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The Effect of Aflatoxin B1 on the Expression of Early Response Genes and Transforming Growth Factor-α in CCl_4 Induced Rat Liver Injury

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Aflatoxin B1(AFB1), a fungal toxin produced by Aspergillus flavus, is known to be a possible hepatocarcinogen. But the molecular biologic changes which may occur following exposure to AFB1 are not known and thus the carcinogenesis is not yet understood. This study was performed to examine the expressions of c-myc, c-fos and TGF-α genes and to investigate the possible role of those molecular biologic changes in hepatic regeneration and in the development of hepatocellular carcinoma (HCC). Sprague-Dawley rats were divided into 3 groups: Carbon tetrachloride (CCl_4) only was administered to group I, AFB1 only was administered to group II and a combination of AFB1 and CCl_4 was administered to group III. The animals were sacrificed at 0.5, 1, 2, 6, 12, 24, 48, and 72 hours after treatment. In addition to the examination of the hematoxylin-eosin stained sections, hepatic regeneration and apoptosis were analyzed quantitatively by bromodeoxyuridine (BrdU)-anti-BrdU immunohistochemistry and TUNEL assay utilizing apoptosis kit, respectively. The hepatic expressions of c-myc, c-fos and transforming growth factor-α (TGF-α) were examined by immunohistochemistry and studied by Western blot. The number of BrdU labelled cells and the degree of necrosis/apoptosis were comparable among the different groups. Livers of the group II rats showed nearly normal histology without regeneration and necrosis/apoptosis. In groups I and III, the number of BrdU- labelled cells showed an increase at 48 hours after treatment, and the increment was significantly higher in group I than in group III. Most BrdU-labelled cells were mature hepatocytes in group I, whereas in group III they appeared to be less mature. In group I, apoptosis showed an increase at around 24 hours, but appeared in group III as early as 12 hours after treatment and persisted through 48 hours. The expressions of c-myc and c-fos were also different between the experimental groups. The expression intensity of c-myc in group I was highest at 1 hour and decreased thereafter. In groups II and III, the expressions were much more intense than in group I, except at 1 hour, and the increased intensity persisted throughout the experiment. Group II in particular showed a peak intensity at 30 minutes and at 6 hours after treatment. In group I, c-fos was strongly expressed only at 24 hours, but in group III, there was progressively increased expression with peak intensity at 24 hours. TGF-α was expressed in similar intensities in all groups throughout the experiment. These results suggest that AFB1 may evoke an intense and protracted expression of c-myc, provocating the CCl_4-induced necrosis of hepatocytes, and a prolonged expression of c-fos, inducing persistent signals for regeneration which in turn may activate the

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replication of immature cells. These findings will aid further investigation of molecular biologic and histologic characteristics of the hepatotoxic and hepatocarcinogenic mechanism of AFB1 in rats. And these results in rats, together with clinico-epidemiologic and molecular biologic investigations in humans and other animals, suggest that AFB1 may supply hepatocarcinogenic background in early exposure time in AFB1-contaminated areas of China and Korea.

Key Words: Aflatoxin B1, CCl_4, hepatocellular carcinoma, c-*myc*, c-*fos*, transforming growth factor, apoptosis

Aflatoxin B1 (AFB1), a metabolite produced by some strains of the mold *Aspergillus flavus*, possess a wide range of biological activities, including a high order of potency as an acute poison for most animal species and as a hepatocarcinogen for rats and ducks (Wogan and Newberne, 1967; Neal and Cabral, 1980).

Particularly in Korea, *Aspergillus flavus* is detected in food and therefore AFB1 contamination and its hepatocarcinogenic role can be expected to be found.

The hepatocarcinogenic mechanism of AFB1 has a known mutation of the p53 gene and Rb1 gene as shown in clinico-epidemiologic and molecular biologic investigations (Murakami, 1993). And it has been reported that the hepatoma induced by AFB1 expresses the c-Ha-*ras* and the c-*myc* gene (Tashiro *et al.* 1986; Sinha *et al.* 1989).

In the liver, regeneration is a necessary and important requirement for the hepatocarcinogenesis model. Regeneration has been mediated by several humoral factors, which include epidermal growth factor (EGF), transforming growth factor (TGF-α) and hepatocyte growth factor (HGF). TGF-α is a complete mitogen for hepatocytes in primary culture. TGF-α mRNA increases at about 4 hours after partial hepatectomy, and reaches a maximum at 18～24 hours (Mead and Fausto, 1989; Fausto and Webber, 1993). Because these growth factors affect the G1 phase hepatocyte only, regeneration requires transition of the cell cycle, G0 to G1. This transition, so-called initial priming, is mediated by the immediate early response gene (Herschman, 1991; Karin, 1992; McMahon and Monroe, 1992). So active expression of these immediate early response genes is associated with the activity of cell proliferation (Mead *et al.* 1990). In a study of primary gene response in regenerating livers, approximately 70 genes were found to be activated during the first 1～3 hours after partial hepatectomy (Fausto, 1986; Diamond *et al.* 1993). A prominent subset among these genes is the class of protooncogenes including c-*fos*, c-*myc*, and c-*jun*, which encode for DNA-binding proteins (Fausto and Shank, 1983; Thomson *et al.* 1986; Morello *et al.* 1990).

