Advances in Brief

Aflatoxin B1-induced Rat Hepatic Hyperplastic Nodules Do Not Exhibit a Site-specific Mutation within the p53 Gene

Janis E. Hulla, Zhi Ying Chen, and David L. Eaton

Molecular Biology Section, Pacific Northwest Laboratory, Richland, Washington 99352 [J. E. H.], and Department of Environmental Health, School of Public Health and Community Medicine, University of Washington, Seattle, Washington 98195 [Z. Y. C., D. L. E.]

Abstract

The weight of accumulated evidence suggests a role for the p53 tumor suppressor gene in the development of human hepatocellular carcinoma (HCC). Most striking is an apparent mutational specificity at codon 249 of the human gene. Aflatoxin B1 (AFB1) is a liver-specific carcinogen which causes G to T substitutions. This transversion was detected at codon 249 in about 50% of the analyzed HCC tumors from African and Asian patients. In these geographic regions aflatoxin exposure and hepatitis B viral infection are risk factors for HCC. In contrast to the human data, no mutations at codon 249 were detected in AFB-induced tumors from nonhuman primates. We have analyzed the p53 gene at the site corresponding to codon 249 of the human gene in AFB-induced preneoplastic hepatic nodules from rats. No mutations were detected in the tissues examined. Our data suggest that, at least in the rat, AFB exposure alone may not be sufficient for the specificity of p53 mutations observed in HCC.

Introduction

The p53 tumor suppressor gene is the gene most often found altered in a wide variety of human cancers (1–4). There is much to suggest a role for the p53 gene in the development of HCC. However, the tumorigenic mechanism of HCC remains undefined. Aflatoxins and chronic hepatitis B viral infection are risk factors for HCC in many parts of the world including southern Africa and eastern Asia. In these regions, AFB is a food contaminant. Recent studies suggest a role for AFB in deactivation of the p53 tumor suppressor gene during development of at least some subset HCC. In one study, tumors from patients in Qidong, China, were analyzed for mutation in the p53 gene (5). Eight of 16 HCC had point mutations at the third base position of codon 249. Seven of these mutations were G to T substitutions; the other was a G to C transversion. Other investigators detected codon 249 mutations in 21 of 36 HCC examined (6). These tissues were also from Qidong patients. In a third study of primary HCC, this time from patients residing in southern Africa, four of five mutations detected were G to T transversions which clustered at codon 249 (7, 8). Together the results suggest a possible mechanism that includes codon 249-specific adduction of a reactive metabolite of AFB. However, of four HCC, two cholangiocarcinomas, a spindle cell carcinoma, and two sarcomas induced by AFB in nonhuman primates, none of the tumors showed mutations at codon 249 (9). These investigators suggest their results may indicate involvement of an environmental carcinogen other than aflatoxin B1, or that the hepatitis B virus is a prerequisite for AFB induction of codon 249 mutations.

Materials and Methods

Primers were selected to amplify exon 6 of the rat p53 gene which contains the region of the rat p53 gene corresponding to codon 249 of the human gene. The sequence of the upstream coding primer that was used is GCCCTTGACT-TATTCCTTCCCTTAG. The sequence of the downstream anticoding primer is CCAAACCTGGCACACAGCTTCCTAC. These sequences are contained within the introns flanking exon 6 and are adjacent to the splice junctions. We used an Applied Biosystems, Inc. (Foster City, CA) model 380A synthesizer to make the primers. The downstream primer was synthesized to contain an amine group on the 5' end. Aminolink II (Applied Biosystems, Inc.) was used for this terminal addition. The linked oligonucleotide was subsequently biotinylated by adding a 50-fold excess of NHS-LC-Biotin II (Pierce Chemical Co., Rockford, IL) according to a previously published procedure (12). The biotinylated primer was purified by reverse-phase high performance liquid chromatography.

