Aflatoxins as Risk Factors for Hepatocellular Carcinoma in Humans

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Abstract

On a global basis, primary liver cancer (PLC) is a very prevalent form of cancer. Wide variation of PLC incidence in different areas of the world suggests the involvement of environmental factors in its etiology. Two major classes of risk factors have been identified. Extensive evidence indicates the importance of infection by the hepatitis B virus as a major risk factor for PLC. Because many organic chemicals induce liver cancer in experimental animals, those to which human exposure is known to occur are also of interest with respect to their possible involvement as risk factors for PLC. Particular emphasis has been placed on aflatoxins because of the frequency with which they occur as food contaminants, together with their potency as liver carcinogens for a large number of experimental animals, including subhuman primates. Other mycotoxins, notably sterigmatocystin and fumonisins, are also relatively potent carcinogens for the liver of animals, but little is known about human exposure to them. Epidemiological surveys carried out over the past 25 years in Asia and Africa have revealed a strong statistical association between aflatoxin ingestion and PLC incidence. The combined experimental and epidemiological evidence has led to designation of aflatoxins as human carcinogens according to International Agency for Cancer Research criteria. Collectively, current evidence strongly suggests that PLC is of multifactorial etiology, with probable interactions between viral and chemical agents in populations concurrently exposed to both classes of risk factors. Recently developed methods that permit individual monitoring of aflatoxin exposure, hepatitis B virus infection, and genetic damage caused by these agents are being applied in the design of molecular and biochemical epidemiological studies of the etiology of the disease. Application of this methodology may contribute to elucidation of the relative importance of interacting etiological agents in different populations.

Introduction

A large amount of evidence is available supporting the multifactorial etiology of primary liver cancer. Of the risk factors that have been identified, HBV is of major significance in populations in which infection by this virus is endemic. Prospective epidemiological studies showed a high incidence of PLC in HBV carriers in such populations, and clinical studies showed that most PLC patients are carriers of HBsAg and have chronic hepatitis. Sequences of the HBV organism have been found to be integrated into the genome of hepatocytes of many patients with PLC, but integration of the virus is not an invariant component of PLC or of chronic hepatitis. Available evidence clearly establishes HBV as a major risk factor for PLC in certain populations, such as the Taiwanese. More recently, infection by the hepatitis C virus has also been shown to be associated with elevated incidence of PLC in Japan. Environmental factors that putatively contribute to the etiology of PLC include aflatoxin contamination of the diet, ingestion of alcoholic beverages, consumption of diets low in selenium, tobacco smoking, and androgen therapy. These have been put forward as putative etiological agents largely on the basis of epidemiological evidence and in some cases with supportive laboratory evidence or data from experimental animal models.

Large numbers of naturally occurring and synthetic organic chemicals have been shown to be carcinogenic in experimental animals, and the list is continuously growing as additional compounds are subjected to bioassay. These carcinogens include representatives of many different chemical classes that induce liver cell carcinoma when administered to experimental animals, generally in lifetime feeding studies at high levels. With respect to possible involvement as risk factors for PLC in humans, stronger evidence exists for aflatoxins than for other members of this broad group of carcinogens. Others to which humans are known to be exposed under specific circumstances include sterigmatocystin and possibly additional mycotoxins; certain pyrrolizidine alkaloids; cycasin and related glycosides; carcinogenic nitrosamines and nitramides formed through the interaction of nitrite and nitrosatable substrates in the environment; heterocyclic aromatic amines formed during the cooking and processing of proteinaceous foods; and components of alcoholic beverages, such as urethan and possibly ethanol. It is impossible to assess with any certainty the importance of other known carcinogens of androgenic origin, such as the chlorinated hydrocarbons, even though some of them are widely distributed at low levels in the environment. Although many other experimental liver carcinogens are known to exist, there is no basis on current evidence to implicate them as etiological agents for PLC in humans.