It has been well established that the regeneration resulting from partial hepatectomy is associated with rapid rises in c-*fos* and c-*myc* mRNA levels which peak at 30 min and 1～2 hours respectively, after surgery, and then quickly return to normal. The c-*fos*, c-*myc*, and c-*jun* expression in hepatotoxin-associated regeneration has been reported (Goytte *et al.* 1984; Sasaki *et al.* 1989; Herbst *et al.* 1991), and the results of these studies were similar to partial hepatectomy-associated regeneration. But another study demonstrated that the c-*fos*, c-*myc*, and c-*jun* expression in hepatotoxin-associated regeneration differs from that in hepatocarcinogen-associated regeneration (Schmiedeberg *et al.* 1993). And the c-*fos*, c-*myc*, c-*jun* and TGF-α expression in AFB1-associated regeneration is not known.

A main histologic finding of regeneration is the proliferation of cells. So an important histologic difference between the hepatotoxin/hepatocarcinogen-associated regeneration and partial hepatectomy-associated regeneration is the type of proliferating cells (Tournier *et al.* 1988; Lemire *et al.* 1991; Sirica *et al.* 1992). However, the exact correlation of c-*fos*, c-*myc*, and c-*jun* expression and histologic differences has not been determined. Also, the relationship of c-*fos*, c-*myc*, and c-*jun* expression and histologic finding in AFB1-associated regeneration has not been studied.

The present study was therefore designed to deter-

mine, first, whether regenerative hepatocyte express c-*fos*, c-*myc* protein and TGF-α protein in AFB1-associated regeneration; second, what is the expression pattern of these genes; and third, whether the expressions of these proteins are related to the histologic finding. To answer these questions, rats which had been administered with a combination of AFB1 and CCl_4 were used and the expression of c-*fos*, c-*myc* and TGF-α protein by the Western blot and immunohistochemical stain was determined. The hepatic regeneration and apoptosis were analyzed quantitatively by bromodeoxyuridine (BrdU)-anti-BrdU immunohistochemistry and TUNEL assay utilizing apoptosis kit, respectively.

MATERIALS AND METHODS

Experimental animals and groups

Experimental animals: Male Sprague-Dawley rats weighing 100~150 gm were used. On arrival, the animals were acclimatized to a daily cycle of alternating 12-hour periods of light and darkness for 1 week, before the start of the experiment. They were given food and water *ad libitum*. The rats were divided into three groups (Table 1).

Experimental groups:

Group I; Administration of carbon tetrachloride; Animals in this group were given CCl_4 (Sigma Chemical Co., St. Louis, MO, USA) intragastrically. The single dose of CCl_4 was 5.0 ml/kg of body weight given as a 1 : 1 dilution with corn oil.

Group II; Administration of aflatoxin B1; Animals in this group were given a total of 7 doses (once each day, for 7 days) of AFB1 during the one-week dosing period. The AFB1 was dissolved in tricaprylin and administered by gastric intubation at a level of 250 μg/kg/dose (Appleton and Campbell, 1983). After the last treatment, they were given a single dose of corn oil.

Group III; Administration of carbon tetrachloride and aflatoxin B1; Animals in this group were given AFB1 as per group II and then given a single dose of CCl_4 as per group I.

Three animals were sacrificed from each group at 30 minutes, 1, 2, 6, 12, 24, 48, 72 hours after the CCl_4 or corn oil treatment (Schmiedeberg *et al.* 1993).

Table 1. Experimental groups

Group I Carbon tetrachloride	(24)
Group II Aflatoxin B1	(24)
Group III Carbon tetrachloride + Aflatoxin B1	(24)

Group I: Single dose (5.0 ml/kg of body weight) of CCl_4 was administered. Group II: A total of 7 doses (250 μg/kg/dose, once each day, for 7 days) of AFB1 was administered during the one-week dosing period. And then corn oil was administered. Group III: A total of 7 doses (250 μg/kg/dose, once each day, for 7 days) of AFB1 during the one week dosing period. And then a single dose (5.0 ml/kg of body weight) of CCl_4 was administered. Three animals were sacrificed from each group at 30 minutes, 1, 2, 6, 12, 24, 48, 72 hours after the CCl_4 or corn oil treatment.

One hour before sacrifice, the rats were injected with 2 ml/kg of body weight of bromodeoxyuridine (BrdU)(Sigma Chemical Co.), (10 mM), intraperitoneally. The animals were sacrificed under light ether anesthesia. The liver tissue was prepared for histologic examination and immunohistochemical and molecular biologic studies while 1cc of heart blood was obtained for serologic examination of liver enzymes (AST, ALT).

