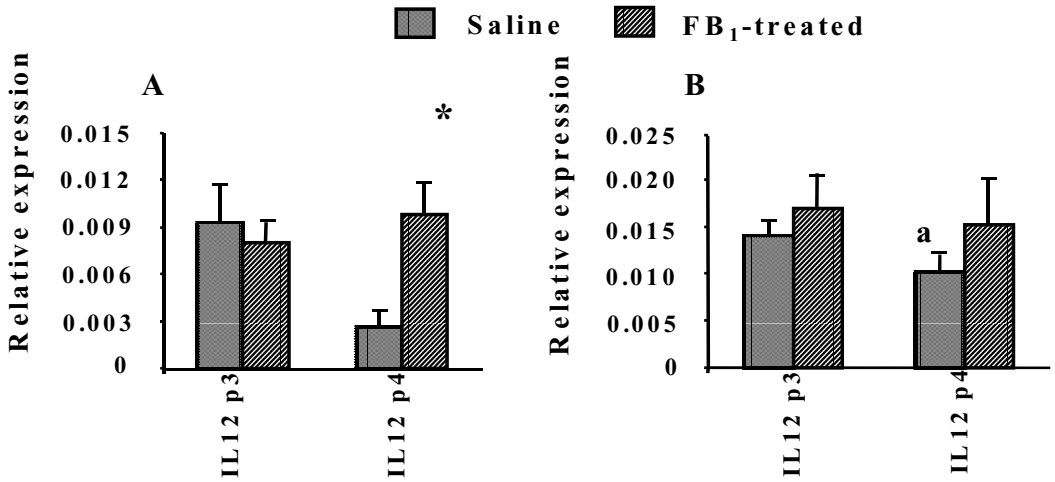

Figure 6.3. Alterations in IL-12 p35 and IL-12 p40 cytokine expression in male (A) and female (B) liver after FB_1 treatment. The relative mRNA expression is indicated and normalized against L32. Results are expressed as mean ± standard error. (n=5) * Indicates significantly different from the control group at $P < 0.05$ and [a] indicates significantly different from the corresponding male group at $P < 0.05$. Note difference in the scale on ordinate for male (A) Vs female (B).

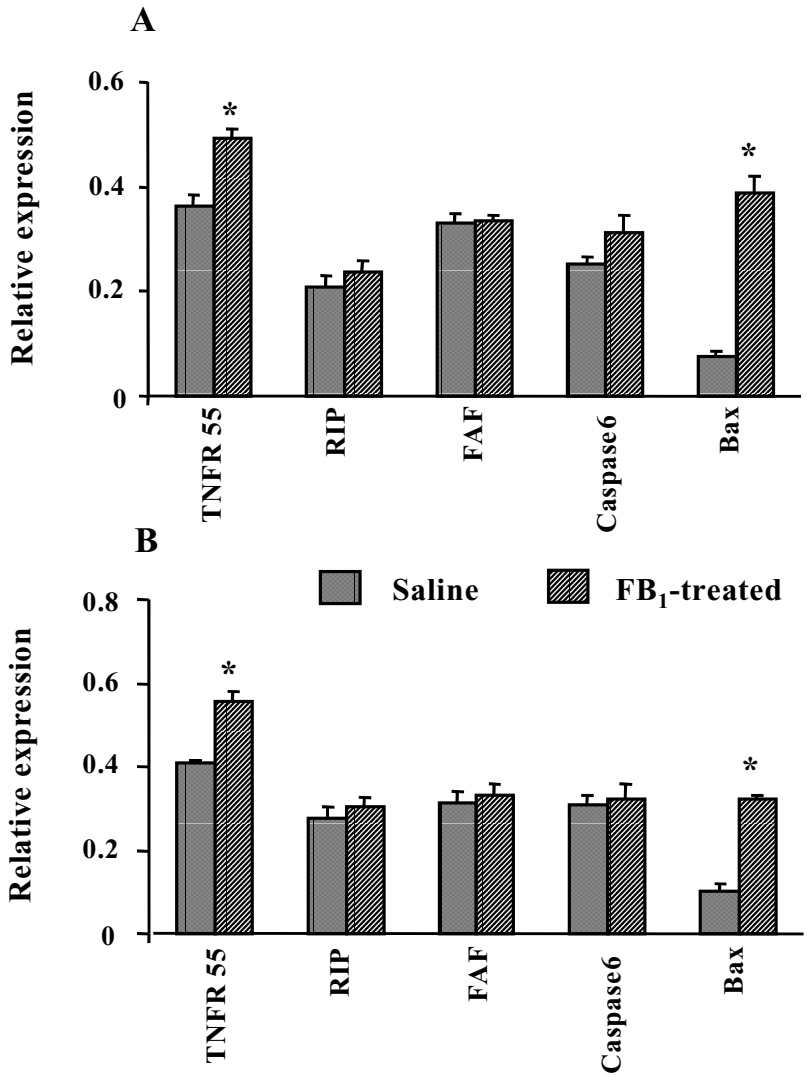

Figure 6.4. Alterations in TNFR55, RIP, FAF, Caspase 6 and Bax expression in male (A) and female (B) liver after FB_1 treatment. The relative mRNA expression is indicated and normalized against L32. Results are expressed as mean ± standard error. (n=5) * Indicates significantly different from the control group at $P < 0.05$.

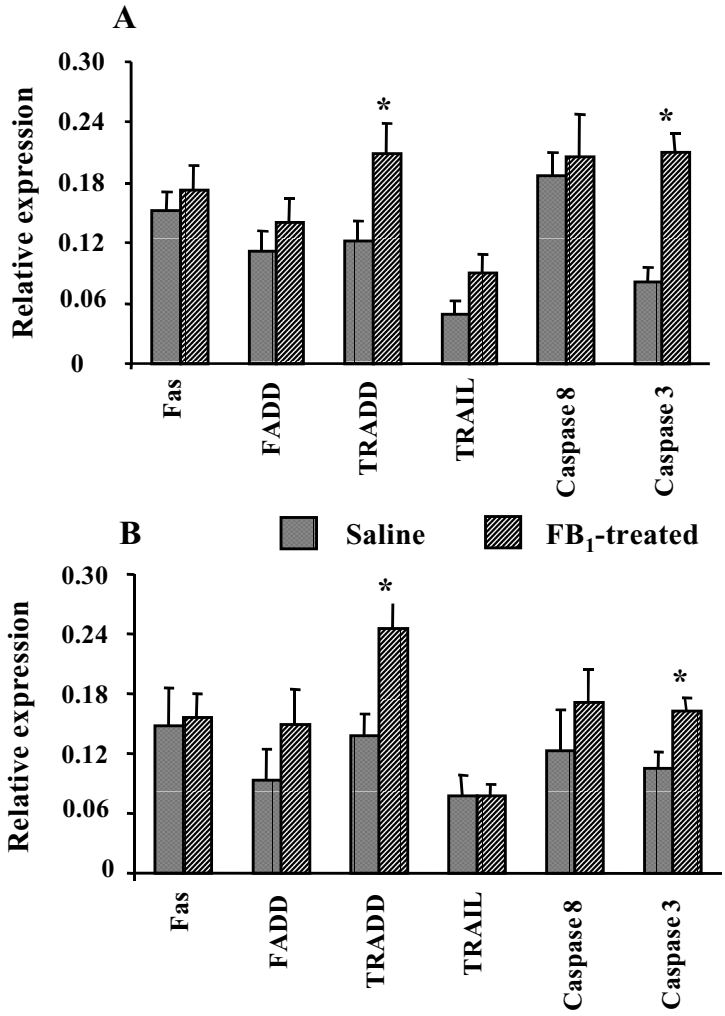

Figure 6.5. Alterations in Fas, FADD, TRADD, TRAIL, Caspase 8 and Caspase 3 expression in male (A) and female (B) liver after FB_1 treatment. The relative mRNA expression is indicated and normalized against L32. Results are expressed as mean ± standard error. (n=5) * Indicates significantly different from the control group at $P < 0.05$.

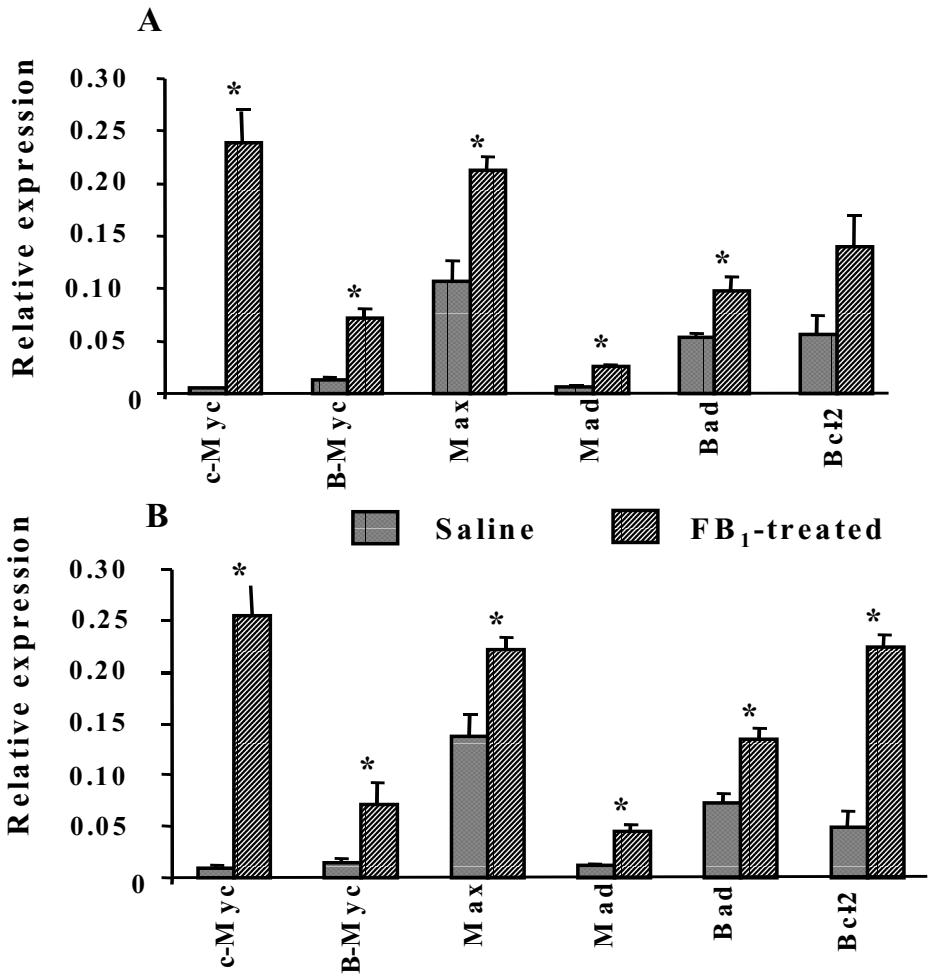

Figure 6.6. Alterations in c-Myc, B-Myc, Max, Mad, Bad and Bcl-2 expression in male (A) and female (B) liver after FB_1 treatment. The relative mRNA expression is indicated and normalized against L32. Results are expressed as mean ± standard error. (n=5) * Indicates significantly different from the control group at $P < 0.05$.

