THE FUMONISIN B_1-FED RAT AS A MODEL FOR LIVER INJURY, OVAL ('PROGENITOR') CELL PROLIFERATION, AND CARCINOGENESIS

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DECLARATION

I, Eric Richard Lemmer, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

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DEDICATION

To my family: Richard, Crinky, Natalie and John, who have stood by me through thick and thin.

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List of abbreviations

AAF/PH	acetylaminofluorene/partial hepatectomy
AAL toxin	*Alternaria alternate lycopersici* toxin
AFP	α-fetoprotein
AIN-76A diet	American Institute of Nutrition-76A diet
ALT	alanine transaminase
AST	aspartate transaminase
CDE diet	choline deficiency and ethionine supplemented diet
cDNA	complementary deoxyribonucleic acid
DEN	diethylnitrosamine
ELEM	equine leukoencephalomalacia
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FA_1	fumonisin A_1
FA_2	fumonisin A_2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GGT	γ-glutamyl transpeptidase
GST pi	pi class glutathione *S*-transferase
HCC	hepatocellular carcinoma
HGF	hepatocyte growth factor
LEC rat	Long-Evans Cinnamon rat
MDA	malondialdehyde
Me-DAB	3'-methyl 4-dimethylaminoazobenzene
mRNA	messenger ribonucleic acid
OV-6 MoAb	OV-6 monoclonal antibody
PBS	phosphate buffered saline
PPO	porcine pulmonary oedema
RH model	resistant hepatocyte model

RLE cells	rat liver epithelial cells
Sa/So ratio	sphinganine to sphingosine ratio
SCF	stem cell factor
TBARS	thiobarbituric acid reacting substances
TCA	tricarballylic acid
TGF	transforming growth factor
TK	thymidine kinase
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling

"Models: All are wrong. Some are useful."
George Box

"It is a capital mistake to theorize in advance of the facts."
Sherlock Holmes
The Adventure of the Second Stain

... thought Alice, and she went on, "Would you tell me, please, which way I ought to walk from here?"
"That depends a good deal on where you want to get to," said the Cat.
Alice's Adventures in Wonderland

ABSTRACT: THE FUMONISIN B_1-FED RAT AS A MODEL FOR LIVER INJURY, OVAL ('PROGENITOR') CELL PROLIFERATION, AND CARCINOGENESIS

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Fumonisin B_1 (FB_1) is a carcinogenic mycotoxin produced by the fungus *Fusarium moniliforme* in maize, and is hepatotoxic and hepatocarcinogenic in rats. The goal of this dissertation was to characterise the FB_1-fed rat as a model for liver injury and carcinogenesis, and to examine the role of oval ('progenitor') cells during these processes. Male Fischer 344 rats were fed FB_1 250 mg/kg diet for five weeks, and this basic feeding regimen was modified in individual experiments. Short-term feeding of FB_1 caused a severe 'toxic' hepatitis, apoptosis and regeneration of hepatocytes, fibrosis, proliferation of OV-6 positive oval cells, and formation of GST pi positive hepatic foci and nodules. Oval cells were noted inside some of the hepatic nodules. There were marked increases in the expression of mRNA transcripts for mature TGF-β1 and c-*myc* in livers of FB_1-fed animals. The overexpression of TGF-β1 by hepatocytes may be responsible for the prominent apoptosis and fibrosis seen with liver injury due to FB_1. Increased expression of c-*myc* and TGF-β1 may cooperate during FB_1-induced promotion of liver tumours, possibly by providing an environment that selects for the growth of TGF-β1-resistant transformed liver cells. In rats given FB_1 in the presence of dietary iron overload, FB_1 augmented iron-induced lipid peroxidation in the liver. However, dietary iron loading appeared to protect against the cancer-promoting properties of FB_1, possibly due to a stimulatory effect on hepatocyte regeneration. Long-term feeding of FB_1 caused fibrosis and regenerative nodules, dysplastic hepatic nodules, cholangiofibrotic lesions, intraductal cholangiocarcinomas, and a hepatocellular carcinoma. 2-Acetylaminofluorene enhanced the effects of FB_1 in the liver, presumably by blocking hepatocyte regeneration in response to FB_1 toxicity. Proliferating oval cells were found inside/adjacent to GST pi positive lesions, dysplastic nodules, and cholangiofibrotic lesions, suggesting that oval cells may be involved in FB_1-induced hepato- and cholangiocarcinogenesis in the liver. Furthermore, the OV-6 antigen was expressed by proliferating oval cells and bile ductules, hepatic nodules, cholangiofibrotic lesions, and cystic lesions, indicating that all of these cells may have a common ('stem') cell of origin. In conclusion, the FB_1-fed rat is a promising model for the study of liver injury, oval ('progenitor') cell proliferation, and carcinogenesis.

SUMMARY OF FUMONISIN B_1-FEEDING STUDIES IN RATS

Animals and diet

In all the feeding studies, male Fischer 344 rats (weight approximately 150 to 200 g) were given FB_1 250 mg/kg diet for five weeks, a dose known to cause severe hepatotoxicity and the generation of 'preneoplastic' liver lesions, and this basic feeding regimen was modified in individual experiments. FB_1 was mixed into American Institute of Nutrition (AIN)-76A control diet for all the experiments (see Appendix C).

5-Week FB_1 Feeding Study: Liver Pathology

In order to study the histopathology in the rat liver during short-term treatment with FB_1, rats were fed either FB_1 250 mg/kg or control diet for 5 weeks. FB_1 caused a predominantly zone 3 'toxic' liver injury, with hepatocyte death due to apoptosis. Hepatocyte injury and death were mirrored by hepatic stellate cell proliferation and marked fibrosis, with progressive disturbance of architecture and formation of regenerative nodules. Despite ongoing hepatocyte mitotic activity, oval cell proliferation was noted from week 2, glutathione *S*-transferase (GST) pi positive hepatic foci and nodules developed and, at later time points, oval cells were noted inside some of the 'atypical' nodules.

5-Week FB_1 Feeding Study: Molecular Mechanisms

There is currently no information on the molecular mechanisms underlying the pathological changes in the liver caused by FB_1. Northern blot (mRNA) analysis was performed on timed rat liver specimens during feeding of FB_1 250 mg/kg for 5 weeks, in order to examine the changes in hepatic gene expression. Changes in gene expression were correlated with the liver histopathology, and immunolocalization of mature transforming growth factor (TGF)-β1 protein was performed using LC(1-30) antibody. There was progressive increase in hepatic gene expression for α-fetoprotein (AFP), hepatocyte growth factor (HGF), TGF-α ,and especially TGF-β1 and c-*myc* during week 3 to 5 of feeding. Immunostaining with LC(1-30) antibody demonstrated a progressive increase in expression of mature TGF-β1 protein by zone 1 and 2 hepatocytes over the 5 week feeding period. The overexpression of TGF-β1 by hepatocytes may be causally related to the prominent apoptosis and fibrosis seen with liver injury due to FB_1. Increased expression of c-*myc* and TGF-β1 may cooperate in the promotion of liver tumour development in the FB_1-fed rat, possibly by providing a milieu that selects for the growth of TGF-β1-resistant transformed oval cells.

5-Week FB_1 Feeding Study: Effects of Dietary Iron Overload

In order to determine whether excess hepatic iron modulates the cancer initiating and promoting properties of FB_1, thirty eight male F344 rats were divided into four dietary treatment groups: (i) control diet (AIN, n = 8); (ii) FB_1 250 mg/kg diet (FB_1, n = 10); (iii) 1 - 2% carbonyl iron (CI, n = 10); or (iv) FB_1 plus iron loading (FB_1/CI, n =10) for 5 weeks. Hepatic iron concentrations (μmol/g dry weight) in iron-loaded animals at five weeks were 444 ± 56 (CI) and 479 ± 80 (FB_1/CI) (mean ± SEM). All the FB_1-fed rats, in the presence or absence of CI, developed a toxic hepatitis with a fourfold rise in serum alanine transaminase (ALT) levels. FB_1 appeared to augment iron-induced hepatic lipid peroxidation, as measured by the generation of thiobarbituric acid reacting substances (TBARS) in liver homogenates ($p<0.0001$). Morphometric analysis

showed that FB_1 caused a significantly greater mean ± SEM number of GST pi positive foci and nodules per cm^2 (5.34 ± 1.42 vs. 1.50 ± 0.52, $p < 0.05$), as well as a greater area (%) of liver occupied by foci and nodules (0.33 ± 0.12% vs. 0.05 ± 0.03%, $p < 0.001$), compared with FB_1/CI. The addition of FB_1 to dietary iron loading caused a shift in distribution of iron from hepatocytes to Kupffer cells, probably due to phagocytosis of necrotic iron-loaded hepatocytes. In conclusion, (i) FB_1 appears to cause toxicity in the liver independently from effects on lipid peroxidation; (ii) FB_1 has a potentiating effect on iron-induced lipid peroxidation; and (iii) dietary iron loading appears to protect against the cancer promoting properties of FB_1, possibly due to a stimulatory effect of iron on hepatocyte regeneration.

25-Week On/25-Week Off FB_1 Feeding Study: Long-Term Effects

In order to determine the long-term effects of FB_1 feeding on rat liver, 54 male F344 rats were allocated to the following treatment groups: (1) FB_1 250 mg/kg for 5 weeks then FB_1 100 mg/kg to 25 weeks (n=12); (2) FB_1 250 mg/kg for 5 weeks then control diet to 25 weeks (n=12); (3) FB_1 250 mg/kg for 5/52 then two weeks of 0.02% acetylaminofluorene (AAF), followed by 100 mg/kg of FB_1 to 25 weeks (n=12); (4) FB_1 250 mg/kg for 5/52 then 0.02% AAF for 2 weeks then control diet till 25 weeks (n=12); (5) control diet (AIN-76A) for 25 weeks (n=6). Rats of all groups were subsequently returned to control diet for a further 25 weeks ('stop study'). Rats from each group were biopsied serially at intervals throughout the study (biopsy results not presented here). Surviving animals were sacrificed at 50 weeks. Post mortem data are given for rats culled at the end of the study and within 6 weeks of the sacrifice date. Liver sections were scored 1 - 4 for the amount of oval cell proliferation. The percentage area of hepatocyte nodules (0 = 0%, 1 = 1 - 25%, 2 = 26 - 50%, 3 = 51 - 75%, 4 = 76 - 100%) was scored and the number of rats with cholangiofibromas (CF) were also counted. Results of post mortem liver data are presented in TABLE I below:

TABLE I. Post mortem liver findings in the different treatment groups in the 25-week on/25-week off FB_1 feeding study[a].

Treatment group	No. of rats per group	Oval cells[b] (mean score)	Nodules (mean score)	Nodule score (range)	No. of rats with CF
FB_1/FB_1	6	2.17	1.17[c]	(0 - 4)	1
FB_1/AIN	6	1.17	0.50	(0 - 2)	0
FB_1/AAF/FB_1	10	2.60	1.40	(0 - 2)	7
FB_1/AAF/AIN	10	2.10	0.50	(0 - 1)	4
AIN	5	0	0	(0 - 0)	0

[a]Post mortem data are given for rats culled at the end of the study and within 6 weeks of the sacrifice date.

[b]Oval cells were identified within nodules.

[c]One animal in this group had a trabecular hepatocellular carcinoma

Thus, (i) dietary FB_1 in the long term resulted in oval cell proliferation with formation of persistent hepatic nodules and a hepatocellular carcinoma; (ii) cholangiofibromas were also introduced by FB_1 in some rats; (iii) addition of AAF enhanced the effects of FB_1 feeding, probably by inhibiting hepatocyte regeneration in response to FB_1 toxicity; and (iv) this study supports the involvement of dietary FB_1 in liver carcinogenesis in male F344 rats.

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Chapter 1
Introduction and aims

1.1. Liver stem cells

My interest in liver stem cells was first stimulated by an immunohistochemical study from the MRC/UCT Liver Research Centre at the University of Cape Town, which showed that in developing human liver the haematopoietic stem cell markers CD34 and c-kit are co-expressed in ductal plate cells in a pattern similar to early cytokeratin markers CAM 5.2 and CK18 (Blakolmer *et al.*, 1995). Results from this and other studies suggested that similarities might exist between hepatic and bile duct precursors and bone marrow stem cells, with respect to the expression of prototypic early cellular markers. My initial 'stem cell' project was to develop a method for the isolation of hepatic 'progenitor' cells from normal human fetal liver (see Appendix D). The identification of ductal plate cells as likely progenitors for both bile duct epithelial cells and hepatocytes and their possible reappearance in the regenerating liver have generated much interest in their pluripotential capacities. The intention was to perform further studies on the 'progenitor' cells that were isolated from human fetal liver, to determine the growth and differentiation capacity of these cells. However, progress was hampered by a severe shortage of supply of 'normal' livers from human fetuses of appropriate gestational age. It was thus decided to look for a suitable animal model for further studies on the role of progenitor cells in the liver.

1.2. The fumonisin B_1-fed rat

There is a long history of research on the toxicology and carcinogenic effects of the mycotoxin fumonisin B_1 (FB_1) at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), a division of the South African Medical Research Council (MRC). The fumonisin B mycotoxins are natural contaminants of maize infected with the fungus *Fusarium*

moniliforme. Ingestion of FB_1, the major fumonisin produced by the fungus, causes a variety of naturally occurring toxicoses in domestic animals. Human dietary consumption of *Fusarium*-contaminated maize products has been linked epidemiologically to increased rates of oesophageal cancer in regions of the world in which maize is the staple grain. In rats, feeding with FB_1 causes severe hepatotoxicity, chronic liver injury progressing to cirrhosis , and sometimes hepatocellular carcinoma or cholangiocarcinoma. To date, however, there have been no studies on the role of 'oval cells', putative liver progenitor cells, during the events that occur in rat liver during feeding with FB_1.

The FB_1-fed rat seemed to hold promise as a model for the study of liver injury, oval ('progenitor') cell proliferation, and carcinogenesis for several reasons: (i) this animal model for hepatocarcinogenesis appears to resemble the scenario in humans, where hepatocellular carcinoma usually occurs in the setting of chronic necroinflammatory liver disease and cirrhosis; (ii) the "bile duct hyperplasia" that has been reported during FB_1-induced hepatotoxicity may in fact represent the proliferation of oval cells; (iii) FB_1 causes both hepatocellular carcinoma and cholangiocarcinoma in rats, indirect evidence for a stem cell origin of liver tumours; and (iv) it is possible that ingestion of FB_1 might be a risk factor for human hepatocellular carcinoma in regions of the world where maize is the staple diet.

1.3. Hypothesis and aims

The hypothesis of this dissertation was that *feeding with FB_1 causes proliferation of oval cells* in rat liver, and that *these putative progenitor cells are the precursors of both the hepatocellular and bile duct tumours* that are seen in this animal model of carcinogenesis. The specific aims were to: (i) describe the histopathology of acute hepatotoxicity due to FB_1 and the role of oval cell proliferation; (ii) study the changes in hepatic gene expression during acute FB_1-induced hepatotoxicity and oval cell activation; (iii) assess the effect of oxidative stress due to dietary iron loading on FB_1-induced cancer induction; and (iv) study the hepatic effects of long-term FB_1 feeding and the role of oval ('progenitor') cells in the development of hepatocellular and bile duct tumours.

Chapter 2

The fumonisin mycotoxins

2.1. Introduction

The fumonisins are natural contaminants of maize (*Zea mays L*) infected with the fungus *Fusarium moniliforme* Sheldon (Gelderblom *et al.*, 1988a; Sydenham *et al.*, 1990a). These food-borne carcinogenic mycotoxins became the spotlight of mycotoxin research in 1988, when researchers at the South African Medical Research Council isolated and structurally characterised the fumonisins (Gelderblom *et al.*, 1988a; Bezuidenhout *et al.*, 1988). Since 1988, there has been intense interest in these mycotoxins, mainly by toxicologists, maize producers, commodity groups, food processors and regulatory agencies (Riley *et al.*, 1993; Norred, 1993; Voss *et al.*, 1995). The reason for the concern regarding the fumonisin mycotoxins is threefold: (i) fumonisins are found in measurable concentrations in maize grown throughout the world (Thiel *et al.*, 1991a; Sydenham *et al.*, 1991; Sydenham *et al.*, 1992a; Ueno *et al.*, 1993 Murphy *et al.*, 1993; Chamberlain *et al.*, 1993); (ii) these compounds have been implicated as the causative agents in a variety of naturally occurring animal diseases, including fatal illnesses in horses (Marasas *et al.*, 1988b; Kellerman *et al.*, 1990) and pigs (Harrison *et al.*, 1990; Colvin *et al.*, 1993); and (iii) there is speculation that fumonisin may in part be responsible for the high incidence of oesophageal cancer in regions of the world in which maize is the staple grain (Marasas, 1994; Chu and Li, 1994). Because of these factors, there is little doubt that these mycotoxins will continue to have a significant, adverse impact on the maize industry, including export markets.

In rats, feeding with fumonisins causes liver and kidney toxicity (Gelderblom *et al.* 1988a; Voss *et al.* 1993; Voss *et al.*, 1994; Bondy *et al.* 1996; Bucci *et al.*, 1998), as well as liver tumours (Gelderblom *et al.*, 1991). Fumonisin-fed rats develop a chronic toxic hepatitis, cirrhosis, cholangiofibrosis, hepatocellular carcinoma and cholangiocarcinoma (Gelderblom *et al.*, 1988a; Gelderblom *et al.*, 1991; Voss *et al.* 1993;). These carcinogenic mycotoxins are nongenotoxic, and act as strong promoters (and possibly weak initiators) of tumours (Gelderblom *et al*, 1991; Gelderblom *et al*, 1992b; Norred *et al.*, 1992).

Fumonisins are sphinganine analogues that inhibit ceramide synthase and block the biosynthesis of complex sphingolipids, promoting accumulation of sphinganine and sphinganine 1-phosphate (Wang *et al.*, 1991; Yoo *et al.*, 1992; Norred *et al.*, 1992b). Disruption of sphingolipid metabolism by FB_1 alters cell-cell interactions, the behaviour of cell-surface proteins, the activity of protein kinases, the metabolism of other lipids, and cell growth and viability (Merrill *et al.*, 1996). This multitude of effects may account for the toxicity and carcinogenicity of these mycotoxins. Naturally occurring inhibitors of sphingolipid metabolism such as fumonisins are proving to be powerful tools for studying the diverse roles of sphingolipids in cell regulation and disease (Merrill *et al.*, 1996).

Ceramide, a recently identified lipid second messenger (*Spiegel et al.*, 1996; Hannun, 1996), has been implicated in the induction of apoptosis in tumour cells (Bose *et al.*, 1995; Jaffrezou *et al.*, 1996) and the ovary (Witty *et al.*, 1996; Kaipia *et al.*, 1996; Martimbeau and Tilly, 1997). Manipulation of apoptosis-associated ceramide signalling pathways with agents such as fumonisin B_1 and sphingosine-1-phosphate has most recently been attempted in order to prevent chemotherapy-induced destruction of oocytes and resultant permanent female sterility (Perez *et al.*, 1997)

2.2. Isolation and chemical characterisation

The fumonisins (diesters of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-polyhydroxy-icosanes) were first isolated in 1988 from cultures of *F. moniliforme* strain MRC 826, known to be hepatocarcinogenic in rats (Gelderblom *et al.*, 1988a), and the structures were subsequently elucidated (Bezuidenhout *et al*, 1988). Six fumonisins have been isolated to date and characterised (Cawood *et al.*, 1991; Gelderblom *et al.*, 1992a). Three of these, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) are the major fumonisins produced in nature, while fumonisin B_4 (FB_4) and fumonisins A_1 and A_2 (FA_1 and FA_2 respectively) are produced in relatively minor quantities. The chemical structures of the three major fumonisins (FB_1, FB_2, and FB_3) are shown in Figure 2.1.

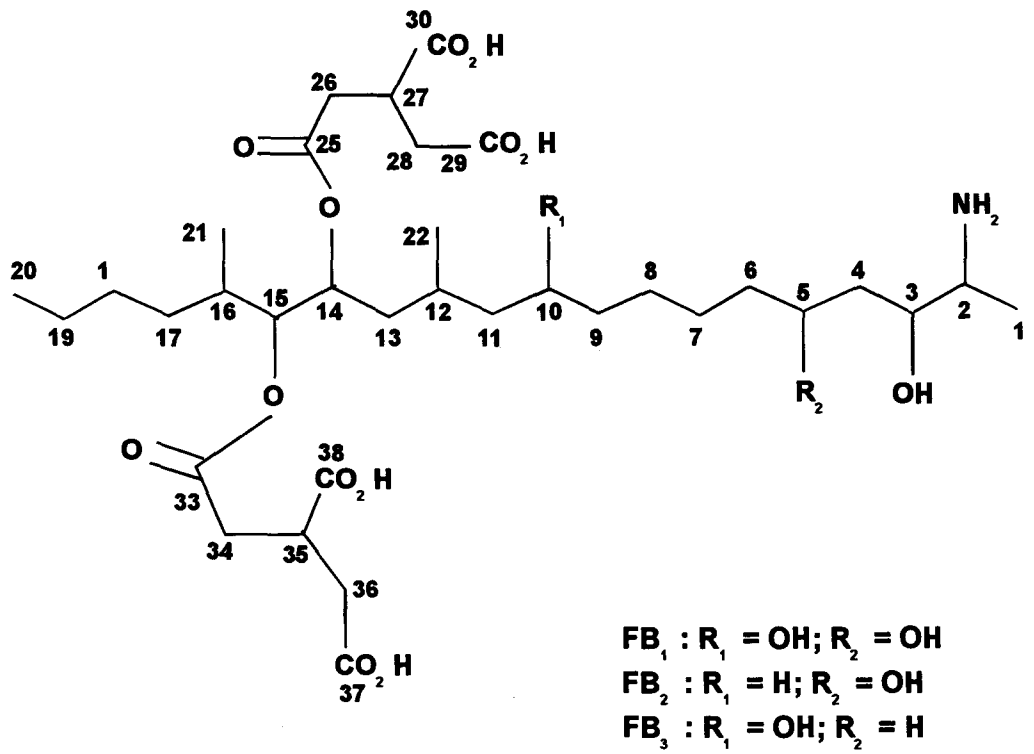

Figure 2.1. Structure of the three major fumonisins (FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3).

The most important producer of fumonisins is *F. moniliforme* (Gelderblom *et al.*, 1988a, Gelderblom *et al.* 1988b; Thiel *et al.*, 1991a; Thiel *et al.*, 1991b; Ross *et al.*, 1990), and most isolates of *F. moniliforme* from maize analysed to date have been found to produce fumonisins. Other *Fusarium* species that produce FB_1 and FB_2 are *F. proliferatum* (Thiel *et al.*, 1991a; Ross *et al.*, 1990) and *F. nygamai* (Thiel *et al.*, 1991a).

A variety of analytical methods have been used by different laboratories for the quantification of naturally occurring levels of fumonisins. These include liquid chromatography (Bennett and Richard, 1994), gas chromatography (GC) (Jackson and Bennett, 1990; Sydenham *et al.*, 1990a), thin layer chromatography (TLC) (Gelderblom *et al.*, 1988a; Jackson and Bennett, 1990; Sydenham *et al.*, 1990a; Wilson *et al.*, 1990), liquid secondary ion mass spectroscopy (LSIMS) (Bezuidenhout et al., 1988; Platttner et al., 1990; Voss *et al.*, 1989), high performance liquid chromatography (HPLC) (Alberts *et al.*, 1990; Gelderblom *et al.*, 1988a; Shephard *et al.*, 1990; Sydenham *et al.*, 1990a; Sydenham *et al.*, 1992b; Wilson *et al.*, 1990), and gas chromatography-mass spectrosopy (GC-MS) (Jackson

and Bennett, 1990; Plattner *et al.*, 1990; Voss *et al.*, 1989; Wilson *et al.*, 1990). The HPLC method of Shephard *et al.* (Shephard *et al.*, 1990) as modified by Sydenham *et al.* (Sydenham *et al.*, 1992b) is currently recommended by the South African Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) for the quantitative analysis of fumonisins in maize-based foods and feeds (Marasas, 1994). This analysis involves fluorescence detection of the o-phthaldialdehyde (OPA) derivatives of FB_1 and FB_2 (Shephard *et al.*, 1990; Sydenham *et al.*, 1992b). All of the available analytical methods are laborious and require extensive extraction, clean-up and derivatisation procedures. Currently, immunoassays are being developed as an alternative rapid, reliable, and relatively inexpensive method for the detection of fumonisins in food and feed samples (Abouzied *et al.*, 1996; Ueno *et al.*, 1997).

2.3. Toxicity in domestic animals

Ingestion of *Fusarium*-contaminated maize causes a variety of toxic effects in domestic animals. Equine leukoencephalomalacia or "mouldy corn poisoning" is a syndrome of acute illness and death in horses (Kellerman *et al.*, 1972) that can be reproduced by feeding animals with FB_1 (Marasas *et al.*, 1988b; Kellerman *et al.*, 1990; Ross *et al.*, 1990, Wilson *et al.*, 1990). Clinical features include incoordination, aimless walking, blindness and head pressing (Marasas *et al.*, 1976; Kriek *et al.*, 1981a). Necropsy characteristically reveals liquefactive necrotic lesions in the white matter of the cerebral hemispheres (Marasas *et al.*, 1976). FB_1 is also responsible for natural outbreaks of porcine pulmonary oedema (Harrison *et al.*, 1990; Ross *et al.*, 1990), a syndrome of pulmonary oedema and pleural effusions in pigs associated pathologically with evidence of damage to the pulmonary microvasculature (Casteel *et al.*, 1994). Contamination of the 1989 US maize crop by *F. moniliforme* caused widespread outbreaks of equine leukoencephalomalacia and porcine pulmonary oedema in the USA during the autumn of 1989 and winter of 1990 (Harrison *et al.*, 1990; Ross *et al.*, 1990; Wilson *et al.*, 1990). In early 1995, an outbreak of equine leukoencephalomalacia killed at least 38 horses in Kentucky and Virginia, and fumonisin was identified in the maize-based feed the horses had consumed (House, 1995). FB_1 has also been shown to cause toxicoses in cattle, sheep, chickens, turkeys and ducks in controlled studies (Miller *et al.*, 1996). While the acute toxicity of FB_1 has been described in several species, the chronic effects of lower doses on

animal health and loss of productivity are less well defined.

2.4. Oesophageal carcinoma in humans

The geographical distribution of oesophageal carcinoma is enigmatic because the disease occurs world-wide with a marked variation in incidence, even within relatively small geographical areas (Rose, 1973; Liu and Li, 1984). In particular, extremely high incidence rates of oesophageal carcinoma of more than 50 per 100 000 population per annum have been recorded in three areas of the world: the south-western districts of Transkei in South Africa (Rose, 1973; Jaskiewicz *et al.*, 1987a), the Linxian County of Henan Province in northern China (Liu and Li, 1984; Li *et al.*, 1980; Yang, 1980), and the Caspian littoral of Iran (Kmet and Mahboubi, 1972; Hormozdiari *et al.*, 1975). This marked geographical variation in incidence rates suggest that environmental factors are involved in the aetiology of oesophageal carcinoma. In the high-risk population in Transkei, the coexistence of deficiencies in vitamin A, vitamin B_{12}, vitamin E, and folic acid (Van Helden *et al.*, 1987; Jaskiewicz *et al.*, 1988a) and selenium (Jaskiewicz *et al.*, 1988b) with exposure to fumonisins in the maize staple diet (Sydenham *et al.* 1990b) is well established. The consumption of maize as a dietary staple and/or as a home-brewed alcoholic beverage has been implicated as a risk factor of oesophageal carcinoma in Africa (Van Rensburg, 1981; Cook, 1971; Marasas, 1982; Marasas *et al.*, 1988a), China (Li *et al.*, 1980; Yang, 1980; Zhen, 1984), Italy (Rossi *et al.*, 1982; Franceschi *et al.*, 1990), and the United States (Brown *et al.*, 1988).

The incidence rate of oesophageal carcinoma in males as well as females in the southern part of Transkei is among the highest in the world, whereas the rate in the northern part of Transkei is moderate to low (Jaskiewicz, 1987a). The staple diet in both areas is home-grown maize. Detailed mycological analyses of home-grown maize samples intended for human consumption from different oesophageal carcinoma rate areas in Transkei during six seasons over the period 1976 - 1989 revealed a statistically significant correlation between the incidence of *F. moniliforme* in maize and oesophageal carcinoma rate (Rheeder *et al.*, 1992; Marasas *et al.*, 1981; Marasas *et al.*, 1988a). In these studies, the correlation between *F. moniliforme* and oesophageal carcinoma rate was based on death certificate cancer registry data. The correlation also was shown to exist between *F. moniliforme* contamination of maize

and premalignant oesophageal brush cytological changes in living individuals (Marasas *et al.*, 1988a).

In the high-incidence area of oesophageal carcinoma in northern China, an association between *F. moniliforme* contamination of maize and the incidence of oesophageal carcinoma also has been found (Li and Cheng, 1984; Li *et al*, 1980; Yang, 1980). The natural occurrence of fumonisins in maize used as the staple diet by people at high risk for oesophageal carcinoma in Linxian County in northern China has recently been reported (Chu and Li, 1994). The association between the incidence of *F. moniliforme* in maize and the oesophageal carcinoma rate that has been established in Transkei and China does not exist in the high-incidence area of Iran, in which the staple diet is wheat, and the most prevalent fungus associated with wheat is *Alternaria alternata* (Fr.) Keissler (Kmet and Maboubi, 1972; Hormozdiari *et al.*, 1975; Van Rensburg, 1981). Thus, it seems that if the fumonisins produced in maize by *F. monoliforme* are foodborne carcinogens involved in the aetiology of oesophageal carcinoma in Transkei and China, other factors must be involved in Iran. Interestingly, the carcinogenic fumonisins produced by *F. moniliforme* are structurally very similar to the *Alternaria* (AAL) toxins, a group of host-specific phytotoxins produced by *A. alternate* f. sp. *lycopersici* (Bezuidenhout, 1988). Preliminary reports suggest that FB_1 and AAL toxin have similar biological effects on plants (Mirocha *et al.*, 1990) and cultured mammalian cells (Shier *et al.*, 1991). The examination of wheat from the high-incidence area of oesophageal carcinoma in Iran for *A. alternata* metabolites related to fumonisins and AAL toxins is clearly indicated.

2.5. Toxicity and tumours in rats

Both culture material of *F. moniliforme* and the fumonisins are *hepatotoxic* (Gelderblom *et. al.*, 1988a; Voss *et al.*, 1993) and *hepatocarcinogenic* (Marasas *et al.*, 1984a; Wilson *et al.*, 1985; Jaskiewicz *et al.*, 1987b; Gelderblom *et al.*, 1991) in rats. The principal pathological change in rats treated with FB_1 in the diet (1000 mg/kg) in short-term toxicity tests (21-33 days) is progressive "toxic hepatitis" characterised by hepatocellular necrosis, bile duct proliferation ("hyperplasia"), and fibrosis (Gelderblom *et. al.*, 1988a), identical to that induced by the culture material of *F. moniliforme* MRC 826 (Kriek *et al.*, 1981b). During a chronic

feeding study over a period of 26 months with FB_1 50 mg/kg and a modified diet, the liver was shown to be the major organ affected (Gelderblom *et al.*, 1991). Pathological changes in the liver were characterised by a "chronic toxic hepatitis" that progressed to cirrhosis and "cholangiofibrosis", and which terminated in hepatocellular carcinoma and cholangiocarcinoma respectively. Ten out of 15 FB_1-treated rats (66%) developed hepatocellular carcinoma from 18 months onwards, and metastases to the lungs and kidneys were present in four of these rats. Although the cholangiofibrotic lesions were non-infiltrative and did not metastasize, some reached a very large diameter (up to 3 cm) and were considered to have progressed to cholangiocarcinoma (Gelderblom *et al.*, 1991). In a preliminary study, Voss *et al.* (1993) reported that FB_1 was hepatotoxic in rats fed a diet containing 150 mg/kg for 4 weeks and also nephrotoxic at 15-50 mg/kg. Microscopic analysis of the livers revealed "scattered single cell necrosis", bile duct proliferation, and an increase in mitotic cells. The focal hepatocellular necrosis was subsequently confirmed as apoptosis, or programmed cell death (Tolleson *et al.*, 1996a; Tolleson *et al.*, 1996b). This is consistent with *in vitro* observations that FB_1 inhibits cell proliferation or induces apoptosis in cultured turkey lymphocytes (Dombrink-Kurtzman *et al.*, 1994), cultured African green monkey kidney cells (Jones *et al.*, 1995), and a renal epithelial cell line (Yoo *et al.*, 1994; Yoo *et al.*, 1995). The increase in mitotic cells noted in the hepatotoxicity studies with high dose FB_1 (Voss *et al.*, 1993; Tolleson *et al.*, 1996b) was thought to reflect hepatocellular proliferation to replace the apoptotic cells.

Cancer induction by FB_1 has been investigated by studying the mechanisms involved during cancer initiation and promotion in rat liver (Farber, 1984; Gelderblom *et al.*, 1996b). FB_1 was shown to be a cancer promoter in a short-term initiation/promotion assay with diethylnitrosamine (DEN)-initiated rats and the induction of γ-glutamyl transpeptidase positive (GGT+) foci as endpoint (Gelderblom *et al.*, 1988a). Although the role of cell proliferation in tumour promotion is controversial, many hepatocarcinogens are known to be inhibitors of cell proliferation (Farber *et al.*, 1989). FB_1 caused inhibition of regenerative hepatocyte proliferation (^{3}H-labelled thymidine incorporation) induced by partial hepatectomy (PH), when fed in the diet at a level of 50 mg/kg and higher for 21 days (Gelderblom *et al.* 1996b). FB_1 is also a cancer initiator, as evidenced by the induction of resistant hepatocytes in rat liver (Gelderblom *et al.*, 1992b; Gelderblom *et al.*, 1994). Although FB_1 is a complete carcinogen in rat liver, it is a poor cancer initiator, requiring prolonged exposure (21 days) to a relatively

high dietary level (250 mg/kg) (Gelderblom *et al.*, 1994). Thus a time and dosage dependent threshold level exists for cancer initiation by FB_1 (Gelderblom *et al.*, 1994). At a dietary level of 1000 mg/kg for 21 days, only the FB fumonisins (FB_1, FB_2 and FB_3) initiated cancer in rat liver whereas the N-acetylated analogues FA_1 and FA_2 and the hydrolysis products AP_1, AP_2 and tricarballylic acid (TCA) did not (Gelderblom *et al.*, 1993). Thus the free amino group and the intact molecule are required for cancer initiation.

FB_1 is not mutagenic in the *Salmonella* mutagenicity test (Gelderblom and Snyman, 1991; Park *et al.*, 1992). Furthermore, FB_1 does not induce unscheduled DNA synthesis in isolated rat hepatocytes (Norred *et al.*, 1991), and is not genotoxic in the *in vivo* and *in vitro* DNA repair assays in rat primary hepatocytes (Gelderblom *et al.*, 1992a; Norred *et al.*, 1992a; Norred *et al.*, 1992b). This contrasts sharply with the carcinogenic effects of aflatoxin B_1, the most intensively studied mycotoxin, which is a potent mutagen (see Appendix E).

2.6. Effect on sphingolipid metabolism

The fumonisins are structurally similar to the long chain base sphingosine, a component of the long chain backbone of sphingolipids (Bell *et al.*, 1993). FB_1 and FB_2 are naturally occurring specific inhibitors of *de novo* sphingolipid biosynthesis and sphingolipid turnover (Merrill *et al.*, 1993b). The site of inhibition is at the formation of ceramides catalysed by sphingosine- and sphinganine N-acetyl-transferase (*ceramide synthase*) (Wang *et al.*, 1991). In primary rat hepatocytes this inhibition occurs at concentrations that are not toxic to the cells (IC_{50} = 0.1 μM) (Wang *et al.*, 1991; Norred *et al.*, 1992b). Similarly, FB_1 and FB_2 inhibit *de novo* sphingosine biosynthesis (IC_{50} = 10 - 15 μM) in a proliferating cell line of pig kidney cells ($LLC\text{-}PK_1$) and cause a remarkable (128 fold) increase in cellular levels of sphinganine (Yoo *et al.*, 1992; Norred *et al.*, 1992b). In these cells inhibition of sphingolipid biosynthesis is an early event in the toxicity of fumonisins, and precedes inhibition of cell proliferation and cytotoxicity. Inhibition of sphingolipid biosynthesis has also been observed in yeast cells (Kaneshiro *et al.*, 1992).

Inhibition of sphingolipid biosynthesis may be the primary effect of fumonisin toxicity in equine leukoencephalomalacia and porcine pulmonary oedema. It may also be responsible for the tumour-promoting ability of the fumonisins. In the latter, fumonisin-induced inhibition

of sphingosine biosynthesis may lead to a deregulation of protein kinase C, which could lead to the proliferation of initiated cells (Norred *et al.*, 1992b). In addition, stimulation of DNA synthesis by FB_1 at concentrations of 10 μM to 100 μM in Swiss 3T3 fibroblasts was attributed to the mitogenic effect of the sphingoid bases that accumulated as a result of a disruption of sphingolipid metabolism (Schroeder *et al.*, 1994). Conflicting results were however obtained from an *in vitro* DNA labelling study in primary hepatocyte cultures (Gelderblom *et al.*, 1995). FB_1 was found to inhibit the epidermal growth factor-induced DNA synthesis by up to 90% when incorporated at concentrations of 150 μM to 300 μM for a period of 44 h (Gelderblom *et al.*, 1995). In this study, no relationship was found between the disruption of sphingolipid biosynthesis by FB_1 and its mitoinhibitory effect or toxicity in primary hepatocytes.

The ratio of free sphinganine to free sphingosine (Sa/So ratio) in serum and tissues were found to increase when rats, ponies and pigs were exposed to fumonisins in their feed (Riley *et al.*, 1994b). In rats, ponies and pigs fed diets that contained 1 ppm of fumonisins the ratio was < 0.35. Levels as low as 5 ppm in the feed of pigs cause statistically significant increases, so that this ratio has been suggested as a specific biomarker for fumonisin exposure (Riley et al., 1994b). Elevations in this ratio were seen before there was evidence of tissue damage as indicated by serum biochemical parameters or histopathology.