Western blot analysis of c-*fos*, c-*myc* and TGF-α

The expression of c-*fos*, c-*myc* and TGF-α genes in nuclear or cytoplasmic protein was assayed by the Western blot technique. Cytoplasmic protein was extracted for transforming growth factor analysis by the usual method. Nucleic protein was extracted for immediate early response gene analysis by a modified Gorski method (Gorski *et al.* 1986).

Equal amounts of the proteins were mixed with the sample loading buffer (Laemmli, 1970) and the mixture was boiled at 100°C for 5 minutes. The mixture was then subjected to SDS-PAGE (6% or 12%). Proteins in the gel were electrotransferred to a nitrocellulose (NC) membrane, blocked with 10% non-fat dried milk in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% tween-10 (TBST), incubated for 1 hour with mouse anti c-*fos* monoclonal Ab (1 : 100 dilution), anti c-*myc* monoclonal Ab (1 : 100 dilution), and anti TGF-α monoclonal Ab (1 : 500

dilution), and then washed twice with TBST for 15 minutes each. The NC membrane was incubated with antimouse horseradish peroxidase conjugate (1 : 2000 dilution) in TBST for 30 minutes. To generate a signal, the NC membrane was incubated with enhanced chemiluminescence (ECL) detection reagents for 1 minute, and the membrane was exposed to Hyperfilm-ECL. The results were calculated by laser densitometer (LKB 2202 ultrascan).

Immunohistochemical stain for c-*fos*, c-*myc* and TGF-α

The immunohistochemical stain was performed using a common method. Briefly, formalin-fixed, paraffin-embedded sections were rehydrated and incubated overnight at 4°C with the primary antibody diluted. Biotinylated anti-mouse and rabbit IgG (DAKO Corp., Carpinteria, CA, USA) and a complex of peroxidase conjugated streptavidin (DAKO Corp.) were added in sequence, followed by 3-amino-9-ethylcarbazole (AEC) in organic solvent and the slides were counterstained with Mayer's hematoxylin.

Used primary antibodies were c-*myc* (Cambridge Research Biochemicals Ltd., Northwich, UK, pan-*myc* monoclonal antibody (Clone m1/4), 1 : 40), c-*fos* (Oncogene Science Inc, San Diego, CA, USA, Ab-2, 1 : 50), and TGF-α (Santa- Cruz Biotechnology Inc., Santa Cruz, CA, USA, Ab-2, 1 : 10).

Histologic examination of regeneration and apoptosis

Tissues were fixed in neutral 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. The evaluation of the histologic changes and degree of necrosis was observed in the liver. The degree of necrosis was measured with a light microscope (Olympus Vanox-T[AHBT-513]) equipped with Optomax V semi-automatic image analyzer and VIDS IV system.

Analysis of regeneration: Staining for BrdU incorporation was performed according to the manufacturer's instructions. Briefly, serial sections were rehydrated, enzymatically digested by trypsin, and incubated overnight at 4°C with anti-BrdU antibody (dilution 1 : 50). Biotinylated anti-mouse IgG (DAKO Corp.) and a complex of peroxidase conjugated streptavidin (DAKO Corp.) were added in sequence, followed by 3-amino-9-ethylcarbazole (AEC) in organic solvent and the slides were counterstained with Mayer's hematoxylin. Cells with a positive reaction were counted in the high-power field. Positive cells per 500 hepatocytes were counted.

Analysis of apoptosis: The *in situ* apoptosis detection kit, ApopTag™ (Oncor, Gaithersburg, MD, USA), was used to check the apoptosis in the lesion. All the procedures were performed according the manufacturer's instructions. Prior to visual inspection, cells were counterstained with methyl green.

Statistical analysis: Statistically significant differences between the experimental groups were determined using two way ANOVA, Kruskal-Wallis Test.

RESULTS

Results of the Western blot assay and immunohistochemical stain of the immediate early response gene and transforming growth factor-α

c-*myc* gene: In the Western blot assay, groups II and III showed high expression of c-*myc* gene at all experimental times with two peak expressions in group III at 30 min and 6 h, but group I showed a peak expression at 1 h without increment at any other experimental time (Fig. 1). The immunohistochemical stain did not demonstrate the gene.

c-*fos* gene: In the Western blot assay, group III showed high expression of c-*fos* gene at all experimental times with a peak expression at 24 h, but group I showed a peak expression at 24 h without increment at any other experimental time. Group II showed a slightly-increased expression at all experimental times (Fig. 2). The immunohistochemical stain did not demonstrate the gene.

TGF-α gene: In the Western blot assay and immunohistochemical stain, intergroup and time interval difference were not noted (Fig. 3, 4).