Table 6.1

Expression of cytokines after FB_1 treatment in mice liver

Gene	GenBank accession number	Fold increase over respective controls		Significance
		Male	Female	
TNFα	M11731	33.3*	4.6*	Pro-inflammatory (hepatotoxic)
IL-1α	X01450	5.4*	2.3*	Pro-inflammatory
IL-1β	M15131	3.7*	1.6	Pro-inflammatory (hepatotoxic)
IL-1Ra	M57525	17.8*	2.9*	Anti-inflammatory (cancer promoter)
IL-6	J03783	11.0*	5.0*	Pro-inflammatory
IL-10	M37897	4.0*	2.5*	Anti-inflammatory
IL-12 p40	M86671	3.7*	1.5	Induce IFNγ
IL-18	D49949	1.6*	1.0	Induce IFNγ
IFNγ	K00083	11.8*	3.8*	Activate Kupffer cells (hepatotoxic)

* Indicates FB_1 treatment caused significant increase in gene expression compared to control group at $P < 0.05$. See Figs. 2 and Fig. 3 for relative high expression of many cytokines in females than in males.

Table 6.2

Expression of apoptosis and signaling genes after FB_1 treatment in mice liver

Gene	GenBank accession number	Fold increase over respective controls		Significance
		Male	Female	
TNFR 55	L26349	1.4*	1.4*	TNFα signaling receptor
TRADD	AA013699	1.7*	1.8*	TNFα signaling adapter protein
B-Myc	W18410	5.4*	4.7*	Inhibit growth (hormonally regulated)
c-Myc	X00195	51.4*	29.5*	Activate transcription, proliferation and apoptosis (cancer promoter)
Max	M63903	2.0*	1.6*	Neutral, heterodimerizes with c-Myc or Mad
Mad	L38926	4.1*	3.9*	Repress transcription
Bcl-2	M16506	2.1	4.6*	Anti-apoptotic
Bax	L22472	5.1*	3.2*	Pro-apoptotic
Bad	L37296	1.8*	1.9*	Pro-apoptotic
Caspase 3	U49929	2.6*	1.5*	Effector caspase (execute apoptosis)

* Indicates FB_1 treatment caused significant increase in gene expression compared to control group at $P < 0.05$.

Discussion

FB_1-induced hepatotoxicity in male and female BALB/c mice is accompanied by induction of several pro-inflammatory cytokines in liver. Expression of TNFα signaling molecules was also induced after FB_1 treatment with no involvement of Fas death receptor activated pathway. Both pro-apoptotic and anti-apoptotic classes of intracellular signaling molecules were induced after FB_1 treatment.

The induction of cytokine network in liver is important because liver has a complete innate immune system consisting of at least Kupffer cells and hepatic natural killer (NK) cells (Seki et al., 2000). Kupffer cells are an essential immunological component of host defense which can induce proinflammatory cytokine like TNFα and IL-1. IL-18, another cytokine produced by Kupffer cells can induce IFNγ production by Th1 cells in presence of IL-12 (Chikano et al., 2000). TNFα, IL-12 and IL-18 can also activate NK cells to produce IFNγ. This IFNγ through a positive feed-back can further activate the Kupffer cells to produce more IL-12 and TNFα (Kaplan and Schreiber, 1999). IL-12 is a disulphide linked heterodimeric cytokine consisting of p35 and p40 kd subunits. Out of the two subunits, p40 is mostly regulated whereas p35 is expressed in an almost constitutive fashion (Chizzonite et al., 1998). This could be the reason why we see changes only in IL-12 p40 expression after FB_1 exposure and not IL-12 p35 expression.

The induced cytokines after FB_1 treatment may play an important role in the severity of the observed liver toxicity in mice. In our previous study, females were shown to be more sensitive to FB_1 toxicity, showing greater increase in circulating alanine aminotransferase and more apoptotic cells in liver after FB_1 treatment (Bhandari

et al., 2001). In the current study, females also showed higher basal expression levels of IL-1α, IL-1Ra, IL-10, IL-12 p40, and IFNγ cytokines in liver compared with males, suggesting the possible role of these increased cytokines in making the females more sensitive to FB_1 induced toxicity. Various cytokines are involved in different experimental mouse models of hepatotoxicity. Neutralizing antibodies to TNFα or IL-1 partially prevented liver damage in mice, initiated by hepatotoxic doses of acetaminophen (Blazka et al., 1995). TNFα, IFNγ and IL-12 were involved in liver toxicity known as Shwartzman reaction caused by repeated lipopolysaccharide challenge (Ogasawara et al., 1998). IFNγ over-expressed mice also develop chronic active hepatitis (Okamoto et al., 1999). TNFα and IL-6 are involved in alcoholic liver disease (Faubion and Gores, 1998). TNFα and IFNγ have also been related to concanavalin A-induced hepatitis (Ksontini et al., 1998).

IL-1Ra expression was also increased 18-fold in males and 3-fold in females after FB_1 treatment. Females also had higher basal levels of IL-1Ra (7.5-fold) as compared with males. IL-1Ra was the primary gene that showed altered expression in mouse hepatocellular carcinoma relative to normal liver (Yamada et al., 1999). IL-1Ra is a natural antagonist of IL-1, where its expression has been clearly correlated with an increase in the proliferating cell nuclear antigen index in hepatocellular adenomas in mice (Yamada et al., 1999). In FB_1 toxicity its increased expression could support a micro-environment for promoting tumors.

TNFR 55 and Fas are two important death receptors whose activation by TNFα and FasL respectively has been shown in various liver diseases. FB_1 toxicity caused increased expression of TNFR 55 and TRADD involved in TNFα signal transduction

pathway, with no alteration in molecules involved in Fas pathway. Expression of TNF type 1 receptor associated protein 2 was also induced in FB_1-sensitive CV-1 cells but repressed in FB_1-resistant COS-7 cells demonstrating the involvement of TNFα-induced death pathway in FB_1-induced apoptosis (Zhang et al., 2001).

TNFR 55 is heavily expressed in hepatocytes and Kupffer cells. The expression of TNFR 55 has been shown to be strongly enhanced in hepatitis B, hepatitis C, autoimmune hepatitis and especially alcohol induced hepatitis (Faubion and Gores, 1999). Signaling via the TNFR 55 is extremely complex, leading to both cell death and cell survival signals (Yuan, 1997). The apoptotic pathway after TNF binding to TNFR 55 involves receptor oligomerization, leading to recruitment of the adapter protein TRADD. TRADD recruits with its self another adapter protein FADD of the Fas signaling pathway, which can activate the caspase 8. An alternative apoptotic pathway by TNFR 55 also involves binding of TRADD with RIP (Yuan, 1997). The RIP expression was unaltered after FB_1 treatment. Another death receptor ligand, TRAIL, which could play a role in the liver toxicity (Faubion and Gores, 1999) showed no change with FB_1.

Fas/FasL receptor interactions also play an important role in induction of apoptosis in liver. The hepatocytes in the liver appear to be especially susceptible to Fas mediated death, because injection of anti-Fas induces massive injury to liver and not elsewhere (Pinkoski et al., 2000). Fas signaling pathway was not involved in the FB_1 hepatotoxicity, because expression of Fas signaling molecules (Fas, FADD and FAF) remained unaltered after FB_1 treatment. Hepatocytes constitutively express Fas and may upregulate expression of this receptor in a variety of liver diseases like viral hepatitis or

alcohol induced liver disease (Kanzler and Galle, 2000). Fas expression has been shown to be induced and upregulated by inflammatory cytokines such as IL-1 (Giordano et al., 1997). At the same time Fas expression has also been found to be reduced in particularly aggressive forms of hepatocellular carcinoma (Ito et al., 1998).

FB_1 treatment caused an induction of Myc/Max/Mad network in both male and female mice. The Myc, Max and Mad proteins form a network centered on Max, which forms transactivating complexes when associated with c-Myc but repressive complexes when bound to Mad (Baudino and Cleveland, 2001). The increase in c-Myc expression after FB_1 treatment was much greater than the increase in Mad, suggestive of c-Myc dominated response. Induced expression of c-Myc after FB_1 exposure has also been reported earlier (Lemmer et al., 1999). The ability of overexpressed c-Myc to facilitate proliferation and inhibit terminal differentiation can thus be involved in the cancer promoting effects of FB_1. Overexpression of cyclin D1 protein has also been suggested as a mechanism for FB_1-induced hepatocarcinoma (Ramljak et al., 2000). There is conflicting data in the litrature on how c-Myc regulates cyclin D1 expression, some suggesting down-regulation and others up-regulation of cyclin D1 by c-Myc (Dang et al., 1999).

Bcl-2 family of genes represents another class of proteins, which are involved in the modulation phase of apoptosis, shifting the balance either to cell survival or cell death. Overexpression of Bad, Bax and Bcl-X_S enhance apoptosis process whereas Bcl-2 and Bcl-X_L expression inhibits apoptosis (Korsmeyer, 1999). In this study, FB_1 treatment caused induction of Bax and Bad in both sexes, whereas Bcl-2 expression was increased only in females after FB_1 treatment. A western blot analysis of mouse liver showed

expression of Bcl-X_L and Bcl-X_S proteins with no detectable levels of Bcl-2 (Ray and Jena, 2000), thus suggesting that expression of Bcl-2 might be of lesser significance in mouse liver than Bcl-X_L.

The caspases are the final executioners of apoptosis. They are expressed as inactive proenzymes in the cytosol and are sequentially activated by proteolysis during apoptosis (Wilson, 1998). Initiator caspases (caspases 8, 9 and 10) activate downstream effector caspases (caspases 3, 6 and 7). Expression of caspase 3 was increased after FB_1 treatment in both males and females, whereas caspase 6 and caspase 8 remained unaltered. Expression of caspase 8, being a initiator caspase in TNFα signaling pathway, was predicted to be increased but was found unaltered. Because the liver samples were analyzed 24 h after last treatment of FB_1, caspase 8 could have been induced at earlier time points and then its expression reduced by I-Flice, an endogenous dominant-negative form of caspase 8 (Hu et al., 1997) or other mechanism.