Ceramide is a lipid second messenger that is believed to be one of the immediate signals for cell death generated in tumour cells treated with the chemotherapeutic agent, daunorubicin (Bose *et al.*, 1995; Jaffrezou *et al.*, 1996). Recent investigations have shown that the production of ceramide and the resultant apoptosis in P388 cells cultured in the presence of daunorubicin, is prevented by pre-treatment with fumonisin B_1 (Bose *et al.* 1995). This inhibition of chemotherapy-induced apoptosis may relate to the result of specific inhibition of ceramide synthase by FB_1 (Wang *et al.*, 1991). In contrast however, the lethality of doxorubicin in haploid mouse oocytes is blocked by pre-treatment with sphingosine 1-phosphate, an endogenous downstream inhibitor of ceramide-promoted intracellular signalling, but not by FB_1 (Perez *et al.*, 1997).

2.7. Risk assessment

The International Agency for Research on Cancer (IARC) recently evaluated the toxins derived from *F. moniliforme* as *possibly carcinogenic to humans* (Group 2B carcinogens) (IARC, 1993; Vainio *et al.* 1993). Risk assessment for humans exposed to carcinogenic mycotoxins consists of exposure assessment and hazard assessment (Kuiper-Goodman, 1990). Human exposure is calculated from estimates of the level of a mycotoxin in foodstuffs and food intake or from direct measurements on humans (Kuiper-Goodman, 1990), and expressed as *probable daily intake* (PDI). Based on the naturally occurring levels of total fumonisins in home-grown maize in Transkei and the assumption that a 70 kg person consumes 460 g maize per day, Thiel *et al.* (1992) calculated a PDI of 14 μg/kg/day for a person eating "healthy" maize and 440 μg/kg/day for a person eating "mouldy" maize. Hazard is an intrinsic property of a mycotoxin with reference to toxicological effects in a specific species at a specific level of exposure (Kuiper-Goodman, 1990). Hazard is calculated from toxicological studies in experimental animals and expressed as the *no observed effect level* (NOEL) and TD_{50} (dose rate at which 50% of animals develop cancer). Extrapolation is done by means of safety factors (NOEL: 100 - 1000 for toxins and 1000 - 5000 for carcinogens; TD_{50}: 50 000) to estimate the *tolerable daily intake* (TDI). TDI values have to be calculated with respect to carcinogenic risk to humans of fumonisins in maize and maize-based foods in order to establish tolerance levels. The mechanism of carcinogenicity of the fumonisins is important in determining the safety factor calculate the TDI (Gelderblom *et al.*, 1996a). Tolerance levels for fumonisins in maize and maize-based foods and feeds should have a firm scientific basis and must be practicable (Marasas *et al.*, 1993a). These levels can only be established following compehensive exposure and hazard assessment and on the basis of reliable PDI and TDI values.

2.8. Conclusions

The fungus *F. moniliforme* is one of the most prevalent seedborne fungi associated with maize intended for human and animal consumption throughout the world. The fumonisin mycotoxins exhibit a remarkable species-specific variation in the target organ(s) affected in different animals, such as the brain in horses, the lungs in pigs, and the liver in rats. Although oesophageal carcinoma has not been reproduced experimentally in animals with either culture material of *F. moniliforme* or pure fumonisins, both the culture material and FB_1 have been proven to cause hepatocellular carcinoma and cholangiocarcinoma in rats. The fumonisin mycotoxins have been found to disrupt sphingolipid metabolism, a finding that has potentially far-reaching implications for understanding the mechanisms of fumonisin toxicity as well as the role of sphingolipids in cell function and regulation.

Chapter 3

Liver stem cells

3.1. Introduction

Stem cells may be defined as immature cells of nondescript morphology with unlimited cycling capacity, clonogenicity, and pluripotency (Pierce *et al.*, 1978). Self-maintenance is a fundamental and common trait of all stem cells (Lajtha, 1970). They are undifferentiated or partially differentiated, and have the potential to proliferate and to develop along several cell lineages. In rapidly renewing adult tissue, such as marrow, skin and gut, stem cells have long since been known to be part of the proliferative compartment, which is ultimately responsible for cell replacement.

There is now a large body of evidence to suggest that the livers of adult animals and humans contain stem cells (Aterman, 1992; Fausto, 1994; Marceau, 1994; Sell 1994; Sell and Pierce, 1994; Sigal *et al.*, 1992; Thorgeirsson, 1993; Thorgeirsson 1996; Golding *et al.*, 1995). In the adult liver, cell turnover is extremely slow and hepatocytes maintain the capacity for replication, and can respond quickly to liver damage associated with mild to moderate cell loss (Reid, 1990; Fausto, 1994). However, when hepatocyte proliferation is impaired or overwhelmed, small periportal cells with scant cytoplasm and ovoid nuclei proliferate and migrate into the surrounding liver parenchyma. In experimental animals, these cells have been termed *oval cells* (Farber, 1956), and their association with defective regeneration has led to the belief that these cells represent a progenitor cell population (Fausto, 1994; Marceau, 1994; Sell and Pierce, 1994; Sigal et al., 1992; Thorgeirsson, 1993; Golding *et al.*, 1996). In humans, these biliary cells have variously been referred to as ductular structures, neoductules, or neocholangioles, and have been observed in many forms of chronic liver disease, including cancer. Oval cells are thought to take over the burden of regenerative growth after substantial liver cell loss, suggesting that they are the progeny of stem cells. The liver is not, however, generally considered as a continually renewing stem cell-fed hierarchy. Rather it appears that the adult liver contains facultative ('potential') stem cells located in biliary ductules that can

function as progenitors for normal and transformed hepatocytes (Golding *et al.*, 1996; Grisham and Thorgeirsson, 1997).

The evidence for the presence of liver stem cells is derived mainly from studies on (i) embryogenesis of the liver; (ii) experimental hepatocarcinogenesis; and (iii) the properties of non-hepatocytic (stem-like) epithelial cells isolated from the liver and examined in culture, and after transplantation into the liver and other sites *in vivo* (Grisham and Thorgeirsson, 1997). Hepatic stem cells have aroused intense interest, because these cells may represent a target population for hepatic carcinogens, and may be useful clinically for the treatment of massive hepatic necrosis and as vehicles for gene therapy. The purpose and fate of stem cells in normal adult liver remain obscure.

3.2. Historical background

The proliferation of bile duct-related cells from the portal tracts was first noted by Opie (1944) in rat carcinogenesis studies using butter yellow. Farber (1956) studied the histological changes induced in the liver by ethionine, 2-acetylaminofluorene (AAF), and 3'-methyl-4-dimethylaminobenzene (Me-DAB), and found that all three agents caused the proliferation of nonparenchymal cells, which he termed 'oval cells' because of their shape. These cells were much smaller than hepatocytes and appeared to be bile ductular cells that proliferated from the portal spaces, starting a few weeks after the administration of the carcinogens. The existence of hepatic stem cells was first postulated by Wilson and Leduc (1958), based on experiments involving liver regeneration in the mouse after chronic injury induced with a methionine-rich basal diet mixed with an equal amount of bentonite. The authors concluded that "prolonged and severe injury may make direct restoration by division of pre-existing parenchymal cells impossible, and that, when this occurs, the new parenchyma is derived from the indifferent cholangiole cells". The "indifferent cholangiole cells" were in fact oval cells. The major support for the existence of hepatic stem cells has since then come from extensive studies of chemical hepatic carcinogenesis in rats. Another landmark observation in the field was made by Desmet (1963), who proposed that hepatocellular carcinomas could derive from either hepatocytes (more differentiated tumours) or oval cells (less differentiated hepatocellular carcinomas and adenocarcinomas with cholangiocellular differentiation). Evidence that oval

cells are precursors of hepatocytes, based on the transformation of ^{3}H-thymidine-tagged oval cells into hepatocytes, has subsequently been provided by Onoé *et al.* (1973), Evarts *et al.* (1987), Lemire *et al.* (1991), and Dabeva and Shafritz (1993).

The most recent approaches to research on liver stem cells have included (i) development of markers to identify stem cells and to distinguish among different types of biliary cells; (ii) study of the growth factors and receptors required for the proliferation and differentiation of progenitor cells; (iv) analysis *in vivo* of the fate of progenitor cells identifiable by expression of genetic markers; (v) identification of stem cells in human livers; and (vi) application of transgenic and knockout mouse models to the study of regulation of stem cell proliferation and differentiation.

3.3. Stem cells and the embryological development of the liver

Embryological studies provide possible explanations for some of the phenomena described in studies of liver regeneration and carcinogenesis. How endodermal cells become determined to form the liver and how these committed cells differentiate into the liver cell lineages are two of the fundamental issues that directly relate to the formulation of stem cell concepts applicable to the adult liver (Fausto, 1994).

The liver is derived from an outgrowth of the primitive foregut, and consists of endodermal and mesodermal components (DuBois, 1963; MacSween and Scothorne, 1985). The hepatic diverticulum is first visible in the mouse embryo on E9.5 and in the rat embryo on E10 - 10.5, as a thickening of the epithelium of the ventral foregut adjacent to the developing heart and projecting into the loose mesenchyme of the septum transversum (Le Douarin, 1975; Houssaint, 1980; Cascio and Zaret, 1991). In the developing human embryo, the hepatic diverticulum develops around the 3rd and 4th week of gestation (Gerber and Thung, 1993). The first functional evidence of the impending development of the liver epithelium is seen at E9 in mice and E10 in rats with the weak expression in the hepatic diverticulum of α-fetoprotein (AFP) (Cascio and Zaret, 1991; DiPersio *et al.*, 1991; Shiojiri *et al.*, 1991), as well as of several liver-enriched transcription factors (Ang *et al.*, 1993; Monaghan *et al.*, 1993).

In both rat and man the differentiation of hepatocytes and biliary epithelial cells from *multipotential endodermal stem cells* of the hepatic diverticulum occurs via the development

of *hepatoblasts*, which have bipotential differentiation capabilities (Van Eyken *et al.*, 1988a; Van Eyken *et al.*, 1988b; Haruna *et al.*, 1996) (Figure 3.1).

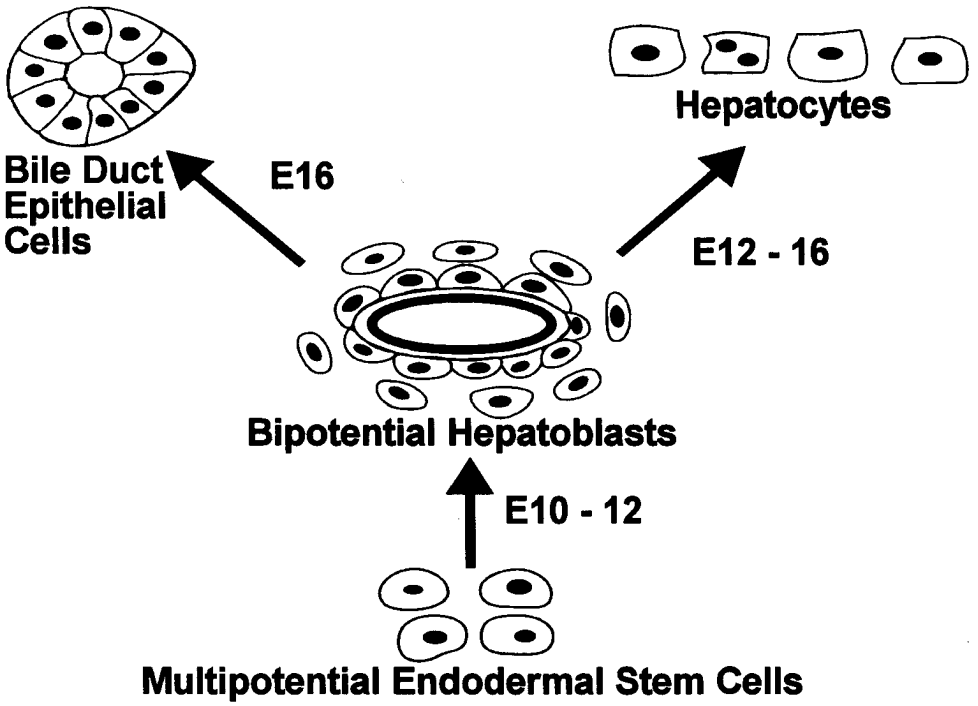

Figure 3.1. Schematic diagram showing the general temporal sequence of the differentiation of hepatocytes and biliary epithelial cells from multipotential endodermal cells of the hepatic diverticulum, through the development of hepatoblasts with bipotential differentiation options (modified from Grisham and Thorgeirsson, 1997).

Hepatoblasts initially bear little structural or functional resemblance to mature hepatocytes or bile duct epithelial cells, both of which morphologically differentiate during the last half of fetal development and the first few postanatal days (Herzfeld *et al.*, 1973; Luzzato, 1981; Feracci *et al.* 1987; Vassy *et al.*, 1988). Morphological and functional changes in cells mirror the development of the extracellular matrix and adhesion molecules (Odin and Öbrink, 1988; Stamatoglou *et al.*, 1992; Martinez-Hernandez *et al.*, 1993).

Most of the hepatoblasts in the liver cell mass located in the septum transversum gradually develop into hepatocytes (Shiojiri *et al.*, 1991). Expression of hepatocyte-enriched transcription factors represents the earliest evidence of hepatoblastic and hepatocellular

differentiation (Lai and Darnell, 1991; Lai, 1992; Xanthopoulos and Mirkovitch, 1993; Zaret, 1996). These transcription factors, including HNF3, HNF4, HNF1α, C/EBP and DBP, are expressed sequentially during the period of gestation, and appear to be essential for embryonic liver development (Ang *et al.*, 1993; Monaghan *et al.*, 1993; Sladek *et al.*, 1990; Tian and Schibler, 1991, Kuo *et al.*, 1992; Nagy *et al.*, 1994). Hepatocyte growth factor (HGF), a growth factor produced by mesenchymal cells which acts in a paracrine fashion on epithelial cells via the c-*met* receptor (Zarnegar *et al.*, 1994; Michalopoulos and DeFrances, 1997), is also essential for embryonic liver development, and mice lacking the HGF gene die *in utero* with a liver that is reduced in size and shows extensive loss of parenchymal cells (Schmidt *et al.*, 1995; Uehara *et al.*, 1995). The developing hepatocytes express the hepatocyte-specific proteins AFP and albumin, as well as the intermediate filaments CK8 and CK18 (TABLE 3.1) (Van Eyken *et al.*, 1988a; Shiojiri *et al.*, 1991)

Hepatoblasts that surround the portal mesenchyme condense to form a double-layered cylinder of cells, the *ductal plate*, which remodels and migrates into the mesenchyme to form the intrahepatic bile ducts (Van Eyken *et al.*, 1988a; Shiojiri *et al.*, 1991). Differentiation of bile duct epithelial cells and formation of bile ducts is also a gradual and continuous process that begins in rats on about E15 - 15.5, and continues postnatally (Van Eyken *et al.*, 1988a; Shiojiri *et al.*, 1991). Little is known about specific changes in expression of transcription factors or synthesis of specific proteins during the differentiation of bile duct epithelium. Biliary differentiation in hepatoblasts that touch portal mesenchyme in rats is heralded by the expression of reactivity for BD.1 antibody, following which the ductal plate then develops into a series of tubules that bind BD.1, OC.2, HBD.1 and CK19; biliary duct epithelium gradually expresses CK7 as ducts develop further (TABLE 3.1) (Van Eyken *et al.*, 1988a; Shiojiri *et al.*, 1991; Hixson *et al.*, 1992). The ductal plate cells initially express hepatocyte-specific proteins AFP and albumin as they migrate into the portal stroma, and additionally begin to express the bile duct specific marker, γ-glutamyl transpeptidase (GGT) (TABLE 3.1) (Shiojiri *et al.*, 1991).

TABLE 3.1

Antibody markers commonly used to assess differentiation and to trace lineage of liver epithelial cells (Modified from Grisham and Thorgeirsson, 1997).

Markers	Hepatoblasts	Oval cells	Hepatocytes	Bile duct cells
CK7	-	-	-	+
CK8	+	+	+	+
CK18	+	+	+	+
CK19	+	+	-	+
CK14	[+][a]	[+]	-	-
Albumin	+	+/-[c]	+	-
AFP	+	+	-	-
GGT	+	+	-	+
OV-6	(+)[b]	+	-	+
OV-1	(+)	+	-	+
BD1	-	-	-	+
OC.2	+	+	-	+
OC.3	+	+	-	+
H.1	-	-	+	-
H.2	+/-[b]	-	-	-
HBD.1	+	-	+	+

[a] [ ] = mRNA transcripts detected by Northern blotting and *in situ* hybridisation, but immunostaining negative

[b] ( ) immunostaining weakly positive

[c] +/- = doubtful positivity of immunostaining

Haematopoiesis and hepatic development share common stages. During fetal development, haematopoietic stem cells move out of the yolk sac and into the developing liver (Kelemen *et al.*, 1987; Sharp *et al.*, 1987). In humans, haematopoiesis can be detected as early as 6 weeks of gestation, expanding exponentially for a few days, then stabilising with a doubling time of 2 days (Paul *et al.*, 1969). The liver remains haematopoietic during the entire fetal period and for approximately the first week after birth in the neonate (Borghese, 1959). Simultaneous with the appearance of haematopoiesis, haematopoietic stem cells can be detected in the fetal liver. This pool of haematopoietic stem cells remains fairly constant throughout fetal life, but the concentration appears to drop as the liver grows to increase its mass. In the latter part of gestation and after birth, the haematopoietic function of the liver is considerably reduced, if not totally absent. As the liver loses its haemopoietic ability, the concentration of stem cells declines. Although the liver loses its haematopoietic functions, hepatic extramedullary haematopoiesis may return in some adult disease states, e.g. myeloproliferative disorders.

There may be a closer relationship between the liver parenchyma and the haematopoietic system than previously thought. Our group has recently shown that in developing human liver haematopoietic stem cell markers CD34 and c-kit are co-expressed in ductal plate cells in a pattern similar to the early cytokeratin markers CAM 5.2 and CK 18 (Blakolmer *et al.*, 1995). Both stem cell factor (SCF) and its c-kit receptor are expressed by early liver progenitor cells in the rat liver during embryonic development and following liver injury (Fujio *et al.*, 1994; Fujio *et al.*, 1996). Similarly, proliferating oval cells in treated livers express the haematopoietic stem cell marker Thy-1 in addition to traditional oval cell markers (AFP, GGT, OV-6, CK-19, and OC.2) (Petersen *et al.*, 1998a). Most recently, cross-sex or cross-strain bone marrow and whole liver transplantation experiments were used to test the hypothesis that oval cells and other liver cells may arise from bone marrow cells during liver regeneration (Petersen *et al.*, 1999). Using markers for Y chromosome, dipeptidyl peptidase IV enzyme, and L21-6 antigen, the investigators were able to show that a proportion of the regenerated hepatic cells were donor derived, thus proving that a stem cell associated with the bone marrow has epithelial lineage capability.

3.4. Normal biliary tree and the stem cell compartment in the adult liver

Periportal hepatoblasts give rise to bile ducts during liver development and, in the adult, the bile ducts remain connected to the hepatocyte parenchyma by the complex anastomoses of the bile canaliculi (Golding *et al.*, 1996). The canalicular network drains the bile produced by the hepatocytes to the portal tract interface. The bile passes through cholangioles composed of hepatocytes and specialised duct cells (Steiner and Carruthers, 1961) and, more distally, cholangioles lined exclusively by squamous ductular cells. From here bile passes into the peripheral or marginal interlobular ducts, and then into larger septal bile ducts (Millward-Sadler and Jezequel, 1992), and ultimately into the duodenum via the extrahepatic bile ducts.

The precise location of the hepatic stem cell compartment in the adult liver is still unclear, but there are several possibilities. Cells located within or in contact with the portal stroma (Yavorkovsky *et al.*, 1995); small nondescript cells around the cholangioles, the 'periductular cells' (Sell, 1993a; Sell and Salman, 1984); the cholangioles (canals of Hering or terminal bile ductules) (Sell, 1990; Sell, 1993a; Grisham and Porta, 1964; Lemire *et al.*, 1991; Factor *et al.*, 1994); and small interlobular ducts (Golding *et al.*, 1995; Alison et al., 1996; Nomoto *et al.*, 1992; Anilkumar *et al.*, 1995) have all been proposed as candidates for the site of stem cells. It is however also possible that any component of the intrahepatic biliary tree can give rise to oval cells (Golding *et al.*, 1995, Lenzi *et al.*, 1992).

3.5. The role of oval cell proliferation during liver regeneration

Rats have been used most extensively to generate experimental models with which to examine the development and outcome of oval cell proliferation. The most commonly used models in rats are produced by (i) treatment with azo dyes (Inaoka, 1967; Rogers, 1978); (ii) feeding of a choline-deficient diet, with or without supplements of ethionine (Shinozuka *et al.*, 1978) or 2-acetylaminofluorene (Sell *et al.*, 1981); (iii) treatment with 2-acetylaminofluorene and partial hepatectomy (AAF/PH) (Tatematsu *et al.*, 1985); (iv) and treatment with D-galactosamine (Lemire *et al.*, 1991). Central to all these experimental models is the extensive destruction and/or compromised function of hepatocytes, coupled with the apparent inability of the residual hepatocytes to proliferate (Solt and Farber, 1976). The common cellular response to all of these regimens is the proliferation of oval cells, also known as ductular cells, ductular oval cells, or ductular epithelial cells. A schematic diagram of the emergence and evolution of oval cells is shown in Figure 3.2.

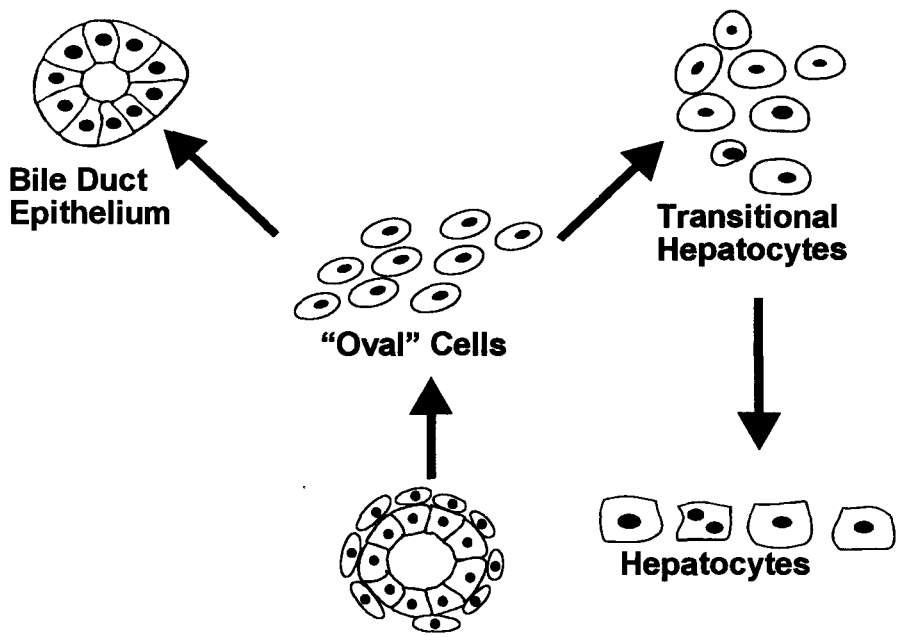

Figure 3.2. Schematic diagram illustrating the hypothesis that oval cells develop from facultative stem cells (bipotential progenitor cells) located in/near the canals of Hering (modified from Grisham and Thorgeirsson, 1997).

Oval cells originate in portal zones in the regions of terminal bile ductules and then rapidly invade the entire liver lobule. As they migrate through the parenchyma, oval cells proliferate rapidly, with labelling rates after pulse doses of ^{3}H-thymidine of 5 - 20% during peak proliferation (Sell *et al.*, 1981; Lemire *et al.*, 1991). As oval cells proliferate, individual and small groups of small, intensely basophilic hepatocytes typically appear among them, after which the oval cells disappear and the parenchyma is gradually reconstructed.

Proliferating oval cells comprise a heterogeneous, expanding population of cells, which has been termed an *oval cell compartment* (Fausto *et al.*, 1992). Ultrastructurally, individual oval cells closely resemble cells that form terminal bile ductules (Lenzi *et al.*, 1992; Sarraf *et al.*, 1994), and, with other oval cells, they form irregular duct-like structures that enclose lumens which connect to adjacent, pre-existing bile ducts (Dunsford *et al.*, 1985; Sarraf *et al.*, 1994). Based on ultrastructural characteristics, the oval cell compartment can be further subdivided into subpopulations, based on degree of differentiation (Sell, 1998). Oval cells express some markers of normal intrahepatic biliary epithelium (Sirica *et al.*, 1990; Lemire *et al.*, 1991; Lenzi *et al.*, 1992), as well as phenotypic properties that distinguish them from biliary epithelial cells (TABLE 3.1). A subset of oval cells expresses mRNA for AFP (Lemire *et al.*, 1991; Fausto *et al.*, 1992), and reacts with antibodies to AFP (Onoé *et al.*, 1973; Germain *et al.*, 1985; Marceau, 1990; Fausto *et al.*, 1992). Similarly, variable numbers of oval cells express albumin mRNA and bind antibodies to albumin (Dabeva and Shafritz, 1993), and some individual oval cells express both AFP and albumin simultaneously (Alpini *et al.*, 1992; Dabeva and Shafritz, 1993). Oval cells also express the bile duct type cytokeratins CK8, CK18 and CK19 (Germain *et al.*, 1985; Alpini *et al.*, 1992 Lemire *et al.*, 1991; Lenzi *et al.*, 1992) and GGT (Sirica and Cihla, 1984; Yokoyama *et al.*, 1986; Marceau *et al.*, 1992; Fausto *et al.*, 1993). Recently, subpopulations of oval cells have been identified in CDE-treated rat liver that express fetal hepatocyte markers (π class glutathione *S*-transferase, M_2-pyruvate kinase) with or without adult hepatocyte (α- glutathione *S*-transferase, L-pyruvate kinase) and bile ductular markers (CK19) (Tee *et al.*, 1996). Oval cells express several of the other antigenic markers that have been shown to react with either hepatocytes or biliary epithelial cells of adult rat livers (TABLE 3.1). OV-6 monoclonal antibody shows strong cytoskeletal staining of bile duct and oval cells, some nodular hepatocytes, and some hepatocellular carcinomas (Dunsford and Sell, 1989; Faris *et al.*, 1991).

Further evidence of *in vivo* differentiation of oval cells into hepatocytes comes from studies combining lineage markers with cell labelling, usually by tagging of replicating DNA with ^{3}H-thymidine. Although fraught with problems that complicate interpretation, labelling studies may help trace the fates of differentiating cells, including oval cells, that change their phenotype. Using the AAF/PH model, Evarts *et al.* (1989) combined ^{3}H-thymidine tagging with the use of phenotypic markers to follow the fate of oval cells. The phenotypic properties expressed by oval cells gradually merged into properties that were typical of hepatocytes, and this change in differentiation was correlated with the transfer of label from oval cells to basophilic hepatocytes (Evarts *et al.*, 1989). Specifically, oval cells expressing several markers of fetal liver epithelial cells, including CK7 and CK19, reactivity to OV-6 antibody, AFP and albumin (weakly), were heavily tagged with a pulse of ^{3}H-thymidine. Residual hepatocytes were not tagged by this pulse, but ^{3}H-thymidine-tagged small hepatocytes later appeared in basophilic foci; these ^{3}H-thymidine-tagged small hepatocytes expressed higher levels of albumin and much lower levels of AFP than did oval cells and, in addition, they expressed other markers of hepatocyte differentiation (Evarts *et al.*, 1989). Employing the galactosamine model, Lemire *et al.* (1991) also combined phenotypic marking of oval cells and hepatocytes with the assessment of time-dependent ^{3}H-thymidine labelling of both cell types. Shortly after administration of ^{3}H-thymidine, label was found in oval cells that expressed CK7 and CK19, GGT, 2.1 kb mRNA for AFP, and bound peanut agglutinin. ^{3}H-thymidine-labelled small hepatocytes expressing albumin and other hepatocytic markers appeared subsequently, together with the loss of the biliary epithelial phenotype (Lemire *et al.*, 1991). Dabeva and Shafritz (1993) analysed the simultaneous occurrence of a programme of hepatocyte differentiation in oval cells and the transfer of ^{3}H-thymidine between oval cells and hepatocytes in the galactosamine model in rats. Tagged oval cells initially expressed fetal AFP mRNA and GGT; during the next few days AFP declined while the expression of albumin and glucose-6-phosphatase increased, as the tagged cells concurrently acquired the morphology of small hepatocytes. During late stages of recovery from galactosamine toxicity, morphologically and phenotypically identifiable hepatocytes also proliferated to augment the production of new hepatocytes. The authors concluded that in the galactosamine model the hepatocyte population is replaced by both differentiation of oval cells and by proliferation of the residual and newly formed hepatocytes (Dabeva and Shafritz, 1993

3.6. Interactions between oval cells and hepatic stellate (Ito) cells

The proliferation of mesenchymal cells in conjunction with oval cells has long been known (Popper *et al.*, 1957). Evarts *et al.* (1993) showed that desmin-positive hepatic stellate (Ito) cells represent an important category of mesenchymal cell intimately involved in the early stages of oval cell proliferation. The earliest cells that proliferate in the AAF/PH model are OV-6 antibody-reactive epithelial cells, as shown by combined ^{3}H-thymidine tagging and immunohistochemistry (Evarts *et al.*, 1993). Both OV-6 positive and desmin positive mesenchymal cells are labelled with ^{3}H-thymidine within four hours after starting the AAF/PH regimen. ^{3}H-thymidine tagged cells are identified as individual desmin positive cells embedded in the portal connective tissue matrix, or as OV-6 positive cells in biliary ductules located in close proximity to branches of the portal vein. These observations showed that the earliest cycling epithelial cells that lead to oval cell proliferation are closely associated with mesenchymal cells, particularly stellate cells.

Interactions between stem cells/oval cells and hepatic stellate cells are mediated by growth factors. Coincident with the initiation of DNA synthesis in OV-6 positive and desmin-positive cells in portal tracts, expression of transforming growth factor alpha (TGF-α), hepatocyte growth factor (HGF) and acidic fibroblast growth factor (aFGF) is observed, and expression of transforming growth factor beta 1 (TGF-β1) begins within 24 hours (Evarts *et al.*, 1990; Nakatsukasa *et al.*, 1991; Marsden *et al.*, 1992; Hu *et al.*, 1993). This group of growth factors continues to be expressed at high levels throughout the period of expansion and differentiation of the oval cell population. Transcripts for TGF-α and aFGF are expressed by both oval cells and hepatic stellate cells (Evarts *et al.*, 1992; Marsden *et al.*, 1992), whereas transcripts for HGF are expressed only by stellate cells (Hu *et al.*, 1993). Transcripts for TGF-β1 are also highest in hepatic stellate cells, although the earliest population of oval cells also expresses lower levels of TGF-β1 mRNA (Nakatsukasa *et al.*, 1991). Oval cells express receptors for all of these growth factors (Lenzi *et al.*, 1992; Marsden *et al.*, 1992; Hu *et al.*, 1993), providing a molecular pathway by which hepatic stellate cells may influence the growth and development of oval cells. Production of matrix proteins by stellate cells may be another mechanism by which they interact with oval cells.

The stem cell factor/c-kit (SCF/c-kit) ligand/receptor system is also involved in the earliest stages of liver stem cell activation and oval cell proliferation (Fujio *et al.*, 1994). In

the AAF/PH model, expression of SCF/c-kit occurs as early as the expression of AFP transcripts, and the levels of SCF/c-kit transcripts reach a peak and decline prior to that of the other growth factors. Individual oval cell precursors express both SCF and c-kit, providing the basis for autocrine stimulation. The SCF/c-kit signal transduction system is believed to play a fundamental role in the survival, proliferation and migration of stem cells during gametogenesis, melanogenesis and haematopoiesis (Morrison-Graham and Takahashi, 1993). Nevertheless, mice which have a mutation for either SCF or c-kit, still respond to bile duct ligation with bile duct proliferation and formation of new bile ducts (Omori *et al.*, 1997). This may be due to the compensatory/additive function of other early acting stem factors, such as the flt-3 ligand (FL)/flt-3 system (Omori *et al.*, 1997).

3.7. Properties of nonhepatocytic (stem-like) epithelial cells isolated from the liver

In addition to the *in vivo* data discussed above, significant support for the existence of a hepatic stem cell has come from results obtained in studies on hepatic cell cultures. Isolation and establishment of long-term cultures of small, morphologically and functionally simple epithelial cells by enzymatic perfusion of normal rat liver (fetal and adult) have been accomplished by several investigators (Tsao *et al.*, 1984; Tsao and Liu, 1988, McMahon *et al.*, 1986; Hampton *et al.*, 1990; Hugget *et al.*, 1991). These rat liver-derived epithelial (RLE) cells are morphologically similar, being small cells (9 - 12 μm in diameter) that grow in closely packed, regular monolayers. The functional and phenotypic properties of these cells lines have been extensively characterised (Grisham and Thorgeirsson, 1997), and while RLE cells share some phenotypic properties with both bile duct epithelial cells and hepatocytes, they are phenotypically much closer to oval cells.

Because of the phenotypic similarities of oval and RLE cells, it has been suggested that RLE cells are also derived from hepatic stem cells. This suggestion is supported by data obtained from extensive use of RLE cells for *in vitro* transformation studies with both chemical carcinogens and oncogenes (see Chapter 4). In addition, a recent insight into the nature of the RLE cells and the potential role of hepatic stem cells in liver biology has been

provided by tagging and transplantation experiments. Coleman *et al.* (1993) genetically tagged RLE cells with the *E. coli* β-galactosidase reporter gene and demonstrated that, following transplantation into the livers of syngeneic rats, the RLE cells integrated into hepatic plates and acquired the size and nuclear structure of mature hepatocytes. Furthermore, hepatocytes and other epithelial cells derived from intrahepatic transplants of the tagged RLE cells could be recovered from the livers of recipient rats, and the liver epithelial cells that were re-established in culture were morphologically identical to the original RLE cells (Grisham *et al.*, 1993). Such tagging and transplantation studies have however not yet provided evidence for the ability of RLE cells to differentiate into bile duct epithelium.

3.8. Conclusions

There now exists a strong body of evidence for the presence of facultative stem cells in the adult liver. The question currently dividing many is no longer whether stem cells exist, but what their exact role is in the liver. Increased understanding of the cellular and molecular biology of the hepatic stem cells and their progeny is likely to have significant impact on diverse areas of clinical hepatology, including hepatocarcinogenesis, artificial liver support, and gene therapy.

Chapter 4
The role of cell types in hepato- and cholangiocarcinogenesis

4.1. Introduction

Cancer must arise from a cell that has the potential to divide (Cohen and Ellwein, 1990). The two major nonexclusive hypotheses of the cellular origin of cancer are that malignancy arises (i) from stem cells due to maturation arrest or (ii) from dedifferentiation of mature cells that retain the ability to proliferate (Sell, 1993). There is no doubt that the hepatocyte frequently is the progenitor cell for liver tumours (Farber, 1992). The involvement of oval cells in the genesis of liver tumours, particularly hepatocellular carcinomas, is still hotly debated. On the one hand, Sell and Pierce (1994) have proposed that "the cell of origin of liver cancer is the putative liver stem cell or its progeny, the transitional duct cell" (Sell and Pierce, 1994). Alternatively, Farber (1992) has stated that "rare original mature hepatocytes in zone 1, 2, or 3 of the adult liver appearing after initiation with genotoxic carcinogens have been shown to be the cell of origin for foci or islands of altered hepatocytes and of nodules derived from these foci."

4.2. The resistant hepatocyte model of experimental hepatocarcinogenesis

The liver serves as an excellent model in studies of cell growth regulation and carcinogenesis, because the normal cell cycle activity is low, with a growth fraction of only 0.02%. Cell replication is increased dramatically by mitogenic stimuli, such as partial hepatectomy, leading to growth fractions of 90%, and regeneration of normal hepatocytes can be blocked by mitoinhibitors such as 2- acetylaminofluorene (AAF). Several liver models for the study of

carcinogenic processes have been developed. The 'resistant hepatocyte' (RH) model was designed by Solt and Farber (1976) to cause rapid (and synchronised) development of foci of altered hepatocytes, nodules, and cancers. As explained by the 'initiation-promotion' hypothesis of carcinogenesis, cells are 'initiated' by a non-necrotic dose of the genotoxic agent diethylnitrosamine (DEN), followed 2 weeks later by a 2-week feeding of AAF, with a partial hepatectomy (PH) performed after 1 week of AAF feeding. The AAF is given to inhibit proliferation of the non-initiated hepatocytes that would other wise be stimulated by the PH, thus allowing the growth stimulus of the PH ('promotion') to act on the DEN-initiated, growth inhibition-resistant cells. In the modified Solt-Farber regimen, the 'initiation' step of DEN injection is omitted. Although the initiation-promotion model is probably an artefact of the laboratory (Cohen, 1998), the Solt-Farber and modified Solt-Farber regimens have nevertheless become the standard methods for the study of experimental hepatocarcinogenesis in the rat.

4.3. Chemical carcinogenesis and premalignant lesions: the role of initiated hepatocytes

One penultimate site of origin of hepatocellular carcinoma in chemical carcinogenesis in the rat is the *persistent hepatocyte nodule* (Farber and Sarma, 1987; Bannasch *et al.*, 1989). This small population is derived from a much larger population of early nodules, the majority of which appear to remodel in a process of redifferentiation. This population of early nodules, in turn, arises from islands of *"enzyme altered" hepatic foci* that have a characteristic spectrum of biochemical changes as part of their phenotype (Farber and Sarma, 1987; Bannasch *et al.*, 1989). These changes include the expression of γ-glutamyl transpeptidase (GGT) and pi class glutathione *S*-transferase (GST-pi) (Moore *et al.*, 1987; Satoh *et al.*, 1989). Early studies with the RH model indicated an origin of foci from hepatocytes (Solt and Farber, 1976; Solt *et al.*, 1977). These could be seen as changes in the appearance of liver parenchymal cells scattered randomly throughout the liver in the three zones of the liver acinus without any apparent geographic relationship to the very early proliferation of oval cells. In several reports since then, it has been proposed that HCC induced by chemical agents may arise in the liver

from oval cells, speculated to be the progeny of liver stem cells. Gindi *et al.* (1994) thus re-examined the cellular origin of the foci, placing reliance on material (linear) continuity between cells rather than the use of phenotypic markers. Two major parameters were examined: (i) the zonal localisation of the foci and oval cells; and (ii) the time of appearance of foci and oval cells after PH. They found no correlation between oval cell proliferation and the time and site of first appearance of foci. The authors concluded that the origin of foci from hepatocytes and the dissociation between their appearance, growth and the proliferation of oval cells was obvious and striking (Gindi *et al.*, 1994).