Histologic changes of liver

Group III was similar to group I except for the

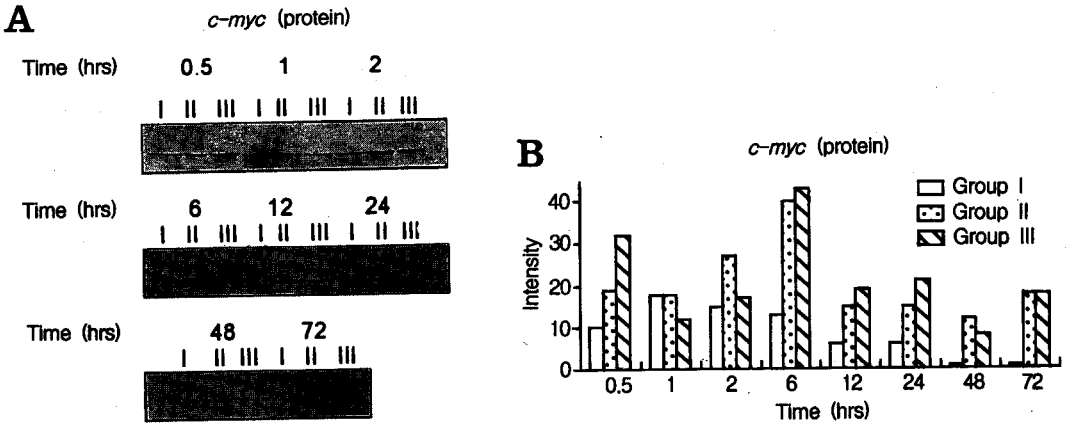

Fig. 1. Result of Western blot assay of nuclear c-myc gene. Western blot results of all experimental groups and times (A) and calculated by scanning densitometry (B). Groups II and III showed high expression at all experimental times with two peak expressions in group III at 30 min and 6 h, but group I showed a peak expression at 1 h without increment at any other time.

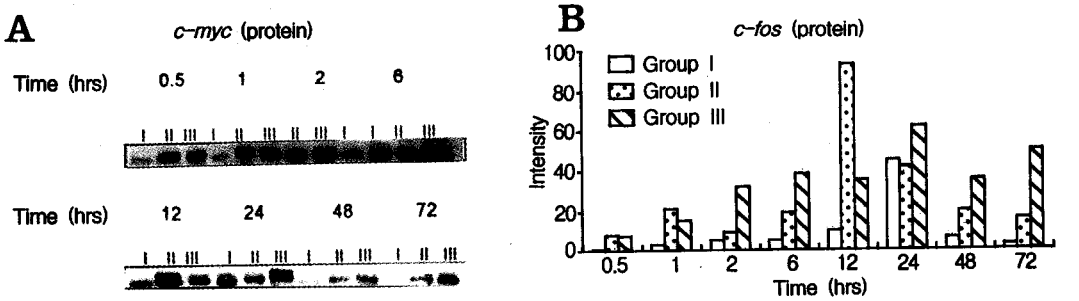

Fig. 2. Result of Western blot assay of nuclear c-fos gene. Western blot results of all experimental groups and times (A) and calculated by scanning densitometry (B). Group III showed high expression at all experimental times with a peak expression at 24 h, but group I showed a peak expression at 24 h without increment at any other time. Group II showed a slightly-increased expression at all experimental times.

following: Group III showed more frequent apoptosis at 12 h after administration of CCl_4 and larger necrotic areas at 48 h (Fig. 5). Group III showed larger necrotic areas than group I at all experimental times (Table 2). Among histologic changes in the liver which were similar in groups I and III, diffuse tiny vacuolation of hepatocytes appeared at the first 1h after administration of CCl_4 and were then followed by massive vacuolar distension of central zone hepatocytes at 12 h after treatment. At 24 h after treatment, this central zone was replaced by necrosis and cytoplasmic vacuolation was noted at the periphery of the necrosis. Until this time, the portal tract was intact. At 48 h after the treatment, necrosis was diminished and replaced by regenerative hepatocytes at 72 h after treatment. The liver tissue of group II was unremarkable except for minimal chronic inflammatory cell infiltration at the portal tract and minimal bile duct proliferation.