A generalized activation of a variety of genes by FB_1 may suggest an effect on transcription factors like nuclear factor-κB (NF-κB), activating protein-1, or nuclear factor of activated T cells, which are known to bind to regulatory elements of TNFα promoter, thus influencing its expression (Zhang and Tracey, 1998). TNFα induction can activate the cytokine network in liver, thus leading to stimulation of multiple intracellular signaling pathways. However, an increased translocation of NF-κB to nuclei may not be relevant in FB_1 hepatotoxicity, because such increase of NF-κB noted in mice carrying a human TNFα gene resulted in a reduced hepatotoxic response (Sharma et al., 2000b).

The gene expression alterations observed in current study showed an induction of cytokine network in liver after FB_1 treatment. FB_1-induced expression of TNFα, IL-1,

IL-6 and IFN γ could play a role in the observed liver toxicity, whereas increased expression of IL-1Ra maybe involved in the cancer promoting effects of FB_1. TNFα death receptor pathway was activated by FB_1 with no alterations in Fas signaling pathway. FB_1 treatment caused increased expression of c-Myc, B-Myc, Max and Mad oncogenes, with a c-Myc dominated response, suggesting c-Myc's involvement in cancer promotion. Both pro-apoptotic and anti-apoptotic Bcl-2 family genes along with caspase 3 expression were increased after FB_1 treatment. Further studies are required to understand the importance of individual altered gene in toxicity caused by FB_1.

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CHAPTER 7

FUMONISIN B_1-INDUCED ALTERATIONS IN CYTOKINE EXPRESSION AND APOPTOSIS SIGNALING GENES IN MOUSE LIVER AND KIDNEY AFTER AN ACUTE EXPOSURE[1]

[1]Bhandari, N., and Raghubir P. Sharma. 2001. To be submitted to Toxicology.

Abstract

Fumonisin B_1 (FB_1), a carcinogenic mycotoxin produced primarily by fungus *Fusarium verticillioides* in corn, causes several fatal animal diseases. In mice, liver is the primary site of toxicity. Our previous study showed that interferon γ (IFNγ) and tumor necrosis factor α (TNFα) To further investigate the time related induction of other cytokines and genes involved induction was maximum at 4 and 8 h, respectively after an acute po FB_1 treatment. in apoptosis signaling, male BALB/c mice were administered po with either saline or 25 mg/kg of FB_1 and sampled 4 or 8 h after treatment. FB_1 treatment caused increased expression of TNFα and interleukin (IL)-1β in both liver and kidney, whereas IL-1α and IL-1 receptor antagonist (IL-1Ra) expression was induced only in liver. Expression of TNFα signaling molecules, TNF receptor 55 and receptor interacting protein, was increased in liver and kidney after FB_1 treatment. Caspase 8 expression was increased only in liver with no changes observed in kidney with FB_1. FB_1 treatment induced expression of Fas in liver and kidney with no alterations in Fas signaling molecules, Fas ligand, Fas-associated death domain and Fas-associated protein factor. Treatment of mice with FB_1 increased the expression of B-Myc, c-Myc and Max oncogenic transcription factors in kidney. FB_1 toxicity caused induction of cytokine network in liver with involvement of TNFα signaling pathway. TNFα-induced caspase 8 expression could be involved in the FB_1-induced liver apoptosis, whereas increased expression of IL-1Ra in liver may be responsible for cancer promoting effects of FB_1.

Key Words: fumonisin; mouse; gene expression; liver; kidney; cytokines

Abbreviations: FADD, Fas-associated death domain; FAF, Fas-associated protein factor; FAP, Fas-associated phosphatase; FasL, Fas ligand; FB_1, fumonisin B_1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFNγ, interferon γ; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; MIF, macrophage migration inhibitory factor; NK, natural killer; RIP, receptor-interacting protein; RPA, ribonuclease protection assay; RT-PCR, reverse transcriptase-polymerase chain reaction; TNFα, tumor necrosis factor α; TNFR, tumor necrosis factor receptor; TRADD, TNF receptor-associated death domain; TRAIL, TNF-related apoptosis-inducing ligand.

1. Introduction

Fumonisins are natural contaminants of corn infected with the fungus *Fusarium verticillioides (=F. moniliforme*, Gelderblom et al., 1988). Consumption of corn molded with *F. verticillioides* leads to leukoencephalomalacia in horses, pulmonary edema in pigs, and liver and kidney toxicity in these and other species (Riley et al., 1998). Human dietary consumption of *Fusarium*-contaminated corn products has been epidemiologically linked to increased rates of esophageal cancer and primary liver cancer in the regions of South Africa and China (Sydenham et al., 1990; Ueno et al., 1997). This mycotoxin has been reported to be carcinogenic in male rat kidney and female mouse liver (Howard et al., 2001). In mice liver is the most sensitive organ, kidney exhibiting little or no effect in long-term feeding studies.

A common feature of exposure to fumonisin B_1 (FB_1) administration is perturbations in sphingolipid metabolism. FB_1 is structurally similar to sphingoid bases and inhibits the enzyme ceramide synthase, resulting in disruption of sphingolipid

metabolism (Riley et al., 1998). FB_1-induced elevation in free sphingoid bases can induce apoptosis, whereas production of sphingosine 1 phosphate or inhibitors of ceramide biosynthesis can also prevent apoptosis (Riley et al., 1998). Alterations of tissue and serum sphinganine and sphingosine levels have been established as early biomarkers of FB_1 toxicity *in vivo*.

FB_1 treatment has been shown to alter a wide range of effector molecules like activation of protein kinase C (Yeung et al., 1996), or mitogen-activated protein kinases at early time points (Pinelli et al., 1999). FB_1 treatment has also been shown to increase expression of transforming growth factor-β1 and c-Myc in rat liver (Lemmer et al., 1999), cause inhibition of serine/threonine phosphatase (Fakuda et al., 1996), and argininosuccinate synthetase (Jenkins et al., 2000), and also alter expression of cyclins and cyclin-dependent kinases (Ciacci-Zanella et al., 1998).

Tumor necrosis factor α (TNFα) has been shown to be a contributor in FB_1 toxicity in various experiments reported previously from our laboratory. Administration of anti-TNFα antibodies partially reversed the *in vivo* hemopoietic effects in FB_1 treated mice (Dugyala et al., 1998). Transgenic mice lacking p75 or p55 TNFα receptor were also resistant to FB_1-induced hepatotoxicity as compared to control mice (Sharma et al., 2000; 2001). Inhibitor of apoptosis protein, an inhibitor of TNFα pathway also prevented FB_1 toxicity in CV-1 cells (Ciacci-Zanella and Jones, 1999). Expression of tumor necrosis factor type 1 receptor associated protein 2 was induced in FB_1-sensitive CV-1 cells but repressed in FB_1-resistant COS-7 cells, demonstrating the involvement of TNFα-induced death pathway in FB_1-induced apoptosis (Zhang et al., 2001). In a recent temporal study we showed that increase in interferon γ (IFNγ) and TNFα expression,

measured by reverse transcriptase-polymerase chain reaction (RT-PCR) in the liver of male mice was greatest at 4 and 8 h, respectively after an acute, po FB_1 treatment (Bhandari et al., 2000).

To further investigate the induction of cytokine network at 4 and 8 h in liver using a quantitative method, expression of various cytokines was measured by ribonuclease protection assay (RPA) after FB_1 treatment. We investigated the expression of various genes involved in cytokine network and signaling molecules, that may play a role in the FB_1-induced apoptosis. In addition expression of signaling molecules involved in TNFα and Fas apoptotic pathways were also investigated. TNFα and Fas death receptor pathways have reported to be involved in a number of liver pathologies (Faubion and Gores, 1998). Intracellular molecules involved in regulation of apoptosis, like caspases (caspase 3, caspase 6 and caspase 8) were evaluated. Increased c-Myc expression after FB_1treatment was suggested as a cancer promoting effect of FB_1 (Lemmer et al., 1999). Therefore, expression of c-Myc, Max and Mad oncogenes, involved in cancer development and apoptosis, was measured in the current study. Bcl-2 family pro-apoptotic (Bax and Bad) and anti-apoptotic (Bcl-2) molecules were also measured.

2. Methods

2.1. Animal and housing

Adult male BALB/c mice, 7 weeks old (about 20 g body weight), were obtained from Harlan Laboratories (Indianapolis, IN). The animals were acclimated in the University of Georgia Animal Resources facility for one week at 23°C and 50% relative humidity, with a 12 h light/dark cycle. Pelleted feed (fumonisin-free) and fresh water

was provided *ad libitum*. Food and water consumption was recorded daily. The protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee.

2.2. Treatment and sampling

Mice (4/group) were treated orally (po) with either physiological buffered saline (PBS) or 25 mg/kg of FB_1 (Sigma Chemical Co., St. Louis, MO; 96% purity, dissolved in PBS), as a single dose and sacrificed at 4 and 8 h after treatment. Dose of FB_1 was selected based on previous dose response studies, in which FB_1 produced liver injury without producing any overt toxicity (Dugyala et al., 1998) and time points selected based on the previous temporal study in which maximum alterations in TNFα and IFNγ expression were observed at 4 and 8 h after po FB_1 treatment (Bhandari et al., 2000). The animals were euthanized with halothane and pieces of liver and kidney were sampled and quickly frozen on dry ice and stored at -85°C until further analysis.

2.3. RNA isolation and RNase protection assay (RPA)

RNA was isolated from frozen tissues using a protocol described earlier (Sharma et al., 2000). Frozen tissues (each 70-80 mg) were grounded in liquid nitrogen in a mortar and then 1 ml TRI® reagent (Molecular Research Center, Cincinnati, OH) added to the mortar and tissues further homogenized. RPA was performed on samples using RiboQuant™ RPA starter kit (Pharmingen, San Diego, CA). Three Pharmingen template sets, one for cytokines (mCK-2b), second for TNFα and Fas signaling molecules (mAPO-3), and a third mouse template set (containing TNFα/Myc/Max/Mad/Bcl-2/Bax/Bad/Caspases) were used. The templates were used for T7 RNA polymerase-

directed synthesis of a high-specific-activity, [α^{32}P]-labeled, anti-sense RNA probe. The probe was labeled using 3000 Ci/mmol and 10 mCi/ml [α^{32}P]UTP (ICN Biomedicals, Costa Mesa, CA). A mouse control RNA (Pharmingen) was used as a positive control for RNA integrity, while yeast tRNA used for negative background. The probe sets were hybridized in excess to the 50 μg of sample RNA in solution by prewarming to 90°C, and then allowing the temperature to ramp down to 56°C and incubating for 12-16 h. The free probe and other single-stranded RNA molecules were digested with RNase A and RNase T1 at 30°C for 45 min, followed by proteinase K digestion at 37°C for 15 min. The RNA duplexes were isolated by extraction/precipitation according to manufacturer's protocol, dissolved in 80% formamide and dyes and electrophoresed in a standard 6% acrylamide/8*M* urea sequencing gel. Dried gels were placed on XAR film (Kodak, Rochester, NY) with intensifying screens and were exposed at -70°C for 48 h. The non-hybridized probe was run to indicate the integrity and labeling efficiency and as a size marker. Using undigested probes as markers, a standard curve of migration distance versus log nucleotide length was plotted on semi-log graph paper, and used to establish the identity of RNase-protected bands. The probe containing polylinker sequences, migrates less than the protected species in the gel. The relative gene expression was normalized against ribosomal protein L32 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) house keeping genes and quantified via densitometric imaging using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT).