Aflatoxin Carcinogenesis in Experimental Animals

The carcinogenic properties of the aflatoxins have been very extensively studied, and much information has been produced concerning various aspects of their mechanisms of action, their occurrence as contaminants of foods, and their putative importance as risk factors for PLC in humans. This information was comprehensively reviewed elsewhere (5). In the context of the present discussion, several characteristics of aflatoxin carcinogenesis are relevant to their putative role in the induction of human PLC. Carcinogenic properties of aflatoxins, in particular, AFB1, have been characterized in many experimental systems. Ingestion of naturally contaminated dietary ingredients or p.o. administration of the purified carcinogen induces PLC in many species of experimental animals, including fish (rainbow trout, sockeye salmon, and guppy), bird (duck), rodents (rats, mice, and tree shrew), a carnivore (ferret), and subhuman primates (rhesus, cynomolgus, African green, and squirrel monkeys). Although the liver is the primary target organ in most species, tumors of other organs have also been observed at differing frequencies in aflatoxin-treated animals. The effective dose of AFB1 for induction of liver tumors varied over a wide range in different animal species, when the carcinogen was administered by continuous feeding, generally for the lifetime of the animals. In fish and bird species, effective doses generally were 10 to 30 ppb in the diet. A particularly wide variation in sensitivity existed within rodent species, with rats responding at levels of 15 to 1,000 ppb, and certain strains of mice showing no response at doses up to 150,000 ppb; the tree

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2 The abbreviations used are: HBV, hepatitis B virus; PLC, primary liver cancer; HBsAg, hepatitis B surface antigen; AFB1, aflatoxin B1; AFM1, aflatoxin M1; PCR, polymerase chain reaction; TD50, 50% tumor-producing dose.
AFB1 potency in induction of liver tumors also differed widely in subhuman primate species (6). Whereas squirrel monkeys developed liver tumors when fed AFB1 at 2,000 ppb for 13 months, average total doses of 99 to 1,225 mg/animal administered p.o. over periods of 48 to 179 months were required to induce a low (7 to 20%) incidence of liver tumors in rhesus, African green, and cynomolgus monkeys. In the latter species, tumors in extrahepatic tissues (including adenocarcinomas of the pancreas, adenomas of the biliary and pancreatic ducts, hemangiocarcinoma of the liver, and osteosarcomas) occurred at a much higher frequency than liver tumors. This indicates that aflatoxin is carcinogenic to extrahepatic tissues in these primates and therefore may also be a risk factor for cancers other than PLC in humans.

Quantitative comparison of the relative carcinogenic potency of AFB1, in animal species is facilitated by calculation of statistical estimates of potency (TD50 values) by the method of Gold et al. (7). By use of data from lifetime feeding studies in rodents, representative TD50 values (expressed as μg/kg body weight/day) for susceptible and resistant species were calculated to be the following: Fischer rat, 1.3 (male) and 7.5 (female); Wistar rat, 5.8 (male) and 6.9 (female); Porton rat, 3.1 (male) and 12.5 (female); C3H mouse, >70 (male); C57BL mouse, >70 (male); and Swiss mouse, >5,300 (male). Data from subhuman primates dosed as summarized above yielded TD50 values of 156 for rhesus monkeys dosed for an average of 3.3 years and 848 for cynomolgus monkeys dosed for 14 years. These values illustrate the extremes of observed sensitivity, reflected in the responses of Fischer rats and Swiss mice. Note that the value calculated for Swiss mice is based not on an effective dose but rather on the highest feeding level at which no tumors of the liver or other tissues were observed.

Aflatoxin, Hepatitis B Virus, and Liver Cancer in Humans

In assessing AFB1 as a risk factor for liver cancer in humans, it is interesting to compare these data from experimental animals with relevant information derived from studies in humans exposed to the carcinogen through dietary contamination. As noted above, combined data from several epidemiological studies have established a strong statistical association between PLC incidence and AFB1 intake (8). These data provide a basis for calculating a hypothetical TD50 value as an estimate of the potency of AFB1 as a liver carcinogen for humans. Analysis of intake versus incidence data by application of the method (7) used in calculation of the above values for experimental animals produced a TD50 value for humans of 132 μg/kg body weight/day. This value is in the same order of magnitude as those for subhuman primates but is substantially lower than those for the most sensitive rodent species. In making this comparison, however, it is important to acknowledge the uncertainty of the TD50 values, especially that for humans, introduced by several assumptions used in making the calculation. First is the assumption that AFB1 was the only cause of PLC and that exposure to the carcinogen was continuous over a hypothetical life span of 50 years. Also of significance is the reliance on crude (i.e., non-age-adjusted) PLC incidence values, derived from cancer registry data. Perhaps most important is the fact that this estimate ignores the impact of HBV infection, established by an extensive body of evidence to be a major risk factor for PLC in humans, as emphasized earlier. In many populations in which PLC occurs at a high incidence, HBV infection and high levels of aflatoxin ingestion exist concurrently. Available evidence suggests that risk for PLC developing in these populations may be amplified through synergistic effects of aflatoxin ingestion and HBV infection. The above estimated TD50 value therefore represents an upper-bound estimate, overestimating the potency of AFB1 as a carcinogen for the liver of humans, probably by a large factor.