Template DNA for the PCR was isolated from six liver nodules and surrounding normal tissues harvested from six male Fischer 344 rats previously exposed to aflatoxin B1 (Sigma Chemical Co., St. Louis, MO). For the induction of large hepatic nodules, rats 6 weeks of age were transferred to the semipurified AIN-76A diet (Teklad, Inc., Madison, WI). After 14 days of acclimation, 10 daily doses, 150 μg/kg/dose AFB, were given i.p. in 2 consecutive weeks (days 1 to 5 and 8 to 12). The rats were submitted to a two-thirds partial hepatectomy 3 weeks after the last dose of AFB and sacrificed 3 weeks later (11). Hepatic nodules were resected and closely trimmed from surrounding normal tissues. Nodular and surrounding tissues were fixed in cold (4°C) acetone and embedded in low melting point paraffin. The paraffin was later extracted from the tissues with xylene. After graded (100, 95, 80, and 70%) ethanol rehydration, the tissues were rinsed and resuspended in 0.5 ml of sterile 20 mM Tris-HCl, pH 8.5. Individual sterilized grinders were used to reduce the tissues prior to overnight digestion at 55°C with proteinase K, 1.4 mg/ml. The DNA was extracted from the tissue digest with an Applied Biosystems model 340A nucleic acid extractor.

PCR used Taq DNA polymerase, buffer, and deoxynucleoside triphosphates from Perkin Elmer Cetus, Norwalk, CT. Final concentrations of 0.2 μM each primer, 0.2 mM each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, and 1 μg of template DNA were used. Each amplification reaction consisted of 38 cycles of 95°C for 1 min, 48°C for 1 min, and 72°C for 1 min. The biotinylated primer was incorporated into the amplicons at this step to facilitate isolation of single-stranded template.
AFB, RAT LIVER NODULES AND p53 CODON 243 MUTATION

We amplified this region using primers consisting of newly defined intron sequences surrounding exon 6 as shown on the rat p53 gene graphic. Line 4 was generated from normal rat liver DNA template and is included as a control. Lane 5 contains a negative control where water was added in place of template DNA. We used thermal cyclers from Perkin Elmer Cetus.

The amplicons generated from the hepatic DNA templates were sequenced directly. Single-stranded DNA was isolated from the PCR assays with strepavidin-coated magnetic beads (Dynal, Inc., Great Neck, NY) according to the manufacturer’s recommendations. Sequenase Version 2 (United States Biochemical, Cleveland, OH) was used for sequencing by the dideoxy method and according to the Sequenase protocol (13).

Results and Discussion

In the rat p53 gene the CGG sequence of codon 243 codes for arginine and corresponds to AGG of codon 249 in the human gene. While human codon 249 is located in exon 7 of the human gene, codon 243 is located in the sixth exon of the rat gene (10). A processed p53 pseudogene is present in the rat genome (14). We have avoided possible interference from this complementary DNA-like sequence by selecting priming sites located within introns flanking exon 6. Amplification of exon 6 of the rat p53 gene yields a single amplicon (Fig. 1). The size of the fragment is approximately 290 bases and is consistent with a size predicted from the complementary DNA. bp, base pairs.

Fig. 1. Amplification of the rat p53 gene region corresponding to the putative hot spot for aflatoxin-induced mutation. Agarose gel is stained with ethidium bromide and contains PCR products. The templates for PCR were harvested from AFB-induced rat liver nodules, surrounding tissue, and normal liver from untreated rats. The putative hot spot in humans is codon 249. This corresponds to codon 243 in the rat gene which is located in exon 6. We amplified this region using primers consisting of newly defined intron sequences flanking exon 6 as shown on the rat p53 gene graphic. Lanes 1–5, amplicons yielded from reactions using DNA isolated from AFB-induced hepatic nodules. The amplicon in Lane 4 was generated from normal rat liver DNA template and is included as a control. Lane 5 contains a negative control where water was added in place of template DNA. Lane 6 contains molecular weight markers. The apparent sizes of the amplicons are consistent with the expected size of 274 predicted from the complementary DNA. bp, base pairs.

suitable for sequencing. All amplifications included negative controls consisting of amplification cocktail and deionized water added in place of template DNA. We used thermal cyclers from Perkin Elmer Cetus.