Anilkumar *et al.* (1995) exploited the antiproliferative effect of AAF in the RH model of carcinogenesis, which results in a co-proliferation of oval cells and initiated hepatocytes, in order to clarify the relationship of oval cell proliferation to the development of early hepatocyte nodules. Using a panel of monoclonal antibodies directed against intermediate filaments, the authors showed that the so-called oval cell response in the first few days after PH in the RH model was in fact a prominent ductular reaction, apparently emanating from portally located bile ducts (Anilkumar *et al.*, 1995). These cells strongly expressed cytokeratins 8 and 19 and vimentin, and from 1 week after PH, they frequently underwent differentiation either into hepatocytes, expressing cytochrome P450 enzymes, or into intestinal-type cells. Five days after PH, numerous basophilic foci were discernible, and these foci expanded rapidly. The ductular cells swirled around the foci, but their antigenic profile clearly indicated that these cells were not involved in the development of the early nodules. The authors concluded that early foci and nodules in the RH model are derived from resistant (initiated) hepatocytes and not ductular (oval) cells, the latter being a facultative multipotential stem cell compartment (Anilkumar *et al.*, 1995).

4.4. Chemical carcinogenesis and oval cell proliferation: the role of liver stem cells

An important role for liver nonparenchymal epithelial cells was provided by Farber (1956), who documented a detailed description of the early histological changes during hepatocarcinogenesis caused by three chemical carcinogens. The carcinogens ethionine, 2-acetylaminofluorene (AAF), and 3'-methyl 4-dimethylaminoazobenzene (Me-DAB), in spite of

being structurally very different, caused similar histological alterations. The common features included (i) oval cell proliferation which progressively involved most of the liver lobule, beginning in the portal areas, (ii) degenerative and hypertrophic changes in hepatocytes adjacent to proliferating oval cells, and (iii) nodular regenerative hyperplasia of liver cells. There were, however, important differences in the time course of appearance and fate of the oval cells induced by these three hepatocarcinogens. Whereas oval cells appeared early following ethionine and AAF administration (7 and 14 days respectively), their appearance occurred significantly later after Me-DAB treatment (first seen at day 21). More importantly, the fate of the oval cells in the Me-DAB treated animals were different from those induced by ethionine and AAF. In the early stages the oval cells induced by Me-DAB were morphologically indistinguishable from those generated by ethionine and AAF. However, at later stages areas of apparent transition between oval cells and hepatocytes were numerous in the Me-DAB treated animals but absent in those receiving ethionine and AAF (Farber, 1956). Since then it has been established that many different chemical compounds capable of producing liver tumours in rats and mice, induce a similar sequence of histological changes in which oval cell proliferation is prominent (Dunsford *et al.*, 1985). If the transition from oval cells to hepatocytes can be morphologically observed after Me-DAB treatment, then it is in principle established that oval cells have the capacity to differentiate into hepatocytes. Furthermore, the fact that a large population of oval cells is cycling during the early stages of chemical hepatocarcinogenesis and that these cells can differentiate into hepatocytes strongly suggests that at least a percentage of the HCCs are derived from oval cell progenitors (Thorgeirsson, 1995).

In recent years there has been accumulating experimental evidence in support of this notion. Hixson and his colleagues (Hixson *et al.*, 1990; Faris *et al.*, 1991) used a battery of monoclonal antibodies specific for antigens associated with bile duct cells, oval cells and fetal, adult, and neoplastic hepatocytes to analyse the phenotypic relationship between oval cells, foci, nodules, and carcinomas during chemical hepatocarcinogenesis. These investigators found, using the RH model, that oval cells, GGT-positive hepatocellular foci, persistent hepatocyte nodules, and primary HCCs express both oval cell and hepatocyte antigens. This finding indicates a precursor-product relationship between oval cells and carcinomas. Similar results were obtained by Dunsford *et al.* (1989) using different monoclonal antibodies raised against oval cells. These lineage relationships between oval cells and HCCs also exist in other

models of liver carcinogenesis. For example, animals maintained on a choline deficient and ethionine (CDE) diet display markers for oval cells and hepatocytes in a significant percentage of nodules and HCCs (Hixson *et al.*, 1990; Faris *et al.*, 1991). Also, metastatic tumours in the lung from the animals harbouring these liver tumours show essentially the same phenotype (Hixson *et al.*, 1990).

The most direct evidence that oval cells can progress to HCCs comes from *in vitro* transformation of rat liver-derived epithelial (RLE) cells (Yoshimura *et al.*, 1983; Tsao *et al.*, 1984; Braun *et al.*, 1987; Garfield *et al.*, 1988; Germain *et al.* 1988b; Fausto 1990b). These simple epithelial cells share some phenotypic properties with both bile duct epithelial cells and hepatocytes, but are phenotypically much closer to some of the oval cell lines (Grisham and Thorgeirsson, 1997). Spontaneous transformation of RLE cells, as well as transformation with chemical carcinogens and dominant oncogenes, results in the tumours displaying a wide range of phenotypes (Tsao and Grisham, 1987; Garfield *et al.*, 1988; Fausto, 1990; Marceau, 1990). When the transformed RLE cells are transplanted into either syngeneic rats or nude mice, a spectrum of tumours is observed that includes highly differentiated HCCs, hepatoblastomas, cholangiocarcinomas, as well as mixed epithelial-mesenchymal tumours (Tsao and Grisham, 1987), demonstrating both the blastic nature of the RLE cells and the potential of the cells to differentiate via both the hepatocytic and biliary lineages (TABLE 4.1).

TABLE 4.1

Classification of tumours produced by chemically transformed rat liver epithelial cells (From Tsao and Grisham, 1987).

Classification of tumours	**Number**
Carcinomas (epithelial)	54
Epidermoid	15
Adenocarcinoma	13
Hepatocellular	4
Poorly differentiated/anaplastic	22
'Sarcomas' (mesenchymal)	19
'Mixed epithelial-mesenchymal' tumours	30
Unclassified	22

4.5. Cellular origin of human hepatocellular carcinoma

Delineation of the sequence of preneoplastic cellular changes and characterisation of the multicellular lesions that precede the emergence of hepatocellular carcinoma (HCC) is less complete in humans than in rodents, because it is more difficult to determine when the process of hepatocarcinogenesis is 'initiated' by natural disease in humans than by experimental manipulation in laboratory rodents (Grisham, 1996). Identification of hepatitis B virus (HBV) and hepatitis C virus (HCV) as aetiologic agents for HCC, together with the availability of sensitive and specific methods to detect the effect of these viruses on hepatocytes, has made it possible to begin to define the cellular pathogenesis of HCC in humans. Preneoplastic cellular changes have been sought most intensively in cirrhotic livers, since most primary liver cancers in humans occur in this setting (Kew and Popper, 1984; Vandersteenhoven *et al.*, 1990). Analysis of preneoplastic changes in humans has emphasised classic morphologic changes of cellular dysplasia (Watanabe *et al.*, 1982; Thomas *et al.*, 1992), and the presence of dysplastic hepatocytes in cirrhotic nodules is correlated with increased risk of HCC in prospective studies (Borzio *et al.*, 1995). The larger cirrhotic nodules (macroregenerative nodules) are an important site of HCC development in human cirrhotic livers (Ferrell *et al.* 1993; International Working Party, 1995). HCCs appear to emerge in populations of aberrant, but non-neoplastic, hepatocytes contained in cirrhotic nodules or multiacinar nodules through the clonal proliferation of new populations that express additional aberrations, eventuating ultimately in frank neoplasia (Grisham, 1996). Hyperplastic nodules containing aggregates of dysplastic hepatocytes, similar to cirrhotic nodules, but not embedded in collagen, are also thought to be the site of cell proliferation and HCC development in noncirrhotic livers affected by chronic hepatitis (Esumi *et al.*, 1986; Unoura *et al.*, 1993).

The cellular elements of an alternative pathway to the development of HCC involving the proliferation and differentiation of stem-like ('oval') cells have been described. In a study on 14 HCC resection specimens from China, Hsia *et al.* (1992) observed the proliferation of a new population of epithelial cells in actively regenerating nodules and in liver tissue surrounding the cancers. These 'oval-like' cells stained strongly positive for cytokeratin 19 and with OV-6 monoclonal antibody against rat oval cells. Oval cells and transitional types of cells appeared to be the principal producers of AFP in the regenerating liver (Hsia *et al.*, 1992). In a subsequent study on 26 hepatocellular carcinoma resection specimens, oval cells

and transitional cells were shown to contain HBsAg and/or HBcAg by immunohistochemistry (Hsia *et al.*, 1994). In contrast with these findings, immunohistochemical staining of 14 surgical resections for hepatocellular carcinoma in noncirrhotic HBV-negative livers from South Africa (Lemmer *et al.*, 1998b) did not demonstrate any proliferation of OV-6 positive "oval-like" cells in non-neoplastic liver tissue surrounding the tumours. The interpretation of these results is that proliferation of oval-like cells in human liver tissue surrounding HCCs is related to the presence of underlying chronic hepatitis and cirrhosis. Furthermore, as in rodents, it is possible that potential cellular pathways to development of HCC in humans may involve either differentiated hepatocytes or stem-like cells.

Further support for the role of stem cells in human hepatocarcinogenesis comes from isolated reports of progenitor cell tumours in human liver. Robrechts *et al.* (1998) recently described a primary liver tumour composed of an immature cell type, displaying features of both hepatocytes and bile duct epithelial cells, and associated with a very rapid clinical course. The intermediate nature of the tumour, as well as the clinical course, was interpreted by the authors as an indication of an immature progenitor cell being the possible cell of origin for the tumour. Ruck *et al.* (1996; 1997), using electron microscopy and immunohistochemistry, described a population of small epithelial cells in human hepatoblastoma that exhibit ultrastructural features of oval cells seen in rats. Small epithelial cells were found to co-express markers for oval cells (OV-1 and OV-6), hepatocytes (albumin) and bile duct cells (cytokeratin 7), suggesting that these cells are closely related to a putative bipotent stem.

4.6. Cholangiofibrotic lesions and cellular origin of cholangiocarcinoma

Bile duct proliferative lesions (cholangiofibromas) have been reported experimentally following treatment with a variety of carcinogens, including coumarin (Evans *et al.*, 1989), polycholorinated biphenyls (Kimbrough *et al.*, 1972), butter yellow (Opie, 1944), congeners of butter yellow, thioacetamide, N,N'-nitrosomorpholine (Bannasch and Massner, 1976), 2-acetylaminofluorene (Teebor and Becker, 1971), methapyrilene (Ohshima *et al.*, 1984), and aflatoxin (Butler, 1965). These puzzling lesions, which do not have an exact counterpart in humans, are characterised histologically by focal proliferation of ducts containing luminal

debris and eosinophilic material, surrounded by dense fibrosis, and accompanied by a heavy inflammatory cell infiltrate (Evans *et al.*, 1989). Although cholangiofibrotic lesions commonly persist throughout the lifetime of the host without further evolution (Edwards and White, 1941), this lesion is considered to be a precursor for cholangiocarcinoma with some carcinogens, such as furan (Elmore and Sirica, 1993) and fumonisin B_1 (Gelderblom *et al.*, 1991), and spontaneously in Long-Evans Cinnamon (LEC) rats with hereditary hepatitis (Masuda *et al.*, 1988). Syrian golden hamsters fed dimethylnitrosamine (DMN) and infected with *Clonorchis sinensis* rapidly develop florid ductular proliferation, cholangiofibrosis, and cholangiocarcinoma (Lee *et al.*, 1993; Lee *et al.*, 1995), whereas ductular proliferation and periductal fibrosis without cholangiocarcinoma are seen after infection with the liver fluke alone. Although there is a paucity of published studies on the role of cell types in cholangiocarcinogenesis, there is some evidence that 'stem' cells may be involved. A review of the morphologic, autoradiographic, and phenotypic analysis of the cellular changes during different AAF-containing regimens supported a stem cell origin for both hepatocellular and cholangiocarcinomas induced by chemical carcinogens (Sell and Dunsford, 1989). And in Syrian golden hamsters fed dimethylnitrosamine and infected with *Clonorchis sinensis*, oval cells were thought to be the precursors of dysplastic duct cells that gave rise to cholangiocarcinomas (Lee *et al.*, 1997).

The aetiological factors for bile duct carcinoma in humans are far less well established than for HCC. Strong clinical associations have been found between cholangiocarcinoma and cystic abnormalities of the bile duct (Todani *et al.*, 1979), infestation with the liver flukes *Clonorchis sinensis* and *Opisthorchis viverrini* (Hou, 1956), gallstones (Jones, 1950), primary sclerosing cholangitis (Rosen and Nagorney, 1991), the radiocontrast agent thorium dioxide (Thorotrast) (Ito *et al.*, 1988), and intrahepatic calculi (hepatolithiasis) (Sato *et al.*, 1991). A common feature of these seemingly disparate predisposing conditions appears to be prolonged bile stasis and/or chronic inflammation, which result in prominent bile duct proliferation (Hou, 1956; Haswell-Elkins *et al.*, 1994)..

Chapter 5

Materials and methods

5.1. Isolation of fumonisin B_1

Fumonisin B_1 (FB_1) was purified from maize cultures of *Fusarium moniliforme* strain MRC 826 according to a method described previously (Cawood *et al.*, 1991). Extraction of FB_1 was achieved with CH_3OH/H_2O (3:1) followed by a solvent-partitioning step using $CHCl_3$. The subsequent purification of the aqueous phase was effected on Amberlite XAD-2, silica gel, and reverse-phase (C_{18}) chromatographic columns. The purity as compared to an analytical standard by high performance liquid chromatography (HPLC) (Alberts *et al.*, 1993) was in the order of 92-95%. The monomethylester derivatives of FB_1, which are artifacts of the purification procedure (Cawood *et al.*, 1991), constituted the remainder of the FB_1 preparation.

5.2. Animals and diets

All the studies were approved by the Ethics and Research committee of the University of Cape Town, and the experiments were conducted in accordance with the laws and regulations controlling experiments on live animals in South Africa. Male Fischer 344 rats weighing between 150 and 200 g were used in all the experiments. The animals were caged individually in a controlled environment at 23 - 24°C and 50% humidity with a 12 h artificial light cycle. Food and water were available *ad libitum*, and rats were weighed weekly. All the rats received the AIN-76 diet (American Institute of Nutrition, 1980) with the following modifications: the maize starch was replaced with glucose/sucrose/maize starch (1:1:1) while sunflower oil was used instead of maize oil as a fat source. Maize products were excluded from the control diet in order to prevent any possibility of contamination by *F. moniliforme*. The cellulose was donated by Sappi Saicor, Umkomaas, Natal, South Africa.

5.2.1. Fumonisin B_1 -containing diet

Gloves and masks were worn for protection while preparing and administering fumonisin-containing diets. The FB_1-containing diet (250 mg FB_1/kg diet) was prepared as follows: FB_1 stock sample dissolved in methanol (50 ml) was evaporated onto a subsample (200 g) of the diet, whereafter it was dried in a fume hood at room temperature for 12 h (Gelderblom *et al.*, 1994). Subsequently the subsample was thoroughly mixed into the diet (6 kg) to obtain the desired concentration of FB_1. The control diet was treated in a similar way using only an equal volume of methanol. Each diet was prepared in 6 kg quantities at a time and stored at 4°C until used.

5.2.2. Acetylaminofluorene-containing diet

The 0.02% 2-acetylaminofluorene (AAF)-containing diet was prepared by mixing 0.2 g AAF (Sigma Chemical Co., St. Louis, MO) per kg AIN diet. Gloves and masks were worn for protection during the preparation of AAF-supplemented diets.

5.2.3. Iron-supplemented diet

The 1% (wt/wt) iron-supplemented diet was prepared by mixing 10 g of carbonyl iron (Sigma Chemical Co., St. Louis, MO) per kg AIN-76 diet. The iron content of the control AIN-76 diet was only 35.7 mg iron per kg diet. Carbonyl iron is an extremely pure form of elemental iron (>98% iron with <0.8% carbon, <0.3% oxygen, and <0.9% nitrogen), prepared by reacting iron at high temperatures with carbon monoxide to form gaseous iron pentacarbonyl, $Fe(CO)_5$.

5.3. Liver histopathology

5.3.1. Light microscopy

For routine light microscopy, thin (4-5 mm) liver slices were immersion fixed in formalin for 24 h before processing and embedding in paraffin wax. Routine processing protocols were adapted to a 4 h cycle to prevent the tissue from becoming brittle. All sections were mounted

5.3.2.4. Alpha-fetoprotein (AFP)

For the detection of AFP, tissues were fixed for 4 h in Bouin's fixative and embedded in paraffin wax (Omori *et al.*, 1997a). Polyclonal rabbit anti-rat AFP (Accurate Chemicals, New York, NY) was used as primary antibody, and fetal rat livers were used as positive controls.

5.3.2.5. Immunolocalization of mature TGF-β1 protein

Antibody to mature TGF-β1 protein was a generous gift from Dr. K. Flanders, National Cancer Institute, Bethesda, MD. Immunohistochemical staining for TGF-β1 was done on 5-μm deparaffinized sections with an indirect immunoperoxidase antiserum detection protocol (Elite kit; Vector Laboratories). Mature TGFβ1 protein was detected by the rabbit polyclonal LC (1-30) antibody, as previously described (Sanderson *et al.*, 1995).

5.3.3. Apoptosis

Cleavage of genomic DNA during apoptosis or programmed cell death yields double stranded low molecular weight DNA fragments (mono- and oligonucleosomes) as well as single strand breaks in high molecular weight DNA. Cells undergoing apoptosis were detected *in situ* by TUNEL-specific labelling of nuclear DNA strand breaks, as described by Gavrieli *et al.* (1992). In this reaction the enzyme deoxynucleotidyl transferase (TdT) is used to label the free 3'-OH terminal with biotinylated-dUTP.

Formalin fixed sections (5 μm) were deparaffinized and digested with 10 μg proteinase K (Boehringer Mannheim)/ml Tris/HCl, pH 7.4, for 15 min at room temperature. Endogenous peroxidase activity was blocked by treating the sections for 10 min with periodic acid in phosphate buffered saline (PBS) followed by a 10 min treatment with sodium borohydride in PBS. The sections were equilibrated for 5 min in reaction buffer (30 mM Tris/HCL pH 7.4, 140 mM sodium cacodylate, 1 mM cobalt chloride, 0.3 % Triton X-100) then incubated (60 min at 37°C in a humidified chamber) with 25 U TdT and 0.3 nM biotinylated dUTP. The reaction was stopped by washing the slides with PBS containing 500 μM EDTA. Biotin labelled DNA was detected using peroxidase conjugated Streptavidin as described above and fast red as substrate for the peroxidase. Haematoxylin was used as a counterstain.

5.4. Liver biopsy technique

Rat liver biopsies were performed under ether anaesthesia according to a modification of a method previously described (Cmielewski *et al.*, 1997). Once fully anaesthetised, the rat was placed on a diathermy plate, the paws were carefully strapped down, and the abdomen was shaved and swabbed with iodine. A small midline incision (2 - 3 cm) was made and the liver exposed (Figure 5.1A). Part of one lobe of the liver was removed using a scalpel, and bleeding from the resected hepatic bed was controlled by diathermy (Figure 5.1B). The abdominal incision was then sutured and the rat allowed to recover. A standard biopsy protocol was followed in all animals, commencing with the posteriorly situated left lobe, and rotating to the more accessible median lobe for repeat biopsy. On average, each rat underwent two liver biopsies during its lifetime before sacrifice.

5.5. Iron studies

5.5.1. Stainable hepatic iron

Perls' Prussian blue stain for trivalent iron was used to assess storage iron content (Williams *et al.*, 1962). Stainable iron in hepatocytes was graded 0 to 4, using a modification of the scale devised by Scheuer *et al.* (1962) (Table 5.1).

Table 5.1

Grading of stainable iron in hepatocytes (modified from Scheuer *et al.*, 1962).

Stainable iron in hepatocytes (%)	Grade
absent	0
less than 25%	1
25% to 50%	2
50% to 75%	3
75% to 100%	4

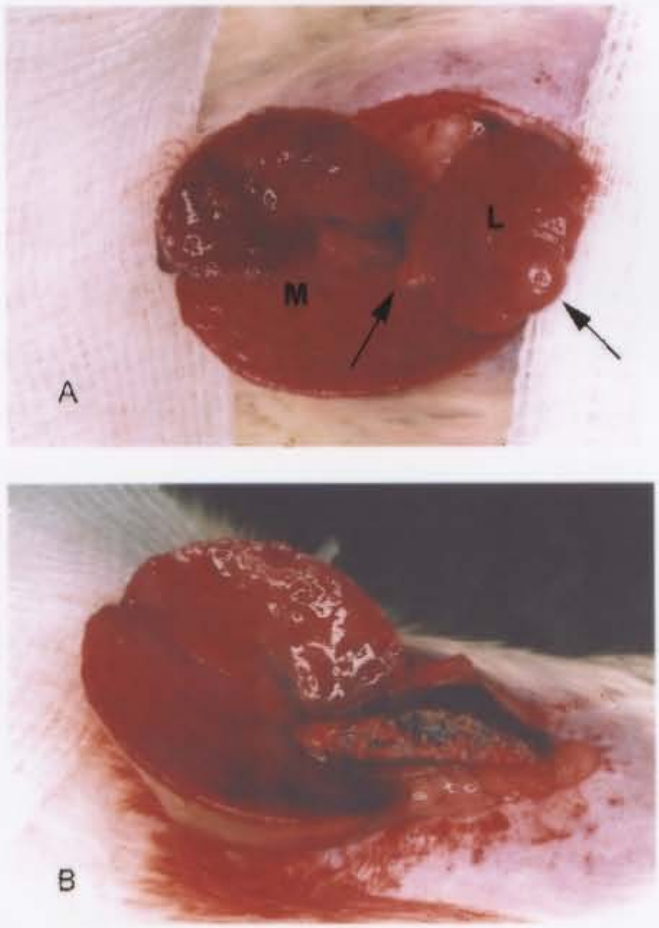

Figure 5.1. Technique for obtaining repeated open wedge liver biopsies from rats. (A) A small midline incision has been made to allow exposure of the posteriorly situated left lobe (L) as well as the more anteriorly situated median lobe (M). This rat has been treated with fumonisin B_1 for 15 weeks (see Chapter 8). The liver has a granular appearance, and multiple subcapsular hepatic nodules are seen in the left lobe (arrows). (B) Same liver as shown in Figure 5.1A following surgical liver biopsy from the left lobe and control of bleeding from the raw edge with diathermy. The head of the animal is on the right of the photograph. See text for details.

5.5.2. Hepatic iron concentration

Liver tissue for determination of iron content was dried for 24 h at 105°C, weighed, digested in 50% nitric acid at 70°C for 1 h, and diluted in 0.2 M sodium acetate buffer pH 4.5. All glassware was rendered iron free and rinsed with iron free water (Torrance and Bothwell, 1980). Iron concentration was determined using a Roche Unimate 5 Iron Kit (Roche Diagnostic Systems, Basel, Switzerland), on a Roche Cobas Fara II Centrifugal Analyser.

5.5.3. Hepatic peroxidative damage

Malondialdehyde (MDA) is formed when polyunsaturated fatty acids of membrane phospholipids undergo peroxidative decomposition. Lipid peroxidation was measured by the thiobarbituric acid (TBA) assay for malondialdehyde (MDA) concentration on samples of liver homogenate, as described by Esterbauer and Cheeseman (1990). Related substances such as sucrose, metal ions, and whole tissue homogenates may also react with TBA or influence the assay procedure, and the term thiobarbituric acid reacting substances (TBARS) more accurately describes the product of this assay (Esterbauer and Cheeseman, 1990). Liver samples were homogenised in 1.15% KCl containing 3 mM EDTA (pH 7.4) to obtain a 10 per cent solution. A mixture of 2 ml of the hepatocyte suspension (in saline) and 2 ml of TBA reagent, consisting of 20% trichloracetic acid (TCA), 0.67% TBA and 0.01% butylated hydroxytoluene (BHT) was incubated at 90°C for 20 min in a waterbath (Kinchington *et al.*, 1993). The samples were centrifuged at 3000 rpm for 10 min, 2 ml of the supernatant was combined with 2 ml of a 0.67% TBA solution and heated for 10 min in boiling water. The mixture was allowed to cool and the absorbance measured at 532 nm (Esterbauer and Cheeseman, 1990). Lipid peroxidation was expressed as nmol MDA equivalents per mg protein, using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ at 532 nm for MDA (Buege and Aust, 1978).

5.5.4. Morphometric analysis of hepatic foci and nodules

Liver sections (5 μm thickness) were stained with GST pi for determination of the number and size of 'enzyme altered' foci and 'premalignant' nodules. Counterstaining was omitted in order to maximize differences in contrast for computerised morphometric analysis. One section from each of the left, right, and median lobes were examined for each rat. The number of hepatic foci and nodules per section were counted, and were considered an estimate of the

cancer initiating effects of FB_1 and/or iron (Solt and Faber, 1976; Farber and Sarma, 1987). For the purposes of this study, a group of GST pi positive cells was classified as a 'focus' if it had an area of less than 100 μm^2, and a 'nodule' if it had an area of 100 μm^2 or greater. The area (%) of liver occupied by GST pi positive foci and nodules was determined using a computerised morphometric analyser (Optimas, Bothell, WA), and was considered a measurement of the cancer promoting effects of FB_1 and/or iron (Solt and Faber, 1976; Farber and Sarma, 1987).

5.6. Molecular studies

Northern (mRNA) analysis was performed on timed rat liver specimens in order to determine the the changes in expression of specific genes during feeding of FB_1. Poly(A)+ RNA was isolated from liver homogenate and separated according to size by electrophoresis through denaturing (formaldehyde) agarose gels. The mRNA was then transferred to nylon membranes by posiblotting, and visualized by autoradiography after hybridization to radiolabelled cDNA (antisense) probes.

5.6.1. Probes

Antisense riboprobes labelled with [^{32}P]CTP were utilized for each of the following. A 429 bp piece of the 5' end rat AFP complementary DNA (cDNA) subcloned into pGEM-4Z (kindly provided by Dr. Thomas D. Sargent, National Institute of Child Health and Human Development, Bethesda, MD) was linearized by Pst 1 and transcribed by SP6 RNA polymerase. A 600 bp cDNA fragment encoding the 3' end of rat HGF subcloned into the pBluescript SK vector (kindly provided by Dr. Brian Carr, University of Pittsburgh School of Medicine, Pittsburgh, PA) was linearized by Hind III and transcribed by T3 RNA polymerase.

A 335 bp fragment of rat TGF-α cDNA was obtained by the reverse transcription polymerase chain reaction (RT-PCR) method and cloned as described previously (Hu *et al.*, 1994). EcoRV and SP6 RNA polymerase were used for its linearization and *in vitro* transcription. A fragment of rat SCF cDNA was also obtained by RT-PCR from poly(A)+ RNA isolated from rat liver as described previously (Fujio *et al.*, 1994). This was linearized and transcribed by Xho 1 and SP6 RNA polymerase respectively. A 985 bp fragment of rat TGF-β1 cDNA cloned in pBluescript II KS+ vector (kindly provided by Dr. Su Wen Qian,

National Cancer Institute, Bethesda, MD) was linearized by Xho 1 and transcribed by T3 RNA polymerase.

Mouse cDNA for c-*myc* subcloned into pGEM4 was linearized by EcoR1 and transcribed by T7 RNA polymerase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe labeled with [^{32}P]dCTP by the random primer extension method was used as an internal control (Fort *et al.*, 1985).

5.6.2. RNA isolation and Northern blot analysis

RNA was extracted from rat liver with guanidium thiocyanate followed by centrifugation in cesium chloride solution. Poly(A)+ RNA was selected by oligo (dT)-cellulose chromatography. Ten micrograms of poly(A) RNAs per lane were electophoresed on 0.8% agarose gels containing 2.22 mol/L formaldehyde and were later transferred to nylon filters. After ultraviolet cross-linking, the filters were hybridized with appropriate probes at 60°C. Blots were washed twice each with 1x standard sodium citrate/0.1% sodium dodecyl sulfate (SSC/SDS) at room temperature (low stringency), 0.1x SSC/SDS at room temperature, and 0.1x SSC/SDS at 60°C (high stringency). Autoradiography was performed on Kodak X-OMAT AR film (Rochester, NY) at -70°C using an intensifying screen.

5.7. Liver cytosolic enzymes

5.7.1. Thymidine kinase activity

The *in vitro* conversion of thymidine to thymidine phosphate by rat liver cytosol was used to determine the level of thymidine kinase activity (Kahn *et al.*, 1980). The reaction mixture contained 100 µl cytosol (30% homogenate), prepared according to the method of Corrigall and Kirsch (1988), 850 µl of incubation buffer consisting of 5 mM adenosine triphosphate and 3.6 mM $MgCl_2$ in 50 mM Tris HCl (pH 8.0), and 50 µl 1µM [^{3}H] thymidine. The reaction was allowed to proceed for 10 min at 37°C and terminated by immersion in boiling water for 2 min. Denatured protein was removed by centrifugation at 1500*g* for 5 min at 4°C. A 100-µl aliquot of the supernatant was spotted on a square of DEAE-cellulose paper. The paper squares were washed in 1 mM ammonium formate for 5 min, followed by distilled water for 3

min and placed in glass scintillation vials. A 0.1 M HCl/0.2 M KCl mixture was added to elute the radioactivity into solution. Ten ml ACS scintillation fluid was added to each vial after 15 min, and the radioactivity present in the vial was counted in a Packard Tri-Carb 460 CD liquid scintillation system (Downers Grove, IL).

5.7.2. Glutathione S-transferase activity

The spectrophotometric measurement of the rate of thioether formation from electrophilic substrates by rat liver cytosol was used to determine the level of glutathione *S*-transferase (GST) activity (Habig *et al.*, 1974; Corrigall and Kirsch, 1988). Whole liver GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as aromatic substrate (Habig *et al.*, 1974) in a Hitachi U 3200 dual beam spectrophotometer (Protea Nuclear Instruments, South Africa). All assays were performed at 25°C at a pH of 6.5. To a 3 ml cuvette the following reagents were added: (1) 2.75 ml 0.1 M potassium phosphate buffer; (2) 0.1 ml stock substrate CDNB (30 mM), freshly prepared in 95% ethanol and kept in the dark; (3) 0.15 ml reduced glutathione (GSH, 20 mM), freshly prepared in degassed H_2O; and (4) rat liver cytosol 0.05 ml, containing the enzyme and prepared as stated above. The reagents were mixed well and the change of absorbance ($\lambda = 340$) was monitored over 1 min. This change was read against an assay blank which included all the reactants except the enzyme in order to correct for the small amount of non-enzymatic activity. Specific activity was calculated from the equation:

$$\frac{\Delta A / \min}{(\varepsilon)} \times \frac{final\ assay\ volume}{sample\ volume} \times \frac{10^3}{protein\ concentration\ (mg / ml)}$$

where ΔA = change in absorbance and $\Delta\varepsilon$ = 9600 =difference in molar extinction coefficient for CDNB ($M^{-1}cm^{-1}$). Results are expressed as μmol/min/mg protein. Protein was measured according to the method of Lowry *et al.* (1951).

Chapter 6

Acute fumonisin B_1-induced hepatotoxicity: Histopathology and biochemical indices of liver injury

6.1. Introduction

FB_1 has been shown to be hepatotoxic (Gelderblom *et. al.*, 1988a) and hepatocarcinogenic (Gelderblom *et al.*, 1991) in rats. The principal pathological change in rats treated with FB_1 in the diet (1000 mg/kg) in short-term toxicity tests (21-33 days) is progressive "toxic hepatitis" characterised by hepatocellular necrosis, bile duct proliferation ("hyperplasia"), and fibrosis (Gelderblom *et. al.*, 1988a). During a chronic feeding study over a period of 26 months with FB_1 50 mg/kg, animals developed a 'chronic toxic hepatitis' that progressed to cirrhosis and cholangiofibrosis, and which terminated in hepatocellular carcinoma and cholangiocarcinoma respectively (Gelderblom *et al.*, 1991). In a preliminary study, Voss *et al.* (1993) reported that FB_1 was hepatotoxic in rats fed a diet containing 150 mg/kg for 4 weeks and also nephrotoxic at 15-50 mg/kg. "Scattered single cell necrosis", bile duct proliferation, and an increase in mitotic cells were described. The "scattered single cell necrosis" was subsequently confirmed as apoptosis (Tolleson *et al.*, 1996a).

The bile duct proliferation ("hyperplasia") noted in rats fed FB_1 (Gelderblom *et al.*, 1988a; Voss *et al.*, 1993) may in fact represent the proliferation of 'oval cells' (Farber, 1956), thought to be the progeny of a liver progenitor ('stem') cell (Grisham and Thorgeirsson, 1997). However, to date there have been no immunohistological studies examining the role of oval cell proliferation during feeding of FB_1 to rats. Another striking histological finding in the short-term FB_1 feeding studies was the rapid development of hepatic fibrosis (Gelderblom *et al.*, 1988a; Voss *et al.*, 1993), but the role of hepatic stellate (Ito) cells in the prominent fibrogenesis has not been examined.

The aim of the present study was to describe in more detail the histopathological and immunohistochemical changes in rat liver during short-term (five weeks) feeding of FB_1 250 mg/kg, with particular emphasis on the role of oval cell and stellate cell proliferation. In addition, time changes in biochemical indices of liver injury, cellular proliferation, and premalignancy were measured during the study.

6.2. Experimental methods

6.2.1. Animals and diet

The purification of FB_1 and preparation of the diets were performed as described in Chapter 5.

6.2.2. Experimental

Thirty male Fischer 344 rats (150 to 200 g) were randomly assigned to two groups and fed for up to five weeks with FB_1 250 mg/kg diet or control AIN-76A diet, as shown in TABLE 6.1.

TABLE 6.1

Treatment and control groups for short-term fumonisin B_1 (FB_1) feeding study.

Group	FB_1 (mg/kg)	No. of rats
Fumonisin	250	20
Control	0	10
Total		30

Four rats from the FB_1 group and two rats from the control AIN group were sacrificed weekly till the end of 5 weeks. At sacrifice, the animals were anaesthetised by the intraperitoneal injection of a sodium pentobarbitone solution (6% m/v). Blood was collected from the abdominal aorta for the measurement of plasma levels of aspartate transaminase (AST), and animals died by exsanguination via the aorta. The liver was removed and weighed. Freshly cut slices of liver were snap frozen in liquid nitrogen and stored at -70°C for analysis of cytosolic thymidine kinase (TK) and glutathione *S*-transferase (GST) activity.

6.2.3. Light microscopy

For routine light microscopy, slices of liver 4-5 mm in thickness were immersion-fixed in 10% neutral buffered formalin for 24 h before processing and embedding in paraffin wax (see Chapter 5). Stains included routine haematoxylin and eosin (H&E), sirius red for collagen, and Gordon & Sweet's method for reticulin. Coded sections were examined for evidence of hepatocyte injury, apoptotic bodies, fatty change, mitoses, architectural distortion, fibrosis, regenerative nodules, and oval cell proliferation. Cells undergoing apoptosis were detected *in situ* by TUNEL-specific labelling of nuclear DNA strand breaks, as described by Gavrieli *et al.* (1992).

6.2.4. Immunohistochemistry

Staining with Desmin D33 (Dako, Copenhagen, Denmark) for hepatic stellate (Ito) cells and rabbit polyclonal GST pi (Novacastra, Newcastle-Upon-Tyne, UK) for 'enzyme-altered' hepatic foci and 'preneoplastic' nodules was performed on paraffin sections. After sequential layering with biotinylated rabbit anti-mouse or swine anti-rabbit (Dako, Copenhagen, Denmark) 1:250 dilution as link antibodies, peroxidase conjugated Streptavidin (Dako, Copenhagen, Denmark) 1:500 was applied for 30 min at room temperature. The OV-6 mouse monoclonal antibody, which stains both oval cells and bile duct cells, was a generous gift from Professor Stewart Sell, Albany, New York. Acetone fixed cryostat sections were brought to room temperature and stained by means of a standard two-stage indirect peroxidase conjugated technique (Dako P161, Copenhagen, Denmark).

6.2.5. Biochemical indices

Serum AST levels, and liver cytosolic TK and GST activity were measured weekly for 5 weeks as biochemical markers of liver injury, cellular proliferation, and premalignancy respectively. Hepatic TK activity was measured as described by Kahn *et al.* (1980), and hepatic GST activity was determined by the method of Habig *et al.* (1974) (see Chapter 5).

6.2.6. Statistics

Statistical analysis was performed using two way analysis of variance, and significance was set at an alpha level (p value) of 0.05.