Unfortunately, none of the earlier epidemiological studies of aflatoxin exposure and PLC incidence included concurrent evaluation of HBV infection in the populations in which aflatoxin exposures were measured. Results of two recent studies are therefore of great interest in this regard, inasmuch as they have been interpreted as providing evidence that PLC incidence was more strongly associated with aflatoxin intake than with HBV carrier status when both risk factors were present simultaneously. In a study of aflatoxin exposure, HBV infection, and PLC incidence in Swaziland, Peers et al. (9) recorded PLC incidence for years 1979 to 1983 through a national system of cancer registry. Prevalence of HBV markers was estimated by analysis of blood donors. Aflatoxin intake was estimated by analysis of dietary samples from households and crop samples collected at representative farms. Across four broad geographic areas, a more than 5-fold variation of estimated aflatoxin intake (from 3.1 to 17.5 μg/person/day) was observed. Prevalence of HBsAg carriers was 23%, with little variation (21 to 28%) across subpopulations. PLC incidence varied over a 5-fold range and was strongly associated with estimated aflatoxin intake. Analysis of data from ten subregions within the country showed aflatoxin exposure to be a more important determinant of variation in PLC incidence than was HBV infection. A similar study of HBV status, aflatoxin exposure, and PLC incidence in southern Guangxi, China, was carried out by Yeh et al. (10) between July 1982 and June 1987. In a cohort of 7,917 men ages 25 to 64 years, a total of 149 deaths occurred, 76 of which (51%) were due to PLC. Prevalence of HBsAg presence in PLC deaths was 91% in contrast to 23% of all members of the cohort, confirming the importance of HBV as a risk factor for PLC. Within the cohort there was a 3.5-fold difference in PLC mortality by place of residence. Estimated mean aflatoxin intakes of subpopulations varied from 0.3 to 51.8 mg/person/year. When aflatoxin intakes were plotted against corresponding PLC mortality rates in subpopulations, a positive and almost perfectly linear correlation was observed. No significant correlation was observed when HBsAg carrier status was compared with corresponding PLC mortality rates.

Biomonitoring of Aflatoxin Exposures in Humans

In these as in earlier studies of similar purpose, monitoring of exposure to aflatoxins was accomplished by analysis of foods ready for consumption or of raw agricultural commodities. This approach suffers from important shortcomings in that it relies on dietary recall for the amounts and types of foods consumed or laborious sampling and analysis of foods prepared for consumption. More importantly, it provides little or no information about interindividual differences in intakes or disposition of the carcinogen after ingestion. Knowledge related to metabolism and mode of action of aflatoxins has made possible the development of procedures for molecular dosimetry of exposure of individuals within study populations. The utility of methodology for detecting and quantifying aflatoxin metabolites and DNA adducts in urine as well as serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis is illus-
Molecular Mechanisms of Aflatoxin Carcinogenesis in Rats

Much research has been carried out in attempts to elucidate molecular and cellular mechanisms through which aflatoxins induce their toxic and carcinogenic effects. Studies of the metabolism of AFB₁ revealed that the compound is activated to its electrophilic DNA-binding form through an epoxidation pathway that is identical in human cells and in all experimental systems that respond to its biological effects. Furthermore, activation and DNA binding produces identical DNA adduct profiles, with the N⁷ position of guanine representing the only site of adduct formation, in humans as well as in experimental animals (5). AFB₁ is a potent mutagen for prokaryotic and eukaryotic cells (including those of human origin) in culture and is also a powerful carcinogen for the liver of many experimental animals, including subhuman primates, as discussed previously (5). Taken together, this evidence strongly suggests that humans consuming diets contaminated with aflatoxins may be at elevated risk for their carcinogenic and other genotoxic effects, in particular for liver cancer. For these reasons, our group has had a long-standing interest in pursuing lines of investigation that may elucidate further the significance of aflatoxins as environmental carcinogens. We have recently been investigating the role of oncogene activation in aflatoxin-induced hepatocellular carcinomas in rodents, and the results of some of these investigations can be summarized as follows (12).

Induction of liver tumors was accomplished in Fischer rats by repeated dosing with AFB₁ during a 2-month period following weaning. As anticipated by results of many earlier studies, all nine AFB₁-treated animals developed overt liver tumors between 1 and 2 years after dosing. Also consistent with earlier findings, no control animals developed liver tumors or other lesions. Histological examination of the AFB₁-induced liver tumors indicated that all were carcinomas containing liver parenchymal exhibiting various degrees of trabecular cord formation, glandular character, cystic degeneration, or liver necrosis. In marked contrast, control rat livers treated with dimethyl sulfoxide alone showed normal liver morphology and hepatocellular histology.

