Low Frequency of p53 Gene Mutation in Tumors Induced by Aflatoxin B1 in Nonhuman Primates

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Abstract

Aflatoxin B1 has been suggested as a causative agent for a G to T mutation at codon 249 in the p53 gene in human hepatocellular carcinomas from southern Africa and Qidong in China. To test this hypothesis, nine tumors induced by aflatoxin B1 in nonhuman primates were analyzed for mutations in the p53 gene. These included four hepatocellular carcinomas, two cholangiocarcinomas, a spindle cell carcinoma of the bile duct, a hemangiopericytoma of the liver, and an osteogenic sarcoma of the tibia. None of the tumors showed changes at the third position of codon 249 by cleavage analysis of the HaeIII enzyme site at codon 249. A point mutation was identified in one hepatocellular carcinoma at the second position of codon 175 (G to T transversion) by sequencing analysis of the four conserved domains (II to V) in the p53 gene. These data suggest that mutations in the p53 gene are not necessary in aflatoxin B1 induced hepatocarcinogenesis in nonhuman primates. The occurrence of mutation in codon 249 of the p53 gene in selective samples of human hepatocellular cancers may indicate involvement of environmental carcinogens other than aflatoxin B1 or that hepatitis B virus-related hepatitis is a prerequisite for aflatoxin B1 induction of G to T transversion in codon 249.

Introduction

Dietary exposure to aflatoxin B1 is an epidemiologically defined risk factor for HCC3 in southern Africa and China (1–3). Aflatoxin B1 is also a potent hepatocarcinogen in different species including nonhuman primates (4–6). Recently, a significant number of HCCs in patients from Qidong province in China and from southern Africa, where hepatitis B virus is endemic and dietary exposure to aflatoxin B1 is high, were found to have a mutation at the third position of codon 249 of the p53 gene (3, 7, 8). It was suggested that this mutation of the p53 gene was caused by aflatoxin B1 and might contribute to the high incidence of HCC in these areas. The absence of mutation at this hot spot in HCCs in patients from Japan, Taiwan, or Australia support this hypothesis since exposure to aflatoxin B1 is very low in these areas (3, 9–11). The specificity of this mutational event represents the first clue as to how an environmental toxin may contribute to tumor development (12). It is therefore important to characterize the frequency and patterns of p53 gene mutations in aflatoxin B1 induced carcinogenesis. We have examined nine tumors previously induced by aflatoxin B1 in nonhuman primates (4, 5) for mutations in the p53 gene by restriction enzyme analysis using HaeIII enzyme site at codon 249 and by sequence analysis for mutations within the highly conserved domains II to V which contain four hot spots for mutations in various tumors (13–15). A predicted amino acid sequence of the p53 gene of nonhuman primates is 95% identical to the human protein and that of conserved domain IV including codon 249 is completely identical (16).

Materials and Methods

Tumors in Nonhuman Primates. Nine tumors including four HCCs, two cholangiocarcinomas, a spindle cell carcinoma of a bile duct, a hemangiopericytoma of the liver, and an osteogenic sarcoma of the tibia were derived from eight nonhuman primates composed of four rhesus (Macaca mulatta) and four cynomolgus (Macaca fascicularis) monkeys (Table 1). Aflatoxin B1 (Calbiochem, Los Angeles, CA; Makor Chemicals, Ltd., Jerusalem, Israel) was administered according to a variety of schedules from 1964 to 1978 (5). Nonhuman primates that died or were sacrificed were carefully necropsied and the tissues were fixed in buffered formalin and embedded in paraffin (5).

DNA Preparation. Extraction of DNA from paraffin embedded tumor tissue was carried out as described previously (17). Briefly, tumor cell areas from two 10-μm sections of each paraffin block were selectively removed based on corresponding hematoxylin and eosin stained sections. The tissues were deparaffinized by xylene, washed with 95% ethanol, and incubated in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.32 M sucrose, 1% Triton X-100, 0.5% sodium dodecyl sulfate, and 0.1 mg/ml proteinase K at 70°C for 1 h and 42°C overnight. DNA was extracted from lysis solution with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and dissolved in 100 μl of 10 mM Tris-HCl, pH 8.0.

PCR Amplification. Oligonucleotide primers for PCR amplification were synthesized with an Applied Biosystems DNA synthesizer (model 380B). Identification of sequences are: P1, ACGTGAATTCCTTACACTCTGTTGCTCCTCACC; P2, TGATGGCAAATTCGGTACCTCCAATCACCA; P3, GGAATTTCTGCAGTACCTGACAACATCCA; P4, ACGTGAATTCTGACTGTACCACCATCCA; P5, ACGTGGAGAATTCTGACTGTACCACCATCCA; P6, ACGTGGAGAATTCGAAGGCAAGCACGGCCT; P7, ACGTGGATCTCCCTCTTCCCT; P8, ACGTGGATCTCCCTCTTCCCT; P9, ACGTGGATCTCCCTCTTCCCT; P10, ACGTGGATCTCCCTCTTCCCT. The eight primers were designed to incorporate extranucleotides comprising EcoRI, or EcoRI and BamHI sites at their 5'ends to facilitate cloning. The PCR products were digested with EcoRI or EcoRI and BamHI, fractionated by 3% low melting agarose gel electrophoresis, and ligated to EcoRI or EcoRI and BamHI digested pGEM vectors (Promega). At least six individual clones were sequenced for each sample by the dyeodeoxy chain termination method (19) and a total of 180 individual clones were examined for mutations of the p53 gene. To confirm a mutation, subclones obtained from two individual amplifications were sequenced.