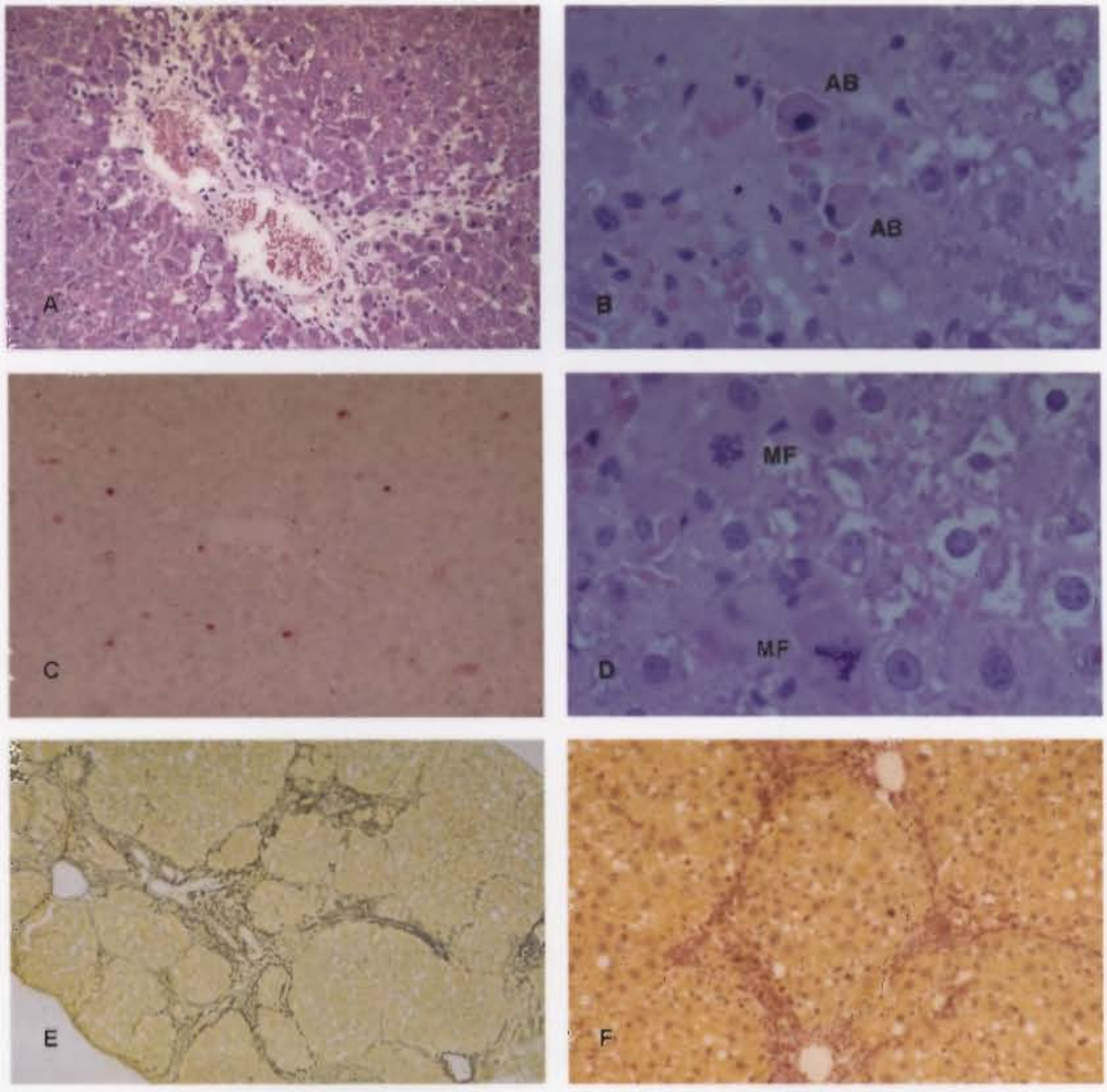

Figure 6.1

week 3 to 5. Some foci were close to the terminal hepatic venules while others abutted on the portal tracts. By weeks 4 and 5 some rats had developed large 'atypical' nodules that were readily seen in the H&E sections and confirmed by GST pi staining. Of note was the close relationship of some hepatic foci and nodules to portal tracts that contained the proliferating oval cells, and the presence of oval cells inside several of these 'atypical' nodules (Figure 6.2F).

Figure 6.1. Liver pathology in the fumonisin B_1-fed rat. (A) Liver at week 1 showing a terminal hepatic vein (centre), with numerous surrounding deeply eosinophilic apoptotic bodies and necrotic hepatocytes in zone 3, as well as a sparse mononuclear cell infiltrate. H&E, objective x 20. (B) High power magnification of liver at week 2 showing two classical apoptotic bodies (AB). These hepatocytes appear as shrunken and deeply eosinophilic cells, with evidence of nuclear chromatin condensation and margination below the cell membrane. H&E, objective x 60. (C) Liver at week 3 showing a terminal hepatic venule (centre) and numerous apoptotic hepatocytes scattered throughout the parenchyma. TUNEL method, objective x 10. (D) High power magnification of liver at week 3 showing prominent mitotic (MF) in two hepatocytes. H&E, objective x 60. (E) Liver at week 5 showing loss of the normal acinar architecture due to the presence of regenerative nodules of hepatocytes which are partially or completely surrounded by bands of fibrous tissue. Gordon and Sweets stain for reticulin, objective x 4. (F) Liver at week 5 showing the features of an early/developing cirrhosis. Sirius red, objective x 10.

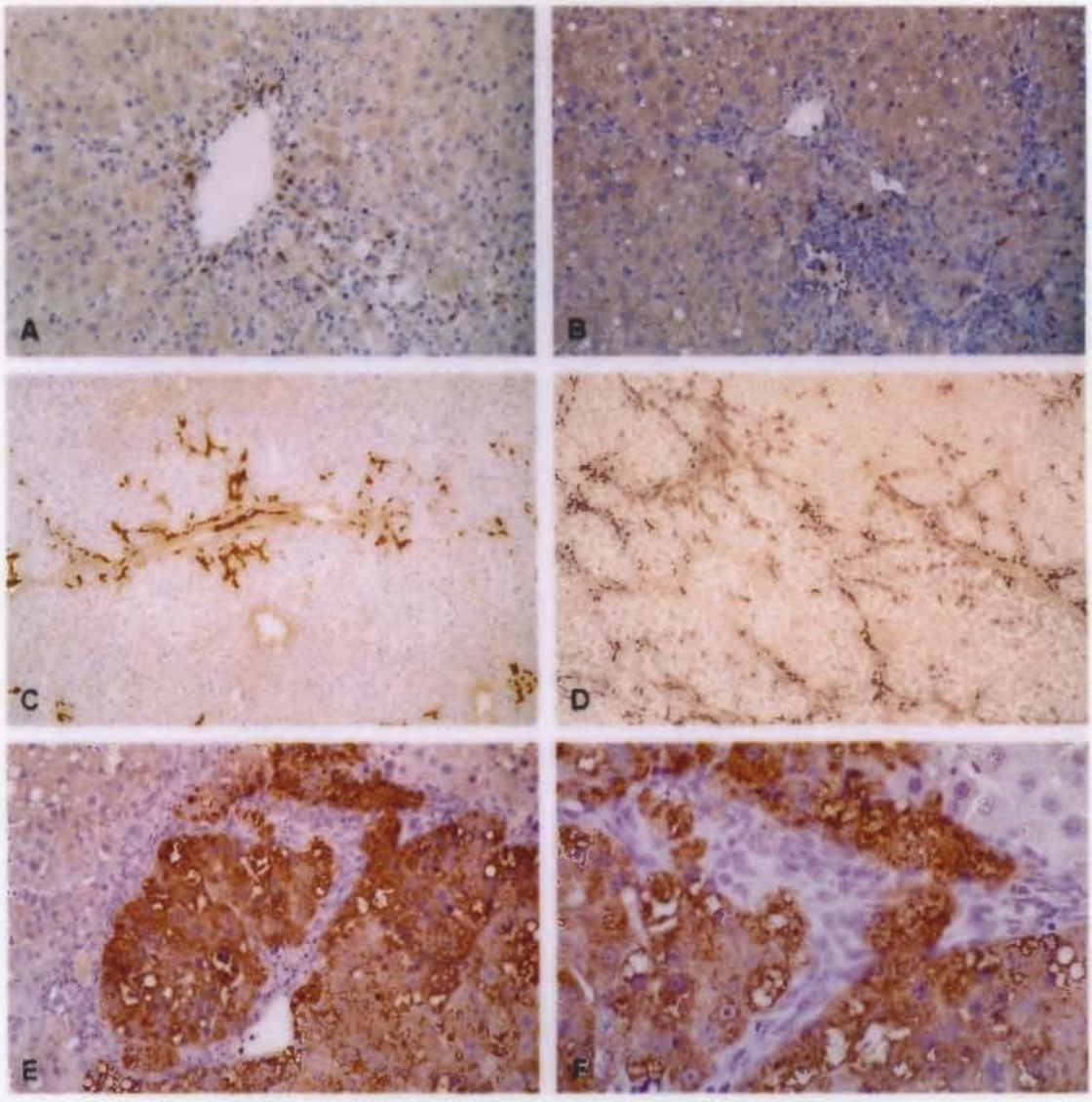

Figure 6.2

Figure 6.2. Immunohistochemical studies of livers from fumonisin B_1-fed rats. (A) Liver at week 1 showing numerous desmin positive hepatic stellate cells which are located in the zone 3 region of the liver with a similar distribution to the liver injury shown in Figure 6.1. P-A-P, objective x 20. (B) Liver at week 3 showing numerous desmin positive hepatic stellate cells which are located in the portal tracts and zone 1 regions. Numerous proliferating oval cells are also seen in the portal tracts, and the hepatocytes show mild fatty change. Objective x 10. (C) Liver at week 2 showing small numbers of OV-6 positive single cells and small ductules in the portal tracts. Objective x 10. (D) Liver at week 5 showing numerous proliferating oval cells, which are seen as single cells and small ductules that are OV-6 positive, located in the portal tracts and adjacent liver parenchyma. Objective x 4. (E) Liver at week 5 showing an 'atypical' nodule composed of GST pi positive hepatocytes which is located next to a portal tract (left). Proliferating oval cells, which are GST pi negative, are seen in the portal tract and within the nodule. Objective x 20. (F) Higher magnification of the liver in Figure 6.2.E showing oval cells with a group of GST pi positive hepatocytes. Objective x 40.

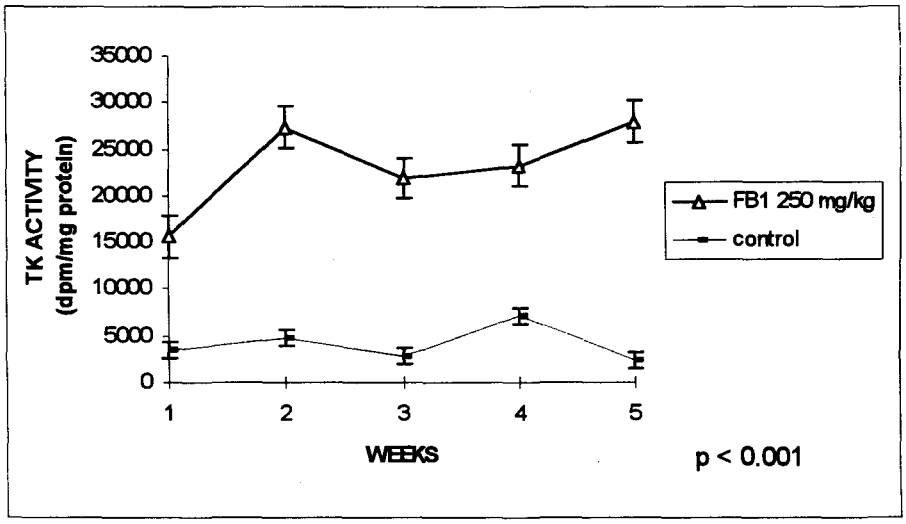

Figure 6.4. Liver cytosolic thymidine kinase (TK) activity in rats fed either FB_1 250 mg/kg or control diet for 5 weeks.

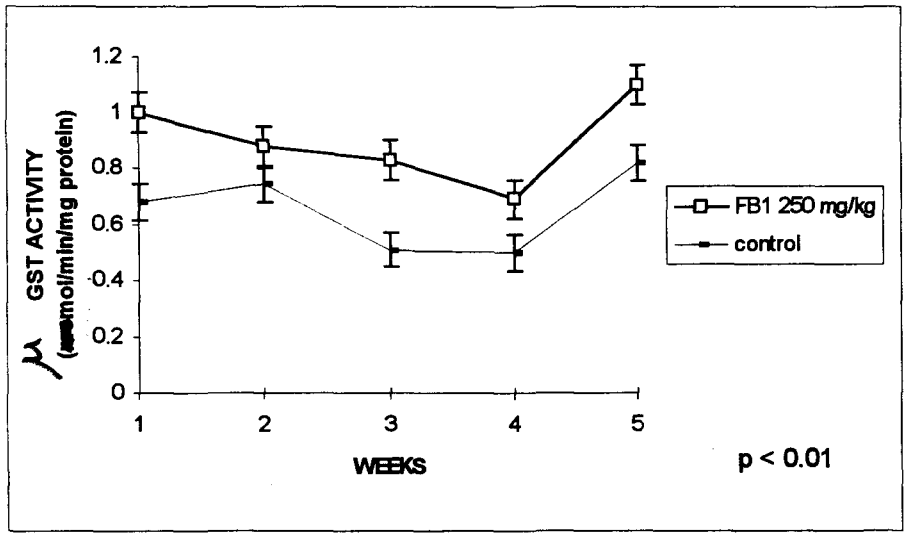

Figure 6.5. Liver cytosolic glutathione *S*-transferase (GST) activity in rats fed either FB_1 250 mg/kg or control diet for 5 weeks.

6.4. Discussion

In this short-term feeding study, FB_1 250 mg/kg caused toxic liver injury, initially with apoptosis in zone 3, followed by apoptosis and mitoses in all zones of the liver, progressive hepatic fibrosis and regenerative nodule formation, and development of 'enzyme-altered' hepatic foci and 'atypical' nodules. The bile duct proliferation ("hyperplasia") previously described by Gelderblom *et al.* (1988a) was confirmed in this study as proliferation of OV-6 positive oval cells, radiating from portal tracts into the adjacent liver parenchyma. Oval cell proliferation occurred despite clear evidence of continued hepatocyte regeneration at all time points. Proliferation of adult hepatocytes has been noted in other models for oval cell activation, including galactosamine (Dabeva and Shafritz, 1993) and dipin (Factor *et al.*, 1994). FB_1 has been reported to cause a dose-dependent inhibition of PH-induced incorporation of [^{3}H]-thymidine in hepatocytes (Gelderblom *et al.*, 1996b), and bile ductular proliferation has been noted at FB_1 dosages of 50 mg/kg diet (Dr. W.C.A. Gelderblom: personal communication). FB_1-induced oval cell proliferation does not appear to be an 'all-or-nothing' phenomenon, which occurs only when hepatocyte regeneration is absent. Complete mitoinhibition of hepatocytes thus does not appear to be an absolute requirement for the activation of the progenitor cell compartment, and other factors (e.g. functional impairment of hepatocytes) may conceivably also play a role.

Immunohistochemical staining demonstrated marked proliferation of desmin positive hepatic stellate cells from week 2, which appeared to mirror the distribution of the hepatocyte injury as well as the development of hepatic fibrosis. Hepatic stellate cells are perisinusoidal non-parenchymal cells which in normal liver are non-proliferative and are the main storage site for vitamin A (Friedman, 1996). Following liver injury of any kind, stellate cells undergo 'activation' and transformation with loss of intracellular retinoid, enhanced production of extracellular matrix proteins, increased contractility, and secretion of a variety of growth factors and cytokines which act in an autocrine and paracrine fashion on cells in the liver (Thorgeirsson *et al.*, 1993). Hepatic stellate cells thus play a major role in hepatic fibrogenesis (Friedman, 1996).

The prominent pro-apoptotic effects of FB_1 in rat liver are intriguing, and contrast with the *in vitro* effects of this mycotoxin on chemotherapy-mediated tumour cell destruction (Bose *et al.*, 1995). FB_1 has been found to have specific, potent activity as an inhibitor of

sphingolipid biosynthesis by blocking the conversion of sphinganine to ceramide (Wang *et al.*, 1991; Yoo *et al.*, 1992; Merrill *et al.*, 1996). Ceramide is a recently identified lipid second messenger that is believed to be one of the immediate signals for cell death generated in tumour cells treated with the chemotherapeutic agent, daunorubicin (Spiegel *et al.*, 1996). The production of ceramide and the ensuing onset of apoptosis in murine leukaemia cells cultured in the presence of daunorubicin is prevented by pre-treatment with FB_1 (Bose *et al.*, 1995). The molecular mechanisms of action of FB_1 are thus complex, and it appears that this fungal toxin may act as either an inhibitor or promoter of apoptosis, depending on the experimental situation (Lemmer *et al.*, 1998a).

At later time points in this study, we noted the close relationship of some 'atypical' hepatic foci and nodules to portal tracts that contained proliferating oval cells and ductules, and the presence of oval cells and ductules inside several of these nodules. It is possible that some of these oval cells were in fact adjacent to ('swirling around') the 'atypical' nodules, depending on the plane of section of the liver specimens. These cells showed typical morphological features of oval cells, but did not express GST pi, thus aiding their recognition within the GST pi positive nodules.

The histological picture of toxic hepatitis, hepatic stellate and oval cell proliferation, and formation of 'enzyme-altered' hepatic foci and nodules was reflected by biochemical indices of liver injury, cellular proliferation, and premalignancy. Both serum AST levels and liver cytosolic TK activity were markedly increased. Coincident with the appearance of GST pi positive foci and nodules, whole liver GST activity was increased in FB_1-fed animals. The glutathione *S*-transferases are a family of multifunctional proteins that catalyse the conjugation of glutathione with electrophilic compounds biotransformed from xenobiotics, including carcinogens (Tsuchida and Sato, 1992; Hayes and Pulford, 1995). In preneoplastic cells as well as neoplastic cells, specific molecular forms of GST are expressed and may participate in the mechanisms of their resistance to drugs (Sato, 1988; Sato 1989). The rat pi class form is strongly expressed in hepatic foci, nodules and carcinomas, and is regarded as one of the most reliable markers for preneoplastic lesions in the rat liver (Satoh *et al.*, 1985; Sato, 1988; Sato 1989). The precise role(s) and function(s) of GST pi in preneoplastic cells is unclear. In addition to participation in conjugation reactions (Tsuchida and Sato, 1992; Hayes and Pulford, 1995), GST pi exhibits selenium-independent glutathione peroxidase activity toward lipid hydroperoxides. Thus, GST pi expression may be related to the prevention of lipid

peroxidation (Meyer *et al.*, 1985; Ketterer *et al.*, 1989), which is considered to play an important role during tumour promotion by various carcinogens, including the fumonisins (Abel and Gelderblom, 1998).

6.5. Conclusions

Short-term feeding with FB_1 causes a severe toxic liver injury characterized by apoptosis, hepatic stellate cell proliferation, fibrosis, oval cell proliferation, and the appearance of 'atypical' hepatic foci and nodules. The histological picture of FB_1-induced hepatotoxicity is reflected by biochemical indices of liver injury, cellular proliferation, and premalignancy. Oval cells closely related to foci and nodules appeared to be undergoing phenotypic changes, and long-term FB_1 feeding studies are required to determine the ultimate fate of these oval cells.

Chapter 7

Hepatic gene expression changes during short-term feeding of fumonisin B_1

7.1. Introduction

Short-term feeding of fumonisin B_1 (FB_1) to rats causes zone 3 toxic liver injury, prominent apoptosis of hepatocytes, oval and stellate cell proliferation, progressive hepatic fibrosis, and development of 'atypical' nodules (see Chapter 6). There is currently no information on the molecular mechanisms underlying these hepatic histopathological changes caused by FB_1. This study employed Northern (mRNA) blot analysis of timed liver specimens from rats fed FB_1 250 mg/kg diet for 5 weeks, in order to examine the hepatic gene expression changes for α-fetoprotein (AFP), hepatocyte growth factor (HGF), transforming growth factor (TGF)-α, TGF-β1, and c-*myc*. Changes in hepatic gene expression were correlated with liver histopathology, and immunolocalization of mature TGF-β1 protein was performed.

It was postulated that TGF-β1 in particular might play an important role in the genesis of some of the hepatic histopathologic changes during feeding of FB_1. TGF-β1 expression in the liver is known to cause both apoptosis and fibrosis, and the two actions may be interrelated (Tsukamoto *et al.*, 1990; Rosser and Gores, 1995; Patel and Gores, 1995). Apoptotic cells secreting TGF-β1 may be the driving force for hepatic fibrogenesis. Stellate cells exposed to TGF-β1, originating from apoptotic hepatocytes, become activated ('myofibroblasts') and contribute to liver fibrogenesis by proliferating and secreting collagen (Rosser and Gores, 1995; Patel and Gores, 1995). Activated stellate cells in turn secrete TGF-β1 inducing apoptosis of neighbouring hepatocytes, via signalling through serine/threonine kinase (Smad) cascades (Massagué *et al.*, 1997).

7.2. Experimental methods

7.2.1. Chemicals and diet

The purification of FB_1 and preparation of the diets were performed as described in Chapter 5.

7.2.2. Experimental

Twelve male Fischer 344 rats were randomly divided into two groups and fed for up to 5 weeks with either diet containing FB_1 250 mg/kg or control AIN diet as shown in TABLE 7.1.

TABLE 7.1

Treatment and control groups for fumonisin B_1-induced hepatic gene expression study.

Group	FB_1 (mg/kg)	No. of rats
Fumonisin	250	9
Control	0	3
Total		12

Three rats from the treatment group and one rat from the control group were sacrificed weekly from weeks 3 - 5 by decapitation. The livers were harvested, and sections were fixed in 10% neutral buffered formalin for light microscopy and immunohistochemistry. The remaining liver was snap frozen in liquid nitrogen and stored at -70°C for mRNA analysis.

Liver samples from week 3 to 5 were examined by Northern blotting for expression of transcripts for AFP, HGF, TGF-α, TGF-β1, and c-*myc*. Liver specimens from day 9 post partial hepatectomy combined with acetylaminofluorene (AAF/PH regimen) from another study (Omori *et al.*, 1996: with permission) were included to serve as positive controls for oval cell proliferation and hepatic gene expression. Maximal oval cell proliferation is known to occur at this time point post AAF/PH (Thorgeirsson *et al.*, 1993).

7.2.3. Light microscopy and immunohistochemistry

Liver sections (5 μm) were fixed in 10% neutral buffered formalin and embedded in paraffin for routine light microscopy and immunohistochemistry (see Chapter 5). Routine stains included haematoxylin and eosin (H&E) and sirius red for collagen. Sections were examined for evidence of hepatocyte necrosis and apoptotic bodies, fatty change, mitoses, architectural distortion, fibrosis and regenerative nodules, and oval cell proliferation. Immunohistochemical stains performed on paraffin sections included desmin for hepatic stellate cells, polyclonal GST pi for 'enzyme-altered' hepatic foci and 'preneoplastic' nodules, and OV-6 monoclonal antibody for oval cells.

7.2.4. Probes

Plasmid vectors containing cDNA sequences to hepatic gene transcripts were linearized by restriction enzymes and transcribed by RNA polymerases. Antisense riboprobes labeled with [^{32}P]CTP were utilised for each of the following. A 429 bp piece of the 5' end rat AFP cDNA subcloned into pGEM-4Z (kindly provided by Dr. Thomas D. Sargent, National Institute of Child Health and Human Development, Bethesda, MD) was linearized by Pst 1 and transcribed by SP6 RNA polymerase (Jagodzinski *et al.*, 1981). A 600 bp cDNA fragment encoding the 3' end of rat HGF subcloned into the pBluescript SK vector (kindly provided by Dr. Brian Carr, University of Pittsburgh School of Medicine, Pittsburgh, PA) was linearized by Hind III and transcribed by T3 RNA polymerase. A 335 bp fragment of rat TGF-α cDNA was obtained by the reverse transcription polymerase chain reaction (RT-PCR) method and cloned as described previously (Hu *et al.*, 1994). EcoRV and SP6 RNA polymerase were used for its linearization and *in vitro* transcription. A 985 bp fragment of rat TGF-β1 cDNA cloned in pBluescript II KS+ vector (kindly provided by Dr. Su Wen Qian, National Cancer Institute, Bethesda, MD) was linearized by Xho 1 and transcribed by T3 RNA polymerase. Mouse cDNA for c-*myc* subcloned into pGEM4 was linearized by EcoR1 and transcribed by T7 RNA polymerase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe labeled with [^{32}P]dCTP by the random primer extension method (Fort *et al.*, 1985) was used as a RNA loading control for for all experiments.

7.2.5. RNA isolation and Northern blot analysis

RNA was extracted from rat liver with guanidium thiocyanate followed by centrifugation in caesium chloride solution. Poly(A)+ RNA was selected by oligo (dT)-cellulose chromatography. Ten micrograms of poly(A) RNAs per lane were electophoresed on 0.8% agarose gels containing 2.22 mol/L formaldehyde and were later transferred to nylon filters. After ultraviolet cross-linking, the filters were hybridised with riboprobes at 60°C and at 42°C with cDNA probes. Blots were washed twice each with 1x standard sodium citrate/0.1% sodium dodecyl sulfate (SSC/SDS) at room temperature, 0.1x SSC/SDS at room temperature, and 0.1x SSC/SDS at 60°C. Autoradiography was performed on Kodak X-OMAT AR film (Rochester, NY) at -70°C using an intensifying screen.

7.2.6. Immunostaining for TGF-β1 protein

Antibody to mature TGF-β1 protein was a generous gift from Dr. K. Flanders, National Cancer Institute, Bethesda, MD. Immunohistochemical staining for TGF-β1 protein was done on 5-μm deparaffinized sections with an indirect immunoperoxidase antiserum detection protocol (Elite kit; Vector Laboratories). Mature TGF-β1 protein was detected by the rabbit polyclonal LC (1-30) antibody, as previously described (Sanderson *et al.*, 1995).

7.2.7. Statistics

Rat weight gain of rats fed FB_1 or control diet was compared by two way analysis of variance. Significance for the acceptance interval was set at an alpha level (p value) of 0.05.

7.3. Results

7.3.1. Rat weight gain

In the total group of 60 rats that were commenced on the long-term feeding study (Chapter 8), animals fed on control AIN diet showed more rapid weight gain during the first 5 weeks than FB_1-fed animals, and their weights crossed the acceptance interval soon after the initiation of the study (Figure 7.1). The effect of FB_1 feeding on weight gain in rats has been noted previously (Gelderblom *et al.*, 1991), and appears to be due to both a feed refusal effect and FB_1 toxicity.

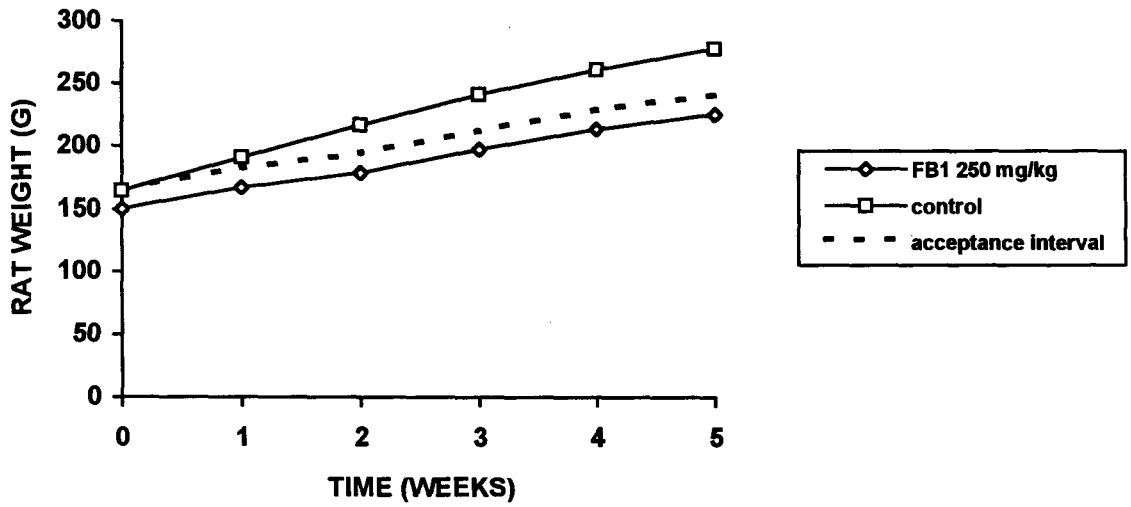

Figure 7.1. Weight gain during the 5 week study period of rats fed either FB_1 250 mg/kg or control AIN diet. Animals fed on control AIN diet showed more rapid weight gain during the 5 week study period than FB_1-fed animals, and their weights crossed the acceptance interval soon after the initiation of the study.

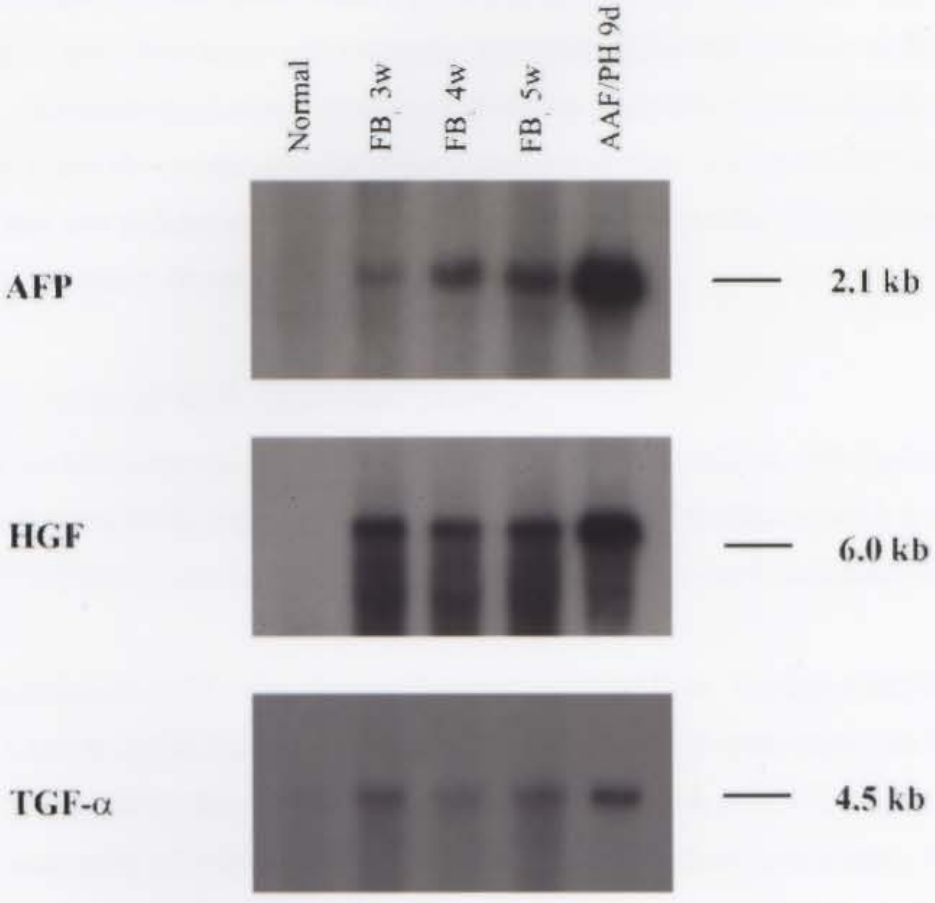

Figure 7.2. Analysis of expression of poly(A)+ RNA (10μg per lane) for α−fetoprotein (AFP), hepatocyte growth factor (HGF), and transforming growth factor alpha (TGF-α) by Northern blotting during weeks 3 to 5 of FB feeding. Timed rat liver specimens from day 9 post acetyl-aminofluorene combined with partial hepatectomy (AAF/PH) served as positive controls.

7.3.2. Light microscopy and immunohistochemistry

The histopathological changes noted in rat liver during feeding with FB_1 250 mg/kg diet were similar to those described previously (see Chapter 7), although the severity of liver injury and degree of resultant oval cell proliferation appeared somewhat less in this study. FB_1 caused ongoing zone 3 toxic liver injury with numerous apoptotic bodies and collapse of the reticulin framework. Immunohistochemistry showed proliferation of desmin-positive hepatic stellate (Ito) cells in zone 3, and also proliferation of OV-6 positive oval cells. Numerous GST pi positive hepatocyte foci and nodules were noted at week 5. Sirius red stains for collagen showed the rapid development of hepatic fibrosis.

7.3.3. Expression of AFP, HGF, and TGF-α

Transcripts for AFP were not detected in normal liver. Feeding with FB_1 250 mg/kg resulted in a progressive increase in the expression of the 2.1 kb AFP transcripts from week 3 to 5. The peak level of AFP expression was however much less than that seen at day 9 post AAF/PH (Figure 7.2).

Transcripts for HGF were also not detected in normal liver. Feeding with FB_1 250 mg/kg resulted in a moderate but sustained increase in expression of HGF from week 3 to 5. HGF expression in FB_1-fed animals was less than that seen at day 9 post AAF/PH (Figure 7.2).

No expression of TGF-α was detected in normal liver. Short-term feeding with FB_1 250 mg/kg resulted in a moderate but fluctuating increase in expression of TGF-α to levels that were similar to that seen at day 9 post AAF/PH (Figure 7.2).

7.3.4. Expression of TGF-β1 and c-myc

Some expression of TGF-β1 mRNA was detected in normal liver. Feeding with FB_1 250 mg/kg resulted in a marked and progressive increase in expression of TGF-β1 from week 3 to 5. The maximum level of expression of TGF-β1 in FB_1-fed rats was similar to that seen at day 9 post AAF/PH (Figure 7.3).

Transcripts for c-*myc* were detectable at a low level in normal liver. Feeding with FB_1 250 mg/kg resulted in a marked increase in expression of c-*myc*, with maximum levels being expressed

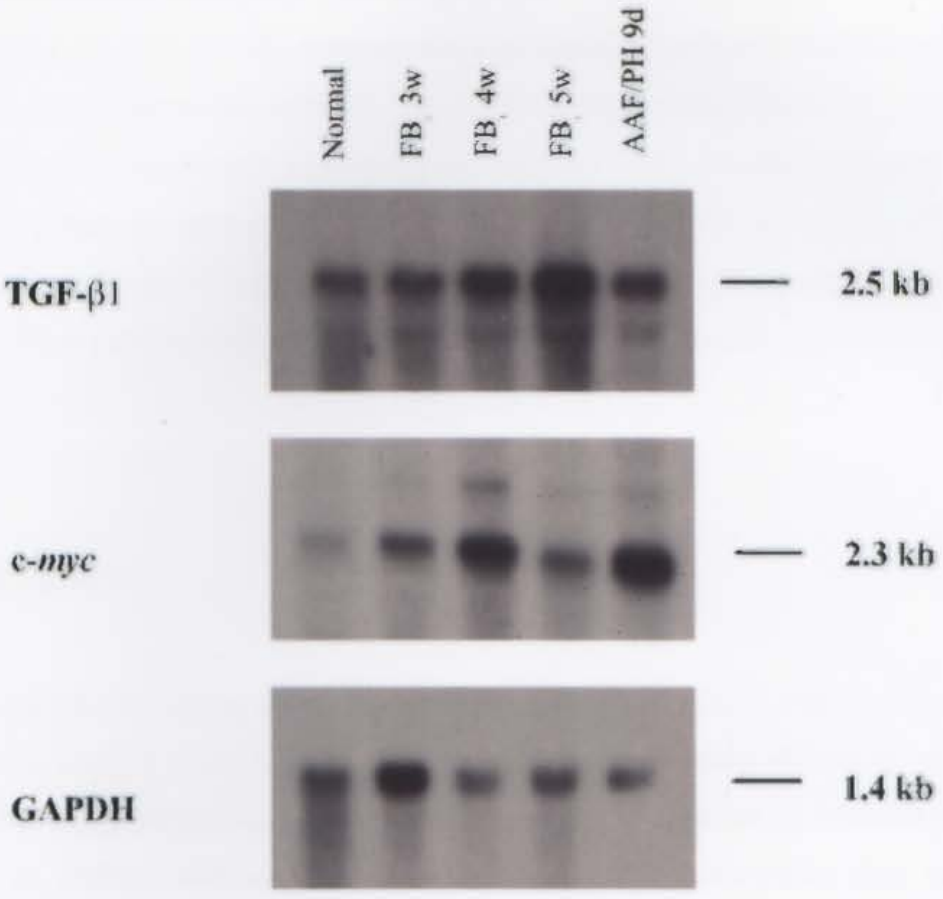

Figure 7.3. Analysis of expression of poly(A)+ RNA (10μg per lane) for transforming growth factor beta 1 (TGF-β1) and *c-myc* by Northern blotting during weeks 3 to 5 of FB_1 feeding. Timed rat liver specimens from day 9 post acetylaminofluorene combined with partial hepatectomy (AAF/PH) served as positive controls. Glyceraldehyde phosphate dehydrogenase (GAPDH) poly(A)+ RNA was used as loading control.

at week 4. This level of expression of c-*myc* was similar that seen at day 9 post AAF/PH (Figure 7.3).

7.3.5. Immunolocalization of TGF-β1 protein

Staining with LC(1-30) antibody demonstrated a progressive increase in expression of mature TGF-β1 protein in the rat liver over the 5 week FB_1 feeding period (Figure 7.4). Expression of TGF-β1 protein by hepatocytes was initially focal (Figure 7.4B), but with continued feeding the protein was abundantly expressed in most hepatocytes (Figures 74.C and 7.4D). TGF-β1 protein was not detected in zone 3 hepatocytes, which showed evidence of frequent mitoses and apoptosis. Oval cells, stellate cells, and Kupffer cells were consistently negative for TGF-β1 protein.

7.4. Discussion

Feeding with FB_1 250 mg/kg resulted in increased expression of HGF, TGF-α, and AFP transcripts at weeks 3, 4 and 5, which coincided with desmin positive stellate cell and OV-6 positive oval cell proliferation. AFP expression was used as a marker for oval cell proliferation (Omori et al., 1997a). HGF and TGF-α are important growth factors in the liver, and are involved in both normal liver regeneration (Michalopoulos, 1990; Michalopoulos and DeFrances, 1997) and activation/proliferation of the oval cell compartment (Thorgeirsson *et al.*, 1994; Grisham and Thorgeirsson, 1997). Expression of transcripts for HGF and TGF-α during feeding of FB_1 was less than seen at day 9 post AAF/PH (time of maximal oval cell proliferation in AAF/PH model). The moderately increased but sustained expression of genes for liver growth factors during feeding with FB_1 presumably reflects ongoing hepatotoxicity. This contrasts with the AAF/PH model, in which massive but transient liver injury and growth factor expression occurs.