2.4. Statistical analysis

Data from these studies were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan's Multiple Range test. The SAS computer program

(SAS Institute, Cary, NC) was employed for all calculations. The level of $P < 0.05$ was considered statistically significant for all comparisons.

3. Results

Single dose treatment of mice with FB_1 by po route produced no obvious gross clinical or behavioral effects. Fig. 7.1 and Fig. 7.2 present representative gels of liver and kidney, respectively, obtained from three RPA templates. The data reported in following figures were normalized to ribosomal protein L32 (Genbank accession # K02060). Identical results were obtained with GAPDH as the normalizing gene (Genbank accession # M32599). Genes from the three template sets have been grouped into different figures based on their relevance.

Gene alterations in the cytokine network were observed in both liver and kidney after acute FB_1 exposure of mice. FB_1 treatment caused increased expression of TNFα and interleukin (IL) -1β in liver at 8 h whereas IL-1α and IL-1 receptor antagonist (IL-1Ra) were induced at both 4 and 8 h after FB_1 treatment (Fig. 7.3). In liver maximum increase was noted for IL-1Ra (15-fold) followed by IL-1α (7-fold), TNFα (5-fold), and IL-1β (3-fold) 8 h after FB_1 treatment. Observed increases in the expression of cytokine genes, which were significantly altered after FB_1 treatment in either organ or time-point, are shown in Table 7.1.

Treatment of mice with FB_1 increased TNFα and IL-1β expression in kidney after 8 h. IL-1α and IL-1Ra expression in kidney was unaltered after FB_1 treatment but their mean values increased more than 2-fold at 4 and 8 h (Table 7.1). FB_1 treatment caused no alteration in expression of IL-18 and macrophage migration inhibitory factor (MIF) in

liver and kidney. In general liver showed a greater induction in cytokines, since IL-1α and IL-1Ra induction was only observed in liver with FB_1. IL-12 p35, IL-12 p40, IL-6, IL-10 and IFNγ expression were not detected in either liver or kidney samples using this array (Fig. 7.1 and Fig. 7.2).

FB_1 treatment caused increased expression of TNFα signaling molecules in liver and kidney. Expression of TNFR 55 and receptor interacting protein (RIP) increased in liver at 4 and 8 h after FB_1 treatment (Fig. 7.4). Table 2 displays calculated increases in expression of apoptosis and signaling genes, which were significantly altered after FB_1 treatment either in male or female. Treatment of mice with FB_1 caused increased expression of TNFR 55 and RIP in kidney at 8 h. The caspase 8 expression was induced in liver at 4 h after FB_1 treatment, with no alterations observed in kidney at either time point. The expression of TNFR-associated death domain (TRADD) was unaltered in both liver and kidney after FB_1 exposure (data not shown).

Fas signaling molecules, namely Fas-associated death domain (FADD) and Fas-associated protein factor (FAF) were unaltered after FB_1 treatment, although FB_1 treatment caused increased Fas expression at 4 and 8 h in liver and at 8 h in kidney (Fig. 7.4). Fas-associated phosphatase (FAP) expression could not be detected in liver, whereas in kidney its expression was unaltered by FB_1. Fas ligand (FasL) expression was also under the detection limit, with no bands observed in control or treated animals (Fig. 7.1 and Fig. 7.2). No alterations were observed in expression of TNF-related apoptosis-inducing ligand (TRAIL) after FB_1 treatment (data not shown).

Gene expression of c-Myc, B-Myc and Max, involved in the Myc/Max/Mad network was increased only in kidney after FB_1 treatment, with no alterations in liver

(Fig. 7.5). FB_1 treatment caused a 5.5-fold increase in liver c-Myc at 4 h, but it was not statistically significant due to variation within animals. B-Myc and Max expression in kidney increased at 4 and 8 h, whereas c-Myc expression increased at only 8 h after FB_1 treatment. In kidney the maximum increase was noted in B-Myc (10-fold), followed by c-Myc (5-fold), and Max (3-fold) 8 h after FB_1 treatment.

FB_1 treatment caused no alterations in the expression of pro-apoptotic Bcl-2 family genes, Bax and Bad and anti-apoptotic gene Bcl-2 (data not shown). Bcl-2 gene expression could not be detected in liver. No alterations were observed in the expression of caspase 3 and caspase 6 after FB_1 treatment (data not shown).

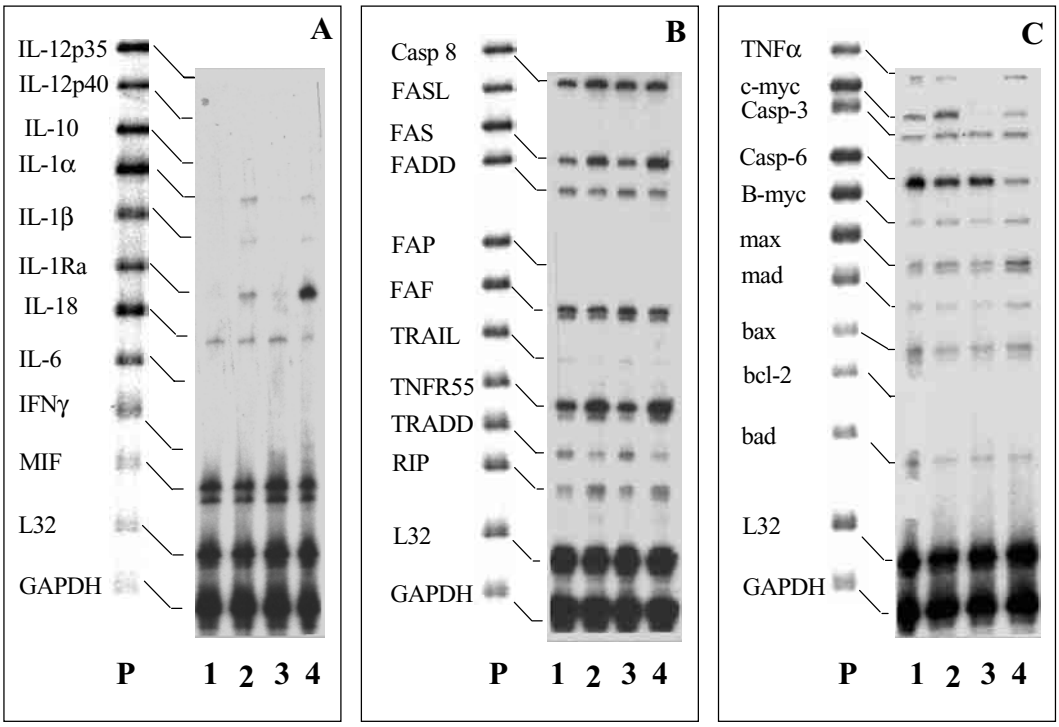

Figure 7.1. Representative gels of RNase protection assay of liver. Three template sets were used (A) for cytokines (mCK-2b), (B) for TNFα and Fas signaling molecules (mAPO-3), and (C) for apoptosis signaling molecules. A non-hybridized probe set (P) was run as a size marker. The genes are listed on the left side of each probe set. Treatment groups are indicated as lane (1) 4 h, saline-treated, (2) 4 h, FB_1-treated, (3) 8 h, saline-treated and (4) 8 h, FB_1-treated.

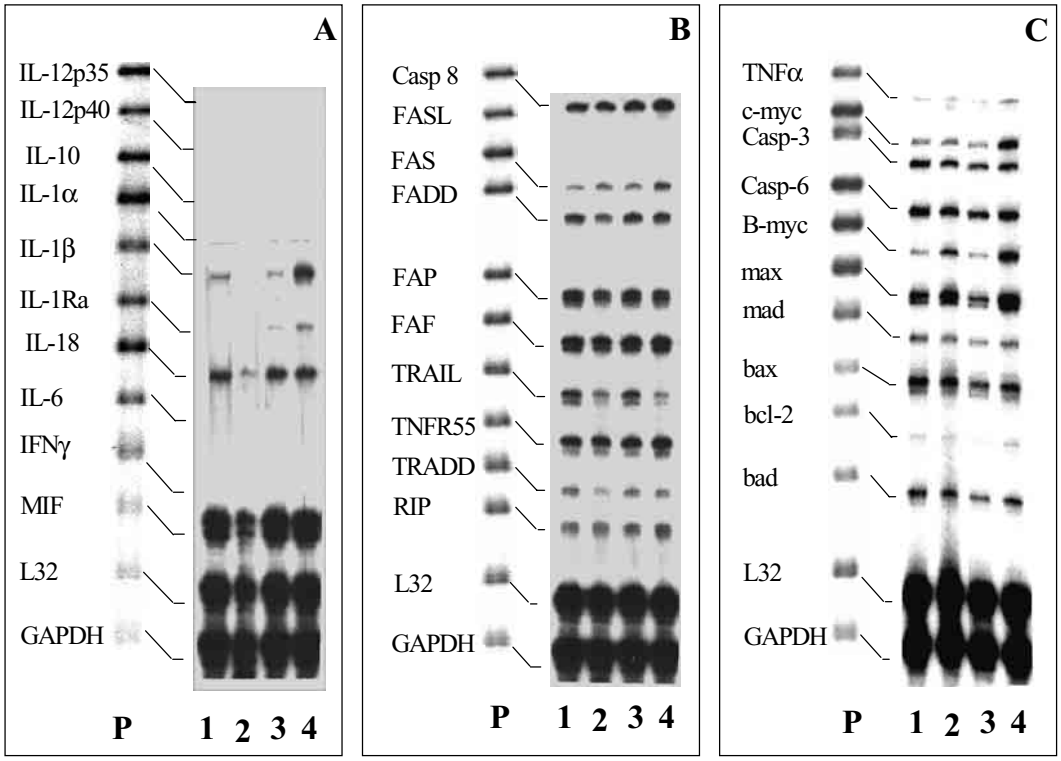

Figure 7.2. Representative gels of RNase protection assay of kidney. The template sets used were same as in Figure 1. (P) A non-hybridized probe set was run as a size marker. The genes are listed on the left side of each probe set. Treatment groups are indicated as lane (1) 4 h, saline-treated, (2) 4 h, FB_1-treated, (3) 8 h, saline-treated and (4) 8 h, FB_1-treated.