Results of BrdU incorporation: These results showed three differences between groups I and III. The first difference was that group I had more proliferation in a short period than group III. Group I showed higher positivity than group III at 48 h and

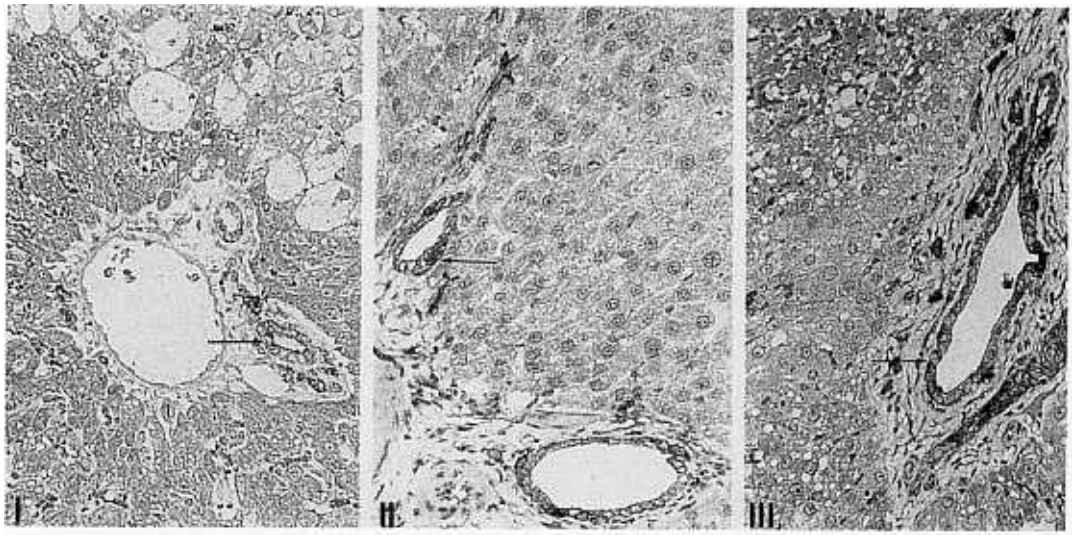

Fig. 3. Result of immunohistochemical stain of TGF-α. All groups showed positive TGF-α stain at bile duct (x100).

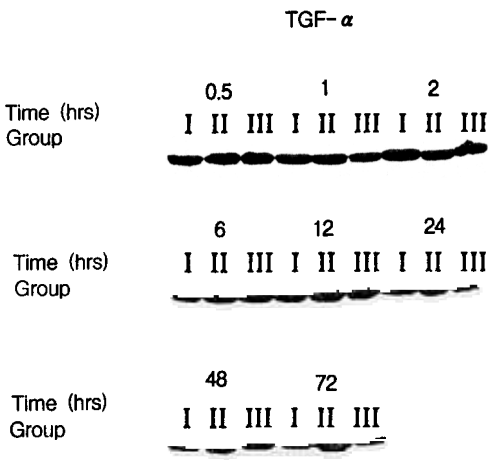

Fig. 4. Result of Western blot assay of TGF-α. Intergroup and time interval differences were not noted.

this difference had statistical value. The second difference was that group III showed relatively prolonged proliferation activity compared to group I. Group I showed a more abrupt decrease in positivity between 48 h and 72 h than group III (Fig. 6). The third difference was that the cell type proliferation in group III was of unknown immature cells but in group I it was well-differentiated hepatocytes. Group I showed positivity on the well-differentiated hepatocytes, but group III showed positivity on the unknown immature cells of the necrotic area (Fig. 7). Group II showed a few positively-stained cells in the portal tract.

Table 2. The area of the necrosis

	24 h	48 h
Group I	17%[a]	21%
Group II	0	0
Group III	26%	31%

[a]: The percentage of tissue necrosis was analyzed by image analyzer. The necrotic area of group III was larger than group I.
But the difference had no statistical significance.

Results of apoptosis: Group III showed frequent apoptosis at 12 h and prolonged apoptosis until 48 h while group I showed frequent apoptosis at 24 h and then an abrupt decrease. Groups I and III had differences at 12 h and 48 h and these were statistically significant (Fig. 8). Apoptosis was mainly noted at the boundary of the viable hepatocytes and the necrotic cells. The apoptotic cells showed chromatin condensation and cell shrinkage (Fig. 9). Group II showed a few positively-stained cells in the