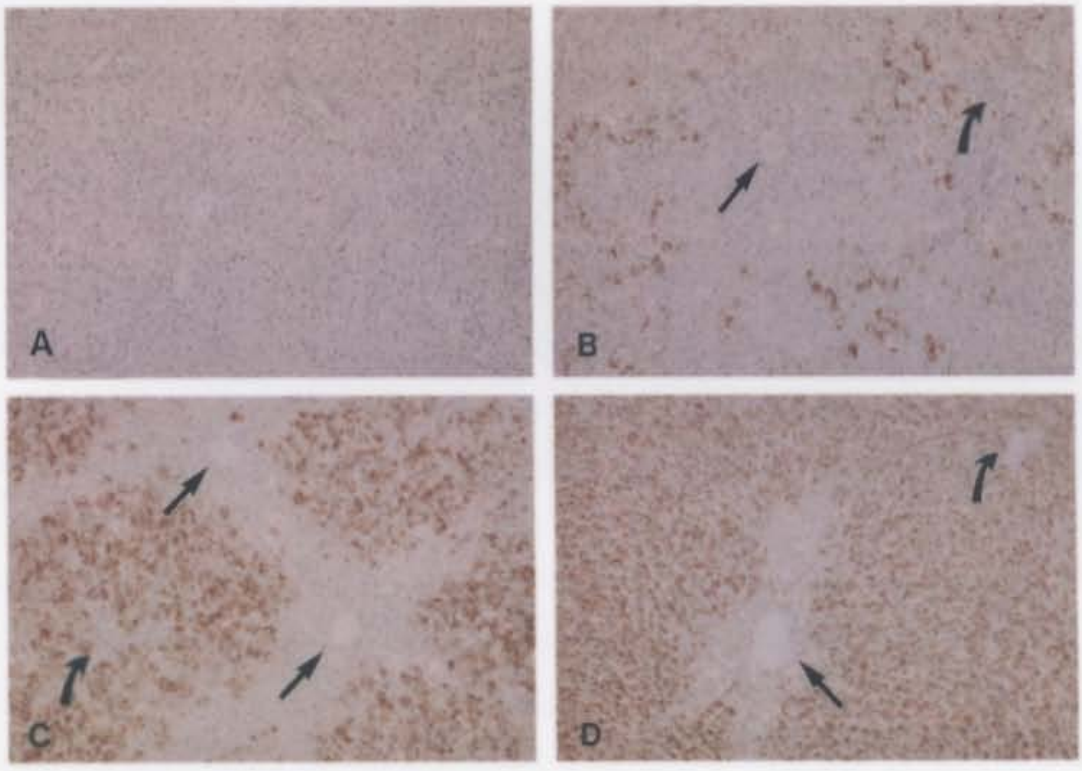

Figure 7.4. Mature TGF-β1 protein in timed liver specimens from FB_1-fed rats, detected by staining with LC(1-30) antibody. (A) Control, with omission of the primary antibody. (B) Liver at 1 week, showing patchy expression of TGF-β1 by hepatocytes. (C) Liver at 2 weeks, showing expression of TGF-β1 by most hepatocytes in zones 1 and 2. (D) Liver at 3 weeks, showing extensive expression of TGF-β1, but persistent lack of staining by zone 3 hepatocytes. DAB, objective x 10. Straight arrows indicate central veins; curved arrows indicate portal tracts.

FB_1 feeding resulted in marked overexpression of TGF-β1 in rat liver. Surprisingly, TGF-β1 protein was expressed almost exclusively by hepatocytes, while stellate cells and oval cells were persistently negative. Immunostaining demonstrated a progressive increase in the numbers of hepatocytes expressing mature TGF-β1 protein during FB_1 feeding, and by 3 weeks most hepatocytes in the liver lobule were stained by LC (1-30) antibody. However, zone 3 hepatocytes remained persistently negative for TGF-β1 protein, and many of these hepatocytes were undergoing mitosis or apoptosis. The markedly increased levels of TGF-β1 expression by hepatocytes may be responsible for the prominent apoptosis and fibrosis seen in the rat liver during feeding with FB_1.

Feeding of FB_1 also resulted in overexpression of c-*myc*, a proto-oncogene that has been characterised as a positive regulator of cell proliferation involved in tumour progression (Garte, 1993; Nagy *et al.*, 1988), and has also been implicated in TGF-β1 signalling (Alexandrow and Moses, 1995). Increased expression of c-*myc* and TGF-β1 may cooperate in the promotion of liver tumours in the FB_1-fed rat. It has been shown that overexpression of c-*myc* in TGB-β1-sensitive cells can result in the loss of sensitivity to TGF-β1 (Alexandrow *et al.*, 1995). Furthermore, co-expression of c-*myc* and mature TGF-β1 in the livers of transgenic mice accelerates hepatocarcinogenesis that is associated with reduced TGF-β type II receptor (TβIIR) expression (Factor *et al.*, 1997). Overexpression of TGF-β1 in transgenic mice may thus produce a mitoinhibitory environment that selects for the growth of TGF-β1-resistant transformed liver cells. Once present, loss of TGF-β1-transduced growth inhibitory signals and up-regulation of c-*myc* expression result in the unrestrained growth of those transformed liver cells and facilitate liver tumour development (Factor *et al.*, 1997). Although it is likely that transformed cells in the livers of FB_1-fed rats have similarly acquired resistance to the antiproliferative effects of TGF-β1, the underlying mechanisms have not been studied.

7.5. Conclusions

Feeding with FB_1 results in increased expression of genes for AFP, HGF, and TGF-α, but levels of these transcripts are lower than following AAF/PH. In contrast, feeding with FB_1 results in marked overexpression of TGF-β1 and c-*myc*. The overexpression of TGF-β1 may be causally related to the prominent apoptosis and fibrosis seen in FB_1 -induced liver injury. Increased expression of c-*myc* and TGF-β1 may cooperate in the promotion of liver tumours in the FB_1-fed rat, possibly due to the provision of a selective milieu for the outgrowth of preneoplastic cells

Chapter 8

The effects of dietary iron overload on fumonisin B_1-induced cancer promotion in the liver

8.1. Introduction

Fumonisin B_1 (FB_1) is hepatotoxic and hepatocarcinogenic in rats (Gelderblom *et al.*, 1988; Gelderblom *et al.*, 1991). FB_1 acts as a strong promoter (and possibly weak initiator) of 'enzyme altered' hepatic foci and nodules in short-term cancer studies (Gelderblom *et al*, 1992b; Gelderblom *et al.*, 1996b). The mechanisms of FB_1-induced toxicity and cancer induction in the liver are unclear, but there is some evidence that peroxidation of lipid membranes (Abel and Gelderblom, 1998; Yin *et al.*, 1998) and oxidative DNA damage (Sahu *et al.*, 1998) might play a role.

Dietary iron overload is common in sub-Saharan Africa, and hepatic iron concentrations rival those occurring in genetic haemochromatosis. Recent studies suggest that dietary iron overload may be a risk factor for HCC in Black Africans (Gordeuk *et al.*, 1996; Mandishona *et al.*, 1998). The development of HCC in humans with iron overload (genetic or dietary) usually occurs in the setting of iron- or alcohol-induced cirrhosis (Niederau *et al.*, 1985), and it is not clear whether iron plays a direct role in the induction of liver tumours or whether the increased cancer risk arises solely from the cirrhotic process. Although mechanisms of iron-induced hepatotoxicity are incompletely understood, free radical-mediated peroxidative damage to cellular lipids, proteins, and DNA is likely (Britton *et al.*, 1994; Abalea *et al.*, 1998). Experimentally, the potentiation of cancer induction due to polyhalogenated hydrocarbons by iron overload in rodents (Smith *et al.*, 1990), would suggest that iron may act as a promoter of initiated hepatocytes. Studies carried out to test this hypothesis have, however, shown conflicting results, with both promoting and inhibiting effects on cancer induction by excess hepatic iron (Stål *et al.*, 1995; Carthew *et al.*, 1997).

Both FB_1 and excess hepatic iron may thus cause peroxidation of membrane lipids and oxidative liver injury (Abel and Gelderblom, 1998; Britton *et al.*, 1994). Furthermore, both

agents may, either directly or indirectly, affect the induction of liver tumours. The aims of this study were to determine whether dietary iron loading (i) enhances FB_1-induced hepatic lipid peroxidation, and (ii) modulates the cancer initiating and promoting properties of FB_1.

8.2. Experimental methods

8.2.1. Chemicals

FB_1 was purified from corn cultures of *Fusarium moniliforme* strain MRC 826 according to the method described by Cawood *et al.* (Cawood *et al.*, 1991) to a purity of 92-95%. Carbonyl iron (CI), an extremely pure form of elemental iron (>98% iron with <0.8% carbon, <0.3% oxygen, and <0.9% nitrogen), was purchased from Sigma Chemical Company (St. Louis, MO).

8.2.2. Animals and diet

The study was approved by the Ethics and Research Committee of the University of Cape Town, and the experiments were conducted in accordance with the laws and regulations controlling experiments on live animals in South Africa. Thirty eight male Fischer 344 rats were fed AIN-76A diet (American Institute of Nutrition, 1980), which has an iron content of 50.6 mg/kg feed (see Appendix C). The diets containing FB_1 250 mg/kg were prepared as described previously (Gelderblom *et al.*, 1994) and stored under nitrogen at -20°C for the duration of the study. Animals that were randomised to receive dietary iron loading, were initially fed 2% (wt/wt) CI when weaned, but this had to be temporarily discontinued after 2 weeks (for 1 week) due to growth retardation, whereafter iron supplementation was resumed with 1% (wt/wt) carbonyl iron (Figure 8.1A).

8.2.3. Experimental

The 5 week FB_1-feeding experiment (250 mg/kg diet) was commenced when the rats weighed approximately 155- 165 g (group 1, 160.9 ± 5.0 g; group 2, 165.2 ± 1.9 g; group 3, 153.2 ± 4.6 g; group 4, 155.7 ± 4.8 g). The 38 animals were divided into four treatment groups according to a 2 x 2 factorial design, i.e. controls (group 1, n = 8); FB_1 250 mg/kg diet

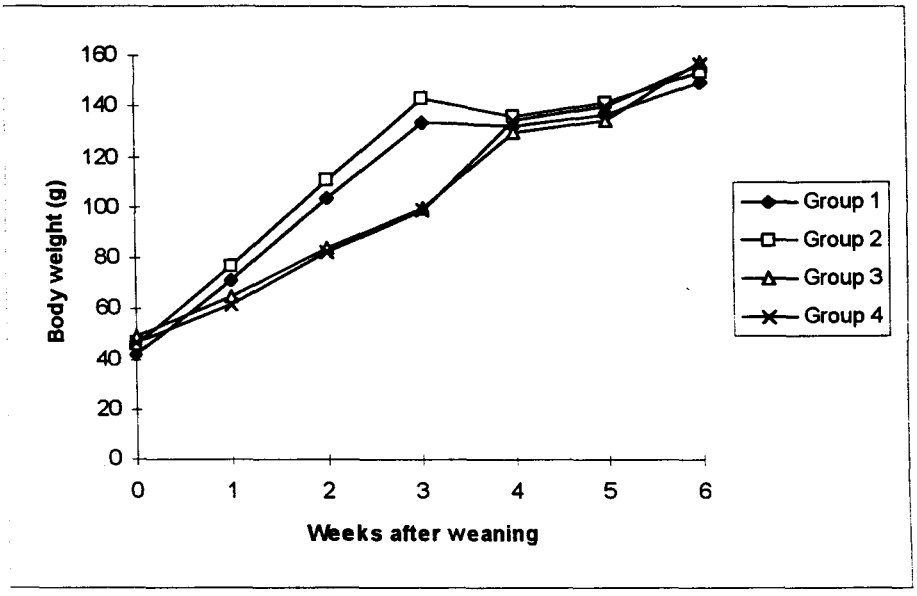

Figure 8.1A. Mean body weights of rats during the period of iron loading from the time of weaning until the start of the FB_1 feeding experiment. Animals that were randomised to receive dietary iron loading (groups 3 and 4), were initially fed 2% (wt/wt) CI from the time of weaning, but dietary iron supplementation was temporarily discontinued after 2 weeks (for 1 week) due to growth retardation. During this seven day period of no iron supplementation, rats in groups 1 and 2 were feed restricted in order to allow for 'catch up' growth of the growth retarded animals in groups 3 and 4. Thereafter, iron loading of group 3 and 4 animals was resumed with 1% (wt/wt) carbonyl iron, and averaged feeding was instituted in order to control feed intake of group 1 and 2 rats, and to allow for comparable rates of body weight gain in all treatment groups. The mean body weights in the different treatment groups had equalised by week 4 after weaning, and total weight gain in the different groups over the six week period preceding the commencement of the FB_1 feeding experiment was comparable (group 1, 111.3 ± 3.7 g; group 2, 108.4 ± 4.3 g; group 3, 107.5 ± 71 g; group 4 110.0 ± 9.0 g). Once instituted, dietary iron supplementation remained constant at 1% until the end of the study. Error bars have been omitted for the sake of clarity of the graph.

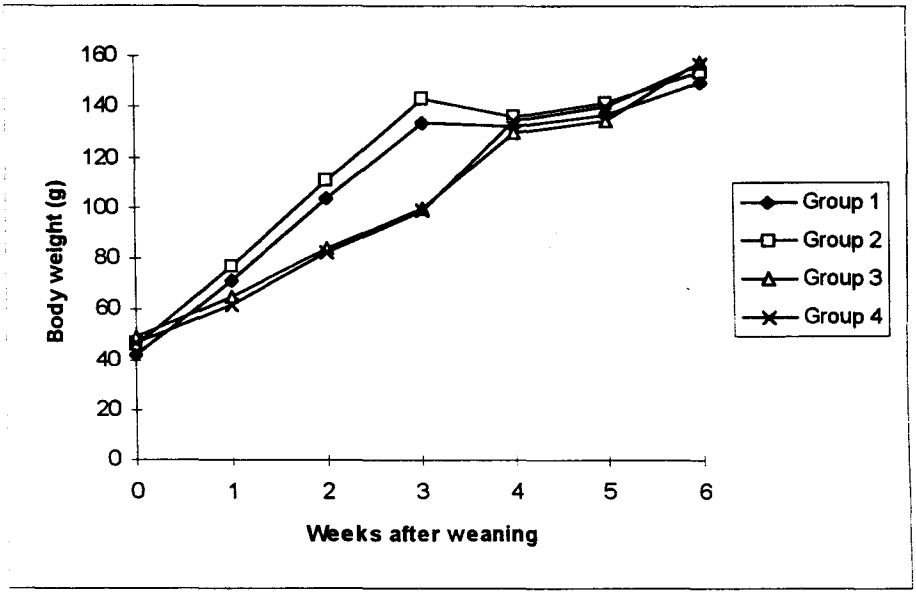

Figure 8.1A. Mean body weights of rats during the period of iron loading from the time of weaning until the start of the FB_1 feeding experiment. Animals that were randomised to receive dietary iron loading (groups 3 and 4), were initially fed 2% (wt/wt) CI from the time of weaning, but dietary iron supplementation was temporarily discontinued after 2 weeks (for 1 week) due to growth retardation. During this seven day period of no iron supplementation, rats in groups 1 and 2 were feed restricted in order to allow for 'catch up' growth of the growth retarded animals in groups 3 and 4. Thereafter, iron loading of group 3 and 4 animals was resumed with 1% (wt/wt) carbonyl iron, and averaged feeding was instituted in order to control feed intake of group 1 and 2 rats, and to allow for comparable rates of body weight gain in all treatment groups. The mean body weights in the different treatment groups had equalised by week 4 after weaning, and total weight gain in the different groups over the six week period preceding the commencement of the FB_1 feeding experiment was comparable (group 1, 111.3 ± 3.7 g; group 2, 108.4 ± 4.3 g; group 3, 107.5 ± 71 g; group 4 110.0 ± 9.0 g). Once instituted, dietary iron supplementation remained constant at 1% until the end of the study. Error bars have been omitted for the sake of clarity of the graph.

(group 2, n = 10); dietary CI 1 - 2% (group 3, n = 10); and FB_1 plus CI (group 4, n = 10). The quantities of feeds in the different groups were adjusted to match the average intake of animals in group 4 (FB_1/CI). Two animals from each treatment group and one animal from the control group were sacrificed at weeks 3 and 4 for analysis of hepatic histopathology, and the remainder of the animals (n = 6, each group) were sacrificed at week 5. The rats were weighed daily and feed intake and wastage were carefully determined. At sacrifice, animals were anaesthetised by the intraperitoneal injection of a sodium pentobarbitone solution (6% m/v). Blood was drawn by cardiac puncture for measurement of alanine transaminase (ALT) levels for biochemical assessment of liver injury, and animals were terminated by exsanguination. The livers were harvested and weighed. A slice of liver was taken from the left, right, and median lobes of each animal, and these were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin for light microscopy. The remaining liver was snap frozen in liquid nitrogen and stored at -70°C.

8.2.4. Light microscopy and immunohistochemistry

Liver sections (5 μm) were stained with haematoxylin and eosin (H&E) for routine light microscopy, and with sirius red for collagen. Perls' Prussian blue stain for trivalent iron was used to assess hepatic iron content (Williams *et al.*, 1962). Stainable iron in hepatocytes was graded 0 to 4, using a modification of the scale devised by Scheuer *et al.* (Scheuer *et al.* 1962). Staining with rabbit polyclonal GST pi (Novacastra, Newcastle-Upon-Tyne, UK) was performed for 'enzyme-altered' hepatic foci and nodules (see Chapter 5).

8.2.5. Morphometric analysis of hepatic foci and nodules

A total of eighteen liver sections (3 sections per rat in 6 rats sacrificed at week 5) each from rats in group 2 (FB_1) and group 4 (FB_1/CI) were stained with GST pi for determination of the number and size of 'enzyme altered' foci and 'premalignant' nodules. One section from each of the left, right, and median lobes was examined for each rat. The number of GST pi positive lesions (foci and nodules) per cm^2 were counted, and were considered an estimate of the cancer initiating effects of FB_1 with or without iron loading (Solt and Faber, 1976; Farber and Sarma, 1987). For the purposes of this study, a group of GST pi positive cells was classified as a 'focus' if it had an area of less than 100 μm^2, and a 'nodule' if it had an area of 100 μm^2 or greater. The percentage area of liver occupied by GST pi positive foci and nodules was

determined using video image analysis (Optimas, Bothell, WA), and was considered a measurement of the cancer promoting effects of FB_1 with or without iron loading (Solt and Faber, 1976; Farber and Sarma, 1987).

8.2.6. Hepatic iron concentration

Liver tissue for determination of iron content was dried for 24 h at 105°C, weighed, digested in 50% nitric acid at 70°C for 1 h, and diluted in 0.2 M sodium acetate buffer pH 4.5 (Torrance and Bothwell, 1980). All glassware was rendered iron free and rinsed with iron free water (Torrance and Bothwell, 1980). Iron concentration was determined using a Roche Unimate 5 Iron Kit (Roche Diagnostic Systems, Basel, Switzerland), on a Roche Cobas Fara II Centrifugal Analyser.

8.2.7. Assessment of oxidative damage

Malondialdehyde (MDA) is formed when polyunsaturated fatty acids of membrane phospholipids undergo peroxidation. Lipid peroxidation was measured by the thiobarbituric acid (TBA) assay for MDA concentration on samples of liver homogenate, as described by Esterbauer and Cheeseman (Esterbauer and Cheeseman, 1990). Related substances such as sucrose, non-ferrous metal ions and whole tissue homogenates may also react with TBA or influence the assay procedure, and the term thiobarbituric acid reacting substances (TBARS) more accurately describes the product of this assay (Esterbauer and Cheeseman, 1990). Addition of EDTA to the liver homogenate and butylated hydroxytoluene (BHT) to the TBA reagent prevent further oxidative changes during the assay procedure. Lipid peroxidation was expressed as nmol MDA equivalents per mg protein, using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ at 532 nm for MDA (Buege and Aust, 1978).

8.2.8. Statistics

The data are presented as the mean ± SEM. Outcome measurements between the treatment groups were compared by one way analysis of variance (ANOVA). Individual comparisons were made using Scheffé's test. FB_1-induced GST pi positive hepatic lesions in groups 2 and 4 were compared using the Mann-Whitney *U* test for nonparametric data. Significance was set at a p value of 0.05.

8.3. Results

8.3.1. Body weight gain and liver weight/body weight ratio

Total feed intake was 222.35 ± 14.26 g per 100 g body weight (mean ± SEM), and equivalent total FB_1 intake was calculated as 55.59 ± 3.56 mg per 100 g body weight. Daily feed intake was 6.57 ± 0.40 g per 100 body weight and daily FB_1 intake was 1.64 ± 0.10 mg per 100 g body weight. Despite control of feed intake there were significant differences in weight gain in the treatment groups, presumably reflecting differential toxic effects (Figure 8.1B).

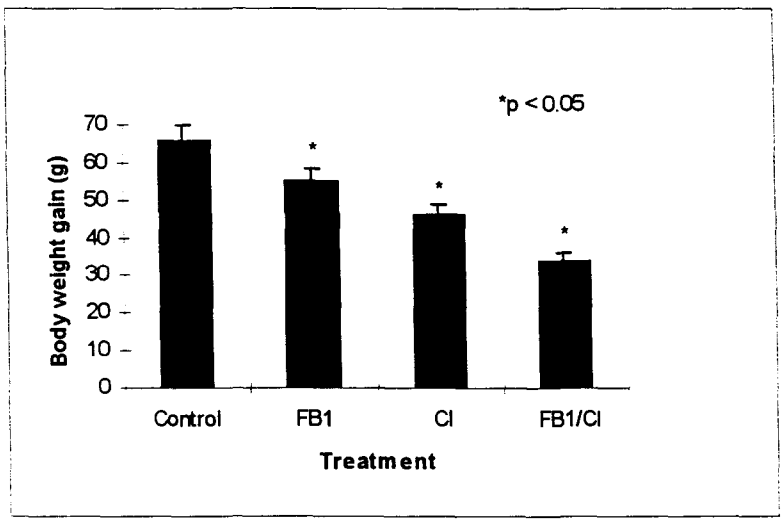

Figure 8.1B. Body weight gain (g) of rats over 35 days according to treatment group.

The liver weight/body weight ratio was reduced only in rats from group 2 (FB_1; $p < 0.0001$) (Figure 8.2).

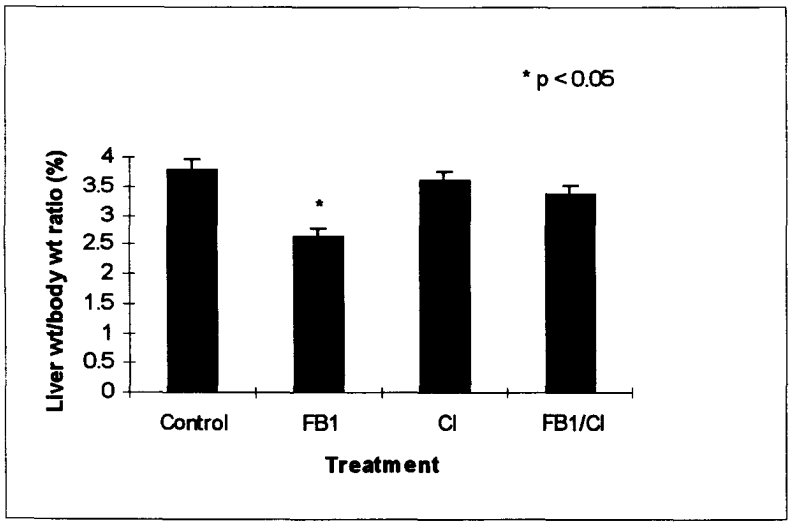

Figure 8.2. Liver weight to body weight (LW/BW) ratio of rats according to treatment group.

8.3.2. Liver injury analysis

Serum levels of ALT in animals that received control AIN diet was 42 ± 2 U/l at 5 weeks. There was a marked increase in week 5 ALT levels in rats fed FB_1 (group 2, 200 ± 28 U/l) and FB_1/CI (group 4, 191 ± 10 U/l), reflecting significant hepatotoxicity of FB_1-containing regimens. Serum ALT levels in animals given CI (group 3) were mildly raised (66 ± 7 U/l) at 5 weeks, indicating minimal hepatocellular injury caused by dietary iron loading alone.

8.3.3. Liver histopathology

Pathological changes in rat liver caused by feeding of FB_1 250 mg/kg diet for 5 weeks are described in Chapter 6, and include severe zone 3 injury with collapse of the reticulin framework, frequent hepatocyte mitoses, and apoptotic bodies, seen after 1 week. These initial changes are followed by the development of 'enzyme-altered' foci and nodules, marked oval cell proliferation, and hepatic fibrosis by week 3. Sequential liver sections from week 3 to 5 in group 2 animals (FB_1) from the present study showed milder liver injury, with cell loss and collapse in zone 3, mitoses, minimal fibrosis, and multiple small GST pi positive foci and nodules. Even though the FB_1 content of the diet was the same compared to the earlier study (Chapter 6), total intake of FB_1 in the present study was reduced because of averaged feeding. Similarly, liver sections of group 4 rats (FB_1/CI) showed evidence of mild liver injury histologically, and there appeared to be fewer hepatic GST pi positive foci in these animals. Liver sections from control animals and group 3 rats (CI) showed no evidence of liver injury.

Perls' Prussian blue staining of sequential liver sections showed progressive hepatic iron loading from week 3 to 5 in animals that received iron supplementation (groups 3 and 4). There was a striking difference in the pattern of iron distribution between animals in group 3 (CI) and group 4 (FB_1/CI). At week 5, livers from group 3 rats (CI) showed grade 3 - 4 parenchymal iron loading and a zonal gradient of iron deposition, with maximum deposition in zone 1 (Figure 8.3A). Livers from group 4 rats (FB_1/CI) showed evidence of marked iron deposition mainly in the Kupffer cells in zone 3, occurring in association with hepatocyte death in this region. (Figure 8.3B).

8.3.4. Morphometric analysis of GST pi-positive foci and nodules

Rats given dietary iron loading alone (CI) did not develop any GST pi positive hepatic foci or nodules. At 5 weeks, 4/6 rats treated with FB_1 (group 2) had developed hepatic foci and 6/6 rats from this group had developed hepatic nodules. In contrast, 3/6 rats treated with FB_1/CI (group 4) had developed foci by 5 weeks, and 4/6 rats from this group had developed nodules. At week 5, corresponding liver sections (3 sections/rat from 6 rats in each group) from rats in group 2 contained an average of 5.34 ± 1.42 GST pi positive lesions per cm^2, as compared with 1.50 ± 0.52 GST pi positive lesions per cm^2 in livers of rats from group 4 ($p < 0.05$; TABLE 8.1). Furthermore, the area (%) of liver sections occupied by GST pi positive lesions in rats from group 2 was $0.33 \pm 0.12\%$, as compared to $0.05 \pm 0.03\%$ in sections of rats from group 4 ($p < 0.05$; TABLE 8.1). These data indicate that there were more, and larger, lesions in the livers of rats that received FB_1 only (group 2).

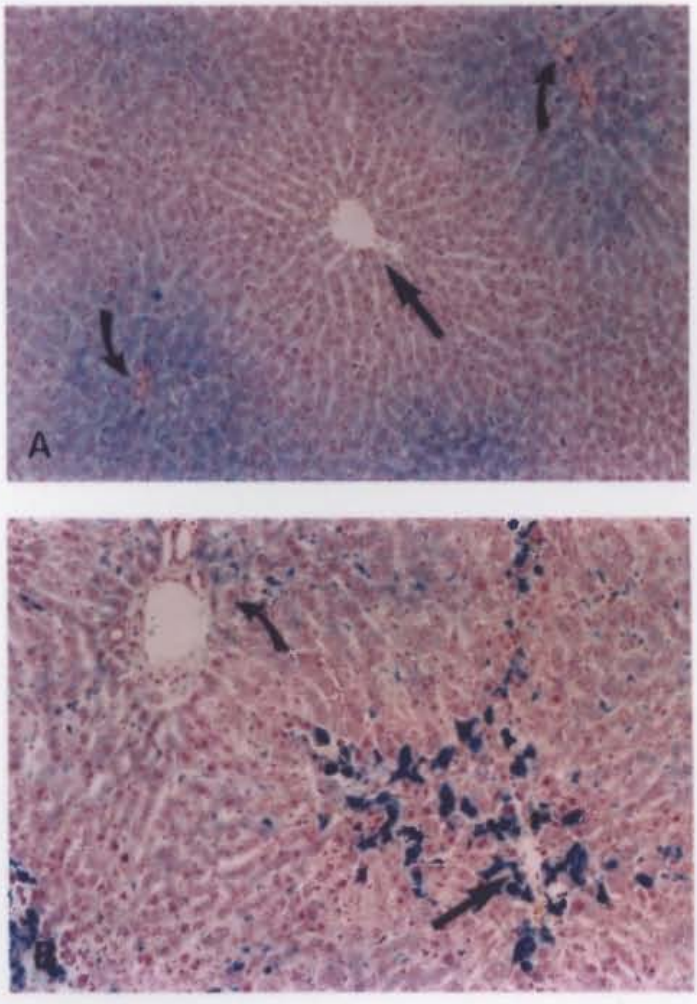

Figure 8.3. Stainable iron in livers from rats in groups 3 and 4. (A) Liver section from a rat in group 3 (Cl), showing grade 3 parenchymal iron deposition with maximal deposition in zone 1. (B) Liver section from a rat in group 4 (FB_1/Cl), showing loss of hepatocytes in zone 3 and abundant iron in Kupffer cells, with lesser amounts of parenchymal iron deposition. Perls' stain for iron, objective x 10. Straight arrows indicate central veins; curved arrows indicate portal tracts.

TABLE 8.1

Glutathione *S*-transferase (GST) pi positive hepatic lesions found at week 5 in rats treated with FB_1 (group 2) and FB_1/CI (group 4) (n = 6, each).

	FB_1 (group 2)	**FB_1/CI (group 4)**
No. of rats with foci[a]	4/6	3/6
No. of rats with nodules	6/6	4/6
No. of lesions per cm^2 [b]	5.34 ± 1.42[c]	1.50 ± 0.52
Area (%) of lesions[b]	0.33 ± 0.12[c]	0.05 ± 0.03

[a]A GST pi positive lesion was classified as a 'focus' if it had an area of less than 100 μm^2, and as a 'nodule' if it had an area of 100 μm^2 or greater.

[b]Data presented as mean ± SEM. [c]$p < 0.05$

8.3.5. Hepatic iron concentration

Hepatic iron concentration (μmol/g dry weight of liver) in the livers of rats treated with CI (group 3) and FB_1/CI (group 4) was 444 ± 56 and 479 ± 80 μmol/g dry weight respectively. In contrast, liver iron concentration in control animals (group 1) and rats treated FB_1 was 48 ± 4 and 48 ± 5 μmol/g dry weight respectively.

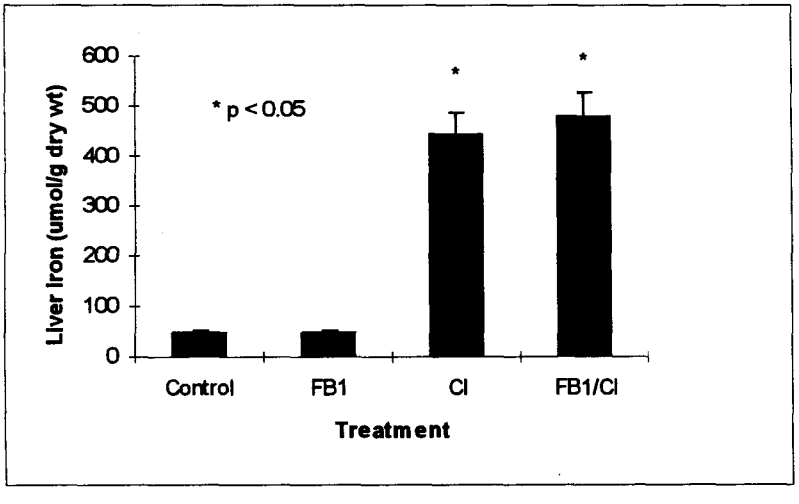

Figure 8.4. Liver iron concentration (μmol/g dry wt) in rats according to treatment group.

8.3.6. Hepatic lipid peroxidation

Generation of TBARS in liver homogenates differed between the treatment groups (Figure 8.5), and these differences persisted when using differences in weight gain as the covariant (*F* ratio = 5.24, $p < 0.01$). Treatment with FB_1 (group 2) slightly increased TBARS generation above control levels, although the increases were not significant ($p < 0.3$). Treatment with CI (group 3), however, caused a significant increase in TBARS generation ($p < 0.001$). Contrast coefficient analysis revealed that hepatic TBARS generation due to FB_1/CI (group 4) was significantly more than the sum of TBARS generation by CI (group 3) plus FB_1 (group 2) ($p < 0.0001$), indicating a potentiating effect of FB_1 on iron-induced lipid peroxidation (Figure 8.5).

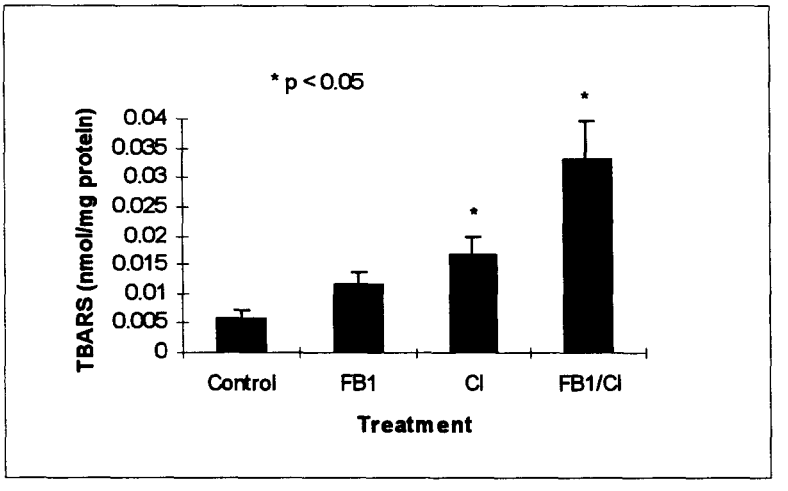

Figure 8.5. Generation of thiobarbituric acid reacting substances (TBARS) from liver homogenates according to treatment group.

8.4. Discussion

FB_1-induced hepatotoxicity may be caused, at least in part, by lipid peroxidation and oxidative damage to hepatocytes (Abel and Gelderblom, 1998; Yin *et al.*, 1998; Sahu *et al.*, 1998). Abel and Gelderblom (1998) recently showed that FB_1 caused a dose-dependent increase in the level of TBARS in rat liver *in vivo* and in primary rat hepatocytes *in vitro*. The *in vitro* effect was further potentiated by the addition of cumene hydroperoxide (CMHP), a potent oxidising agent. The authors suggested that lipid peroxidation with generation of TBARS appeared to be a secondary effect, rather than a causative mechanism, of FB_1-induced hepatic injury (Abel and Gelderblom, 1998). Sahu *et al.* (1998) found no effect of metals (iron or copper) on FB_1-induced peroxidation of lipid membranes and oxidative DNA damage of isolated rat nuclei. The present *in vivo* study, however, shows that excess liver iron (mean content 479 μmol/g dry weight) has a significant potentiating effect on FB_1-induced lipid peroxidation, measured as generation of TBARS, in liver homogenates. In contrast to the study by Abel and Gelderblom (1998), FB_1 250 mg/kg diet alone did not cause a significant increase in hepatic TBARS generation, although the level appeared to be slightly higher than in controls. This might have been due to the lower total dose of FB_1 administered in the present study due to averaged feeding of the animals. FB_1-induced hepatotoxicity was reflected by a reduction in the liver/body weight ratio and a four-fold rise in serum ALT levels, and was confirmed histologically.

Agents may increase the risk of cancer by causing DNA damage (genotoxicity) and/or by causing increased proliferation (increased DNA replications) in a pluripotential cell population of the tissue (Cohen and Ellwein, 1990). FB_1 acts as a strong promoter, and causes apoptosis together with regeneration of hepatocytes and proliferation of oval cells (Lemmer *et al.*, 1999b), putative precursor cells for liver tumours (Grisham and Thorgeirsson, 1997). Although FB_1 was found to be nongenotoxic in the Ames mutagenicity test (Gelderblom and Snyman, 1991, Park *et al.*, 1992), recent studies have reported the DNA damaging potential of FB_1 (Tolleson *et al.*, 1996b; Knasmuller *et al.*, 1997; Sahu *et al.*, 1998). FB_1-induced lipid peroxidation might result in oxidative damage to DNA and errors in replication. These effects might be expected to be enhanced by oxidative injury due to iron loading. However, in the present study, iron overload significantly enhanced lipid peroxidation in the absence of hepatotoxic injury, whereas FB_1-induced hepatocyte injury was

associated with no significant change in lipid peroxidation. These findings are in accordance with the hypothesis of Abel and Gelderblom (1998) that lipid peroxidation is secondary to FB_1-induced liver injury. With respect to cancer induction, this study showed that moderately severe hepatic iron overload (nine-fold increase in liver iron content) decreased the number and size of GST-pi positive foci and nodules in FB_1-fed rats. A similar protective effect of hepatic iron loading (6- to 13-fold increase) was reported by Stål *et al.* (1995), who added or substituted dietary iron loading for the initiating and promoting events in the Solt Faber model of chemical hepatocarcinogenesis. They showed a mild mitostimulatory effect of iron on normal hepatocytes, which may have protected against the promotion of resistant hepatocytes. Although hepatocyte proliferation was not measured in this study (e.g. BrdU labelling, PCNA), the liver weight/body weight ratio was maintained in animals treated with FB_1/CI, suggesting that iron may augment the hepatic regenerative response to FB_1-induced loss of hepatocytes and hence may counteract the selection (mitoinhibition of normal hepatocytes) that effects the outgrowth of initiated cells into GST pi positive foci and nodules. Under the present conditions, where cancer promotion was apparently impaired, the effect of excess hepatic iron on FB_1-induced cancer initiation could not be determined.

There was a striking difference in the pattern of distribution of liver storage iron between group 3 (CI) and group 4 (FB_1/CI). Dietary supplementation with CI is known to cause predominantly parenchymal (zone 1) hepatic iron deposition, with a zonal gradient, similar to that seen in genetic haemochromatosis (Park *et al.*, 1987; Roberts *et al.*, 1993). Addition of FB_1 feeding to iron loading resulted in a shift of liver iron from parenchymal cells to Kupffer cells, presumably due to ingestion of dead hepatocytes by liver macrophages. A similar shift in the distribution of iron from hepatocytes to reticuloendothelial cells is seen in alcohol-iron-CCl_4-treated rats (Mackinnon *et al.*, 1995), and also occurs in genetic haemochromatosis with episodes of alcoholic hepatitis (Powell *et al.*, 1995). Although African dietary iron overload is also characterized by massive deposition of reticuloendothelial iron (Bothwell and Bradlow, 1960), the pathogenesis of the hepatic siderosis is different, and appears to relate to associated ascorbic acid deficiency with impaired release of iron from Kupffer cells (Lipschitz *et al.*, 1971).

8.5. Conclusions

FB_1 had a potentiating effect on iron-induced lipid peroxidation in the liver. However, the effects on cancer induction by the fumonisin mycotoxins still need to be elucidated, particularly as dietary iron overload appears to protect against the promotion of GST pi positive hepatic lesions by FB_1, possibly due to a stimulatory effect on hepatocyte regeneration. These findings need to be interpreted with caution, however, as only a single marker of cancer promotion was used in this study, and a direct inhibitory effect of iron on GST pi expression cannot be excluded.