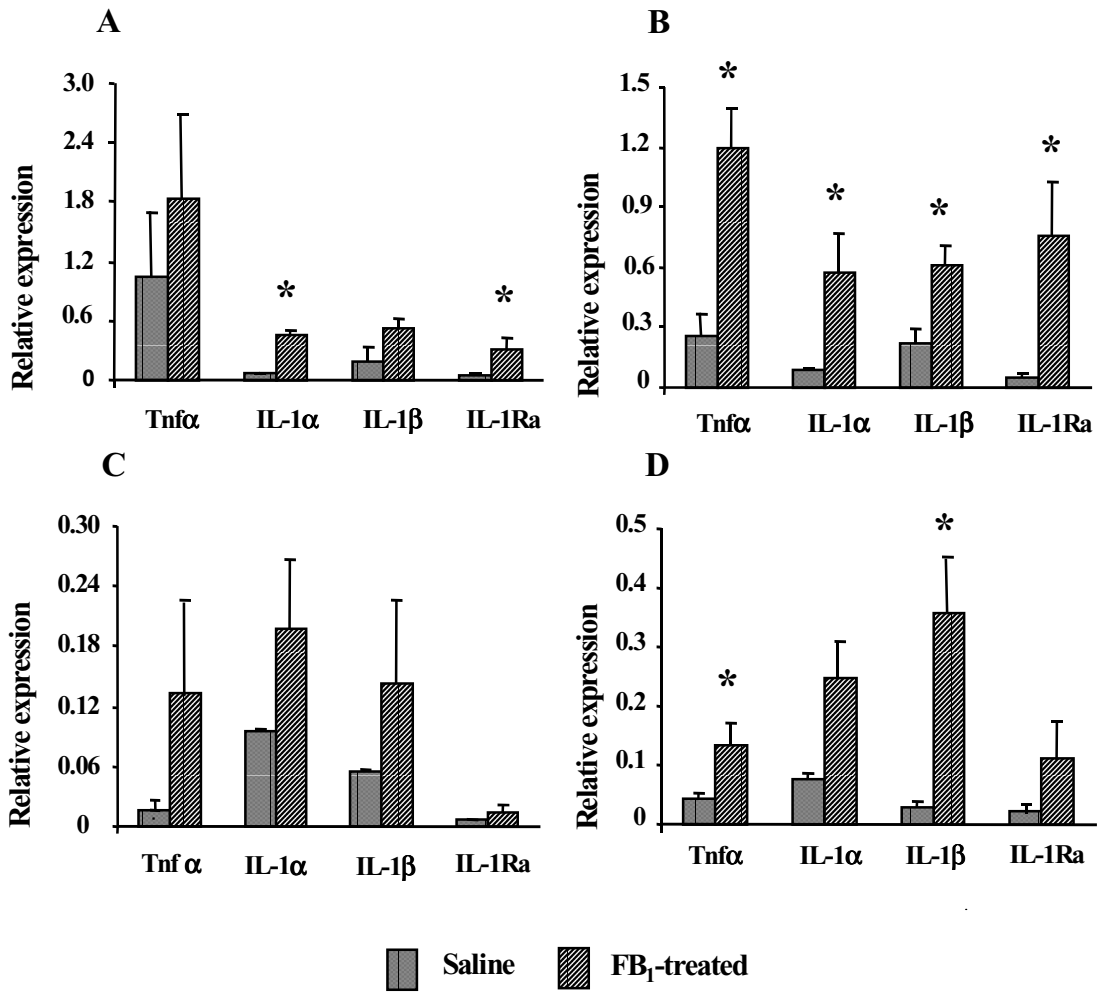

Figure 7.3. Alterations in TNFα, IL-1α, IL-1β and IL-1Ra expression in liver at 4 h (A), and 8 h (B), and in kidney at 4 h (C), and 8 h (D) after acute FB_1 treatment. The relative mRNA expression was normalized against L32. Results are expressed as mean ± standard error. (n=4). * Indicates significantly different from the control group at $P < 0.05$. Because of low abundance a multiplication factor of 10 has been applied to the relative expression values of TNFα, IL-1α and IL-1β in liver and TNFα in kidney, and a factor of 100 has been applied to relative expression values of IL-1α in kidney.

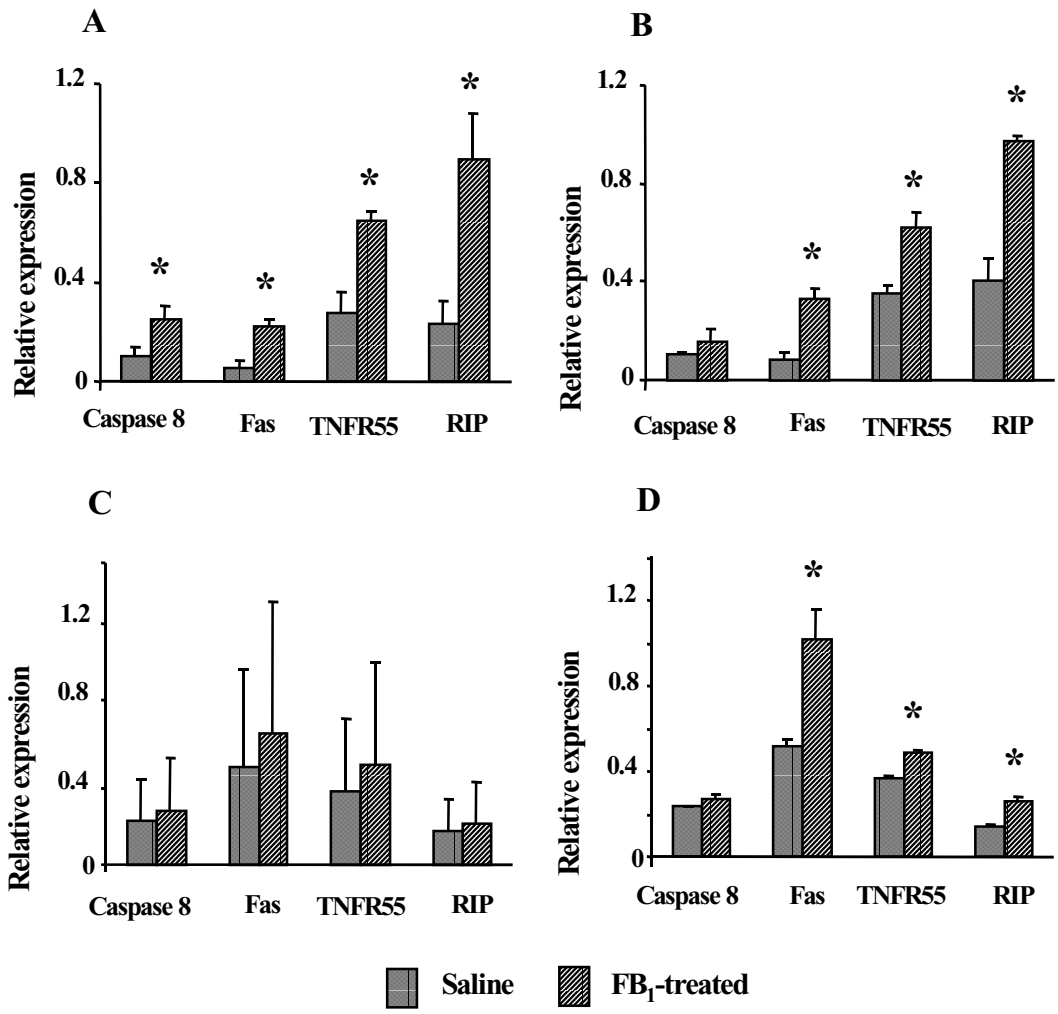

Figure 7.4. Alterations in TNFR 55, RIP, caspase 8 and Fas expression in liver at 4 h (A), and 8 h (B), and in kidney at 4 h (C), and 8 h (D) after acute FB_1 treatment. The relative mRNA expression was normalized against L32. Results are expressed as mean ± standard error. (n=4). * Indicates significantly different from the control group at $P < 0.05$. Because of low abundance a multiplication factor of 10 has been applied to the relative expression values of RIP in liver and Fas in kidney.

