In Vivo Activation of Aflatoxin B₁ in C57BL/6N Mice Carrying a Human Fetus-specific CYP3A7 Gene

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ABSTRACT

The in vivo activation of aflatoxin B₁ (AFB₁) was assessed by using two transgenic mouse lines, M2 and M10, in which the human fetus-specific CYP3A7 was expressed in the kidney (M2) and the liver (M10), respectively. Male mice of 8 weeks old from these two lines were treated with a single i.p. injection of AFB₁ (4 mg/kg body weight). AFB₁-N₂-guanine adduct was quantified by high-performance liquid chromatography. DNA damage was measured using the alkaline elution technique 2 and 6 h after AFB₁ treatment. Administration of AFB₁ resulted in a significantly higher level of AFB₁-N₂-guanine in the livers of M10 transgenic mice compared with their nontransgenic littermates (16.5 ± 4.2 versus 10.4 ± 1.2 ng/mg DNA, P < 0.01). The level of this biomarker was also significantly higher in the kidney of the M2 mice compared with control mice (73.0 ± 6.3 versus 50.2 ± 9.5 ng/mg DNA, P < 0.01). Similar results were also observed with DNA damage expressed as normalized area above curve (NAAC) in the two transgenic lineages, e.g., NAAC values were significantly higher in the livers of M10 and the kidneys of M2 mice. A dose-response relationship of NAAC values was observed in the livers of M10 mice when treated with AFB₁ at different doses ranging from 1 to 16 mg/kg body weight, whereas in nontransgenic mice, only slight but not statistically significant increases of NAAC values were observed. Both the mouse CYP3A11 and GST-Yc subunit were expressed at identical levels in these transgenic lines. The results of this study further present evidence that human fetuses are also under the carcinogetic attack of AFB₁, as adults if exposed to this potent carcinogen.

INTRODUCTION

AFB₁ is a potent carcinogen to many species, ranging from fish to nonhuman primates (1, 2). A great deal of evidence from extensive studies of epidemiology and laboratory experiments incriminates AFB₁ as a hepatocarcinogen in man (3). Besides the well-documented great variations of susceptibility among different species to the toxicity/carcinogenesis of AFB₁, this mycotoxin is also characterized by its more potent toxic/carcinogenic effects on young animals than adults (4). Of particular concern is the toxicological and carcinogenic significance of AFB₁ in utero in humans. AFB₁ is a ubiquitous contaminant in the human environment and has been classified into group 1 carcinogens in humans by the IARC (5). AFB₁ and its DNA adducts were detected in human placenta and cord blood (5, 6). Experiments on animals revealed in utero carcinogenesis in rats (7, 8) and embryotoxicity and teratogenicity in two resistant species, hamsters and mice (6, 9).

It is well established that AFB₁ exhibits its toxicity after undergoing bioactivation mainly by the CYP-mediated oxygenation system. To date, multiple isofoms of CYP have been found to contribute to the bioactivation of AFB₁, although there is some ambiguity concerning which CYP isozyme is the most important one (10). On the other hand, little data are available regarding the possible involvement of CYP(s) expressed in the human fetus in the biotransformation of this potent carcinogen. We have demonstrated that CYP3A7 is a CYP isozyme expressed predominantly, if not exclusively, in human fetal liver (11, 12). Our earlier work using an in vitro assay has shown that CYP3A7 possessed the ability to activate AFB₁ (13, 14). Recently, we established several transgenic mouse lines carrying a murine MT-I-CYP3A7 transgene, with an attempt to clarify the in vivo toxicological significance of this human fetal CYP isoform (15). In this report, we provide evidence for in vivo bioactivation of AFB₁ by CYP3A7 expressed in our transgenic mice using an AFB₁-Gua adduct as a biomarker, and DNA damage was measured with the alkaline elution technique.

MATERIALS AND METHODS

Chemicals. AFB₁ was purchased from the Makor Chemical (Jerusalem, Israel). Hoechst 33258 was obtained from the Wako Pure Chemical Industries (Tokyo, Japan). Other chemicals and reagents used were of the highest quality available commercially.

Animals and Treatment. Male mice of 8 weeks old from the M2 and M10 transgenic lines and their respective NT littermates were used. A mouse MT-I promoter-CYP3A7 cdNA hybrid transgene was integrated into the genome of these transgenic mice. Expression of the transgene was detected in the kidneys and livers of M2 and M10 transgenic lines, respectively (15). All of the animals were pretreated with 25 mm ZnSO₄ in their drinking water for 1 week to induce the expression of the transgene followed by a single i.p. injection of AFB₁, dissolved in DMSO at different doses. At different time points after the treatment, animals were euthanized and the livers and kidneys removed for further analysis. The dose and the time of sampling after AFB₁ treatment are specified in the legends to Figs. 1–5 or in the text.

