The Effect of Aflatoxin B1 on the Expression of Early Response Genes and Transforming Growth Factor-α in CCl₄ Induced Rat Liver Injury

Soon Won Hong¹ and Chanil Park²

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 (CCl₄) only was administered to group I, AFB1 only was administered to group II and a combination of AFB1 and CCl₄ 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, provoking the CCl₄-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, CCl4, 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 (Goyette 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 CCl4 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 CCl4 (Sigma Chemical Co., St. Louis, MO, USA) intragastrically. The single dose of CCl4 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 CCl4 as per group I.

Three animals were sacrificed from each group at 30 minutes, 1, 2, 6, 12, 24, 48, 72 hours after the CCl4 or corn oil treatment (Schmiedeberg et al.

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<td>Group I  Carbon tetrachloride</td>
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<td>Group II Aflatoxin B1</td>
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<td>Group III Carbon tetrachloride + Aflatoxin B1</td>
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Group I: Single dose (5.0 ml/kg of body weight) of CCl4 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 CCl4 was administered. Three animals were sacrificed from each group at 30 minutes, 1, 2, 6, 12, 24, 48, 72 hours after the CCl4 or corn oil treatment.

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).
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 en-
hanced 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 apop-
tosis

Tissues were fixed in neutral 10% formalin, em-
bedded in paraffin, sectioned at 5 μm, and stained
with hematoxylin and eosin. The evaluation of the
histologic changes and degree of necrosis was obser-
ved 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 in-
corporation was performed according to the manu-
facturer’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 or-
ganic solvent and the slides were counterstained
with Mayer’s hematoxylin. Cells with a positive
reaction were counted in the high-power field. Posi-
tive cells per 500 hepatocytes were counted.

Analysis of apoptosis: The in situ apoptosis de-
tection 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 inspec-
tion, cells were counterstained with methyl green.

Statistical analysis: Statistically significant dif-
fferences between the experimental groups were de-
termined using two way ANOVA, Kruskal-Wallis
Test.

RESULTS

Results of the Western blot assay and immuno-
histochemical 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 immunohisto-
chemical 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 expe-
mental 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 expe-
nmental times (Fig. 2). The immunohistochemical
stain did not demonstrate the gene.

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

Histologic changes of liver

Group III was similar to group I except for the
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**B**

![Graph](image)

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.

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**B**

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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 CCl₄ 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 CCl₄ 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
TGF-α

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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.

Table 2. The area of the necrosis

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<tr>
<td>Group I</td>
<td>17%*</td>
<td>21%</td>
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<tr>
<td>Group II</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Group III</td>
<td>26%</td>
<td>31%</td>
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*: 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.

tocytes. 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.

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
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).
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**Fig. 4. 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).**

Regeneration is related to apoptosis is that the AFB1-pretreated, CCl₄-injured group showed more frequent apoptosis than the CCl₄-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, CCl₄-injured group showed lower proliferation activity than the CCl₄-only treated group at the starting point of proliferation, but the decrease in proliferation activity was lower in the AFB1-pretreated, CCl₄-injured group than in the CCl₄-only treated group. Evidence that AFB1-pretreated regeneration was related to the proliferation of unknown immature cells was that the AFB1-pretreated, CCl₄-injured group showed BrdU positivity on the ovoid or spindle-shaped cells with a high N/C ratio in the necrotic area, but the CCl₄-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 CCl₄-only treated group, was supported by
the previous report that the expression of the immediate early response gene in the early stage of hepatocarcinogenesis induced by 2-acetylaminofluorene, was different from that of CCl₄ 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 CCl₄ only was well matched to previous reports of toxin induced and partial hepatectomy induced regeneration (Goyette et al. 1984; Sasaki et al. 1989; Herbst et al. 1991). So we think that the CCl₄-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™ (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-pre-treated 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 clinicobiologic 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.

ACKNOWLEDGMENTS

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