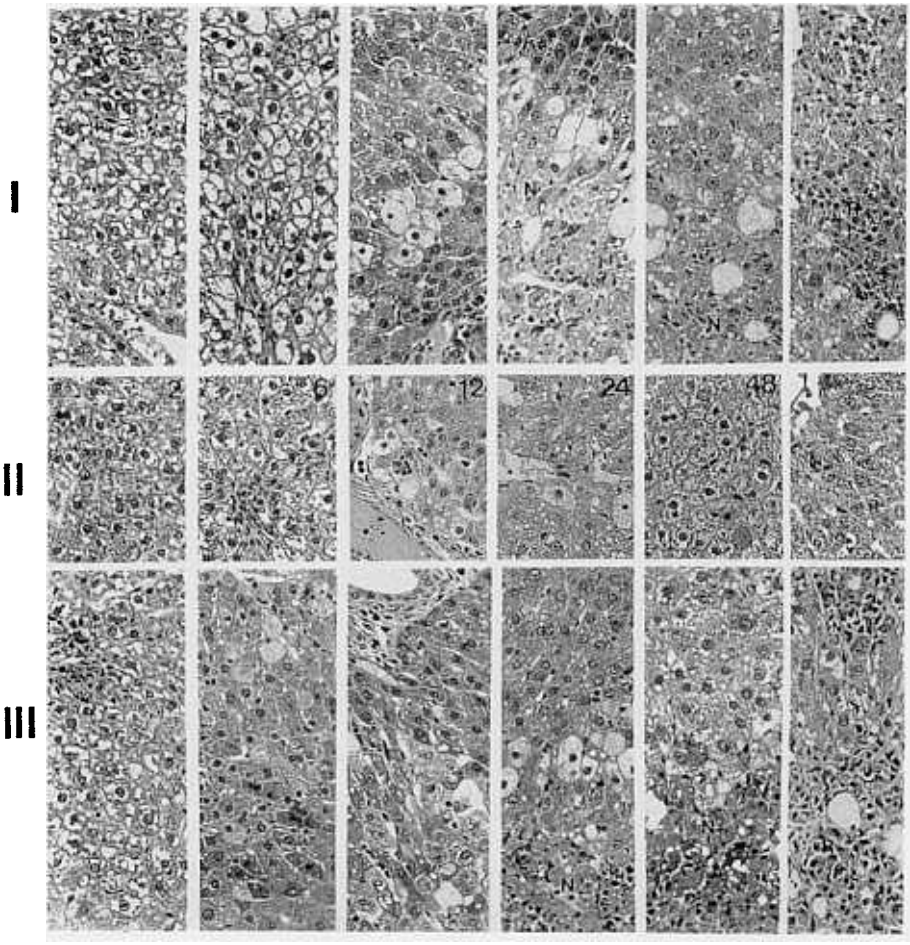

Fig. 5. Histologic changes of the liver. Group I (CCl_4-only treated group): Diffuse tiny vacuolation of hepatocytes which occurred at the first 1 h after administration of CCl_4. The central necrosis was started at 24 h and then this necrosis was replaced by regenerative cells. Group II (aflatoxin B1 pretreated group): There was no remarkable change. Group III (aflatoxin B1 pretreated CCl_4 injured group): The central necrosis was started at 24 h and then the necrosis was developed until 48 h (H&E, ×200).
N; necrosis,
R; regeneration

central zone.

Results of serum liver enzyme

Groups I and III began to increase the level of the serum liver enzyme at 6 h after treatment and this corresponded to histologic changes. Group II showed a normal range over the period of the experiment.

DISCUSSION

This study in rats demonstrates that AFB1- pretreated regeneration is related to apoptosis, delayed proliferation and proliferation of unknown immature cells and these histologic findings are mediated by c-*fos* and c-*myc* protein. Evidence that AFB1-pret-

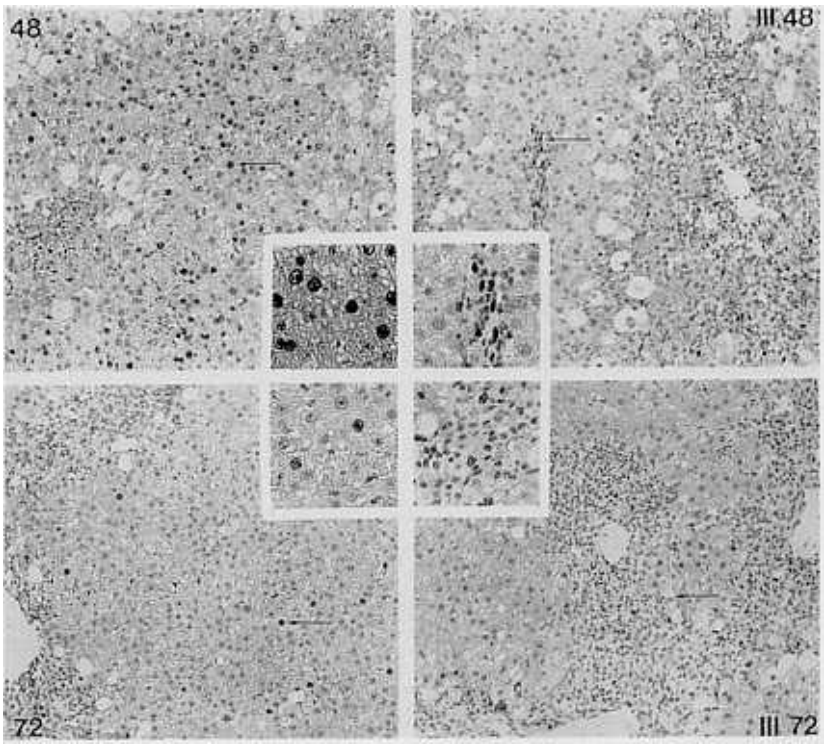

Fig. 7. Location and morphology of BrdU-positive cells (arrow) in group I and group III. Peak incorporation of BrdU was noted at 48 h in both groups and BrdU-positive cells of group I were well-differentiated hepatocytes, but not in group III. Decreased incorporation of BrdU was noted at 72 h in both groups and BrdU- positive cells were noted at same type cells as at 48 h (×100, inset ×200).