DNA Preparation from Mouse Tissues. DNA was prepared from the livers and kidneys of the mice as described by Groopman et al. (15). With some modifications, briefly, mouse tissues were quickly removed, minced, and homogenized in 4 volumes of 50 mm Tris-HCl buffer (pH 7.0) containing 0.25 m sucrose. The supernatant was discarded after centrifugation at 800 × g for 10 min. After resuspension in the Tris-sucrose buffer containing 0.05% Triton X-100, the pellets were recenterfuged and washed twice. The resulting nuclear pellets were resuspended in 5 ml of lysate buffer containing 50 mm Tris-HCl (pH 7.0), 0.1 m NaCl, 20 mm EDTA, 1% SDS, 150 μg/ml protease K, and 1.0 mg/ml Pronase E and incubated at 40°C for about 8 h with gentle shaking until the solutions appeared to be clear. DNA in the solutions was extracted with phenol-chloroform, precipitated with cold ethanol, and then dissolved in water. The DNA purity was judged by using the absorbance ratio A260/A280 of 1.8 or higher as a criterion. The DNA content was determined spectrophotometrically using a 260-nm extinction of 1.0 for 50 μg of DNA/ml.

AFB₁-Gua Adduct Analysis. About 1 mg of DNA was hydrolyzed in 0.15 m HCl at 100°C for 1 h and then chilled quickly on ice. After neutralization with 1.0 m potassium acetate to yield a pH 5.5, methanol was added to give a final concentration of 5%. The hydrolysates were applied to a methanol/prewashed Bond Elute C₁₈ cartridge (Varian, Harbor City, CA), washed with

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* The abbreviations used are: AFB₁, aflatoxin B₁; AFB₁-Gua, AFB₁-N₂-guanine; AFB₁-Fapy, 2,3-dihydro-2-(N₂-formyl-2,3,6-triamino-4-oxopyrimidine-N'-yl)-3-hydroxy AFB₁; AFB₁-Gua, AFB₁-N₂-guanine; CDNB, 1-chloro-2,4-dinitrobenzene; CYP, cytochrome P450; GST, glutathione S-transferase; MT-I, metallothionein type 1; MZ, mida złom; NAAC, normalized area above curve; NT, nontransgenic; RT, reverse transcription; SSB, single-strand break; SS, single stranded; TEAH, tetrathyrammonium hydroxide.

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The eluates were monitored with a fluorescent detector (RF-530; Shimadzu, Kyoto, Japan) with emission at 420 nm and excitation at 360 nm. Quantification of AFB1-Gua adducts was achieved by comparing the peak areas in DNA samples with that of an authentic standard synthesized as described previously (17).

Alkaline Elution Assay. DNA SSB was measured using the alkaline elution technique described by Brunborg et al. (18) and Holme et al. (19), with minor modifications. Briefly, mice were sacrificed 6 h after AFB1 treatment, and organs were quickly excised and frozen in liquid nitrogen and stored at —80°C until use. For the preparation of nuclei, organs were thawed on ice and minced in ice-cold Merchant’s solution containing 10 mM EDTA and homogenized in 10 volumes of Merchant’s solution. After centrifugation at 10,000 × g for 4 min, the pellets were resuspended in the same solution. A cell/nuclei mixture (2.5 × 10⁹) was loaded onto a polycarbonate filter (pore size, 2.0 μm; Millipore, Watford, UK), washed with 3 ml of Merchant’s solution, and lysed on the filter in the dark for 30 min with a lysis buffer containing 2.0 mM NaCl, 20 mM EDTA, 0.2% sodium lauroylsarcosinate, and 0.5 mg/ml proteinase K at pH 10.0. After washing with 5 ml of 20 mM EDTA, the damaged DNA on the filter was eluted in the dark with 20 mM EDTA adjusted to pH 3.0 with 20% TEAH. Five fractions of the eluates were collected with a flow rate of 2.4 ml/h using a multichannel peristaltic pump (MS-4 REGLO; Ismatec, Switzerland). The amounts of SS DNA in each fraction and on the filter were quantified using a fluorochrome Hoechst 33258 (20). The elution rates of SS DNA are expressed as NAACs, calculated as described by Brunborg et al. (18). To increase reliability and sensitivity, care was taken during the assay process as demonstrated by Koch and Giandomenico (21).