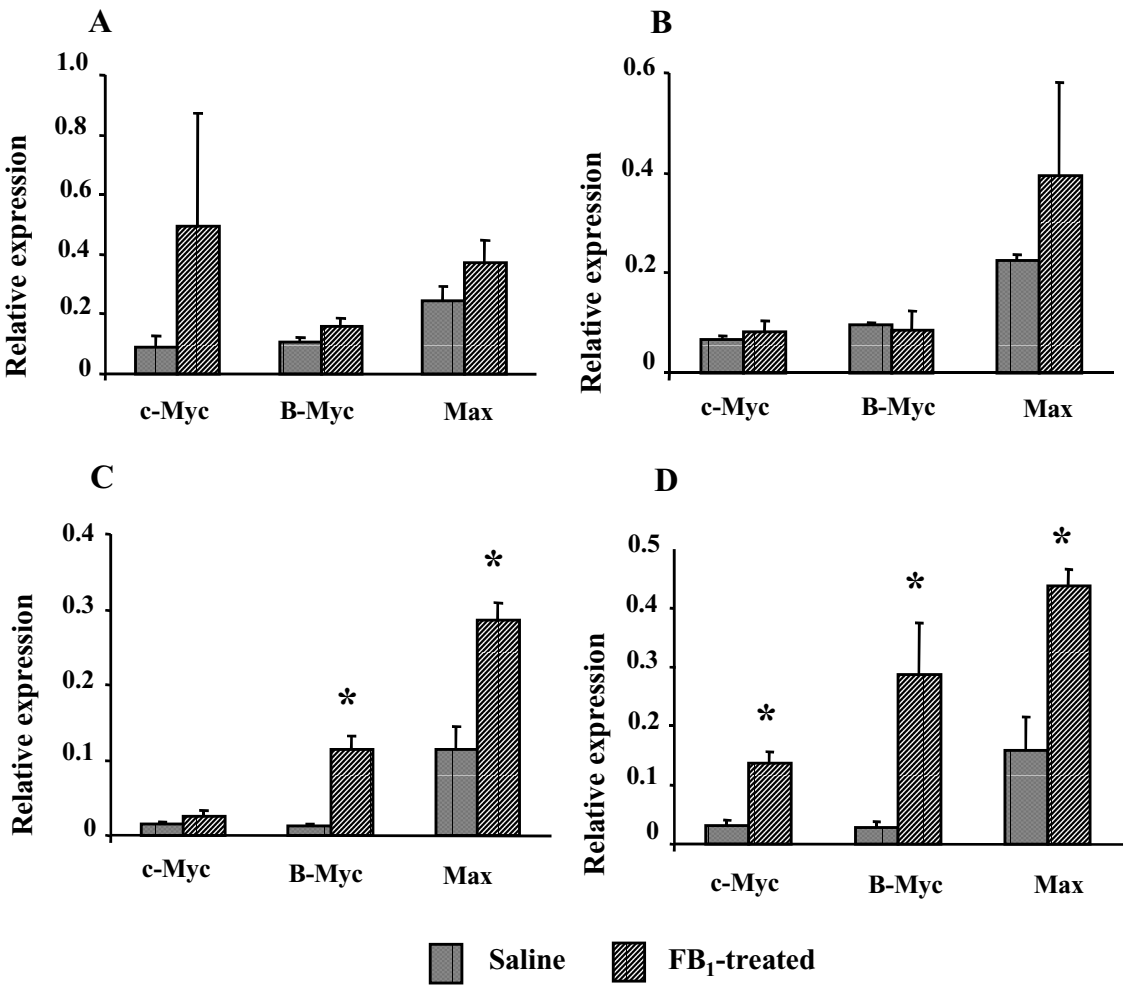

Figure 7.5. Alterations in c-Myc, B-Myc and Max expression in liver at 4 h (A), and 8 h (B), and in kidney at 4 h (C), and 8 h (D) after acute FB_1 treatment. The relative mRNA expression was normalized against L32. Results are expressed as mean ± standard error. (n=4). * Indicates significantly different from the control group at $P < 0.05$. Because of low abundance a multiplication factor of 10 has been applied to the relative expression values of c-Myc and Max in liver and c-Myc in kidney, and a factor of 100 has been applied to relative expression values of B-Myc in liver.

Table 7.1

Cytokines altered after FB_1 treatment in liver and kidney of mice.

Gene	Accession number	Fold-increase over respective control				Significance
		Liver		Kidney		
		4 h	8 h	4 h	8 h	
TNFα	M11731	1.8	4.7*	7.9	3.3*	Pro-inflammatory (hepatotoxic)
IL-1α	X01450	7.0*	6.5*	2.1	3.3	Pro-inflammatory
IL-1β	M15131	2.6	2.7*	2.6	11.9*	Pro-inflammatory (hepatotoxic)
IL-1Ra	M57525	5.8*	14.8*	2.1	5.6	Anti-inflammatory (cancer promoter)

* Indicates FB_1 treatment caused significant increase in gene expression compared to control group at $P < 0.05$.

Table 7.2

Apoptosis signaling genes altered after FB_1 treatment in liver and kidney of mice.

Gene	Accession number	Fold-increase over respective control				Significance
		Liver		Kidney		
		4 h	8 h	4 h	8 h	
TNFR 55	L26349	2.3*	1.8*	1.4	1.3*	TNFα signaling receptor
RIP	U25995	3.9*	2.4*	1.3	1.8*	TNFα signaling protein
Caspase 8	AA071802	2.6*	1.4	1.3	1.2	Initiator caspase (execute apoptosis)
Fas	M83649	3.7*	3.8*	1.4	1.9*	Fas signaling death receptor
B-Myc	W18410	1.5	0.9	9.6*	10.1*	Inhibit growth (hormonally regulated)
c-Myc	X00195	5.5	1.2	1.8	4.5*	Activate transcription, proliferation and apoptosis (cancer promoter)
Max	M63903	1.5	1.8	2.5*	2.7*	Neutral, heterodimerizes with c-Myc or Mad

* Indicates FB_1 treatment caused significant increase in gene expression compared to control group at $P < 0.05$.

4. Discussion

This study involved alterations in the expression of selected genes after acute FB_1 treatment. Two distinct advantages of the multi-probe RPA approach are its sensitivity and its capacity to simultaneously quantify several mRNA species in a single sample of total RNA. This assay is highly specific and quantitative due to the sensitivity of ribonuclease for mismatched base pairs and the use of solution phase hybridization driven toward completion by the excess probe.

Single acute treatment of BALB/c mice with FB_1 by po route caused induction of proinflammatory cytokine network in liver. TNFα, IL-1α and IL-1β expression was increased, suggesting their possible involvement in the FB_1-induced toxicity. Observed induction in IL-1Ra expression could be involved in cancer promoting effects of FB_1. Over-production of TNFα and IL-1 has been associated with number of clinical conditions, such as alcoholic hepatitis (Bird et al., 1990) and fulminant liver failure (Muto et al., 1988). Neutralizing antibodies to TNFα or IL-1α partially prevented liver damage in mice initiated by hepatotoxic doses of acetaminophen (Blazka et al., 1995). In addition TNFα and IL-1 cause a variety of pathophysiological conditions in liver, including increased expression of adhesion molecules on endothelial cells (Springer et al., 1990), migration and activation of neutrophils (Ferrante et al., 1988) and nitric oxide production in hepatocytes (Kitade et al., 1996).

FB_1 treatment in mice caused an increased expression of IL-1Ra in liver. Proinflammatory effect of IL-1 can be neutralized by its natural antagonist IL-1Ra, which also binds with IL-1 receptor without any induction of intracellular signaling. IL-1Ra expression was increased in liver of mouse with hepatocellular adenoma, where its

expression correlated with an increase in the proliferating cell nuclear antigen index (Yamada et al., 1999). Pretreatment of IL-1Ra in mice decreased TNFα production in liver (Shito et al., 1997). TNFα and IL-1 induction after FB_1 treatment may therefore be involved in the observed FB_1 liver toxicity, whereas IL-1Ra induction, having a negative feedback control on TNFα production and IL-1 activation, can lead to a micro-environment for promoting tumors.

We recently showed that increased TNFα expression after FB_1 treatment was localized mainly in Kupffer cells (unpublished data). Kupffer cells are essential immunological component of host defense which when further activated by TNFα can secrete various proinflammatory cytokines like IL-1 and IL-6 (Abbas et al., 1997). It has been established that liver has its own innate immune network, which can cause localized induction of various cytokines (Seki et al., 2000). TNFα and IL-12 produced by macrophages can also stimulate the natural killer (NK) cells and $NK1^+$ T cells to secrete IFNγ, which through a positive amplification loop can further increase TNFα and IFNγ (Kaplan et al., 1999). In this study expression of IL-6, IL-10, IL-12 and IFNγ cytokines by RPA could not be detected, although in our previous study we showed increased IFNγ expression by RT-PCR at 4 h after a similar FB_1 treatment (Bhandari et al., 2000). The increased TNFα expression noted at 8 h by RPA correlated with the peak of TNFα observed at 8 h by RT-PCR in the previous study. The TNFα induction was also related with the increase in alanine aminotransferase and aspartate aminotransferase level also noted at 8 h after po FB_1 treatment (Bhandari et al., 2000). Male BALB/c mice after a five day repeated sc dose of FB_1 also showed increased TNFα, IL-1β, IL-6, IL-12 p40 and IFNγ expression in liver, measured by RT-PCR (Bhandari et al., 2001).

FB_1 treatment caused increased expression of TNFα signaling molecules, namely TNFR 55, RIP and caspase 8. These death receptor-ligand signaling pathways are involved in different liver diseases. TNFα/TNFR 55 signaling is extremely complex and can lead to both cell death and cell survival signals. Apoptosis induced by TNFR 55 requires receptor oligomerization, leading to recruitment of the adapter protein, TRADD to the death domain of TNFR 55. TRADD recruits with its self another adapter protein FADD of the Fas signaling pathway, which further activates the caspase 8 (Ledgerwood et al., 1999). An alternative apoptotic pathway by TNFR 55 also involves binding of TRADD with RIP (Yuan, 1997). Another member of the death receptor family, TRAIL that could play a role in the liver toxicity, (Faubion and Gores, 1999) showed no alteration after acute FB_1 treatment.

TNFR 55 is abundantly expressed in hepatocytes and Kupffer cells. Its expression in liver was shown to be greatly induced in hepatitis B, hepatitis C, autoimmune hepatitis and especially alcohol induced hepatitis (Faubion and Gores, 1999). Similarly Fas/FasL pathway also plays an important role in induction of apoptosis in liver. Anti-Fas treatment selectively caused liver toxicity affecting the hepatocytes with less effects on other organs (Pinkoski et al., 2000). Hepatocytes also constitutively express Fas and can up regulate its expression in a variety of liver diseases like viral hepatitis or alcohol induced liver disease (Kanzler and Galle, 2000). In this study Fas pathway was not altered in FB_1 toxicity, since expression of Fas signaling molecules (FADD, FAF and FAP) remained unchanged after FB_1 treatment, although Fas expression was increased in liver and kidney. Fas expression has been shown to be induced and up regulated by inflammatory cytokines such as IL-1 (Giordano et al., 1997),

which could explain for the observed induction in this study. At the same time Fas expression is also reduced in particularly aggressive forms of hepatocellular carcinoma (Ito et al., 1998). Although mere expression of Fas does not imply that the cell is susceptible to apoptosis by Fas ligation, Fas-R signaling in many tissues, and likely in the liver, can be inhibited by a variety of processes depending on the cell type, including expression of I-Flice (an endogenous dominant-negative form of caspase 8), Bcl-2 (a cytoprotective molecule), and X-linked inhibitor of apoptosis molecules that directly inhibit downstream effector caspases (Faubion and Gores, 1999).

Increased c-Myc and cyclin D1 expression in liver after FB_1 treatment have been reported to be involved in the cancer promoting effects of FB_1 (Lemmer et al., 1999; Ramljak et al., 2000). c-Myc was shown to both up-regulate as well as down-regulate cyclin D1 expression (Dang et al., 1999). Deregulated c-Myc expression has also been linked to increased cyclin E and cyclin A expression (Dang et al., 1999). In this study FB_1 treatment caused an induction of Myc/Max/Mad network in kidney. The Myc/Max/Mad network comprises a group of transcription factors whose distinct interactions result in gene-specific transcriptional activation or repression. Myc requires Max to activate transcription of genes, whereas Mad-Max heterodimers act as transcriptional repressors at the same binding sites (Baudino and Cleveland, 2001). Since FB_1 treatment caused only c-Myc induction with no alterations in Mad expression, this suggests a c-Myc dominated response. Deregulation of c-Myc expression has been implicated in the development of a variety of experimentally induced and naturally occurring tumors, including hepatocellular carcinoma (Kanzler and Galle, 2000). Transgenic mice with specific overexpression of c-Myc in liver exhibit increased levels

of apoptosis, and a higher incidence of hepatocellular carcinoma (Henriksson and Luscher, 1996). The ability of overexpressed c-Myc to facilitate apoptosis could be involved in decreased kidney weights observed after repeated FB_1 treatment (Sharma et al., 1997).