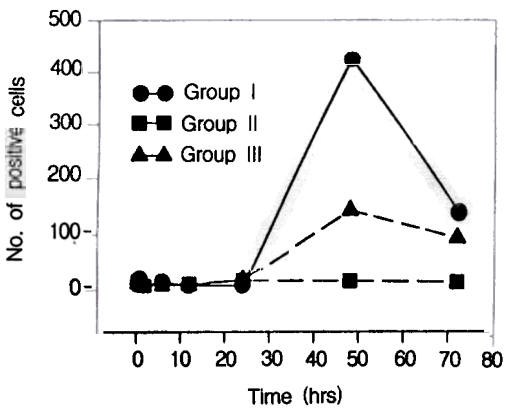

Fig. 6. Sequential change of BrdU-positive cell number. Group I had more proliferation in a short period than group III. Group I showed higher positivity than group III at 48 h and this difference had statistical value ($p < 0.005$). Group III showed relatively prolonged proliferation activity compared to group I. Group I showed a more abrupt decrease of positivity between 48 h and 72 h than group III.

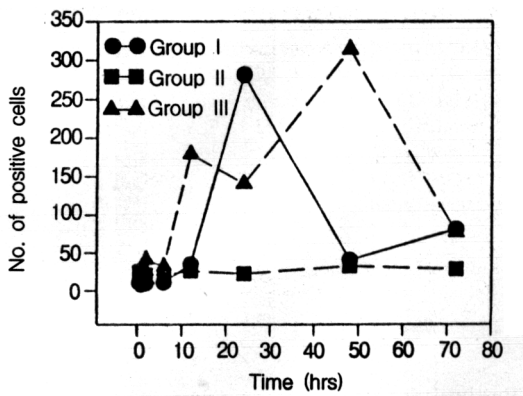

Fig. 8. Sequential change of apoptotic cell number. Group III showed frequent apoptosis at 12 h and prolonged apoptosis until 48 h, but group I showed frequent apoptosis at 24 h and then an abrupt decrease. Groups I and III had differences at 12 h and 48 h and these were statistically significant ($p < 0.005$).

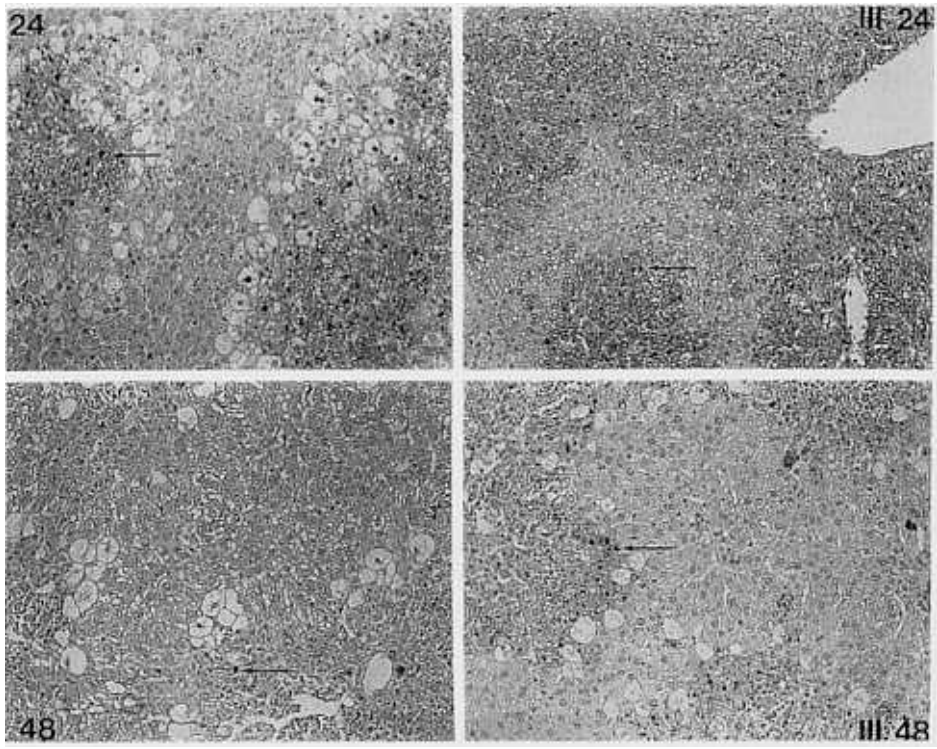

Fig. 9. Location of apoptotic cells (arrow) of group I and group III. Apoptosis was mainly noted at the boundary of the viable hepatocytes and the necrotic cells (×100).