Expression of CYP3A11 and GST in Transgenic Mice. Total RNA was prepared from the livers of M10 and M2 transgenic mice and subjected to Northern blot analysis as detailed previously (15) using CYP3A11 cDNA as a probe (22). To detect the mouse GST-Yc mRNA, a cDNA probe was synthesized and amplified from mouse liver total RNA with RT-PCR using a sense primer 5′-GCT GGT GTG GAT TGA AA-3′ and an antisense primer 5′-GCG AAT ATC AGC CCT CA-3′ corresponding to the nucleotide positions 103–122 and 485–504 of the mouse GST-Yc gene, respectively (23). Briefly, 2 μg of RNA, 1.2 μg of random primer pd(N)₆ (Pharmacia, Uppsala, Sweden), 0.4 mM deoxynucleotide triphosphates, and 100 units of reverse transcriptase (Toyobo, Tokyo, Japan) were added, mixed (total volume, 30 μl), and incubated at 37°C for 1 h. The expected 402-bp cDNA was subsequently amplified with PCR using an aliquot of the above sample as a template, 5 units of Taq DNA polymerase, and 25 pmol of each primer. PCR was carried out by repeating 35 cycles as 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min. The PCR product was subsequently fractionated electrophoretically with a 1.0% agarose gel and recovered from the gel using a GeneClean II kit (LabJolla, CA). This cDNA was labeled with [³²P]dCTP and used as a probe in RNA hybridization analysis as described previously (15).

GST Activity toward CDNB in the Livers of Transgenic Mice. Mice were pretreated with ZnSO₄ as described above, and the GST activity was measured using CDNB as a substrate according to the published procedures (24, 25).

RESULTS

AFB1-Gua Adduct Levels. A single dose of AFB1 resulted in a significantly higher AFB1-Gua adduct level in the livers of M10 mice 2 h after the AFB1 treatment, which diminished rapidly in 6 h to a level identical to that in NT mice. On the other hand, M2 mice showed comparable adduct levels to their NT littermates both at 2 and 6 h (Fig. 1A). We showed previously that among the six transgenic lines established, M10 was the only line to express the transgene in the liver, at both mRNA and protein levels, whereas M2 mice expressed the transgene in the kidney and many other extrapathic tissues, but not in the liver (15).

Compared with their NT littermates, M2 mice showed a significantly higher adduct level in the kidneys 2 h after AFB1 treatment. As expected, the M10 mice had identical adduct levels in this tissue both at 2 and 6 h with their NT littermates (Fig. 1B). In general, the adduct levels were significantly higher in the kidneys than in the livers of both the transgenic and NT mice. Unlike the adduct levels in the livers, there was no substantial difference in adduct levels in the kidneys between 2 and 6 h after AFB1 injection (Fig. 1B).

DNA Damage in the Livers and Kidneys. The alkaline elution technique remains a high sensitive assay in measuring DNA damage caused by UV light and some chemical agents (21, 26). In this study, treatment with AFB1 resulted in significant DNA damage in the livers of M10 mice 6 h after a single i.p. injection. Fig. 2 shows a representative elution profile of the damaged DNA from AFB1-treated mice. After a 100-min elution, there was significantly less DNA retained on the filter in AFB1-treated M10 mice compared with their NT littermates, indicating more pronounced DNA damage occurred. The DNA damage expressed as NAAC calculated from the elution profile is shown in Fig. 3. Particularly, NAAC values of M2 mice treated with either AFB1 or the vehicle DMSO were much higher than those of the NT littermates (Fig. 3A), suggesting nonspecific DNA damage probably caused by the DMSO. We repeated the experiment and observed the same results. DMSO has been known to be toxic to erythrocytes and vascular epithelium (27) and even caused increased DNA damage (28). However, no effort was made to further investigate the underlying mechanism for this phenomenon.

![AFB1-Gua adduct levels in the livers and kidneys. Male mice from each line were given AFB1 in a single i.p. injection (4 mg/kg body weight in DMSO), and the AFB1-Gua adducts were determined in the livers (A) and kidneys (B) 2 and 6 h after AFB1 treatment. Each column represents the mean of eight mice. Bars, SD. **, significantly different from the value of NT mice within the respective sampling time (P < 0.01, two-tailed, unpaired Student’s t test).](attachment:image)
DNA SSBs were detected in the kidneys of M2 mice at 6 h after AFB1 treatment (Fig. 3B), consistent with the high expression of the CYP3A7 transgene in the kidney of this transgenic line (15). No DNA damage was detected in the kidneys of M10 mice compared with the NT mice.