The amplicons generated from the hepatic DNA templates were sequenced directly. Single-stranded DNA was isolated from the PCR assays with strepavidin-coated magnetic beads (Dynal, Inc., Great Neck, NY) according to the manufacturer’s recommendations. Sequenase Version 2 (United States Biochemical, Cleveland, OH) was used for sequencing by the dideoxy method and according to the Sequenase protocol (13).

Results and Discussion

In the rat p53 gene the CGG sequence of codon 243 codes for arginine and corresponds to AGG of codon 249 in the human gene. While human codon 249 is located in exon 7 of the human gene, codon 243 is located in the sixth exon of the rat gene (10). A processed p53 pseudogene is present in the rat genome (14). We have avoided possible interference from this complementary DNA-like sequence by selecting priming sites located within introns flanking exon 6. Amplification of exon 6 of the rat p53 gene yields a single amplicon (Fig. 1). The size of the fragment is approximately 290 bases and is consistent with a size predicted from the rat gene structure.

None of the sequences that we analyzed from the six hepatic nodules or surrounding normal tissues differed from normal p53 gene sequence at codon 243 (Fig. 2). Since the third position of each of the corresponding codons is occupied by guanine, this neighboring target specificity of AFB would not discriminate between the two sequences; nor is it likely that the difference at the first position of the codon is responsible for the lack of target specificity in the rat. No mutations at codon 249 were detected in the nine AFB-induced HCC from nonhuman primates even though the codon and surrounding sequence are identical to those of humans (9). Our results suggest that mutation at the site corresponding to the codon 249 hot spot associated with human hepatocellular carcinoma may not be involved during the early stages of AFB-induced rat liver tumorigenesis. Because the majority of preneoplastic lesions induced by other carcinogen treatment protocols undergo remodeling and never develop into tumors, the possibility remains that mutation at codon 243 in the rat p53 gene is a somewhat rare, but important, early determinant of progression of hyperplastic nodules to hepatocellular carcinoma (15). The development of allele-specific in situ PCR techniques to evaluate the presence of codon 243 mutations in histological sections containing many foci and nodules will be required to fully evaluate this hypothesis. With this limitation in mind, the data are consistent with alternative explanations for the codon 249 clustering of mutations in HCC. Fujimoto et al. (9) suggest that environmental factors coincident with AFB may be responsible for the mutations. Since the selective mutations have been identified only in populations at risk for hepatitis B, it is also possible that both AFB and chronic hepatitis are essential for mutation at codon 249 in the human p53 gene (9).

Fig. 2. Sequence analysis of the region of the rat p53 gene corresponding to the putative hot spot for aflatoxin-induced mutation. Left, normal sequence of the rat p53 gene. Reaction sets labeled C, A, T, and G contain dideoxycytosine, dideoxyadenine, dideoxythymine, and dideoxyguanine stop reactions, respectively. The template DNA used in Lanes 1, 3, and 5 of each set was amplified from aflatoxin-induced hepatic nodules. The templates in Lanes 2 and 4 were amplified from tissues surrounding the nodules. Arrows, codon 243 of the rat gene corresponding to the putative hot spot for aflatoxin-induced mutation of the human gene. None of the sequences shown differs from the normal p53 gene.
Acknowledgments

We gratefully acknowledge R. P. Schneider for his expert advice and review.

References

Aflatoxin B$_1$-induced Rat Hepatic Hyperplastic Nodules Do Not Exhibit a Site-specific Mutation within the $p53$ Gene

Janis E. Hulla, Zhi Ying Chen and David L. Eaton