reated regeneration is related to apoptosis is that the AFB1-pretreated, CCl_4-injured group showed more frequent apoptosis than the CCl_4-only treated group at 12 h and 48 h and this had statistical value. Evidence that AFB1-pretreated regeneration is related to delayed proliferation is that the AFB1-pretreated, CCl_4-injured group showed lower proliferation activity than the CCl_4-only treated group at the starting point of proliferation, but the decrease in proliferation activity was lower in the AFB1-pretreated, CCl_4-injured group than in the CCl_4-only treated group. Evidence that AFB1-pretreated regeneration was related to the proliferation of unknown immature cells was that the AFB1-pretreated, CCl_4-injured group showed BrdU positivity on the ovoid or spindle-shaped cells with a high N/C ratio in the necrotic area, but the CCl_4-only treated group showed BrdU positivity on well-differentiated hepatocytes. And evidence that these histologic findings were mediated by c-*fos* and c-*myc* protein was that the delayed-proliferation group III showed delayed c-*fos* protein expression and higher c-*myc* protein expression than group I and that the frequent-and-prolonged apoptotic group III showed prolonged higher expression of c-*myc* protein than group I.

The result that AFB1 induced high expression of c-*myc* protein was matched with the report that untransformed cultured hepatocytes with administration of AFB1 demonstrated relatively high expression of c-*myc* protein (Sinha *et al.* 1989).

The result that regeneration and apoptosis are mediated by c-*fos* and c-*myc* protein can be explained by previous reports that c-*fos* protein has a key role for cell proliferation but not for cell death (Fausto and Webber, 1993) and that c-*myc* protein triggers the regeneration (Mareu *et al.* 1992) and has a relationship with apoptosis (Evan *et al.* 1992; Oren, 1992; Shi *et al.* 1992).

The result, that delayed and prolonged expression of the immediate early response gene in the early stage of AFB1-pretreated regeneration was different from the CCl_4-only treated group, was supported by

the previous report that the expression of the immediate early response gene in the early stage of hepatcarcinogenesis induced by 2-acetylaminofluorene, was different from that of CCl_4 induced regeneration and was delayed and prolonged (Schmiedeberg *et al.* 1993).

The result that AFB1 induced the proliferation of unknown immature cells can be explained by the appearance of immature cells with toxin administration, but not with partial hepatectomy (Tournier *et al.* 1988; Lemire *et al.* 1991; Sirica *et al.* 1992).

In this study, the difference between TGF-α gene expression and pattern was minimal, because TGF-α gene was stained in the bile duct epithelium in all groups and at all experimental times with the immunohistochemical stain and expressed without any difference among the groups and the experimental times in the Western blot assay. In a recent study, TGF-α was highly expressed in fetal bile duct epithelium without external stimuli and then it was expressed but diminished in neonatal animals and disappeared in adult animals. This study concluded that TGF-α has an intimate relationship with hepatocyte proliferation and differentiation (Polimeno *et al.* 1995). We could explain the results by the fact that the animals used were relatively young and expressed the gene normally.

The immediate early response gene expression pattern of the group treated with CCl_4 only was well matched to previous reports of toxin induced and partial hepatectomy induced regeneration (Goytte *et al.* 1984; Sasaki *et al.* 1989; Herbst *et al.* 1991). So we think that the CCl_4-induced injury model for the evaluation of immediate early response gene expression is optimal.

In this study, we used the *in situ* apoptosis detection kit, ApopTag^{TM} (Oncor, Gaithersburg, MD, USA), to check the apoptosis in the lesion. Some reports have said that this method showed false positive results due to DNA strand breaks of necrosis and autolysis (Ansari *et al.* 1993; Grasl-Kraupp *et al.* 1995). So we analyzed the results carefully.

Some investigations on AFB1 have already been made. It was found that the c-Ha-*ras*, c-Ki-*ras*, c-*myc* and p53 gene were associated with the hepatocarcinogenesis of AFB1 in the molecular-biologic aspect (McMahon *et al.*1986; Tashiro *et al.* 1986; Sinha *et al.* 1989), but the expression pattern of these genes and their histologic relationship were not shown in the early stage of hepatocarcinogenesis. In this study, we reported the expression pattern of the immediate early response gene and related histologic findings in AFB1-associated regeneration.

In summary, our results indicate that AFB1-pretreated regeneration is related to apoptosis, delayed proliferation, proliferation of unknown immature cells and that these histologic findings are mediated by c-*fos* and c-*myc* protein. These findings will aid the investigation of molecular biologic and histologic characteristics of the hepatotoxic and hepatocarcinogenic mechanism of AFB1 in rats. And these results in rats, together with previous clinico-epidemiologic investigation in humans and molecular biologic investigation in humans and animals (Tashiro *et al.* 1986; Sinha *et al.* 1989; Murakami, 1993), suggest that AFB1 may supply hepatocarcinogenic background in early exposure time in AFB1 contaminated areas of China and Korea.

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