Increase of DNA Damage in the Livers of M10 Mice as a Function of the Dose of AFB1. In the M10 mice, treatment of AFB1 at a dose of 2 mg/kg body weight resulted in detectable DNA damage in the livers compared with the DMSO-treated control mice. The DNA damage was measured using the alkaline elution technique as detailed in "Materials and Methods." Each curve represents the mean of six mice with duplicate determinations. Bars, SD. TG, transgenic.

In vivo Activation of AFB1 by CYP3A7 in Transgenic Mice

DISCUSSION

The metabolism of AFB1 is characterized by multiple pathways, multiple enzyme systems involved, and different and sometimes conflicting outcomes. To date, more than five CYP isoforms belonging to the CYP1A, 2A, 2B, 2C, and 3A subfamilies have been reported to be involved in the metabolism of AFB1 in vitro, and one isozyme is usually involved in more than one metabolic pathway of AFB1 to yield different products (3, 29, 30). Thus, it is not surprising that different and/or contradicting data exist in the literature regarding the possible role of a particular CYP isozyme in the metabolism of AFB1 using different metabolites as criteria. In human adults, both CYP1A2 and 3A4 seem to play equally important roles in the metabolic activation of AFB1, with different affinities at different substrate concentrations (31, 32). Humans are believed to be exposed to AFB1 in utero (5, 6, 33, 34). Nevertheless, the source of reactive AFB1 metabolites in the human placenta-fetus unit remains relatively unclear. We have demonstrated previously that CYP3A7 was a major P450 isozyme expressed in human fetal livers (11, 12) and was capable of bioactivating AFB1 in vitro (13, 14). To gain an insight into the in vivo role of CYP3A7, we measured the AFB1-Gua adduct level and DNA damage as criteria in two of our transgenic mouse lines harboring this CYP isozyme. AFB1-Gua adduct is a biomarker that gives direct evidence of AFB1 attack to the macromolecules in vivo and has found comprehensive applications in both experimental animal and epidemiological studies (35-37).

It is well established that the AFB1-Gua adduct is unstable and may either undergo spontaneous nonenzymatic depurination or be stabilized by the opening of the imidazole ring to yield AFB1-Fapy, which is believed to be subject to enzymatic removal (30). The repair/removal of the adducts usually will give rise to apurinic sites which are alkaline labile and readily converted into nicks detectable with the alkaline elution technique (38, 39). Following a single dosing with AFB1, maximal DNA adduct level was observed in the liver at 2 h. By 24 h, 88% of the adducts had been removed (40). The differences in adduct levels in 2 h disappeared in 6 h after AFB1 treatment (Fig. 1A). The detectable DNA damage in the livers of M10 mice at 6 h when the adduct level was no longer significantly different from those in the other mice does not necessarily mean discrepancy since the DNA damages (apurinic sites and nicks) detected by alkaline elution were later events following the repair and/or removal of the DNA adduct. It seems that the adduct repairing rate was higher in the M10 mice, although a higher rate of adduct modification to form AFB1-Gua adducts were detected in the kidneys of M2 mice at 6 h after AFB1 treatment (Fig. 3B), consistent with the high expression of the CYP3A7 transgene in the kidney of this transgenic line (15). No DNA damage was detected in the kidneys of M10 mice compared with the NT mice.

Expression Levels of CYP3A11 and GST-Yc in Mice. The results of Northern blot analysis for the mouse CYP3A11 revealed that this isozyme was expressed at identical levels in both transgenic and NT mice, as was the mouse GST-Yc subunit (Fig. 5). The activities of GST in the livers of transgenic and NT mice were also comparable (Table 1), indicating that the mouse CYP3A subfamily as well as GST have not been altered by the integration of the MT-I-CYP3A7 transgene.
were almost as high as those at 2 h after AFB1 treatment (Fig. 1). This may be explained by a lower adduct repair/removal rate, and by a lower renal GST activity (23), leading to adduct accumulation in the kidney. AFB1 is a well-known hepatocarcinogen to many species including nonhuman primates (2). However, little information is available linking AFB1 as a renal carcinogen to humans, although there were scattered reports about renal carcinogenesis of AFB1 in animals (50, 51). The significance of high and persistent AFB1-Gua adduct levels in the mouse kidneys is unclear.

The mouse has a high GST activity in the liver which was found to be approximately 50-fold that in the rat, although the murine liver microsomes possess approximately a 4-fold greater capacity to activate AFB1. Therefore, it is of general opinion that GST-catalyzed detoxification, rather than microsomal activation, is the crucial determinant of susceptibility to the toxicity of AFB1 (10). Among the mouse GST isoenzymes, the Yc subunit of the α class has been found to possess the highest activities toward AFBO (52). The constitutive expression of GST-Yc has been suggested to be responsible for the high capacity to detoxify AFBO in the mouse (23). However, the expression of GST-Yc has not been altered in our transgenic mice by the integration of the CYP3A7 gene nor has the mouse CYP3A11 (Fig. 5 and Table 1). Thus, our data indicate that the integration of the CYP3A7 gene into the mouse genome has elevated the sensitivity of our transgenic mice to the toxicological effect of AFB1.