Chapter 9

Liver injury due to prolonged feeding of fumonisin B_1, and the effect of inhibition of hepatocyte regeneration on oval cell proliferation and carcinogenesis

9.1. Introduction

In humans, hepatocellular carcinoma (HCC) usually occurs in the setting of chronic necroinflammatory liver disease and cirrhosis, most commonly due to chronic hepatitis B virus (Kew, 1978; Beasley *et al.*, 1981; Moradopour and Wands, 1996; Okuda, 1997) or hepatitis C virus (Di Bisceglie, 1997; El-Serag and Mason, 1999; Ince and Wands, 1999) infection. In rodents, on the other hand, experimental HCC is usually produced by chemical carcinogens, and occurs in the absence of cirrhosis (Goldsworthy and Hanigan, 1986). Although animal models have greatly aided the understanding of the molecular and cellular events underlying hepatocarcinogenesis, their applicability to the study of human HCC remains uncertain (Grisham, 1996). Furthermore, numerous chemical agents cause HCC in rats (Goldsworthy and Hanigan, 1986), and some cause cholangiofibrosis/cholangiocarcinoma, but only a very limited number of carcinogens cause both types of liver tumour (Teebor and Becker, 1981; Maronpot *et al.*, 1991; Masuda *et al.*, 1988).

Prolonged administration of fumonisin B_1 (FB_1) to rats causes a chronic toxic hepatitis and fibrosis, which progresses to cirrhosis, and sometimes terminates in HCC (Gelderblom *et al.*, 1991). This model of hepatocarcinogenesis may thus more closely resemble the process in humans. In addition, treatment with FB_1 results in the development of cholangiofibrotic lesions (cholangiofibromas). These fibrotic bile duct lesions have no exact counterpart in humans, and are caused by a number of carcinogen regimens (Teebor and Becker, 1981; Kimbrough *et al.*, 1972; Bannasch and Massner, 1976; Oshima *et al.*, 1984; Evans *et al.*, 1989). Although cholangiofibrotic lesions commonly remain static throughout the lifetime of

the animal (Edwards and White, 1941), progression to cholangiocarcinoma has been demonstrated with some carcinogens, particularly furan (Maronpot *et al.*, 1991) and FB_1 (Gelderblom *et al.*, 1991), and also in Long-Evans Cinnamon (LEC) rats with hereditary hepatitis (Masuda *et al.*, 1988). FB_1 thus causes both hepatocellular and cholangiocellular carcinomas in rats, indirect evidence for a common ('stem') cell of origin of these tumours.

Hepatic oval cells proliferate under certain conditions, mainly when hepatocytes are prevented from proliferating in response to liver damage. 2-acetylaminofluorene (AAF) given before a partial hepatectomy (PH) of two thirds results in suppression of hepatocyte proliferation and allows sustained proliferation of oval cells (Solt and Farber, 1976; Evarts *et al*, 1987; Evarts *et al*, 1989; Thorgeirsson *et al.*, 1993). Similarly, chemical injury with carbon tetrachloride (CCl_4) or allyl alcohol in the presence of AAF results in a marked oval cell response (Petersen *et al.* 1998b).

The present study describes the sequential changes in hepatic histopathology during prolonged feeding of FB_1. In order to test the hypothesis that inhibition of hepatocyte regeneration in the face of liver injury enhances FB_1-induced oval cell proliferation and liver tumorigenesis, animals were treated with different FB_1 feeding regimens alone and in the presence AAF. Scoring systems were devised for selected hepatic histological parameters, and the time course changes in these parameters were followed by serial liver biopsies. All treatments were discontinued after 25 weeks, and the animals were followed for a further 25 weeks on control AIN-76A diet, in order to distinguish reversible from persistent lesions ('stop study') (Bannasch *et al.*, 1982; Tatematsu *et al.*, 1988; Bannasch and Zerban, 1990, Maronpot *et al.*, 1991). Post mortem livers were examined at the time of sacrifice at the end of the study, or where animals died unexpectedly during the course of the study

9.2. Experimental methods

9.2.1. Animals and diet

The study was approved by the Ethics and Research Committee of the University of Cape Town, and the experiments were conducted in accordance with the laws and regulations controlling experiments on live animals in South Africa. The purification of FB_1 and preparation on the diets were performed as described in Chapter 5.

9.2.2. Experimental

Fifty four male Fischer 344 rats (150 g) were randomly divided into four treatment groups (Figure 9.1) and a control group. The treatment regimens were administered for 25 weeks, after which animals in all groups were returned to control AIN-76A diet and followed for an additional 25 weeks.

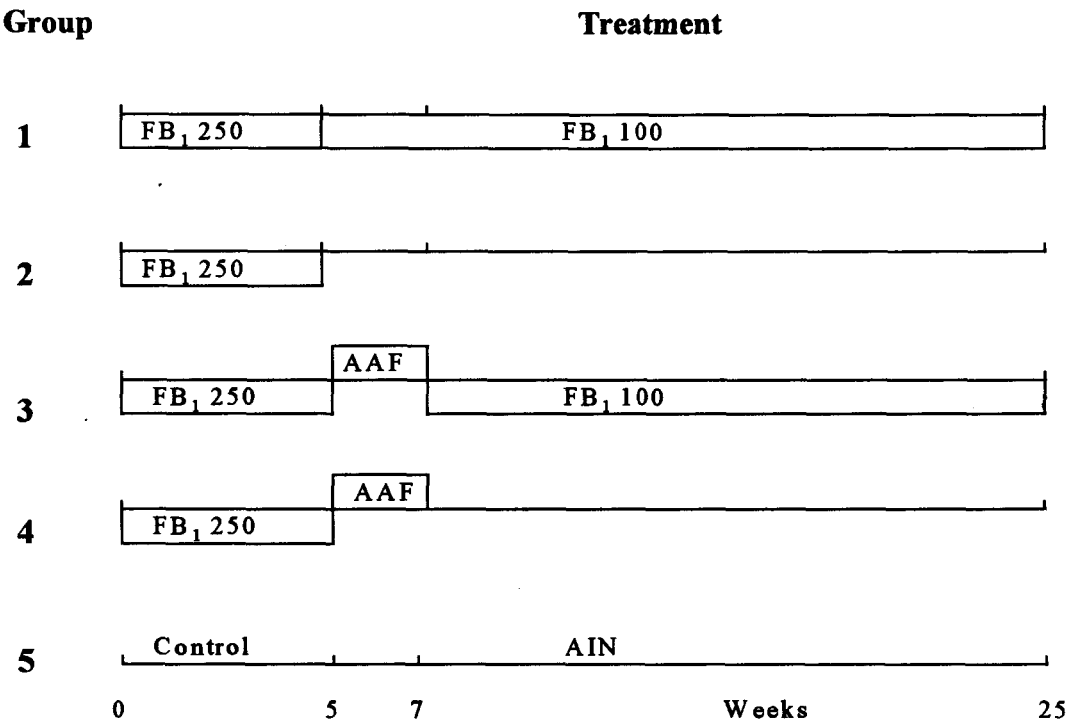

Figure 9.1. Treatment regimens (groups 1 - 5) for 25 week 'on/off' study (see text for details). FB_1 250, fumonisin B_1 250 mg/kg; FB_1 100, fumonisin B_1 100 mg/kg; AAF, 0.02% 2-acetylaminofluorene; AIN, American Institute of Nutrition-76A (control) diet.

Details of the treatments groups were as follows: *Group 1* (FB_1/FB_1; n=12). FB_1 250 mg/kg diet (initial dose) for 5 weeks, and thereafter FB_1 100 mg/kg diet (maintenance dose) until 25 weeks; *Group 2* (FB_1; n=12). FB_1 250 mg /kg diet for 5 weeks, and thereafter AIN-76A diet only; *Group 3* (FB_1/AAF/FB_1; n=12). FB_1 250 mg/kg diet for 5 weeks, followed by AAF 0.02% for 2 weeks, and then FB_1 100 mg/kg resumed until 25 weeks; *Group 4* (FB_1/AAF; n=12). FB_1 250 mg/kg diet for 5 weeks, followed by AAF 0.02% for 2 weeks, and then AIN-76A diet only; *Group 5* (control; n=6). Control group fed AIN-76A diet only.

The sequential development of hepatic histopathological changes in individual rats were studied by repeated open liver biopsies as described in Chapter 5, and animals were sacrificed at the end of the study. Liver biopsies were performed at five and seven weeks, and then monthly (Figure 9.1). At each session, two rats from each of the treatment groups and one control animal were biopsied. Rats receiving biopsies were rotated at each session until all the animals in the different groups had undergone liver biopsy, whereafter repeat biopsies were performed in the same order. All 54 rats had one open liver biopsy, and 32 rats underwent a second biopsy before sacrifice at 50 weeks. Because of the high procedure-related mortality rate encountered, no liver biopsies were performed during the last two months of the study. At sacrifice, the animals were anaesthetised by the intraperitoneal injection of a sodium pentobarbitone solution (6% m/v). The livers were removed, and slices of tissue were taken from the left, right, and median lobes of the liver for histopathological analysis.

9.2.3. Light microscopy

For routine light microscopy, blocks of liver 4-5 mm in thickness were immersion-fixed in 10% neutral buffered formalin overnight before processing and embedding in paraffin wax (see Chapter 5). Stains included routine haematoxylin and eosin (H&E) and sirius red for collagen.

9.2.4. Immunohistochemistry

Immunohistochemical stains performed on paraffin sections included desmin D33, OV-6 monoclonal antibody, and polyclonal GST pi (see Chapter 5). Staining with Desmin D33 (Dako, Copenhagen, Denmark) for hepatic stellate (Ito) cells and rabbit polyclonal GST pi (Novacastra, Newcastle-Upon-Tyne, UK) for 'enzyme-altered' hepatic foci and nodules were performed on paraffin sections. After sequential layering with biotinylated rabbit anti-mouse

or swine anti-rabbit (Dako, Copenhagen, Denmark) 1:250 dilution as link antibodies, peroxidase conjugated Streptavidin (Dako, Copenhagen, Denmark) 1:500 was applied for 30 min at room temperature. The OV-6 mouse monoclonal antibody, which stains both oval cells and bile duct cells, was a generous gift from Professor Stewart Sell, Albany, New York. Acetone fixed cryostat sections were brought to room temperature and stained by means of a standard two-score indirect peroxidase conjugated technique (Dako P161, Copenhagen, Denmark). For the detection of α-fetoprotein (AFP), tissues were fixed for 4 h in Bouin's fixative and embedded in paraffin wax (Omori *et al.*, 1997a). Polyclonal rabbit anti-rat AFP (Accurate Chemicals, New York, NY) was used as primary antibody, and fetal rat livers were used as positive controls.

9.2.5. Scoring of fibrosis, oval cell proliferation, GST pi positive lesions and dysplastic nodules

In order to follow selected histological features with time in each group, numerical scoring systems were devised for hepatic fibrosis, oval cell proliferation, and the extent of the GST pi positive lesions/dysplastic nodules (TABLE 9.1). Liver sections from the different treatment groups were scored for each of these parameters by two independent assessors, who were unaware of the treatment administered.

TABLE 9.1

Scoring of hepatic fibrosis, oval cell proliferation, and the extent of GST pi positive lesions and dysplastic nodules.

Hepatic fibrosis (modified from Scheuer, 1991)	Score
None	0
Enlarged, fibrotic portal tracts	1
Periportal or portal-portal septa	2
Fibrosis with disturbed architecture, but no obvious cirrhosis	3
Probable or definite cirrhosis	4
Oval cell proliferation	**Score**
None	0
Confined to portal tracts	1
Radiating out from portal tracts into fibrous septa	2
Infiltrating liver parenchyma	3
Diffuse proliferation	4
GST pi positive lesions[a]	**Score**
None	0
< 25% of liver section	1
25 - 50% of liver section	2
> 50% but < 75% of liver section	3
75% - 100% of liver section	4
Dysplastic nodules[a]	**Score**
None	0
< 25% of liver section	1
25 - 50% of liver section	2
> 50% but < 75% of liver section	3
75% - 100% of liver section	4

[a] % area occupied by GST pi positive lesions and dysplastic lesions was a simple visual assessment by the histopathologist.

Dysplastic liver nodules were labelled as low-grade dysplastic, high-grade dysplastic or well-differentiated HCC, based on a modification of the International Working Party (Wanless *et al.*, 1995) criteria for the classification of human nodular hepatocellular lesions (TABLE 9.2). In practice, the features that were found to be most useful in distinguishing low-grade from high-grade dysplastic nodules were the presence of mitoses, occasional pseudogland formation, and the compression of surrounding liver parenchyma. The predominant cytoplasmic change encountered in high-grade dysplastic nodules in the rat appeared to be eosinophilic large cell change.

TABLE 9.2

Histological criteria to distinguish between dysplastic nodules (low-grade and high-grade) and well-differentiated hepatocellular carcinoma. Modified from Wanless *et al.*, (1995).

Histological feature	Dysplastic nodule, low-grade	Dysplastic nodule, high-grade	Well-differentiated HCC
Mitotic figures[a]	-	+	++
Cell density[b]	-	+	++
Nuclear hyperchromasia	-	+	+
Irregular nuclear contour	-	+	+
Clone-like populations	+	+	+
Hepatic plates > 3 cells wide	-	-	+
Pseudogland formation	-	+	+
Eosinophilic large cells	-	+	+
Reticulin less than normal	-	-	+
Compression of surrounding liver	-	+	+
Invasion of stroma or portal tracts	-	-	+

[a]Mitotic figures: + = 1-5/10 HPFs, ++ = greater than 5/10 HPFs;

[b]Cell density: + = greater than 1.3 times normal; ++ = greater than 2 times normal.

Abbreviations: HCC = hepatocellular carcinoma; HPF = high power field.

9.2.6. Data presentation

Although formal statistical analysis of the data was not performed because of the small number of animals from different groups at each biopsy time-point, histological parameters were compared using cumulative scores at each time-point. Post mortem histological data for hepatic nodules and bile duct lesions in the different groups were presented in the form of scatter plots.

9.3. Results

9.3.1. Rat deaths

The 54 rats underwent 77 open liver biopsies. There were 22 deaths during the 50 week study period. Thirteen deaths were related to the biopsy procedure and anaesthetic (TABLE 9.3). These included uncontrolled haemorrhage from the raw liver surface (2), bowel perforation due to adhesions related to previous surgery (1), and 'anaesthetic deaths' presumably due to the effects of general anaesthesia and surgery in animals with chronic FB_1-induced hepatotoxicity (10). Identified causes of deaths not related to surgery included pneumonia (3), sepsis (1), or poor general condition necessitating early termination (3). There were two rats for which the cause of death was unknown: rat #9 (group 1) died at week 38 just before it was due for a second biopsy, and rat #44 (group 4) died at week 45 shortly before the end of the study. Unfortunately, in these two cases, the animals died out of normal working hours and the carcasses were disposed of in error before the deaths were reported to the project team.

Seven deaths occurred in group 1, eight deaths in group 2, four deaths in group 3, three deaths in group 4, and one death in the control group. The timing and causes of death in the animals are depicted in TABLE 9.3 below.

TABLE 9.3

Causes of rat deaths in the different treatment groups, sorted according to timing of mortality.[a]

Rat #.	Group	Week	Cause
36	1	5	perioperative (1)[b]
33	2	6	perioperative (1)
27	2	12	biopsy bleed (1)
52	3	15	perioperative (1)
43	5	15	perioperative (1)
45	4	15	biopsy bleed (1)
47	1	23	perioperative (1)
51	2	24	otitis (culled)
32	4	31	perioperative (2)
13	2	35	perioperative (2)
23	1	35	perioperative (2)
12	1	39	perioperative (2)
17	2	39	perioperative (2)
31	2	39	bowel perforation (2)
40	3	42	pneumonia
34	2	45	poor condition
16	3	47	pneumonia
6	2	48	poor condition
22	1	48	poor condition
42	3	49	pneumonia

[a]Cause of death was not recorded for two animals (see text)

[b]For 'perioperative' deaths, the numbers in parentheses indicate during which liver biopsy procedure (1 or 2) the death occurred.

9.3.2. Hepatic histopathology

The hepatic histopathology is reported for each treatment group (1 - 5) according to a general schema. The sequential histological changes in liver biopsy findings are described. Cumulative scores are shown for fibrosis, oval cell proliferation, GST pi positive lesions, dysplastic nodules, and cholangiofibrotic lesions seen in liver biopsy specimens (Figures 9.2 to 9.6). The liver post mortem findings in the livers are described separately, and the extent of GST pi positive lesions/dysplastic lesions, as well as the number of cholangiofibrotic lesions are depicted as scatter plots (Figures 9.7 to 9.9).

9.3.2.1. GROUP 1 (FB_1/FB_1)

Biopsy findings:

(i) Fibrosis and hepatic stellate cells. A progressive increase in the cumulative score for hepatic fibrosis was noted over the course of the study (Figure 9.2). A prominent proliferation of desmin-positive hepatic stellate cells, radiating out from portal tracts into the surrounding hepatic parenchyma, was identified at weeks 5 and 7. Thereafter, the proliferation of stellate cells appeared to decline over the remainder of the study period. Sirius red staining for collagen showed early development of hepatic fibrosis, and portal-portal linkage with architectural distortion (score 3) was evident by week 7. Further progression however appeared to occur more gradually, and only one liver section showed established cirrhosis.

(ii) Oval cell proliferation. A progressive increase in the cumulative score for oval cell proliferation was noted till week 23, whereafter the score appeared to level out somewhat (Figure 9.3). Staining with OV-6 monoclonal antibody showed score 2 - 3 oval cell proliferation during the course of the study. Proliferating oval cells were noted to radiate out from the portal tracts along the fibrous septa, and to extend into the hepatic parenchyma. Staining with GST pi demonstrated cells with the morphology of oval cells and not expressing GST pi, that were proliferating inside developing GST pi positive lesions (Figures 9.10C and 9.10D). Conversely, hepatocytes within some dysplastic nodules and bile duct cells within cholangiofibrotic lesions showed expression of OV-6 (see below).

(iii) GST pi positive lesions. A progressive increase in the cumulative score for GST pi positive lesions was noted over the course of the study (Figure 9.4). GST pi positive lesions were seen as early as week 5. The GST pi positive lesions were larger at later time points, and

by week 19 these lesions occupied 25 -50% (score 2) of the liver sections. As described above, a striking feature was the presence of oval cells (GST pi negative) within almost all GST pi positive lesions.

(iv) Dysplastic nodules. There was a marked increase in the cumulative score for dysplastic nodules, particularly from week 15 onwards (Figure 9.5). At routine H&E staining, the hepatocytes within the nodules showed predominantly large cell eosinophilic change of the cytoplasm. The nodules invariably showed features of high-grade dysplasia, as evidenced by the presence of mitoses, occasional pseudogland formation, and compression of the surrounding parenchyma. These 'atypical' nodules were infiltrated by nonparenchymal (oval) cells, which radiated out from the portal tracts (Figure 9.10E). From week 19 onwards, some of the hepatocytes in the nodules showed strong cytoplasmic expression of OV-6 at the periphery of the cells. However, no expression of AFP by cells in the dysplastic nodules could be demonstrated at any stage in paraffin sections, and no unequivocal HCCs were diagnosed in liver biopsy specimens.

(v) Cholangiofibrotic lesions. Two cholangiofibrotic lesions were identified in liver biopsy sections stained with sirius red for collagen (Figure 9.6). At routine light microscopy, these focal lesions were seen as proliferating and dilated bile ducts, which showed no evidence of dysplasia. The bile duct lesions were surrounded by desmin-positive hepatic stellate cells and dense fibrosis. The bile ducts within the cholangiofibrotic lesions expressed OV-6.

Post mortem findings:

(i) Fibrosis. Most livers showed evidence of score 2 - 3 fibrosis at post mortem, but one liver showed established cirrhosis (score 4).

(ii) Oval cell proliferation. Oval cell proliferation in post mortem livers varied between score 1 - 3.

(iii) GST pi positive lesions. There was a wide scatter in percentage liver involvement by GST pi positive lesions at post mortem (Figure 9.7 and Appendix A), which ranged from less than 25% (score 1) to more than 75% (score 4).

(iv) Dysplastic nodules. There was a wide scatter in the scores for dysplastic nodules in post mortem livers, and most of the dysplastic nodules showed features of high-grade dysplasia (Figure 9.8 and Appendix A). An unequivocal HCC (mixed trabecular/pseudoglandular pattern) was found in Rat #28 (Figure 9.11F), and the rest of the liver was virtually replaced

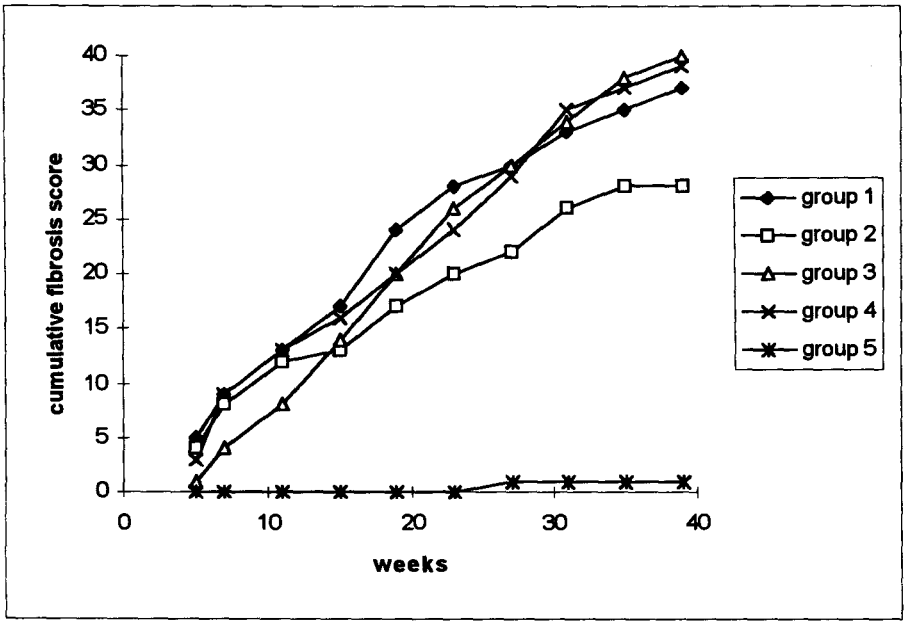

Figure 9.2. Cumulative scores for fibrosis in rat liver biopsies from the different treatment groups.

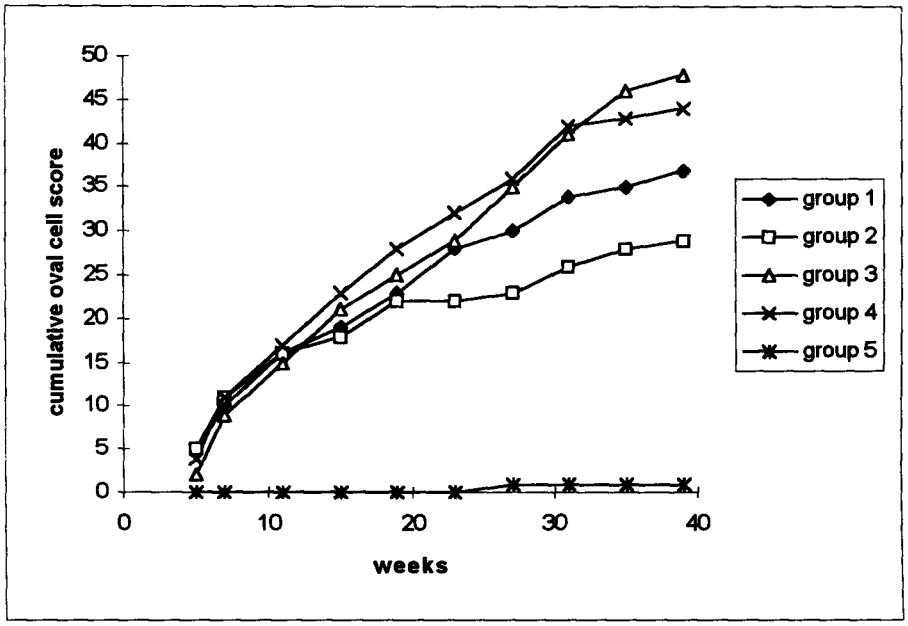

Figure 9.3. Cumulative scores for oval cell proliferation in rat liver biopsies from the different treatment groups.

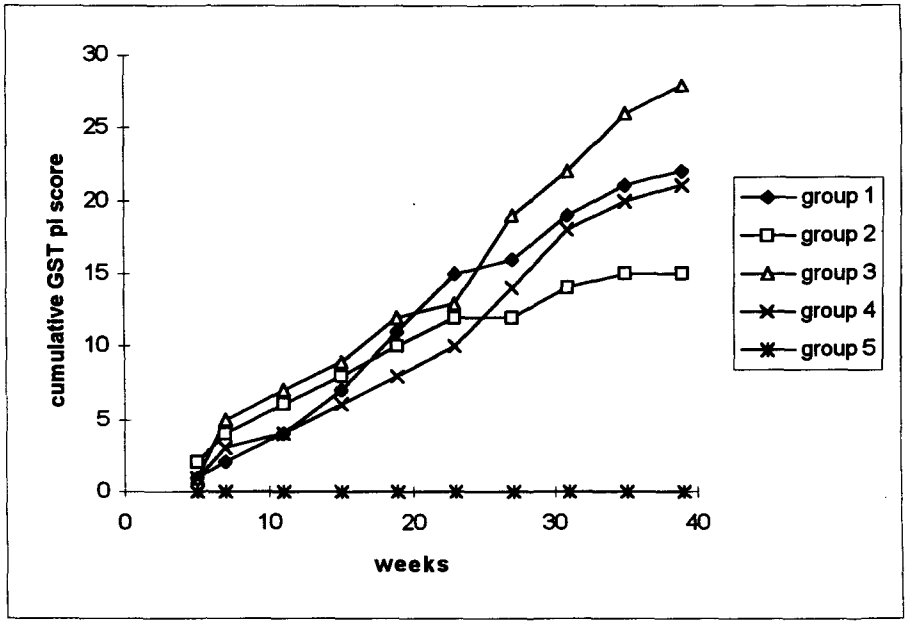

Figure 9.4. Cumulative scores for GST pi positive lesions in rat liver biopsies from the different treatment groups.

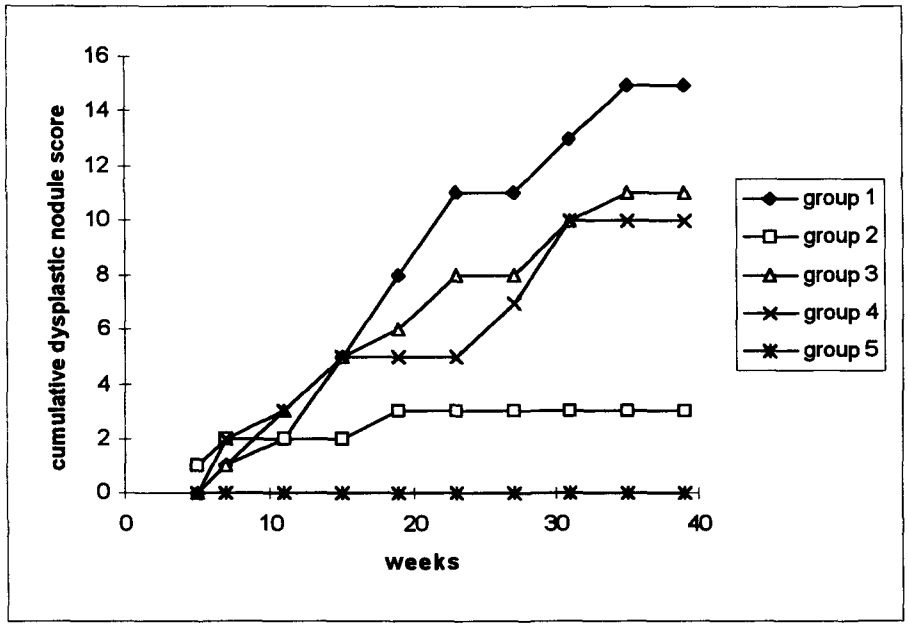

Figure 9.5. Cumulative scores for dysplastic nodules in rat liver biopsies from the different treatment groups.

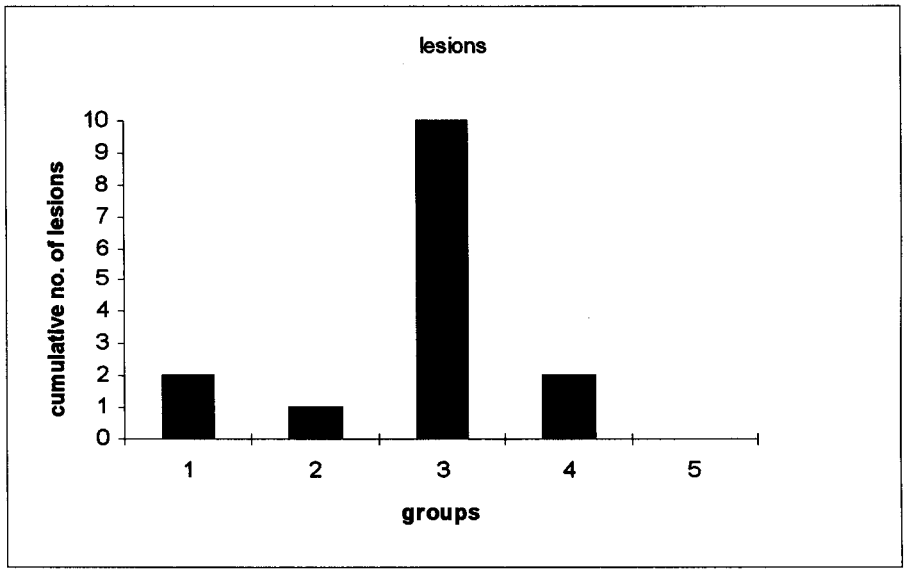

Figure 9.6. Cumulative number of cholangiofibrotic lesions in the different treatment groups at the end of liver biopsy procedures.

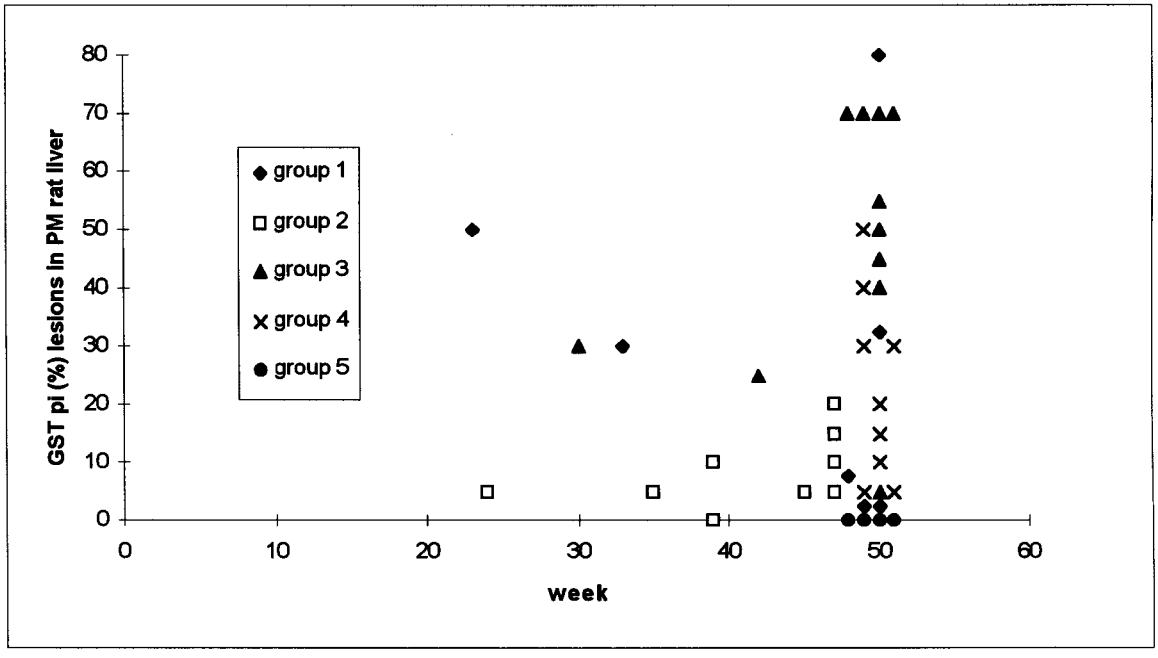

Figure 9.7. Scatter plot of GST pi positive lesions (%) in rat livers at post mortem in different treatment groups.

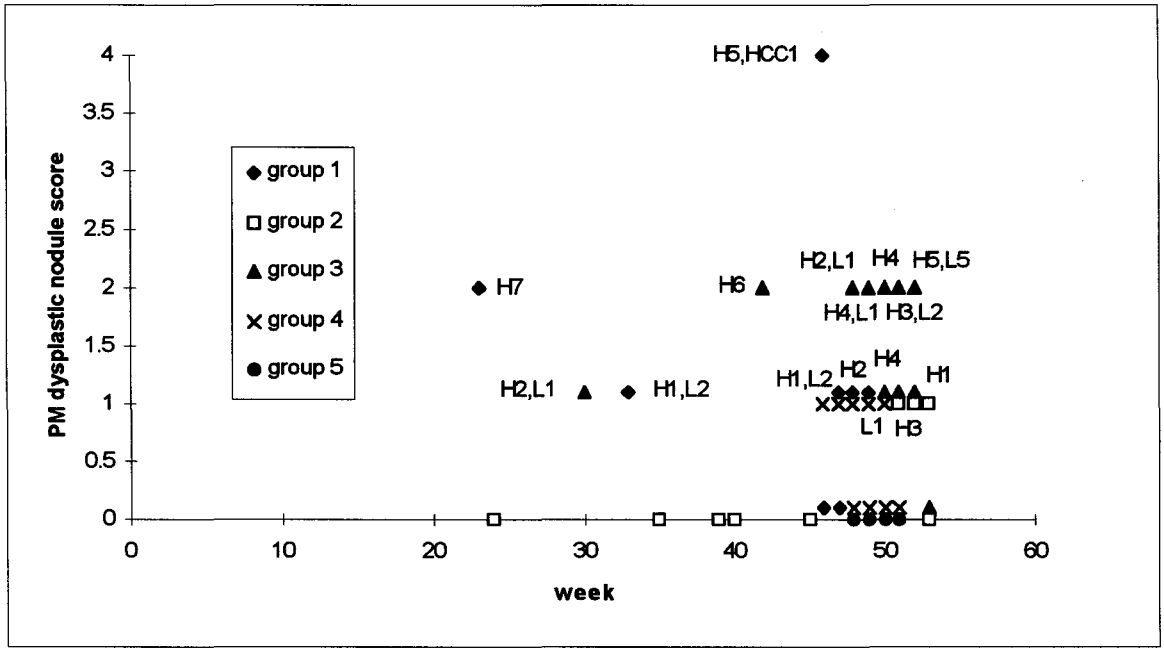

Figure 9.8. Scatter plot of scores for dysplastic nodules in rat livers at post mortem for the different treatment groups. The number of low-grade (L) dysplastic nodules, high-grade (H) dysplastic nodules, and hepatocellular carcinomas (HCC) in livers of animals from group 1 and group 3 are indicated.

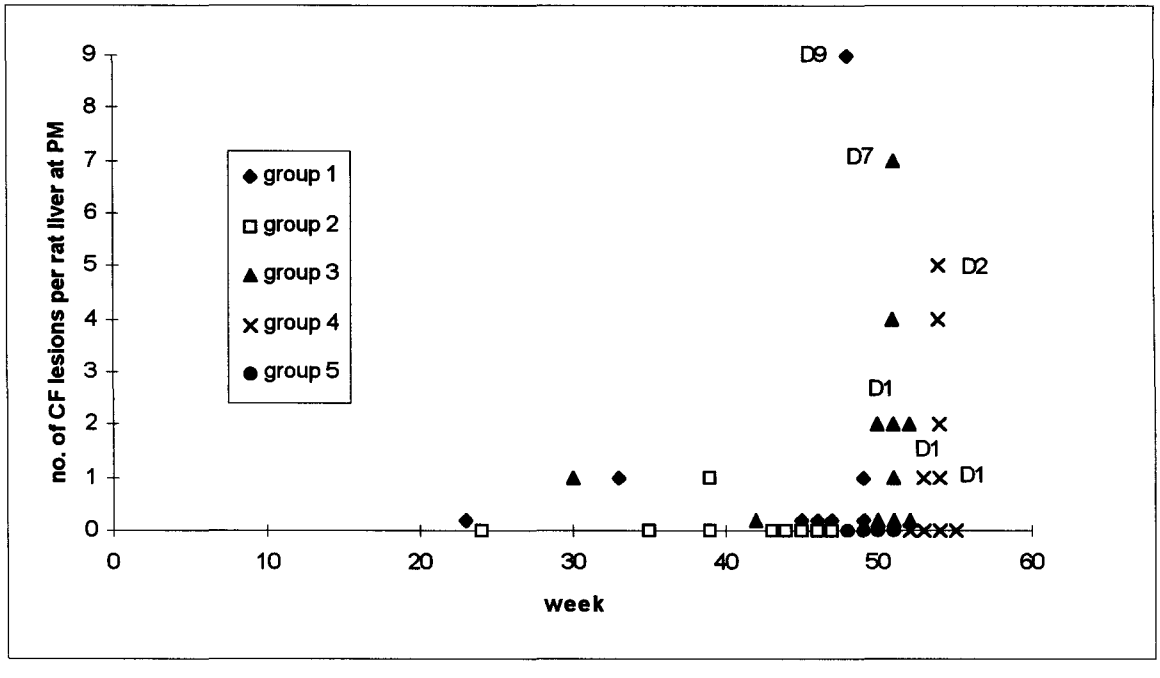

Figure 9.9. Number of cholangiofibrotic (CF) lesions per rat liver at post mortem in the different treatment groups. D = number of lesions with features of dysplasia.

by high-grade dysplastic nodules.

(v) Cholangiofibrotic lesions. One post mortem liver (Rat #4) contained a single cholangiofibrotic lesion and another (Rat #22) contained 9 lesions, all of which showed features of dysplasia (Figure 9.9 and Appendix A). Dysplastic cholangiofibrotic lesions showed irregularity of ducts, and there was evidence of severe cytological atypia of bile duct cells (e.g. crowding of cells, nuclear hyperchromasia, and mitoses), suggestive of intraductal cholangiocarcinoma. One of the dysplastic cholangiofibrotic lesions in Rat #22 produced pools of mucin, additional evidence for a cholangiocarcinoma.