Members of the Bcl-2 gene family, which include Bcl-2, Bcl-X_L, Bcl-X_S, Bax, Bid and Bad are the most prominent genes involved during the modulation phase of apoptosis. Dimerization of the Bcl-2 family members has been proposed as a model of action of these proteins, where the presence of equal or greater amounts of one of the cytoprotective proteins (Bcl-2, Bcl-K_L) pushes the balance towards cell survival, but an excess of cytotoxic proteins (Bad, Bax, Bcl-X_S) leads to cell death (Korsmeyer, 1999). No changes were observed in the expression of Bax or Bad in liver and kidney after acute FB_1 treatment. Bcl-2 expression could only be detected in kidney with no changes observed with FB_1. A western blot analysis of mouse liver showed expression of Bcl-X_L and Bcl-X_S proteins with no detectable levels of Bcl-2 (Ray and Jena, 2000), thus suggesting that expression of Bcl-2 might be of lesser significance in the mouse liver.

Apoptosis induced by TNFα and Fas involves activation of procaspase 8, which is the apical component of the proteolytic cascade. Most caspases are constitutively expressed as inactive proenzymes in the cytosol, and are sequentially activated by proteolysis during apoptosis. Caspase 3 and caspase 6 are considered to be the downstream effector caspases, activated by the initiator caspases (Caspases 8, 9 and 10, Wilson, 1998). Expression of caspase 8 was increased after FB_1 treatment only in liver with no changes in kidney. Caspase 3 and caspase 6 expression remained unaltered in both liver and kidney. Increased expression of caspase 8 further supports the

involvement of receptor-induced apoptosis in FB_1 liver toxicity. Mouse liver thus may be susceptible to FB_1 toxicity via activation of TNFα downstream signaling pathways.

In the current study, FB_1 treatment caused an induction of cytokine network in liver. Acute po FB_1 treatment after 8 h, caused an increased expression of TNFα and its signaling molecules, TNFR 55, RIP, and caspase 8. Acute FB_1 treatment caused increased expression of c-Myc/Max in kidney and to a less extent in liver. Increased expression of TNFα, IL-1α and Il-1β may be involved in FB_1-induced hepatotoxicity, whereas IL-1Ra expression may contribute to the cancer promoting effects of FB_1.

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CHAPTER 8

SUMMARY AND CONCLUSION

Fumonisin B_1 (FB_1) is a naturally occurring mycotoxin produced primarily by *Fusarium verticillioides* and related fungi, which are common contaminants of corn throughout the world. FB_1 is a carcinogen and causative agent of lethal animal diseases. Liver is the primary target organ in mice. Our previous studies indicated the involvement of tumor necrosis factor-α (TNFα) in FB_1-induced toxic responses.

To further investigate the time-course of TNFα production and signaling, mice (4/group) were treated subcutaneously (sc) or orally (po) with either vehicle or 25 mg/kg of FB_1 as a single dose and sacrificed at 0, 2, 4, 8, 12 and 24 h after treatment. The TNFα expression was increased in liver and kidney after both routes of FB_1 exposure without any alterations in spleen. The po route FB_1 treatment caused greater hepatotoxicity as compared to sc route, as depicted by increased alanine aminotransferase and aspartate aminotransferase level in plasma, observed only after po FB_1 treatment. The increase in enzymes at 8 h after po treatment correlated with the highest TNFα expression also noted at 8 h after po treatment, thus further confirming the involvement of TNFα in FB_1 toxicity. The Interferon γ (IFNγ) expression was increased in liver at 4 h after po FB_1 treatment, suggesting a possible combined role of TNFα and IFNγ in their induction and hepatotoxicity. These changes were closely correlated with changes in free sphingoid bases indicative of fumonisin inhibition of ceramide synthase.

Male B6,129 mice (5/group) were injected subcutaneously with vehicle or 2.25 mg/kg/day of FB_1 for 5 days and sampled one day after the last treatment. FB_1 treatment caused an increased expression of TNFα, IFNγ and interleukin (IL)-12 p40 in liver without any changes in kidney or spleen, suggesting the localized site of their production. IL-1β cytokine expression was increased in liver and kidney after FB_1 exposure. Cells

involved in TNFα production after FB_1 treatment in liver were identified as Kupffer cells. FB_1 increased alanine aminotransferase in plasma and increased apoptotic cells in liver. Selective increase in proinflammatory T helper (Th)1-cytokines (IL-12 and IFNγ) and TNFα with no alteration in Th2-cytokines (IL-4, IL-6 and IL-10) suggest the involvement of IL-12, produced by Kupffer cells, in induction of IFNγ production by natural killer (NK) cells and/or $NK1^+$ T cells, which can undergo a positive amplification loop with TNFα produced by macrophages or other hepatic cells to elicit the toxic reaction.

Species and sex variations to chronic FB_1 toxicity have been reported. Free sphingoid bases and cytokine levels are two biochemical alterations of FB_1 *in vivo* and may explain any sex differences in FB_1 toxicity. Male and female BALB/c mice (5/group) were injected subcutaneously with either vehicle or 2.25 mg/kg/day of FB_1 for 5 days. One day after the last injection females showed a greater increase in circulating alanine aminotransferase and greater number of apoptotic cells in liver after FB_1 treatment than males, suggesting greater hepatotoxicity. Peripheral leukocytic counts, including neutrophil counts, were increased in females only after FB_1 treatment. The increased toxicity in females correlated with a greater increase of sphinganine and sphingosine levels in liver after FB_1 treatment compared to males. No sex differences in kidney sphinganine and sphingosine levels were observed after FB_1 treatment. Previously we have shown the induction of tumor necrosis factor α (TNFα) in FB_1-induced hepatotoxicity. While in males FB_1 treatment caused increased expression of TNFα, interleukin (IL)-12 p40, interferon γ (IFNγ), IL-1β, IL-6 and IL-10, females showed an increased expression of IL-6 only, and a decreased expression of IFNγ,

indicating gender differences in cytokine pathways activated by FB_1. There were higher basal expression levels of TNFα, IL-12 p40, IL-1β and IFNγ in liver of females as compared to males. Gender differences in alterations in sphingoid bases and cytokine expression after FB_1 treatment, suggests their possible mechanistic involvement in liver toxicity.

In order to further investigate the genes involved in the cytokine network and apoptosis signaling, male and female BALB/c mice (5/group) were injected subcutaneously with either saline or 2.25 mg/kg/day of FB_1 for 5 days. FB_1 treatment caused increased expression of TNFα, IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-6, IL-10, IL-12 p40, IL-18 and IFNγ in male liver, with a similar increase in females except that IL-1β and IL-18 were unaltered. Control females showed higher basal levels of IL-1α, IL-1Ra, IL-10, IL-12 p40 and IFNγ as compared to males. Expression of TNF receptor 55 and TNF receptor associated death domain (TRADD) was increased, with no changes in Fas signaling molecules, Fas, Fas ligand, Fas associated death domain (FADD) and Fas-associated protein factor (FAF). Expression of c-Myc, B-Myc, Max and Mad oncogenic transcription factors and apoptotic genes, namely Bcl-2, Bax and Bad, was also increased after FB_1 treatment. FB_1 caused an activation of cytokine network in liver along with TNFα signaling pathways. Induction of IL-1Ra and oncogenes could contribute to the cancer promoting properties of FB_1.

To further investigate the time related induction of other cytokines and genes involved induction was maximum at 4 and 8 h, respectively after an acute po FB_1 treatment. in apoptosis signaling, male BALB/c mice were administered po with either saline or 25 mg/kg of FB_1 and sampled 4 or 8 h after treatment. FB_1 treatment caused

increased expression of TNFα and interleukin (IL)-1β in both liver and kidney, whereas IL-1α and IL-1 receptor antagonist (IL-1Ra) expression was induced only in liver. Expression of TNFα signaling molecules, TNF receptor 55 and receptor interacting protein, was increased in liver and kidney after FB_1 treatment. Caspase 8 expression was increased only in liver with no changes observed in kidney with FB_1. FB_1 treatment induced expression of Fas in liver and kidney with no alterations in Fas signaling molecules, Fas ligand, Fas-associated death domain and Fas-associated protein factor. Treatment of mice with FB_1 increased the expression of B-Myc, c-Myc and Max oncogenic transcription factors in kidney. FB_1 toxicity caused induction of cytokine network in liver with involvement of TNFα signaling pathway. TNFα-induced caspase 8 expression could be involved in the FB_1-induced liver apoptosis, whereas increased expression of IL-1Ra in liver may be responsible for cancer promoting effects of FB_1.

Overall, the data from these studies indicates that FB_1 toxicity can lead to a localized induction of TNFα, mainly produced by the Kupffer cells in liver. Expression of both pro-and anti-inflammatory cytokines was increased in liver after FB_1 treatment, where TNFα, IFNγ and IL-1β may play a role in liver toxicity, whereas IL-1Ra may contribute to development of hepatocellular carcinoma. Gender related differences in FB_1 toxicity correlated with similar differences in sphingoid bases alterations and cytokine expression in liver, suggesting their involvement in FB_1 toxicity. FB_1 treatment induced TNFα apoptotic pathway with no alterations in Fas signaling molecules. Increased c-Myc expression was suggested in cancer promoting effects of FB_1.

APPENDICES

Histochemistry protocol

1. Heat slides at 70^{o}C for 10 min
2. Microclear/Citrasolv for 5 min
3. Microclear/Citrasolv for 5 min
4. Microclear/Citrasolv for 5 min
5. Air dry until tissue is chalky white and encircle tissue with PAP (hydrophobic) pen.
6. Endogenous Peroxidase quenching: Immerse slides in 3% Hydrogen peroxide for 10 min
7. Rinse under running water for 10 min.
8. Antigen retrieval: by one o0f the following methods
    a. Trypsin treatment: Add .01% Trypsin to 0.1M Tris ph 7.5, with 1% calcium chloride for 30 min in humid chamber for 30 min. Inactivate trypsin by washing in 2X5 min in 0.2M Tris pH 7.5 with 0.1 M glycine.
    b. Autoclave in above solutions-15 min 121°C. Cool, and then wash in PBST 2X5 min.
    c. Microwave in above solutions 2X5 min. Cool, and then wash in PBST 2X5 min.
    d. 4.2 g of Citric acid in 20 ml water, 814.7 g Sodium Citrate in 500ml water. Add 18 ml of citric acid sol and 82 ml of Sodium citrate solution. Fill to 1 liter and ph to 6.0. Pre-warm citrate buffer in steamer and steam slides for 25 min.
9. Block with 2% normal animal serum (in PBST) of species in which secondary biotinylated antibody was made and incubate for 30 min at 37C in humid chamber
10. Blot off excess blocking buffer, add Primary antibody at appropriate dilution and incubate at 4°C overnight in humid chamber or at 37°C for 2 h in humid chamber.
11. Rinse in PBST for 5 min
12. Rinse in PBST for 5 min
13. Add Secondary antibody diluted 1:250 in PBST and incubate for 1 hour in humid chamber at 37°C.
14. Rinse in PBST for 5 min

15. Rinse in PBST for 5 min
16. Add Avidin -Biotin peroxidase solution. The solution is made 30 min in advance and made to sit at room temperature. 5 ml of PBST, add 2 drops of Vector ™ Sol A and mix. Add Sol B and mix and incubate for an hour in humid chamber at 37^{o}C
17. Rinse in PBST for 5 min
18. Rinse in PBST for 5 min
19. Add DAB (5mls water and tablet A and B mix well). Incubate for 5-15 min.
20. Rinse in DD water.
21. Counter-stain with hematoxylin, (dip and take out) and wash in running tap water.
22. Air-dry the slide.
23. Coverslip with Permount.