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Table 1 Mutation of the p53 gene in tumors induced by aflatoxin B1 in nonhuman primates

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Histology</th>
<th>Metastasis</th>
<th>Monkey</th>
<th>Species</th>
<th>Mutation atCodon 249</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hepatocellular carcinoma</td>
<td>-</td>
<td>692I</td>
<td>Rh</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Hepatocellular carcinoma</td>
<td>+</td>
<td>692I</td>
<td>Rh</td>
<td>175 (G to T)</td>
</tr>
<tr>
<td>3</td>
<td>Hepatocellular carcinoma</td>
<td>-</td>
<td>497F</td>
<td>Cyno</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Hepatocellular carcinoma</td>
<td>-</td>
<td>454F</td>
<td>Rh</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Cholangiocarcinoma</td>
<td>+</td>
<td>500F</td>
<td>Rh</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Cholangiocarcinoma</td>
<td>+</td>
<td>473F</td>
<td>Rh</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Spindle cell carcinoma</td>
<td>-</td>
<td>518G</td>
<td>Cyno</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Hemangiopericytoma sarcoma</td>
<td>+</td>
<td>498F</td>
<td>Cyno</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Osteogenic sarcoma</td>
<td>+</td>
<td>508F</td>
<td>Cyno</td>
<td>-</td>
</tr>
</tbody>
</table>

* Indicates metastases to distant organs at time of autopsy.
* Identification of monkeys corresponding to a previous paper of Sieber et al. (5).
* Rh, rhesus; Cyno, cynomolgus.
* Tumors 1 and 2 are from the same animal. Tumor 1 was from a liver biopsy taken 5 months prior to sacrifice of monkey, whereas tumor 2 was from liver tumor taken at autopsy.
* No mutations found at codon 249.

Results and Discussion

Nine tumors derived from eight nonhuman primates treated with aflatoxin B1 were analyzed for mutations in the p53 gene, particularly at the third position of codon 249 (7, 8). PCR products for exon 7 were amplified from DNAs extracted from paraffin sections using primers P3 and P4 and were digested with the restriction enzyme HaeIII. Wild-type p53 alleles are cleaved by HaeIII at codon 249, giving three products of 74, 66, and 41 base pairs. A mutation at the second or third position of codon 249 would result in loss of the HaeIII cleavage site and yield two products of 140 and 41 base pairs. In contrast to human HCCs from Qidong and southern Africa (3, 7, 8), all nine nonhuman primate tumor samples were cleaved by HaeIII at codon 249 of the p53 gene (Fig. 1). To test for mutations at different positions within the p53 gene, exons 5, 7, and 8, which include the highly conserved domains II to V that contain four mutation hot spots (13–15), were amplified from the nine tumors. At least six individual clones for each PCR product were sequenced. A sequence polymorphism was observed at the third position of codon 186, a substitution of T to C, resulting in no amino acid change (Fig. 2). Analysis of the polymorphism revealed no allele loss in eight informative tumors. Only one HCC showed a point mutation at the second position of codon 175, a substitution of G to T, resulting in an amino acid change from arginine to leucine (Fig. 2). The mutation was confirmed by sequence analysis of subclones obtained from two individual amplifications. Although the substitution of G to T at codon 175 might be caused by the preferential binding of aflatoxin B1 to G residues (20), the frequency of mutations in the p53 gene in the tumors was very low (Table 1). These data suggest that, unlike the proposed involvement in human HCCs (3, 7, 8), aflatoxin B1 infrequently induces mutations in the p53 gene in nonhuman primates. Consequently mutations in the p53 gene are not necessary in aflatoxin B1-induced carcinogenesis in nonhuman primates. As to the suggestion that aflatoxin B1 is responsible for the G to T mutation at codon 249 of the p53 gene in human HCCs from Qidong and southern Africa (7, 8), we would like to suggest two possible explanations. One is that aflatoxin B1 may indeed cause the mutation at codon 249 in the p53 gene in nonhuman primates. This does, however, not occur in nonhuman primates because of species differences possibly in gene structure (21) metabolism (22, 23), and in the absence of HBV-induced chronic active hepatitis. Since G to T mutation in codon 249 has only been identified in HBV-related human...
HCC, it is possible that both aflatoxin B₁ exposure and HBV-induced chronic active hepatitis are necessary for induction of the mutation (3, 7). The other explanation is that environmental carcinogens other than and coincident with aflatoxin B₁ are responsible for the mutation at codon 249 in the p53 gene in human HCCs.

Acknowledgments

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References

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