9.3.2.2. GROUP 2 (FB_1/AIN)

Biopsy findings:

(i) Fibrosis. There was an initial progressive increase in the cumulative score for hepatic fibrosis until week 11, but thereafter the score appeared to level off somewhat in this group (Figure 9.2). Sirius red staining for collagen showed the presence of score 2 hepatic fibrosis by week 5, but the fibrosis scores of individual liver sections did not progress beyond score 2 during the remainder of the study.

(ii) Oval cell proliferation. There was an initial progressive increase in the cumulative score for oval cell proliferation until week 11, but thereafter the cumulative score appeared to reach plateau levels (Figure 9.3). Staining with OV-6 showed score 2 - 3 oval cell proliferation at weeks 5 to 11, but this proliferation appears to have levelled out by week 23.

(iii) GST pi positive lesions. There was a progressive increase in the cumulative score for GST pi positive lesions until week 23, whereafter the score appeared to reach plateau levels (Figure 9.4). Small (score 1) GST pi positive lesions were seen at week 5, but the extent (%) of liver involvement by these lesions did not increase at subsequent time points.

(iv) Dysplastic nodules. There was almost no increase in the cumulative score for dysplastic nodules (Figure 9.5). Furthermore, features of high-grade dysplasia were found in only three liver biopsies during the course of the study period.

(v) Cholangiofibrotic lesions. Only a single small cholangiofibrotic lesion was detected in liver biopsy sections stained with sirius red for collagen (Figure 9.6).

Post mortem findings:

(i) Fibrosis. Post mortem livers showed mild (score 1 to 2) fibrosis only, and in no liver did

by high-grade dysplastic nodules.

(v) Cholangiofibrotic lesions. One post mortem liver (Rat #4) contained a single cholangiofibrotic lesion and another (Rat #22) contained 9 lesions, all of which showed features of dysplasia (Figure 9.9 and Appendix A). Dysplastic cholangiofibrotic lesions showed irregularity of ducts, and there was evidence of severe cytological atypia of bile duct cells (e.g. crowding of cells, nuclear hyperchromasia, and mitoses), suggestive of intraductal cholangiocarcinoma. One of the dysplastic cholangiofibrotic lesions in Rat #22 produced pools of mucin, additional evidence for a cholangiocarcinoma.

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(iii) GST pi positive lesions. There was a progressive increase in the cumulative score for GST pi positive lesions until week 23, whereafter the score appeared to reach plateau levels (Figure 9.4). Small (score 1) GST pi positive lesions were seen at week 5, but the extent (%) of liver involvement by these lesions did not increase at subsequent time points.

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(v) Cholangiofibrotic lesions. Only a single small cholangiofibrotic lesion was detected in liver biopsy sections stained with sirius red for collagen (Figure 9.6).

Post mortem findings:

(i) Fibrosis. Post mortem livers showed mild (score 1 to 2) fibrosis only, and in no liver did

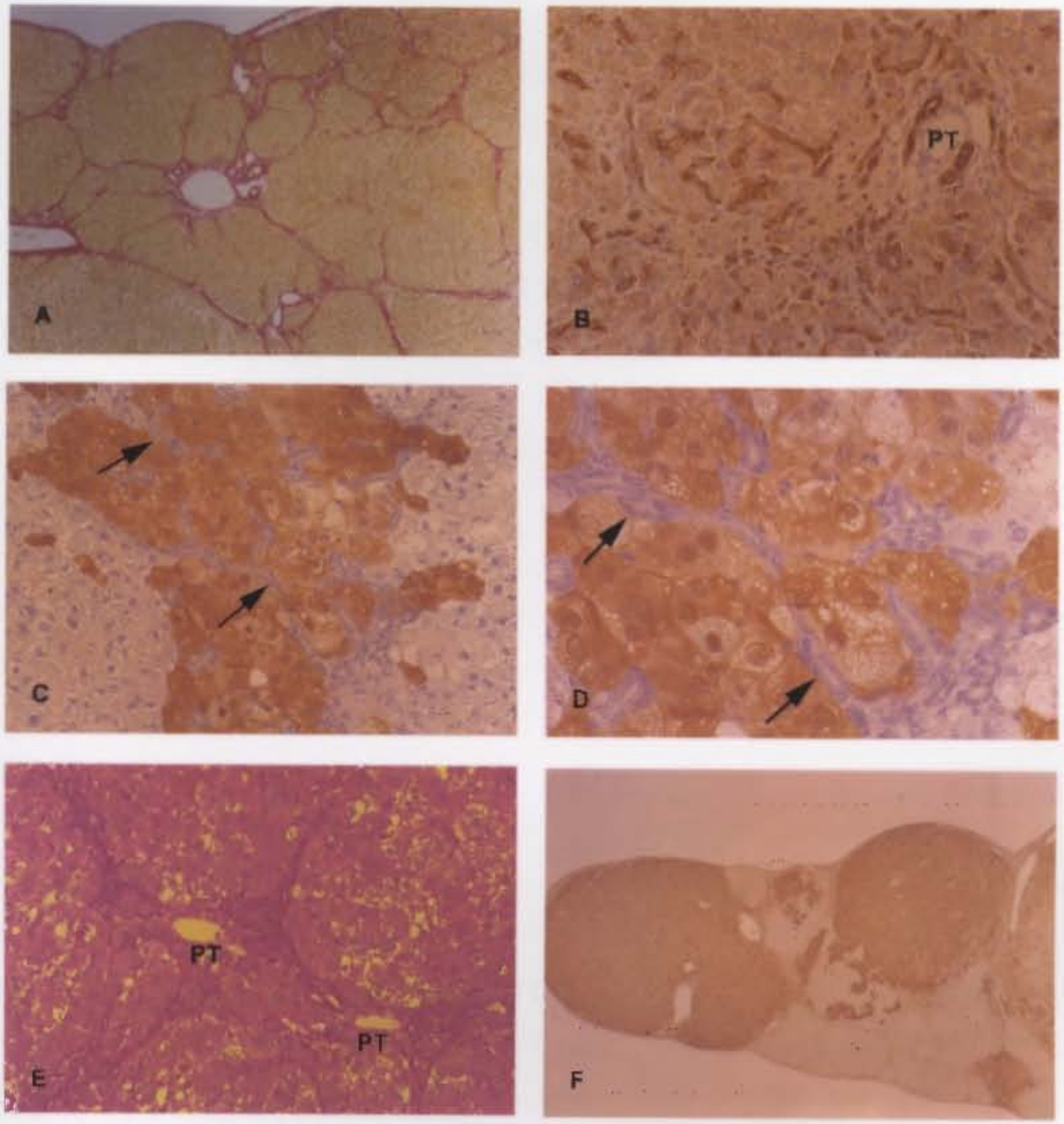

Figure 9.10

the amount of fibrosis exceed score 2.

(ii) Oval cell proliferation. Oval cell proliferation was either absent (score 0) or minimal (score 1) in most post mortem livers, and only one liver showed score 2 oval cell proliferation.

(iii) GST pi positive lesions. The involvement (%) of liver by GST pi lesions at post mortem was consistently low (0 - 20%) (Figure 9.7 and Appendix A).

(iv) Dysplastic nodules. Most post mortem livers did not contain any dysplastic lesions (Figure 9.8 and Appendix A). Two post mortem livers contained one low-grade dysplastic nodule each, and only one liver (Rat #5) contained high-grade dysplastic nodules.

(v) Cholangiofibrotic lesions. A single cholangiofibrotic lesion was found in one post mortem liver (Rat #17), and this lesion showed no evidence of dysplasia (Figure 9.9 and Appendix A).

Figure 9.10. Liver pathology during prolonged feeding of fumonisin B_1-containing treatment regimens to Fischer 344 rats (see text for details). (A) Liver biopsy from Rat #23 (group 3) at week 23 showing loss of the normal acinar architecture due to the presence of regenerative nodules of hepatocytes which are partially or completely surrounded by bands of fibrous tissue (fibrosis score 4, established cirrhosis). Sirius red stain for collagen, objective x 4. (B) Liver biopsy from Rat #32 (group 4) at week 7 showing extensive (score 4) proliferation of OV-6-positive oval cells and ductules, which are radiating out from a portal tract (PT) as ribbons and cords into the hepatic parenchyma. Objective x 20. (C) Liver biopsy liver from Rat #22 (group 1) at week 19 showing cells with the morphology of oval cells (arrows) which are proliferating inside a lesion composed of GST pi positive hepatocytes. The oval cells are GST pi negative, which aids their identification within the GST pi positive lesion. Objective x 20. (D) Higher magnification of the liver in Figure 9.10C showing oval cells (arrows) within a group of GST pi positive hepatocytes. (E) Liver biopsy from Rat #36 (group 1) at week 5 showing the proliferation of oval cells, which are radiating out from two portal tracts (PT) and infiltrating into surrounding 'atypical' nodules of dysplastic (low-grade) hepatocytes. H&E, objective x 20. (F) Liver biopsy from Rat #25 (group 3) at week 23 showing loss of the normal acinar architecture due to the presence of regenerative nodules of hepatocytes, suggestive of established cirrhosis. Hepatocytes within some of the 'atypical' nodules stain positive for GST pi. Objective x 4.

9.3.2.3. GROUP 3 (FB_1/AAF/FB_1)

Biopsy findings:

(i) Fibrosis. A progressive increase in the cumulative score for hepatic fibrosis was noted during the course of the study (Figure 9.2). Prominent proliferation of desmin-positive hepatic stellate cells, radiating out from the portal tracts, was noted at weeks 5 and 7. Most liver biopsies during the study period showed score 2 - 3 fibrosis, but in three animals the biopsies showed established cirrhosis (Figure 9.10A).

(ii) Oval cell proliferation. A progressive increase in the cumulative score for oval cell proliferation was noted during the course of the study (Figure 9.3). Staining with OV-6 showed score 2 - 3 oval cell proliferation throughout the study period, and one liver biopsy showed score 4 (diffuse) proliferation.

(iii) GST pi positive lesions. A progressive increase in the cumulative score for GST pi positive lesions was noted during the course of the study (Figure 9.4). In most biopsies, these lesions occupied 50% or less of the section (score 1 - 2), but in one rat the GST pi positive lesions had virtually replaced the biopsy section (score 4). GST pi positive lesions were sometimes seen within regenerative nodules (Figure 9.10F). Proliferating oval cells were seen inside GST pi positive lesions in liver biopsy specimens taken throughout the study.

(iv) Dysplastic nodules. There was a progressive increase in the cumulative score for dysplastic nodules during the study period (Figure 9.5). Most of the nodules showed cytological features of high-grade dysplasia, but in one biopsy section a low-grade dysplastic nodule was found. Some hepatocytes in nodules showed strong cytoplasmic expression of OV-6 at the periphery of the cells, and almost all dysplastic nodules contained proliferating OV-6 positive oval cells and ductules. No expression of AFP could be detected, and no cases of unequivocal HCC were found in liver biopsy specimens.

(v) Cholangiofibrotic lesions. Ten cholangiofibrotic lesion were identified in liver biopsy sections from group 3 animals (Figure 9.6). These cholangiofibrotic lesions were particularly striking in the biopsy taken from Rat #52, and were seen as proliferating bile ducts surrounded by hepatic stellate cells and dense fibrosis. Staining with desmin D33 demonstrated the presence of vascular proliferation, as well as proliferating hepatic stellate

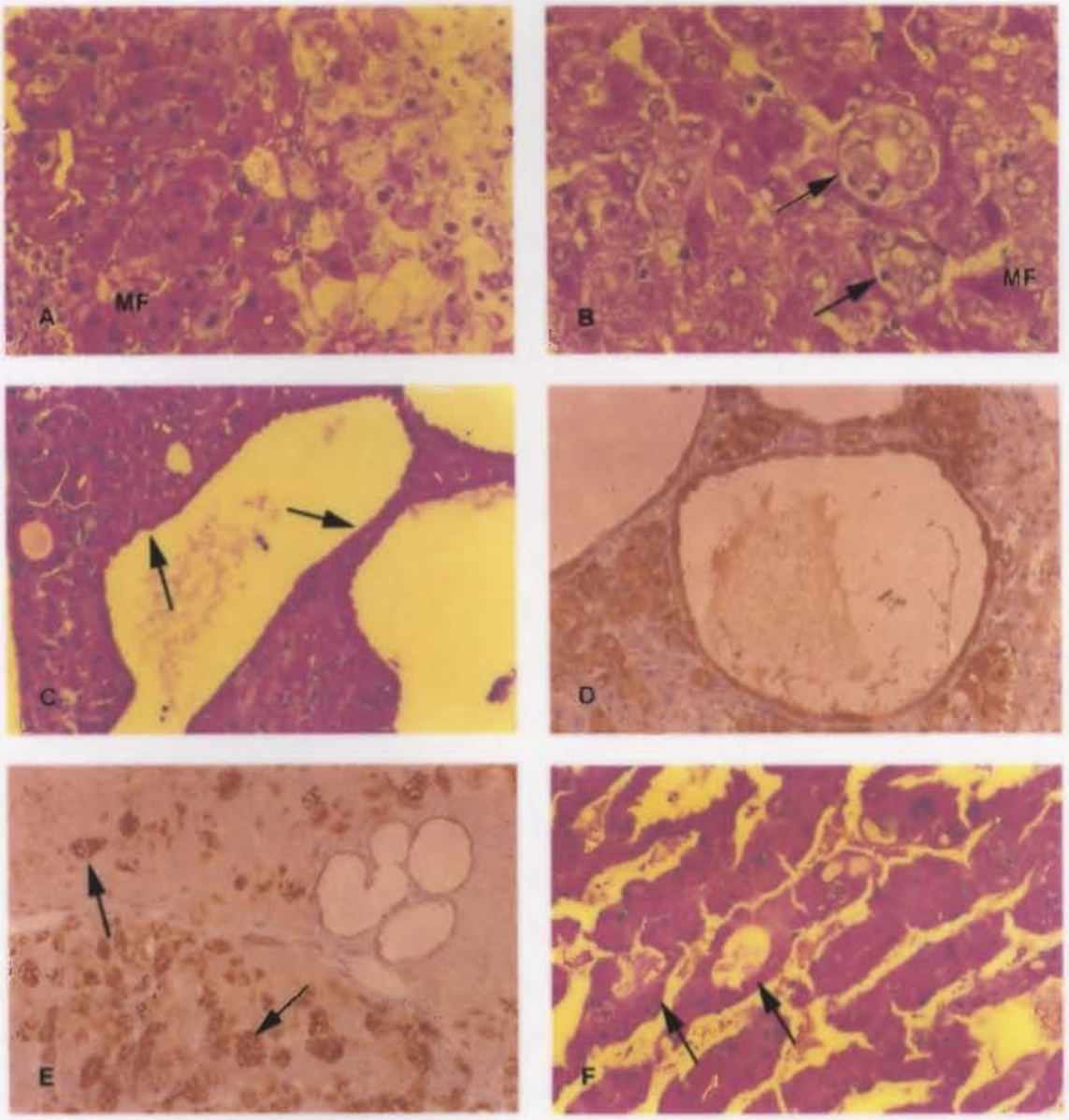

Figure 9.11

cells within the fibrous tissue (Figure 9.12B). Some of the cholangiofibrotic lesions showed features of dysplasia (as described for group 1), without unequivocal evidence of invasion, and this was felt to be consistent with a diagnosis of intraductal cholangiocarcinoma (Figure 9.12C).

Post mortem findings:

(i) Fibrosis. Most livers showed evidence of score 2 -3 fibrosis, and none of the livers showed established cirrhosis.

(ii) Oval cell proliferation. The amount of oval cell proliferation in post mortem livers varied between score 1 to 3.

Figure 9.11. Liver pathology during prolonged feeding of fumonisin B_1-containing treatment regimens to Fischer 344 rats (see text for details). (A) Post mortem liver from Rat #24 (group 3) at week 50 sacrifice showing an 'atypical' nodule with features of high-grade dysplasia. The dysplastic nodule contains both eosinophilic and clear hepatocytes, and these cells show evidence of cytological atypia. A mitotic figure (MF) is seen in an eosinophilic hepatocyte. H&E, objective x 20. (B) Post mortem liver from Rat #40 (group 3) at week 50 sacrifice showing a higher magnification view of a dysplastic hepatocellular nodule. Within the dysplastic nodule, cells with the morphology of hepatocytes appear to be forming ductules (arrows). H&E, objective x 40. (C) Post mortem liver from Rat #25 (group 3) at week 50 sacrifice showing curious cystic lesions that are lined by cuboidal cells with the morphology of hepatocytes (arrows). H&E, objective x 20. (D) Immunostaining of the liver shown in Figure 9.11C with OV-6 monoclonal antibody demonstrates that the cuboidal cells surrounding the cystic lesions express OV-6. These cystic lesions are inside an 'atypical' nodule, and some of the hepatocytes stain with OV-6. Objective x 20. (E) Immunostaining of post mortem liver from Rat #14 (group 3) at week 50 sacrifice shows expression of OV-6 by cystic liver lesions, which are adjacent to an 'atypical' nodule containing hepatocytes that are expressing OV-6 in a peripheral pattern. Some of the OV-6 positive hepatocytes within the nodule appear to be forming ductules (arrows). (F) Post mortem liver from Rat #28 (group 1) at week 50 sacrifice showing an unequivocal hepatocellular carcinoma. The malignant tumour is moderately differentiated and has a predominantly trabecular pattern, but in areas the formation of pseudoglands can be seen (arrows).

(iii) GST pi positive lesions. Most post mortem livers showed prominent involvement by GST pi positive lesions, which comprised up to 70% of the liver (Figure 9.7 and Appendix A). Of note was the presence of proliferating oval cells (GST pi negative) and columnar-lined cystic lesions within GST pi positive nodules (see below).

(iv) Dysplastic nodules. The scores for dysplastic nodules ranged between 1 and 2, and individual nodules showed a mix of low-grade and high-grade dysplasia (Figure 9.8 and Appendix A). Features of high-grade dysplasia included the presence of mitoses, occasional pseudogland formation, and compression of the surrounding parenchyma. High-grade dysplastic nodules showed predominantly eosinophilic large cell change, but occasional clear cell or mixed eosinophilic/clear cell (Figure 9.11A) change was seen (e.g. Rat #25). Within some high-grade dysplastic nodules, there were ductular structures formed by cells with a hepatocellular phenotype (Figure 9.11B). In some liver specimens there were curious cyst-like lesions that were lined by cuboidal cells resembling hepatocytes (Figure 9.11C). Both the ductular structures within dysplastic nodules and the cuboidal cell-lined cystic lesions showed expression of OV-6 (Figures 9.11D and 9.11E), and some cystic lesions were also found within dysplastic nodules (Figures 9.12E and 9.12F).

(v) Cholangiofibrotic lesions. A total of 19 cholangiofibrotic lesions were found in post mortem livers, and eight of these showed features of dysplasia (Figure 9.9 and Appendix A). Dysplastic cholangiofibrotic lesions showed irregularity of ducts, and there was evidence of severe cytological atypia of bile duct cells, suggestive of intraductal cholangiocarcinoma. Of note was the presence of some cholangiofibrotic lesions adjacent to GST pi positive lesions/dysplastic nodules.

9.3.2.4. GROUP 4 (FB_1/AAF/AIN)

Biopsy findings:

(i) Fibrosis. A progressive increase in the cumulative score for hepatic fibrosis was noted during the study period (Figure 9.2). There was however a wide range in the score for fibrosis (score 0 - 3) noted in individual liver biopsies, and one biopsy showed established cirrhosis (score 4).

(ii) Oval cell proliferation. A progressive increase in the score for oval cell proliferation was noted during the study period (Figure 9.3). Proliferation of OV-6 positive oval cells and ductules were prominent (Figure 9.10B) until week 31, whereafter the proliferation appeared

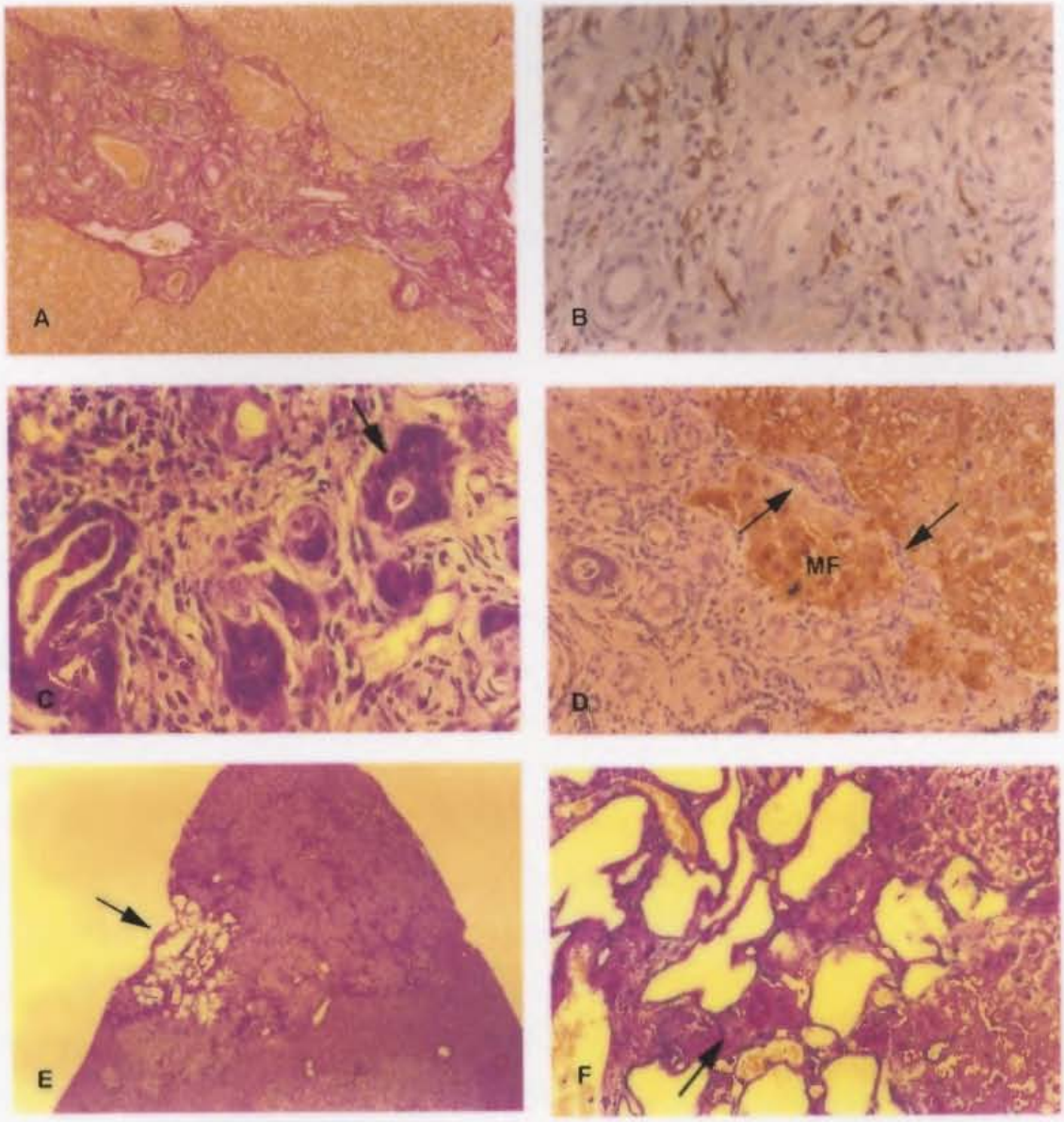

Figure 9.12

to settle down (score 0 - 1).

(iii) GST pi positive lesions. There was a progressive increase in the cumulative score for GST pi positive lesion during the study period (Figure 9.4). Although there was a wide range in the involvement (%) of liver sections by these lesions (score 0 - 3), most GST pi positive *lesions were small and occupied less than 25% of the section (score 1).*

(iv) Dysplastic nodules. The increase in cumulative score for dysplastic lesions appeared to occur in a stepwise fashion (Figure 9.5), and there was a mix between low-grade and high-grade dysplastic lesions. However, no unequivocal HCCs were found in liver biopsy specimens.

Figure 9.12. Liver pathology during prolonged feeding of fumonisin B_1-containing treatment regimens to Fischer 344 rats (see text for details). (A) Post mortem liver from Rat #25 (group 3) at week 50 sacrifice showing a cholangiofibrotic lesion with dense fibrosis surrounding proliferative bile duct lesions. Strands of fibrotic tissue extend into the surrounding liver, which shows disturbance of the normal acinar architecture. Sirius red, objective x 10. (B) Liver biopsy from Rat #52 (group 3) at week 15 showing the proliferation of desmin-positive hepatic stellate cells around bile duct lesions in a cholangiofibrotic lesion. Objective x 40. (C) Liver from the same animal as in figure 9.12B showing a cholangiofibrotic lesion containing irregular bile ducts with features of severe cytological atypia, suggestive of intraductal cholangiocarcinoma. A mitotic figure is seen in a dysplastic bile duct cell (arrow). (D) Post mortem liver from Rat #25 (group 3) at week 50 sacrifice (same liver as in Figures 9.11C and 9.11D) showing a lesion made up of GST pi positive hepatocytes, which is directly adjacent to bile duct lesions (GST pi negative) of a cholangiofibrotic lesion. Furthermore, cells with the morphology of oval cells (also GST pi negative) are proliferating within the GST pi positive lesion (arrows). A mitotic figure (MF) is seen within one of the GST pi positive hepatocytes. Objective x 20. (E) Low-power magnification of post mortem liver from Rat #49 (group 4) at week 50 sacrifice showing cystic lesions (arrow) within a subcapsular nodule of pale staining 'atypical' hepatocytes. H&E, objective x 4. (F) Higher magnification of the same liver as shown in Figure 9.12E showing deeply eosinophilic ('atypical') hepatocytes (arrows) located between the irregular cystic lesions. H&E, objective x 40.

(v) Cholangiofibrotic lesions. Two cholangiofibrotic lesions were identified in liver biopsy sections by sirius red staining (Figure 9.6), and neither lesion showed cytological features of dysplasia.

Post mortem findings:

(i) Fibrosis. There was a wide variation in the amount of amount of fibrosis of post mortem livers (score 0 - 3), but no liver showed established cirrhosis.

(ii) Oval cell proliferation. Oval cell proliferation in post mortem livers ranged from score 1 - 3.

(iii) GST pi positive lesions. There was a wide scatter in the involvement (%) of liver by GST pi lesions at post mortem (Figure 9.7 and Appendix A), which ranged up to 50% (score 2). Of note was the presence of cells with the morphology of oval cells within some GST pi positive lesions (e.g. Rat #49).

(iv) Dysplastic nodules. Six dysplastic nodules were found in post mortem livers, and four of these showed features of high-grade dysplasia (Figure 9.8 and Appendix A).

(v) Cholangiofibrotic lesions. Thirteen cholangiofibrotic lesions were found in post mortem livers, and four of these showed features of dysplasia (Figure 9.9 and Appendix A).

9.3.2.5. GROUP (AIN control)

Biopsy findings: Liver biopsies from control animals all showed normal liver histology, apart from a blinded review of a biopsy on one rat (Rat #26) that reported score 1 fibrosis (Figure 9.2) and score 1 oval cell proliferation (Figure 9.3). However, at subsequent post mortem examination the liver of this animal was found to be normal, with no evidence of fibrosis or oval cell proliferation.

Post mortem findings: Post mortem livers showed normal histology, apart from two cases (Rat #3 and Rat #7) that contained a few cells that stained GST pi positive (technically score 1 as liver involvement > 0% but considered to be insignificant).

9.4. Discussion

The present study is the first to demonstrate that prolonged feeding of FB_1 in a normal diet causes dysplastic hepatocellular nodules and bile duct lesions in rats. Male Fisher 344 rats fed different FB_1-containing regimens for 25 weeks and returned to normal AIN-76A diet for another 25 weeks developed a variety of preneoplastic and neoplastic hepatic lesions, including GST pi positive lesions, dysplastic liver nodules, cholangiofibrotic lesions, intraductal cholangiocarcinomas, and one unequivocal HCC. The single HCC showed a trabecular pattern histologically and was found in a post mortem liver specimen from a group 1 (FB_1/FB_1) animal. Two other long-term FB_1 studies have been performed in rats and mice, but there are problems in the designs of both of these trials. In a 26 month feeding study performed by the South African Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) (Gelderblom *et al.*, 1991), BD IX rats were fed FB_1 50 mg/kg in a semi-purified maize-based diet and showed the development of chronic toxic hepatitis, cirrhosis, HCCs, cholangiofibromas, and cholangiocarcinomas (see Appendix B). The basal diet, which was deficient in multiple vitamins and lipotropes (see Appendix C), was used in an attempt to induce oesophageal lesions in the animals (Van Rensburg *et al.*, 1985). Diets deficient in lipotropes such as choline and methionine have however been shown to induce liver cancer in rats (Ghoshal and Farber, 1984), and also render the liver more susceptible to the carcinogenic action of carcinogens (Newberne, 1986). Thus, the diet used in the PROMEC study may have had an enhancing effect on the action of FB_1 in the liver. The results of recently completed 2-year studies on the toxicology and carcinogenesis in rats and mice performed by the United States National Toxicology Program (NTP) have appeared in a preliminary report (Howard *et al.*, 1999). Male F344/N rats fed diets containing 0, 5, 15, 50, or 150 ppm FB_1 for 2 years developed renal tubular neoplasms (adenoma and carcinoma) but no hepatocellular neoplasms only, and there was no evidence of carcinogenic activity of FB_1 in females (see Appendix B). In contrast, female $B6C3F_1$ mice fed diets containing 0, 5, 15, 50, or 80 ppm FB_1 for 2 years developed hepatocellular neoplasms (adenoma or carcinoma), while there was no evidence of carcinogenic activity of FB_1 in males (see Appendix B). The reasons for the lack of hepatocarcinogenic effect of FB_1 in F344/N rats is not clear, but the NTP feeding studies employed the ammonium salts of FB_1. Although this chemical compound was apparently not tested for efficacy before the start of the experiments, the absence of any differences in body

weight gain between treated and control animals would suggest lack of toxic effect.

Open wedge liver biopsies were performed under ether anaesthesia, according to a modification of the technique described by Cmielewski *et al.* (1997). The technique offers a number of advantages for histological studies: (i) relatively large amounts of liver can be obtained under direct vision; (ii) repeated liver biopsies can be taken, permitting longitudinal studies and limitation of the number of animals used; and (iii) liver histology at sites distant from previous biopsy sites is essentially unaffected (Cmielewski *et al.*, 1997). The success of the technique is dependent on the experience and technical skills of the operator, and with repeated biopsies the procedure can become more difficult as adhesions gradually develop at the site of biopsy. In this study, the overall perioperative mortality rate was significant at 16.8% (13 deaths from 77 biopsy procedures). The high mortality rate may in part be ascribed to anaesthesia and surgery in the face of ongoing hepatotoxicity and chronic liver disease, since there was only one postoperative death recorded in the control group. However, there were wide variations in the perioperative mortality rates among the different treatment groups, and mortality rates in individual groups did not appear to reflect the perceived intensities of the treatment regimens. The large number of biopsy-related deaths in this study unfortunately limited the potential advantages over conventional sacrifice at predetermined time-points, and open liver biopsies were discontinued during the last two months of the study.

The dysplastic hepatic nodules and bile duct lesions occurred in a setting of chronic hepatic fibrosis and regenerative nodules. Prolonged feeding with FB_1 resulted in a progressive increase in the cumulative scores for hepatic fibrosis in sequential biopsy specimens in all the treatment groups, apart from group 2, where the score appeared to level off after week 11. Nevertheless, established cirrhosis was found in only 1 biopsy specimen from group 1, three from group 3, and one from group 4. At post mortem examination, only one liver from group 1 was cirrhotic. It is possible that the maintenance dose of FB_1 100 mg/kg was too low, and that a higher dose of FB_1 and/or a longer duration of treatment is required to produce cirrhosis in all animals. Although not specifically quantitated in this study, hepatic stellate cell proliferation appeared to subside rapidly after discontinuation of treatment 25 weeks in all the groups. In a previous long-term FB_1 feeding study (50 mg/kg diet), cirrhosis was seen in all animals that were sacrificed from 18 months onwards, but in no animals sacrificed before that time period (Gelderblom *et al.*, 1991).

In order to study whether inhibition of hepatocyte proliferation in the face of FB_1-induced liver injury enhances oval cell proliferation, animals in the present study were treated with different FB_1 feeding regimens alone and in the presence of the potent mitoinhibitor AAF. Although a treatment group fed with AAF alone for 2 weeks was not included in the present study, this regimen is reported to produce little cellular change (Sell and Dunsford, 1989). Indeed, cumulative oval cell scores appeared to indicate that rats which received FB_1 in the presence of AAF (groups 3 and 4) had a more sustained hepatic oval cell response, as opposed to rats were given FB_1 alone (groups 1 and 2).

In order to determine the effect of AAF-induced mitoinhibition and oval cell proliferation on cancer induction, GST pi positive lesions and dysplastic nodules in the different groups were analysed in sequential liver biopsy specimens and in post mortem liver tissue. There were striking discrepancies between the cumulative scores for GST pi positive lesions and dysplastic nodules in biopsy specimens, indicating that these two histological features are not identical. The cumulative scores for GST pi positive lesions were greater (in most instances by factor 2 or more) than those for dysplastic lesions in all treatment groups, indicating that only a proportion of 'enzyme-altered' lesions form dysplastic nodules. The slopes of the curves for GST pi positive lesions differed among the treatment groups, and presumably the curves with the steeper slopes (groups 1, 3 and 4) were the more 'toxic' ones. Cumulative scores for dysplastic lesions appeared to increase in a stepwise fashion in most treatment groups, and after week 15 the cumulative scores increased most rapidly in group 1, while there was a plateau effect in group 2. The slope of the curves for cumulative scores in liver biopsies for focal lesions relates to the chance of including lesions in the biopsy specimen, and depends on the number and size of the lesions, as well as sample variation. A linear increase in the cumulative score for a particular focal lesion may thus reflect either a change between biopsies or even a steady state, depending on sample variation. Likewise, a flattening out of the curve for a cumulative score could represent either a decrease in the number of focal lesions or else lesions being 'missed' in the biopsy. AAF-induced mitoinhibition appeared to increase the development of GST pi positive lesions in both group 3 and 4, while the effect on the development of dysplastic nodules was noted only in group 4. Some caution is needed in interpreting these results, as the number of animals biopsied at each time point was small, and marked inter-animal variation for the different scores was found at the different time points.

Post mortem livers from animals that either died from non-operative causes or were culled at the end of the study were examined for GST pi positive lesions and dysplastic nodules. The extent of liver involvement by both of these 'preneoplastic' lesions was scored according to specially designed scoring systems. In addition, dysplastic lesions were classified into low-grade dysplasia, high-grade dysplasia, or well-differentiated HCC, based on a modification of the International Working Party (Wanless *et al.*, 1995) criteria for the classification of human nodular liver lesions. The distinction between high-grade dysplasia and well-differentiated HCC may be difficult (Ferrell *et al.*, 1993; International Working Party, 1995), and additional criteria such as loss of reticulin staining or expression of AFP may be helpful in such cases. Reticulin staining was unsuccessful, possibly due to problems with fixation/storage of the liver specimens, and AFP protein could not be detected in the nodules, despite positive fetal rat liver controls (reason unclear). At post mortem, group 3 and 4 livers were most extensively involved by GST pi positive lesions, which suggested a potentiating effect on the development of these lesions by AAF-induced mitoinhibition. These findings were reflected in the sequential liver biopsies, and it thus appears as if there was a good correlation between biopsy and post mortem findings for GST pi positive lesions. The highest scores for dysplastic nodules in post mortem livers were recorded in groups 1, 3, and 4, which also contained the most high-grade dysplastic lesions. These three groups also contained the highest cumulative scores for dysplastic nodules in sequential liver biopsies. Thus, there was a good correlation between the biopsy and post mortem findings for dysplastic nodules, and sampling error did not appear to be a problem.

In order to study the effect of AAF-induced mitoinhibition on the development of FB_1-induced bile duct lesions, the cumulative number of cholangiofibrotic lesions in the different treatment groups at the end of the liver biopsy procedures were analysed and compared. This method of analysis was employed because biopsy specimens at week 15 from two animals in group 3 that contained three cholangiofibrotic lesions each otherwise skewed the data presented as cumulative scores. Cholangiofibrotic lesions developed in all the treatment groups, but were particularly prominent in biopsies from group 3 livers. These lesions appeared as focal areas of bile duct proliferation, surrounded by desmin-positive hepatic stellate cell proliferation within dense fibrous tissue. It is likely that the proliferating stellate cells were responsible for the prominent fibrosis surrounding the bile ducts. AAF had a clear potentiating effect on the development FB_1-induced cholangiofibrotic lesions, suggesting that

inhibition of hepatocyte proliferation and activation of the oval cell compartment may be important in the generation of these lesions. Post mortem livers were also examined for the presence of cholangiofibrotic lesions. Most post mortem cholangiofibrotic lesions were found in group 3 and 4 livers. There was thus a discrepancy between the post mortem and biopsy findings for cholangiofibrotic lesions, and the likely explanation is that potential sampling error becomes an important factor for isolated focal hepatic lesions. Some cholangiofibrotic lesions from group 1 (n = 1), group 3 (n = 4), and group 4 (n = 3) livers showed features of severe cytological atypia, and were considered to have progressed to intraductal cholangiocarcinoma. Dysplastic bile duct lesions within dense surrounding fibrous tissue were reminiscent of sclerosing cholangiocarcinoma in humans (MacSween and Scothorne, 1985).

This immunohistochemical study attempted to gain some insights into the role (if any) of proliferating oval cells in the genesis of 'preneoplastic' hepatocellular lesions and bile duct lesions in the FB_1-fed rat. A striking finding in all treatment groups was the intimate spatial relationship between proliferating oval cells, bile ductules, GST pi positive lesions/dysplastic nodules, and cholangiofibrotic lesions. Cells with the morphology of oval cells were noted inside GST pi positive foci/dysplastic nodules, and also within/adjacent to cholangiofibrotic lesions. Furthermore, particularly in group 3, columnar cell-lined cystic lesions were found inside some high-grade dysplastic nodules, and in other dyplastic lesions cells with the morphology of hepatocytes appeared to be forming ductular structures. Many of these different liver cells expressed the OV-6 antigen, suggesting that all of these cell types may have a common cell of origin. In addition to proliferating oval cells, OV-6 was expressed by ductules within high-grade dysplastic nodules, bile ducts in cholangiofibrotic lesions, and columnar cells lining cystic lesions. Furthermore, some hepatocytes within nodules showed peripheral cytoplasmic staining for OV-6, and the antibody presumably detected cytokeratin filaments just below the hepatocyte plasma membranes. Observations from this and other immunophenotypic studies (Sell and Dunsford, 1989; Tee *et al.*, 1996; Tian *et al.*, 1997) suggest that these closely related cells that are all expressing OV-6 might be the progeny of a common 'liver stem cell'. There is now strong evidence that the 'oval cell compartment' in the liver contains a heterogeneous population of cells, based on both immunophenotypic (Tee *et al.*, 1996) and ultrastructural characteristics (Sell, 1998). It is also postulated that increased proliferation of some/all of these putative pluripotential progenitor cells due to chronic FB_1-induced hepatotoxicity (Cohen and Ellwein, 1990) may underlie the risk of both the

hepatocellular and bile duct tumours that arise during prolonged feeding of FB_1.