Terminal UTP nucleotide transferase end-labeling (TUNEL) protocol

1. Microclear/Citrasolv for 5 min.
2. Microclear/Citrasolv for 5 min.
3. Microclear/Citrasolv for 5 min.
4. 100% ethanol for 5 min.
5. 100% ethanol for 5 min.
6. 95% ethanol for 3 min.
7. 70% ethanol for 3 min.
8. PBS for 5 min.
9. Proteinase K (20 μg/ml) for 15 min.
10. DD H_2O for 2 min.
11. DD H_2O for 2 min.
12. 3% H_2O_2 for 5 min.
13. PBS for 5 min.
14. PBS for 5 min.
15. Blot.
16. Equilibration buffer, at least 10sec.
17. Blot.

18. TdT enzyme(working strength) 40 µl/slide, 1-2 hr (at 37°C).
19. Stop/Wash buffer, agitate for 15 sec.
20. Stop/Wash buffer, incubate for 10 min.
21. PBS for 1 min.
22. PBS for 1 min.
23. PBS for 1 min.
24. Blot.
25. Anti-Dig Peroxidase conjugate for 30 min (45 µl/slide).
26. PBS for 2 min.
27. PBS for 2 min.
28. PBS for 2 min.
29. PBS for 2 min.
30. Peroxidase Substrate (working strength) 55 µl/slide, for 15 min.
31. DDH_2O for 1 min.
32. DDH_2O for 1 min.
33. DDH_2O for 1 min.
34. DDH_2O for 5 min.
35. Hematoxylin for 5 sec.
36. DDH_2O, 10 dip.
37. DDH_2O, 10 dip.
38. DDH_2O for 30 sec.
39. 100% N-Butanol, 10 dips.
40. 100% N-Butanol, 10 dips.
41. 100% N-Butanol for 30 sec.
42. Xylene for 2 min.
43. Xylene for 2 min.
44. Xylene for 2 min.
45. Blot.
46. Air-dry the slide.
47. Coverslip with Permount.

RiboQuant™ multi-probe RNase protection assay protocol

Probe synthesis:

1. Add the following in order to a 1.5 ml tube: 1 µl Rnasin, 1 µl GACU pool, 2 µl DTT, 4 µl 5X transcription buffer, 1 µl RPA Template set, 10 µl [α^{-32}P] UTP, and 1 µl T7 RNA polymerase. Mix by flicking and quick spin.
2. Mix and incubate at 37°C for 1 hr.
3. Add 2 µl DNase. Mix by flicking and quick spin. Incubate at 37°C for 30 min.
4. Add 26 µl 20 mM EDTA, 25 µl Tris-saturated phenol, 25 µl chloroform:isoamyl alcohol (50:1) and 2 µl yeast tRNA and vortex into an emulsion.
5. Spin in microfuge 15,000 g for 5 min at room temp.
6. Transfer the upper aqueous phase to a new tube and add 50 µl chloroform:isoamyl alcohol (50:1). Mix by vortexing and spin in microfuge 15,000 g for 2 min at room temp.
7. Transfer the upper aqueous phase to a new tube and add 50 µl 4 M ammonium acetate, 2 µl (20 mg/ml) Glycogen and 250 µl ice-cold 100% ethanol.
8. Invert the tubes to mix and incubate for 30 min at –70°C.
9. Spin in a microfuge for 15 min at 4°C.
10. Carefully remove the supernatant (use western blot gel loading tip), and add 100 µl of ice-cold 90% ethanol to the pellet. Spin in a microfuge for 5 min at 4°C.
11. Carefully remove the supernatant and air-dry the pellet for 5-10 min.
12. Add 50 µl of hybridization buffer and solubilize the pellet by gently vortexing for 20 sec. Quick spin in a microfuge.
13. Quantitate duplicate 1 µl samples in the scintillation counter.
14. Store probe at -20°C until needed.

RNA preparation and hybridization:

15. After quantitating the sample RNA, freeze aliquots of sample RNA (50 µg for tissues) at -70°C.
16. Dry completely for 15-20 min in a vacuum evaporator centrifuge.
17. Add 8 µl hybridization buffer to each sample. Solubilize the RNA by gently vortexing for 3 min and quick spin in the microfuge.

18. Dilute probe to appropriate concentration (approx 4 x 10^5 cpm/ µl).
19. Add 2 µl diluted probe to each RNA sample and mix by pipetting.
20. Add a drop of mineral oil to each tube.
21. Quick spin in the microfuge.
22. Place the samples in a heatblock pre-warmed to 90°C. Immediately turn the temperature to 56°C and incubate overnight (12-16 h).

RNase treatments:

23. Turn the heat block to 37°C for 15 min.
24. Prepare the RNase cocktail.
25. Remove RNA samples from heat block and lower temp of heat block to 30°C.
26. Pipet 100 µl of RNase cocktail underneath the oil into the aqueous layer (use gel loading tips). Spin in microfuge for 10 sec.
27. Incubate for 45 min at 30°C and prepare proteinase K cocktail.
28. Add 18 µl of proteinase K cocktail to new tubes.
29. Extract the RNase digests from underneath the oil (use gel loading tips) and transfer to the tubes containing proteinase K solution.
30. Quick vortex, quick spin in the microfuge, and incubate for 15 min at 37°C.
31. Add 65 µl Tris-saturated phenol and 65 µl chloroform:isoamyl alcohol (50:1).
32. Vortex into an emulsion and spin in the microfuge 15,000 g for 5 min at RT.
33. Carefully extract the upper aqueous phase (set pipettor at 120 µl) and transfer to a new tube.
34. Add 120 µl 4 M ammonium acetate, 2 µl of 20 mg/ml glycogen and 650 µl ice-cold 100% ethanol. Mix by inverting the tubes.
35. Incubate for 30 min at –70°C.
36. Spin in the microfuge 15,000 g for 15 min at 4°C.
37. Carefully remove the supernatant (use gel loading tips) and add 100 µl ice-cold 90% ethanol.
38. Spin in the microfuge for 5 min at 4°C.
39. Carefully remove the supernatant and air-dry the pellet completely.
40. Add 4 µl of 1X loading buffer, vortex for 2 min and quick spin in the microfuge.

41. Prior to loading the samples on the gel, heat the samples for 3 min at 90°C and then place them immediately in an ice bath.

Gel electrophoresis:

42. Make the denaturing polyacrylamide gel (5% acrylamide, 8 M urea, 5mm x 30 cm).
43. Remove the comb and flush the wells thoroughly with 0.5X TBE.
44. Prerun at 20-25 mA for 40 min, gel temp should be 50°C.
45. Flush the wells again and load the samples, and also a dilution of probe set in the loading buffer (5000-8000 cpm/lane).
46. Run the gel around 18 mA. Let the first dye front overflow the gel, till the second dye front has run 2/3 of the gel.
47. Preheat the gel dryer to 80°C.
48. Disassemble the gel mold, adsorb the gel to filter paper, cover the gel with saran wrap.
49. Place it in the gel dryer under vacuum for ~1 h at 80°C.
50. Place the dried gel on film in a cassette with an intensifying screen and develop at -70°C (exposure times will vary from 4 h to 3 days).

RNA isolation and reverse transcriptase-polymerase chain reaction protocol

1. Between 50-100 mg of frozen tissue, was transferred to a mortar and reduced to powder in liquid nitrogen.
2. 1 ml of TRI® reagent was added to the mortar and tissue further homogenized.
3. Transfer to a tube and store at RT for 5 min.
4. Spin at 12,000 g for 10 min at 4°C to separate all ungrounded tissue and transfer the solution phase to a new tube.
5. Add 200 µl chloroform and shake vigorously for 15 sec.
6. Store at RT for 2-15 min.
7. Spin at 12,000 g for 15 min at 4°C.
8. Transfer the upper aqueous phase to a fresh tube and add 500 µl of isopropanol.
9. Mix and store at RT for 5-10 min.

10. Spin at 12,000 g for 15 min at 4°C.
11. Remove supernatant and wash RNA pellet once with 75% ethanol by vortexing and subsequent centrifugation at 7,500 g for 5 min at 4°C.
12. Remove the ethanol wash and air-dry completely the RNA pellet for 3-5 min.
13. Dissolve the RNA pellet in 50-100 µl DEPC water (depending on the pellet size) and quantitate RNA.

cDNA synthesis

14. Take 5 µg RNA, add 1 µl oligo(dt)12-18 (500µg/ml) and sufficient quantity DEPC water to make 12 µl mixture.
15. Heat at 70 °C for 10 min and quick chill on ice.
16. Briefly centrifuge and add 4 µl 5X first strand buffer, 2 µl 0.1 M DTT and 1 µl 10 mM dNTP mixture.
17. Mix gently and incubate at 42 °C for 2 min.
18. Add 1 µl (200U) Superscript II, mix by pipetting gently.
19. Incubate at 42°C for 50 min.
20. Stop the reaction by heating at 70 °C for 15 min.
21. Chill on ice, store at –70 °C, or directly to PCR.

Polymerase chain reaction

22. Prepare the PCR mixture: 5 µl 10X PCR buffer, 1 µl dNTP, 1 µl sense primer, 1 µl antisense primer, Taq polymerase 1 µl and sufficient quantity DD water to make 50 µl.
23. Add 1 µl cDNA.
24. Perform PCR.
25. Add 20 µl PCR product to 2 µl DNA dye buffer.
26. Run 10 µl of PCR product with dye on a 2% agarose gel prepared in 0.5X TBE buffer, at 150 V for ~ 1 h.
27. Take a picture using digital camera and quantitate using UN-SCAN-IT software.