Fig. 4. Dose-dependent response of transgenic M10 mice to the DNA-damaging effect of AFB1 in vivo. Male mice from the M10 transgenic line as well as NT littermates were treated with a single i.p. injection of AFB1 at dosages ranging from 0 to 16 mg/kg body weight in DMSO. Each symbol represents the mean of six mice with duplicate determinations. Bars, SD. ** significantly different from the respective control mice with DMSO treatment (P < 0.01; ANOVA Scheffe F test).

Fapy cannot be ruled out. The reasons for this phenomenon are unknown.

Mouse liver microsomes were found to be approximately five times as efficient as human adult liver microsomes in activating AFB1 into AFBO (41). Using MZ as a substrate, we also found that the 1'-hydroxymidazolam formation, which is believed to be catalyzed exclusively by the CYP3A isozymes, was much higher in mouse liver microsomes than that in humans. These may explain the high background level of AFB1-Gua in the mouse and relatively narrow difference of adduct levels as well as NAAC values between the transgenic mice and their NT littermates.

It is not unusual that an endogenous gene is interrupted by the integration of a foreign gene (42). We showed previously that the steroid 5α-reductase might have been affected, leading to a significant increase of serum testosterone in the M10 male mice (15). To test the possible interaction between the CYP3A7 transgene and the mouse endogenous CYP3A isoforms, we carried out Northern blot analysis for the CYP3A11, a major isozyme of the mouse CYP3A subfamily (43). The result revealed that CYP3A11 was expressed at comparable levels in both transgenic and NT mice (Fig. 5). On the other hand, the CYP1A2 does not seem to play a crucial role in AFB1 biotransformation (30). The results of this study are in agreement with this notion (Fig. 1). Nevertheless, the kidney was found to be refractory to this rule. The adduct levels at 6 h

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\* Unpublished observation.

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** Fig. 5. Northern blot analyses for mRNA expressions of CYP3A11 and GST-Yc in mouse livers. Total RNAs were prepared from the livers of 8-week-old M2 and M10 mice pretreated with 25 nmol ZnSO4 for 1 week. An aliquot (20 µg) of RNA was formaldehyde denatured and separated electrophoretically in a 1.0% agarose gel. The RNA was blotted onto a nylon membrane and hybridized with 32P-labeled CYP3A11 cDNA and visualized by autoradiography at −80°C for 2 weeks (upper panel). The same membrane was rehybridized with 32P-labeled mouse GST-Yc cDNA obtained with RT-PCR as detailed in “Materials and Methods” after the removal of the CYP3A11 probe (bottom panel).

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** Table 1 GST activity in the livers of M2 and M10 transgenic mice**

<table>
<thead>
<tr>
<th>Line</th>
<th>Activity (µmol/min/mg)</th>
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<tbody>
<tr>
<td>NT</td>
<td>4.48 ± 0.46</td>
</tr>
<tr>
<td>M2</td>
<td>4.54 ± 0.30</td>
</tr>
<tr>
<td>M10</td>
<td>4.40 ± 0.72</td>
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* Four 8-week-old male mice from each line were used.

b Values represent means ± so of duplicate determinations.

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** Fig. 5. Northern blot analyses for mRNA expressions of CYP3A11 and GST-Yc in mouse livers. Total RNAs were prepared from the livers of 8-week-old M2 and M10 mice pretreated with 25 nmol ZnSO4 for 1 week. An aliquot (20 µg) of RNA was formaldehyde denatured and separated electrophoretically in a 1.0% agarose gel. The RNA was blotted onto a nylon membrane and hybridized with 32P-labeled CYP3A11 cDNA and visualized by autoradiography at −80°C for 2 weeks (upper panel). The same membrane was rehybridized with 32P-labeled mouse GST-Yc cDNA obtained with RT-PCR as detailed in “Materials and Methods” after the removal of the CYP3A11 probe (bottom panel).
Taken together, our results indicate that the human CYP3A7 expressed in our M2 and M10 mice activated AFB1, in vivo, leading to significantly higher DNA adduct levels as well as pronounced DNA damage in the kidneys and livers of M2 and M10 transgenic mice, respectively. The results reported here reinforce our earlier work in which the human CYP3A7 was predominantly responsible for the bioactivation of AFB1, in human fetal livers (53).

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