9.5. Conclusions

Long-term feeding of FB_1 in a normal diet resulted in oval cell proliferation with the formation of dysplastic hepatic nodules, dysplastic bile duct lesions, and a trabecular/pseudoglandular HCC. AAF enhanced the effects of FB_1 feeding, probably by blocking hepatocyte regeneration. Proliferating oval ('progenitor') cells may be the source of both the dysplastic nodules and bile duct lesions induced by FB_1. This study supports the involvement of dietary FB_1 in hepatocarcinogenesis in male Fischer 344 rats.

Chapter 10

Overview and future directions

10.1. Fumonisin feeding studies in rats

For the present series of studies, male Fischer 344 rats were fed fumonisin B_1 (FB_1) 250 mg/kg in normal AIN-76A diet for 5 weeks, a dose known to cause severe hepatotoxicity and cancer induction (Gelderblom *et al.*, 1994), and this basic feeding regimen was modified in individual experiments. Although this basic regimen proved to be a very efficacious one, some difficulties were experienced during the course of the individual feeding studies. Problems not anticipated at the time of design of the studies included the marked sensitivity of our rat strain to the gastrointestinal side-effects of oral iron loading and the high operative mortality rate in the animals undergoing open liver biopsies during chronic FB_1 feeding. Another problem was the high cost and short supply of FB_1, mainly due to the slow and labour intensive methods required for its purification. Nevertheless, observations from the present studies have provided new insights into the cellular and molecular mechanisms of FB_1-induced hepatocellular and bile duct tumours in the rat. Furthermore, these studies come at a time of rapidly increasing interest in these carcinogenic mycotoxins in other countries, including the USA. FB_1 was recently nominated by the Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition for study, because of its occurrence in maize and maize-based products in the United States, the toxicity of FB_1 in field exposure of horses and pigs, and the reports of carcinogenicity in rats (Howard *et al.*, 1999).

10.2. Cellular origin of liver tumours caused by fumonisin B_1

Agents may increase the risk of cancer by causing DNA damage (genotoxicity) and/or by causing increased proliferation (increased DNA replications) in a pluripotential cell population of the tissue (Cohen and Ellwein, 1990). To increase the number of DNA replications, an agent can either increase cell births (direct mitogenesis or toxicity and regenerative proliferation) and/or decrease cell deaths (inhibition of apoptosis) (Cohen, 1998). There is currently little evidence for any genotoxic effects by FB_1, and it is more likely that this strong cancer promoter increases the risk of liver tumours by causing chronic hepatotoxicity and cellular proliferation. FB_1 appears to be a unique carcinogen which causes marked apoptosis together with regeneration of hepatocytes and proliferation of oval cells (Lemmer *et al.*, 1999b), putative precursor cells for liver tumours (Grisham and Thorgeirsson, 1997).

To date, *in vivo* studies of liver carcinogenesis in rats during feeding of FB_1 have focused exclusively on the role of 'initiated' hepatocytes in the formation of hepatocellular carcinomas via the hepatic foci/nodule sequence, according to the 'resistant hepatocyte' model (Solt and Farber, 1976). In contrast, the role (if any) of oval cell proliferation during FB_1-induced carcinogenesis has been ignored, and there have been no studies on the cellular origin of the bile duct tumours that are seen in this model of carcinogenesis. In this dissertation, the "bile duct hyperplasia" during FB_1-induced hepatotoxicity is identified as proliferation of OV-6 positive oval cells. Proliferating cells with the morphology of oval cells were found inside/adjacent to GST pi positive lesions, dysplastic nodules, and cholangiofibrotic lesions, suggesting that oval cells may be involved in FB_1-induced hepato- and cholangiocarcinogenesis in the liver. Furthermore, the OV-6 antigen was expressed by proliferating oval cells and bile ductules, hepatic nodules, cholangiofibrotic lesions, and cystic lesions, indicating that all of these cells may have a common ('stem') cell of origin. While the present series of observations do not provide direct proof that oval cells are the source of the dysplastic nodules and hepatocellular carcinomas seen in FB_1-fed rats, they add to the growing weight of evidence from other carcinogenesis models of such a concept. Furthermore, it is proposed that both the hepatocellular and bile duct tumours induced by FB_1 are derived from a common ('stem') cell of origin.

10.3. Isolation of liver progenitor cells

Adequate data have been gathered that show that oval ('progenitor') cells exist in the rat and human liver, but their place of origin and their role in liver development, regeneration, and carcinogenesis remain enigmatic. Specific problems that still need to be addressed include (i) understanding the mechanisms involved in oval cell activation; (ii) developing a system to track these cells *in vivo* to determine their final fate in the liver; (iii) development of an *in vitro* system to understand differentiation mechanisms; and (iv) the possibility of being able to use these cells as a target for gene therapy. These questions and many other could be addressed if a reasonably pure population of progenitor cells could be routinely obtained, something that has been difficult to achieve in the past. It now appears that there may be a close relationship between the liver parenchyma and the haematopoietic system (Fujio *et al.*, 1994; Blakolmer *et al.*, 1995; Petersen *et al.*, 1999; Baumann *et al.*, 1999). This intriguing observation has led to the development of an immunoaffinity method for the enrichment from human fetal liver of 'progenitor' cells that co-express CD34 haematopoietic stem cell and CAM 5.2 epithelial cell markers (Lemmer *et al.*, 1998c; see Appendix D). Similarly, using Thy-1 antibody (another cell surface marker expressed by primitive haematopoietic stem cells) as a new marker for the identification of oval cells in combination with flow cytometry, Petersen *et al.* (1998a) were able to obtain a highly enriched population of 'progenitor' cells from rat livers treated with chemical carcinogens. Efforts are currently underway at our institution to isolate oval cells from FB_1-treated rats with immunomagnetic beads for *ex-vivo* labelling and transplantation experiments (Dr. C. Vessey, personal communication).

10.4. Molecular mechanisms of fumonisin B_1-induced carcinogenesis

The fumonisin mycotoxins are strong cancer promoters, and as such are predicted at a molecular level to cause deregulation of cell cycle control, resulting in alterations in the balance between cellular proliferation and apoptosis. Liver cells *in vivo* are impinged on both by growth inhibitors, such as transforming growth factor (TGF)-β1, and growth stimulators, such as hepatocyte growth factor (HGF) and TGF-α (Mills *et al.*, 1995). When the effects of these two opposing types of growth factors balance, the cells remain in the resting phase (G_0). In contrast, an imbalance in growth factor signals can either stimulate liver cells to enter the cell cycle, thereby causing cell proliferation, or induce cells to undergo apoptosis. Once in the cell cycle, further regulation of progression through the cycle occurs by cell cycle control factors (e.g. tumour suppressor genes, oncogenes, cyclins, cyclin dependent kinases), which exert their control independently from external influences and act on cell cycle restriction (**R**) checkpoints, such as G_1/S.

The molecular mechanisms underlying FB_1-induced hepatotoxicity and carcinogenesis have not previously been studied. Northern blot (mRNA) analysis showed increased hepatic expression of HGF, TGF-α, and especially TGF-β1 and c-*myc* during short-term feeding of FB_1. Overexpression of mature TGF-β1 by zone 1 and 2 hepatocytes may be responsible for the prominent pro-apoptotic effects of FB_1 in the liver. The proto-oncogene c-*myc* is a positive regulator of cell proliferation that is involved in tumour progression (Garte, 1993; Nagy *et al.*, 1988), and has also been implicated in TGF-β1 signalling (Alexandrow and Moses, 1995). Increased expression of c-*myc* and TGF-β1 may cooperate in the promotion of liver tumours during feeding of FB_1, possibly by providing an environment that selects for the growth of TGF-β1 resistant transformed liver cells. Future immunohistochemical studies should include staining of liver specimens from FB_1-fed rats for expression of TGF-β type II receptor (TβIIR) protein by preneoplastic/neoplastic lesions and surrounding liver tissue.

Oncogenesis due to overexpression of both c-*myc* and TGF-α appear to involve disruption of the Rb/E2F pathway and deregulation of cell cycle control (Santoni-Rugiu *et al.*, 1998). Both c-*myc* and TGF-α contribute to induction of cyclin D1 expression and resultant

inactivation of retinoblastoma (Rb) tumour suppressor protein, and c-*myc* may directly induce E2F (Santoni-Rugiu *et al.*, 1998). Short-term feeding of FB_1 has recently been shown to cause overexpression of cyclin D1 in the nucleus (Ramljak *et al.*, 1997). Increases have also been observed in FB_1-treated liver samples of cyclin dependent kinase 4 (cdk4) complexes with cyclin D1, and consequently elevated cdk4 activity, as shown by increased phosphorylation of Rb (Dr. D. Ramljak, personal communication). Future studies in this field should be directed at unravelling the molecular events involved in the disruption of the Rb/E2F cell cycle control pathway by FB_1. Specifically, the changes in cell signalling that transduce the epigenetic toxic effects of FB_1 on the cell membrane, such as disruption of sphingolipid (Merrill *et al.*, 1996) or fatty acid (Gelderblom *et al.*, 1999) metabolism, to the molecular events in the nucleus need to be elucidated.

10.5. Fumonisins and human health

Chronic liver disease, cirrhosis, hepatocellular carcinoma, exposure to mycotoxins, and dietary iron overload are important health problems in Africa. Although epidemiological studies have focused on the possible association between human exposure to fumonisins and oesophageal carcinoma, these carcinogenic mycotoxins might also be a risk factor for hepatocellular carcinoma in certain regions of the world where maize is the staple diet (Ueno *et al.*, 1997). Well-conducted epidemiological studies are required to assess any association between dietary ingestion of FB_1 with human hepatocarcinogenesis in southern Africa, as well as possible interactions with known major risk factors (e.g. chronic hepatitis B virus infection, aflatoxin exposure) or minor risk factors (e.g. African siderosis) for human HCC. The ingestion of traditional maize-based alcoholic beverages that are brewed in iron pots or drums in certain regions of Africa (Bothwell and Bradlow, 1960) could potentially result in exposure to increased levels of FB_1 in the presence of dietary iron overload. Surprisingly, data from our study in rats indicates that dietary iron overload may in fact protect against the cancer promoting effects of FB_1, possibly due to a mitostimulatory effect on normal hepatocytes (Lemmer *et al.*, 1999a). Thus, the interactions between FB_1 and iron appear to be complex and deserve further study.

10.6. Conclusions

Feeding with FB_1 causes marked proliferation of oval cells in rat liver. Observations presented in this dissertation support the hypothesis that oval cells are the precursors of both the hepatocellular and bile duct tumours that are seen in this model of carcinogenesis. The FB_1-fed rat is a promising model for the study of liver injury, oval ('progenitor') cell proliferation, and carcinogenesis.

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Appendix A

Summary of hepatic nodules and bile duct lesions found at post mortem in male F344 rats during prolonged feeding of fumonisin B_1

TABLE A1

Summary of hepatic nodules and bile duct lesions seen at post mortem in male F344 rats in the 25 week on/25 week off fumonisin B_1 feeding study (data presented in Chapter 9). Post mortem liver tissue was available from rats that either died from non-operative causes or were culled at the end of the study.

Rat #	Group[a,b]	Week	Dysplastic score	No. of nodules	GST pi + % (score)	No. of CF lesions
47	1	23	2	H7	50 (2)	0
51	2	24	0	-	1 (1)	0
16	3	30	1	H2, L	30 (2)	1
4	1	33	1	H1, L2	30 (2)	1
13	2	35	0	-	1 (1)	0
17	2	39	0	-	-	1
31	2	39	0	-	1 (1)	0
34	2	40	0	0	0	0
40	3	42	2	H6	25 (2)	0
22	1	48	0	-	5 (1)	9 (D9)
42	3	49	1	H3	70 (3)	4
4	1	50 (cull)	1	H1, L2	-	1
18	1	50 (cull)	0	-	1 (1)	0
20	1	50 (cull)	1	L1	30 (2)	0
28	1	50 (cull)	4	H5 (HCC)	80 (4)	0
29	1	50 (cull)	1	H2	5 (1)	0
2	2	50 (cull)	1	L1	5 (1)	0
5	2	50 (cull)	1	H2	10 (1)	0
10	2	50 (cull)	1	L1	5 (1)	0
11	2	50 (cull)	0	-	5 (1)	0
8	3	50 (cull)	2	H2, L1	70 (3)	0
14	3	50 (cull)	2	H4, L1	50 (2)	1
24	3	50 (cull)	1	H1	40 (2)	0
25	3	50 (cull)	1	H4	45 (2)	7 (D7)
30	3	50 (cull)	2	H5, L5	70 (3)	2
35	3	50 (cull)	2	H3. L2	45 (2)	2 (D1)
46	3	50 (cull)	2	H4	70 (3)	2
54	3	50 (cull)	0	-	2 (1)	0

H = high-grade dysplasia; L = low-grade dysplasia; CF lesion = cholangiofibrotic lesion; D = dysplastic ductal lesion

[a]The data has been sorted according to timing of death and group.

[b]Group 1 (FB_1/FB_1); Group 2 (FB_1/AIN); Group 3 (FB_1/AAF/FB_1); Group 4 (FB_1/AAF/AIN); Group 5 (AIN control).

TABLE A1 (Continued)

Rat #	Group[a,b]	Week	Dysplastic score	No. of nodules	GST pi + % (score)	No. of CF lesions
1	4	50 (cull)	1	H1	40 (2)	5 (D2)
15	4	50 (cull)	0	-	30 (2)	2
37	4	50 (cull)	0	-	10 (1)	0
38	4	50 (cull)	0	-	-	0
39	4	50 (cull)	1	H1	30 (2)	1 (D1)
48	4	50 (cull)	1	L1	2 (1)	0
49	4	50 (cull)	1	H2	40 (2)	4
50	4	50 (cull)	1	L1	20 (1)	0
53	4	50 (cull)	0	-	5 (1)	1 (D1)
3	control	50 (cull)	0	-	1 (1)	0
7	control	50 (cull)	0	-	1 (10	0
19	control	50 (cull)	0	-	0 (0)	0
26	control	50 (cull)	0	-	0 (0)	0

H = high-grade dysplasia; L = low-grade dysplasia; CF lesion = cholangiofibrotic lesion; D = dysplastic ductal lesion

[a]The data has been sorted according to timing of death and group.

[b]Group 1 (FB_1/FB_1); Group 2 (FB_1/AIN); Group 3 (FB_1/AAF/FB_1); Group 4 (FB_1/AAF/AIN); Group 5 (AIN control).

Appendix B

Summary of long-term fumonisin B_1 feeding studies in rats and mice

TABLE B1

Summary of the pathologic changes in the livers of male BD-IX rats given a diet containing 50 ppm[a] fumonisin B_1 in the South African Programme of Mycotoxins and Experimental Carcinogenesis (PROMEC) 26 month feeding study (data presented in Gelderblom *et al.*, 1991)

Duration (months)	Body weight gain (g)	Liver weight (% body weight)	Regenerative nodules	Cholangio-fibrosis	Cirrhosis
6 months					
Control[b]	381.6 ± 25 4	ND	0/5	0/5	0/5
50 ppm FB_1	330.2 ± 14.5	ND	5/5	4/5	0/5
12months					
Control	434.0 ± 60.6	ND	0/5	0/5	0/5
50 ppm FB_1	353.0 ± 18.4	ND	5/5	5/5	0/5
20 months					
Control	482.2 ± 53.7	2.6 ± 0.3	0/5	0/5	0/5
50 ppm FB_1	404.2 ± 24.5	4.2 ± 0.3	5/5	5/5	5/5
26 months					
Control	618.4 ± 56.8	2.3 ± 0.2	0/5	0/5	0/5
50 ppm FB_1	454.8 ± 88.8	8.6 ± 3.4	5/5	5/5	5/5
18 - 25 months					
Control	ND	ND	0/5	0/5	0/5
50 ppm FB_1[c]	ND	ND	5/5	5/5	5/5

FB_1 = fumonisin B_1; HCC = hepatocellular carcinoma; ppm = parts per million; ND = not determined

[a]1 ppm is equivalent to 1 mg/kg diet

[b]The control feed was found to contain 0.5 ppm FB_1 and 0 ppm aflatoxin B_1, and was marginally deficient in some nutritional components (see Appendix C).

[c]These animals died during the 18 to 25 month period.

TABLE B2

Summary of the United States National Toxicology Program (NTP) 2 year carcinogenesis fumonisin B_1 feeding studies in F344/N rats and $B6C3F_1$ mice (data presented in Howard *et al.*, 1999)

	Male F344/N rats	Female F344/N rats	Male $B6C3F_1$ mice	Female $B6C3F_1$ mice
Concentration in feed	0, 5, 15, 50, or 150 ppm	0, 5, 15, 50, or 150 ppm	0, 5, 15, 50, or 150 ppm	0, 5, 15, 50, or 150 ppm
Body weights	Exposed groups similar to controls	Exposed groups similar to controls	Exposed groups similar to controls	Exposed groups similar to controls
Survival rates	16/48, 17/40, 25/48, 18/48, 25/48	25/48, 22/40, 24/48, 30/48, 29/48	41/48, 39/48, 45/48, 37/48, 42/48	35/48, 44/48, 46/48, 39/48, 28/48
Nonneoplastic effects	Kidney: renal tubule epithelial hyperplasia, focal (2/48, 1/40, 4/48, 14/48, 8/48)	None	Liver: hepatocellular hypertrophy (10/47, 9/47, 24/48, 25/48, 30/48)	Liver: hepatocellular hypertrophy (0/47, 0/48, 27/47, 31/45); hepatocellular apoptosis (0/47, 0/48, 0/48, 7/47, 14/45)
Neoplastic effects	Kidney: renal tubule adenoma (0/48, 0/40, 0/48, 2/48, 5/48); renal tubule carcinoma (0/48, 0/40, 0/48, 7/48, 10/48); renal tubule adenoma or carcinoma (0/48, 0/40, 0/48, 9/48, 15/48)	None	None	Liver: hepatocellular adenoma (5/47, 3/48, 1/48, 16/47, 31/45); hepatocellular carcinoma (0/47, 0/48, 0/48, 10/47, 9/45); hepatocellular adenoma or carcinoma (5/47, 3/48, 1/48, 19/47, 39/45)
Level of evidence of carcinogenic activity	Clear evidence	No evidence	No evidence	Clear evidence

Appendix C

Dietary compositions of feeds used in fumonisin B_1 feeding studies

TABLE C1

Comparison of dietary composition of AIN-76A diet with semi-purified diet used in short-term and long-term fumonisin B_1 feeding studies

	AIN-76A	SEMI-PURIFIED		AIN-76A	SEMI-PURIFIED
PROTEIN (g/kg)	214.3	94	**VITAMINS**[a]		
Soy protein		30	Thiamine (mg)	6	3.9
Casein	200	10	Riboflavin (mg)	6	3.55
Egg albumin		10	Nicotinic acid (mg)	30	32
Methionine	3	0.5	Vitamin B6 (mg)	7	0.6
			Folate (mg)	2	0.5
CHO (g/kg)	605.1	659.1	Vitamin B12 (μg)	10	8
Corn starch	150	750	Pantothene (mg)	16	3
Sucrose	500		Biotin (mg)	0.2	0.1
Glucose		111.25	Vitamin A (IU)	66000	55567
Dextrin		54.3	Vitamin D (IU)	1000	250
			Vitamin E (mg)	80	41.33
FAT (g/kg)	46.4	47.6	Vitamin K (mg)	5	0.25
Saturated	5.9	6.33			
MUFA	9.0	11.7	**MINERALS**[b]		
PUFA	29.3	27.0	Calcium	5122.5	515
Sunflower seed oil	44	30	Iron	50.6	17.3
			Magnesium	514.5	488
ENERGY			Phosphate	4059	1133
kcal	3639	3448	Potassium	3707	2307
kJ	15235	14426	Sodium	1083	1028
			Zinc	44.9	20.8
FIBRE (g/kg)	54.8	37.2	Copper	5.7	

Abbreviations: CHO = carbohydrate, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

[a]mg/kg or units/kg

[b]mg/kg

Appendix D
Isolation from human fetal liver of cells co-expressing CD34 haematopoietic stem cell and CAM 5.2 pancytokeratin markers

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Isolation from human fetal liver of cells co-expressing CD34 haematopoietic stem cell and CAM 5.2 pancytokeratin markers

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Background/Aims: **Ductal plate and bile duct cells in developing human liver express haematopoietic stem cell markers, such as c-kit and CD34, in association with cytokeratin markers CAM 5.2 and CK 18. The identification of such ductal plate cells as likely progenitors for both bile duct epithelial cells and hepatocytes and their possible reappearance as oval cells in the regenerating liver have generated much interest in their pluripotential capacities. This study aimed to isolate cells from human fetal liver that co-express haematopoietic stem cell and epithelial cell markers.**
Methods: **Human fetal liver was harvested following legal termination of pregnancy at week 14–22. CD34+ mononuclear cells were isolated from liver cell suspensions with immunomagnetic beads. Immunofluorescent staining, using anticytokeratin CAM 5.2 against CK 8 and 18, was performed on permeabilised CD34+ cells for flow cytometry and fluorescent microscopy. CD34+ cells were also stained for other stem cell markers (HLA-DR, c-kit) and committed haematopoietic cell markers (CD33, CD38).**
Results: **Approximately 0.9% (range 0.07–4.0%) of the mononuclear cells isolated were CD34+ cells. The number of mononuclear cells isolated correlated with fetal liver weight (r=0.508). About 3–8% of these CD34+ cells stained positively for CAM 5.2. In addition, CD34+ cells were positive for HLA-DR, but only a small percentage was positive for c-kit. Staining for the committed haematological markers, CD33 and CD38, was consistently negative.**
Conclusions: **This study describes an immunoaffinity method for the enrichment from human fetal liver of cells that co-express haematopoietic stem cell and epithelial cell markers. Such cellular subsets may correspond to pluripotential ductal plate and bile duct cells.**

Key words: **Anticytokeratin CAM 5.2; Bile duct cells; CD34+ haematopoietic stem cells; Ductal plate cells; Human fetal liver.**

THE HUMAN liver is derived from an endodermal outgrowth of the primitive foregut into the mesenchyme of the septum transversum at around the third and fourth weeks of gestation (1). In both rat and man, the two major types of hepatic epithelial cells, hepatocytes and bile duct cells, arise from the primitive hepatoblast, a progenitor cell that has pluripotential differentiation capabilities (2,3). The hepatoblasts that surround the portal mesenchyme condense to form a double-layered cylinder of cells, the ductal plate, which remodels and migrates into the mesenchyme to form intrahepatic ducts (4,5). These cells initially express the hepatocyte-specific proteins α-fetoprotein (AFP) and albumin as they migrate into the portal stroma, and additionally begin to express the bile duct-specific marker γ-glutamyl transpeptidase (GGT). Initially intermediate filament expression is restricted to cytokeratin 8, but during the later stages of ductular morphogenesis, the ductal cells begin to express the characteristic biliary cytokeratins 7, 8, 18, and 19 (6). These differentiated ductal cells continue to express hepatocyte traits during the early neonatal period (7). In contrast, hepatoblasts not in contact with the portal mesenchyme differentiate into hepatocytes that form the liver cell plates. The hepatocyte intermediate filament expression is restricted to cytokeratin 8 and 18, but these cells continue to express GGT until birth (4).

The fetal liver becomes the main site of haema-

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topoiesis by the 12th to 16th week of gestation (8). Haematopoietic cells of the fetal liver are derived from stem cells that migrate from the yolk sac prior to the 15th week (9,10). Erythropoiesis is established first, followed by myelopoiesis, and further migration of cells ensues to populate the spleen and bone marrow (11). Ultimately, as bone marrow haematopoiesis is established, the role of the fetal liver is diminished (8).

Our group has recently shown that in developing human liver haematopoietic stem cell markers CD34 and c-kit are co-expressed in ductal plate cells in a pattern similar to the early cytokeratin markers CAM 5.2 and CK18 (12). Similarities appear to exist between hepatic and bile duct precursors and bone marrow stem cells with respect to the expression of prototypic early marker, viz. the presence of stem cell factor (SCF) and the specific receptor c-kit have been demonstrated in both embryonic and adult rat liver (13). This signalling system appears to be uniquely associated with the early activation of hepatic stem (oval) cells (14,15). Conversely, hepatocyte growth factor (HGF) appears to have a synergistic effect on the proliferation of CD34+ haematopoietic progenitor cells isolated from human cord blood, that express the *c-met* receptor (16,17). Hepatoblasts in the developing human liver, that may be in paracrine communication with haematopoietic cells, could thus have a closer relationship with the haematopoietic system than hitherto suspected (5).

This study reports on the isolation from human fetal liver of cells that we can show co-express stem cell and epithelial cell markers. Our techniques are based on methods for positive immunomagnetic selection of CD34+ haematopoietic stem cells from bone marrow (18,19) and fetal liver (20), and were highly reproducible. This procedure has the potential to generate sufficient quantities of precursor cells and hence develop techniques of cellular transplantation.

Materials and Methods

Tissues

Human fetal livers were obtained from legal termination of pregnancy at week 14– 22. The fetal livers were obtained after termination had been performed by induction of labour using intravaginal prostaglandin E2α. All patients gave written informed consent and had no known family history of an inherited condition. The research team were not involved in the clinical decision to terminate the pregnancy. Authority to obtain fetal tissue was granted by the Department of National Health and Population Development, South Africa, according to the Human Tissue Act, 1983 (Act 65 of 1983), as amended by the Human Tissue Amendment Act, 1989 (Act 51 of 1989). The study was approved by the Ethics Committee of the Faculty of Medicine of the University of Cape Town.

Preparation of cell suspensions

Liver cell suspensions were obtained in Hepes isolation buffer pH 7.4 (Hepes 20 mM, $CaCl_2 \cdot 2H_2O$ 1.2 mM, NaCl 140 mM, KCl 4 mM, D-glucose 5 mM, $MgCl_2 \cdot 6H_2O$), Na_2HPO_4 0.5 mM) by thorough forceps disruption of the fresh fetal liver specimen. The liver suspension was then dissociated by adding collagenase 0.1% final concentration (Sigma Chemical Co., St. Louis, MO, USA) and incubating for 10 min at 37°C. The reaction was stopped by adding an equal volume of fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY, USA), and the suspension was filtered through a very fine Swiss silk filter. The cell filtrate was subjected to Ficoll (Histopaque®, Sigma Diagnostics, St. Louis, MO, USA) density gradient centrifugation for 20 min at 800 *g*. Mononuclear cells were collected from the interface, and these cells were washed with Hepes isolation buffer and then citrate buffer (phosphate-buffered saline, 0.6% citrate, 2% bovine serum albumin). Aliquots of the cell suspension were counted and assessed for viability, using the trypan blue exclusion technique.

Isolation of CD34+ stem cells

CD34+ mononuclear cells in citrate buffer were separated with Dynabeads® M-450 CD34 immunomagnetic beads (Dynal, Oslo, Norway), according to the manufacturer's instructions, using a bead concentration of 40×10^6/ml and a cell concentration of $25–50\times10^6$/ml. Briefly, the cell bead suspension was mixed in a U-bottomed tube, and incubated for 30 min at 4°C with gentle tilt rotation, using the Dynal sample mixer (Dynal, Oslo, Norway), to allow rosettes to form. The tube was exposed to the magnet, and the non-rosetted cells were aspirated and discarded. Cells were detached from the beads by incubation with polyclonal anti-murine Fab Detachabead® CD34 (Dynal, Oslo, Norway) 100 μl per 40×10^6 beads for 45 min at room temperature and gentle tilt rotation. The detached cells were washed twice in citrate buffer and counted, and viability was checked using the trypan blue exclusion method.

Flow cytometry and fluorescent microscopy

Characterization of the CD34+ cell suspensions was performed using a Coulter Epics-Profile II flow cytometer (Coulter Electronics, Hialeah, FL, USA). Morphology of the cells was assessed by immunofluorescent microscopy on cytospin preparations. Intracytoplasmic cytokeratin 8 and 18 was detected by indirect immunofluorescent staining, after permeabilization of

TABLE 1

Details of fetal weight, liver weight, gestational age, mononuclear cells and CD34+ cells isolated in 17 successful experiments. Approximately 0.9% of the mononuclear cells isolated were CD34+ cells. The number of mononuclear cells isolated correlated with fetal liver weight (r=0.508)

	Fetal wt (g)	Liver wt (g)	Gestation (weeks)	Mono cells (million)	CD34+ (million)
1	120	8.2	15	920	8.6
2	400	17.9	16	1760	11.2
3	480	26	21	1640	1.2
4	200	7.8	20	350	1.6
5	250	13.9	19	552	1.2
6	100	4.6	14	440	1.2
7	280	13.6	19	270	11.2
8	390	19.9	16	1108	1.3
9	500	18.3	22	2880	4.5
10	110	4.9	15	270	2
11	200	11.6	20	710	6.4
12	200	8.2	15	408	6.4
13	120	4.4	14	363	1.8
14	660	12.2	18	680	5.4
15	110	4.5	16	520	2.6
16	400	12.4	18	780	5.8
17	350	25.5	20	296	6

the cells with methanol, using mouse monoclonal antihuman cytokeratin CAM 5.2 (Becton Dickenson, San Jose, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (DAKO, Copenhagen, Denmark). Using direct immunofluorescent staining with FITC, cells were also analysed for stem cell markers HLA-DR (clone C243, Becton Dickenson, San Jose, CA, USA) and c-kit (Immunotech, Marseille Cedex, France), with other indicators of haematologic lymphoid and myeloid lineage specificity, CD38 (Leu 1™-17, Becton Dickinson, San Jose, CA, USA) and CD33 (MY9, Coulter Immunology, Hialeah, FL, USA).

Statistics

The correlation between fetal weight, liver weight, gestational age, mononuclear cells and CD34+ cells was calculated by Pearson's correlation coefficient, using Statgraphics® software.

Results

Tissue collection and isolation of stem cells

The data from 17 successful experiments are shown in Table 1. Gestational ages ranged from 14–22 weeks, fetal weight ranged from 110–660 g, and liver weight ranged from 4.4–25.5 g. The total number of mononuclear cells isolated ranged from 270–2880×10^6, and the number of CD34+ cells isolated with immunomagnetic beads ranged from 1.2–11.2×10^6. Approximately 0.9% (range 0.07–4.0%) of the mononuclear cells isolated were CD34+ cells. Pearson's correlation coefficient was used to test any correlation between the variables fetal weight, liver weight, gestational age, number of mononuclear cells and CD34+ cells. The number of mononuclear cells isolated correlated with liver weight (r=0.508). There was, however, no correlation between the number of CD34+ cells isolated and any of the other variables examined.

Flow cytometry and immunofluorescent microscopy

Characterization of the CD34+ cells by flow cytometry revealed that about 3–8% of these cells showed intense immunofluorescence for CAM 5.2 (Fig. 1). This was confirmed with immunofluorescent microscopy on the CD34+ cell suspensions, which demonstrated occasional cells with bright intracytoplasmic fluorescence (Fig. 2). In addition, most CD34+ cells showed fluorescence for HLA-DR (Fig. 3), but only occasional cells stained positive for c-kit. Staining for the committed lymphoid and myeloid haematological markers, CD38 and CD33, was consistently negative.

Discussion

Currently the most widely used technique for separating early haematopoietic progenitor cells has been based on immunoaffinity techniques (19). Once labelled with antibody, cells can be separated using a range of methods, including solid phase immunological methods, immunomagnetic particles or flow cytometry. The characterisation of the CD34 antigen, which is expressed on only 0.5–5% of human bone marrow cells, has been central to the development of this approach for use on clinical samples (21). CD34 is expressed on early progenitor cells but not on their

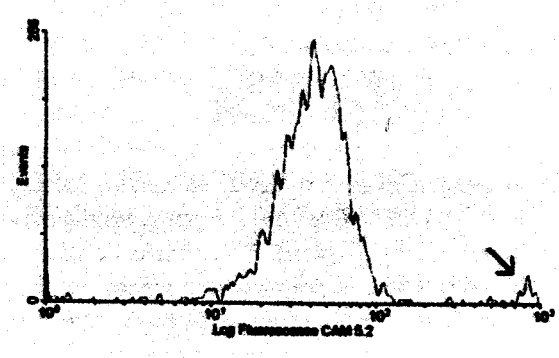

Fig. 1. Single parameter histogram following immunofluorescent staining of CD34+ cells isolated from fetal liver with monoclonal antihuman cytokeratin CAM 5.2. A small percentage (about 6.7%) of the cells shows intense fluorescence following immunostaining with CAM 5.2 (arrow).

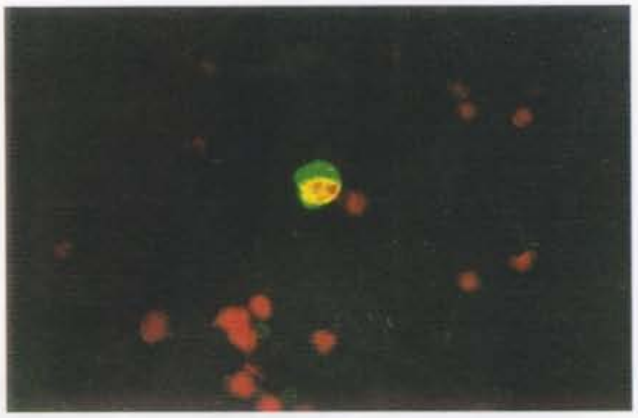

Fig. 2. Immunofluorescent microscopy of CD34+ mononuclear cells isolated from human fetal liver, showing intense intracytoplasmic staining of a single cell for CAM 5.2. Counterstaining with ethidium bromide demonstrates the nuclei of the CD34+ cells (orange).

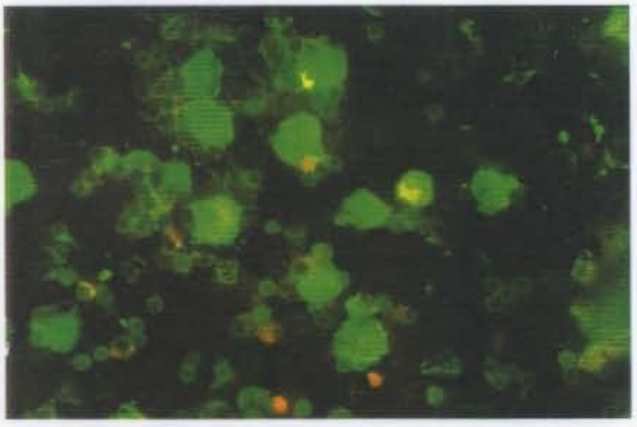

Fig. 3. Immunofluorescent microscopy of CD34+ mononuclear cells isolated from human fetal liver, showing immunoreactivity for HLA-DR (large green/yellow stained cells). Counterstaining with ethidium bromide demonstrates the cellular nuclei (orange). Aggregates of residual immunomagnetic beads (small, round, green bodies) are intermingled with CD34+ cells.

more mature counterparts (22), and there are now numerous commercially available antibodies and devices for separating CD34+ cells from bone marrow- and cytokine-mobilised peripheral blood progenitor and stem cells. For research purposes paramagnetic beads that can be directly conjugated to antibody have been widely used (18,23,24). Cells labelled with CD34+ conjugated beads can be easily separated from unlabelled cells using a magnet. Afterwards the isolated cells are detached from the beads with a polyclonal antibody against murine Fab fragments. Recently this technique has also been utilised to isolate putative progenitor endothelial cells, that are known to express CD34 (25,26), from the leukocyte fractions of human peripheral blood (27).

We have adapted this technique for the isolation from human fetal liver of cells which we show to co-express haematopoietic stem cell and epithelial cell markers, based on purification with CD34+ magnetic beads and characterization with CAM 5.2 anticytokeratin antibody. Fetal human livers from second trimester terminations of pregnancy were utilised in order to isolate sufficient numbers of CD34+ stem cells. The paucity of cells co-expressing stem cell and epithelial cell markers provides a major challenge to their isolation and characterisation. In this study only 0.07–4.0% of the mononuclear cells isolated were CD34+ cells, and only about 3–8% of these CD34+ cells stained for CAM 5.2. We attempted to identify variables that would predict the yield of CD34+ cells from fetal livers. Although the number of mononuclear cells isolated correlated with fetal liver weight, there was no correlation between the number of CD34+ cells isolated and any of the other variables examined.

Although these CD34+ cells stained for HLA-DR (21), they did not appear to represent true stem cells in that they were clearly already committed to an epithelial cell lineage, as evidenced by their intense staining for CAM 5.2. In contrast, the absence of staining for CD33 and CD38 suggested that there was no commitment of the CD34+ progenitor cells to either the myeloid or lymphoid lineage (28). This may reflect the decline in the role of the fetal liver as a haematopoietic organ after week 16 of gestation. A surprising finding was the difficulty in demonstrating c-kit, the receptor for SCF which is expressed by progenitor cells in human fetal liver (12) and oval cells in rat liver (13), albeit at low levels. Similar problems have been reported during the isolation of progenitor cells from rat liver, and may relate to interference with the c-kit receptor by the CD34+ labelled immunomagnetic beads (Dr. R. Padmanabhan: personal communication).

Conclusion

We report a technique for the isolation from human fetal liver of cells that co-express CD34 stem cell and CAM 5.2 pancytokeratin markers, based on immunoaffinity isolation methods for haematopoietic stem cells. It is possible that some of these cells correspond to the pluripotential ductal plate and bile duct cells identified in our previous immunohistochemical study. This study confirms the close relationship between epithelial and haematopoietic cells in the developing human liver. Although liver and haematological progenitor cells have a different embryological derivation (ec-

toderm vs. mesoderm), it is conceivable that these primitive cells may share phenotypic markers and growth factor receptors during embryogenesis.

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Appendix E

Comparison of carcinogenic effects of fumonisin B_1 and aflatoxin B_1

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