Elucidation of the importance of oncogene activation in carcinogenesis, and in particular the critical role of base substitution mutations in activation of members of the ras gene family, encouraged us to investigate oncogene activation in these AFB₁-induced tumors. For detection of oncogenes, DNA isolated from excised tumors was transfected into NIH3T3 mouse fibroblasts and assayed for its potential to induce transformed foci or the formation of s.c. tumors after injection of transfected cells into athymic nude mice. DNA was purified from morphologically transformed foci or from s.c. nude mouse tumors and was analyzed by Southern hybridization for the presence of novel c-ras genes of rat origin. In a total of nine liver tumors analyzed, the presence of c-Ki-ras oncogenes was indicated in two and the presence of N-ras oncogenes was shown in an additional two. The detection of these two classes of genes confirmed our own previous work (13) as well as that of others.

The apparent absence of oncogenes identifiable by these assays in the remaining liver tumors prompted us to undertake a more extensive analysis of DNA sequence changes in the c-Ki-ras and N-ras genetic loci of tumor and control rat livers using PCR DNA amplification. DNA amplification procedures were used to survey genetic changes in the vicinity of the 12th codon of both c-Ki-ras and N-ras genes because previous studies in our own laboratory and that of others indicated that the 12th codon of the c-Ki-ras gene was a potential site of AFB₁-induced mutagenesis in tumor DNA. By use of primer sets delineating regions of the first exon, PCR amplification of DNA was performed with Taq polymerase. The types of c-Ki-ras and N-ras alleles detected can be summarized as follows. Sequence analyses of c-Ki-ras protooncogenes from control rat liver DNA revealed gene regions identical to the previously published DNA sequence of the rat gene. Two different Ki-ras oncogene alleles were detected in DNA derived from three of eight AFB₁-induced liver tumors. In liver tumors and tumor-derived transformants, identical oncogenes containing single nucleotide changes (G-C to T-A or A-T) in the 12th codon resulting in single amino acid substitutions at this site (glycine to cysteine or aspartate) were detected. The lack of such mutations in livers of normal and DMSO-treated rats suggests that the c-Ki-ras alleles detected in the primary tumors were oncogenes in which the nucleotide changes reflect somatic events resulting from exposure of rats to AFB₁, followed by mutation of a germline protooncogene.

Analysis of the corresponding N-ras gene regions in AFB₁-induced liver tumors and control livers revealed several important differences from the findings of c-Ki-ras alleles. First, three N-ras alleles were detected that contained different nucleotide sequences in codons 8, 13, 14, and 18. The N-rasA allele was found to be homologous to both mouse and human N-ras gene and no divergence in amino acid sequence was found for this gene region. The N-rasB allele was identical to N-rasA except for a single nucleotide difference (G-C to A-T) in codon 14 that resulted in an amino acid substitution (isoleucine for valine). The N-rasC allele, when compared with N-rasA, revealed four nucleotide differences (G-C to A-T in codon 8; G-C to A-T and C-G to T-A in codon 13; and C-G to T-A in codon 18). These differences resulted in two amino acid substitutions at codon 13 (valine for glycine) and codon 18 (valine for alanine). A second feature differentiating N-ras from c-Ki-ras is the finding that nude mouse tumors contained a rat N-ras gene identical to the N-rasC allel detected in livers of control as well as tumor-bearing rats. This indicates that the N-rasC allele is, by definition, an oncogene because of its transforming activity in the nude mouse tumorigenicity assay. Importantly, the presence of the valine-for-glycine substitution at codon 13 is consistent with published activating mutations of the N-ras gene in other
systems. The frequency distribution of N-rasA, N-rasB, and N-rasC alleles in both AFB₁-induced liver tumors and normal rat livers is strikingly similar. This unexpected finding, confirmed independently by DNA sequencing of representative clones and the plaque-screening assay, suggests that the N-rasC oncogene allele is a germline gene in the Fischer rat. However, we were unable to detect its presence by the nude mouse assays in control liver samples. In summary, the nature of the mutations that impart oncogenicity can be inferred by analysis of PCR-amplified DNA from tumor-derived NIH3T3 transformants and confirmed by analysis of primary liver tumors. Putative activating mutations in the c-K-ras genetic locus involved a single-base modification of either G-C base pair in codon 12 leading to aspartate or cysteine substitutions for glycine. The oncogenicity of the N-rasC allele, present in both normal livers and AFB₁-induced liver tumors, may be related to an amino acid substitution of valine for glycine in codon 13 in the gene product.

Our experimental evidence suggests that some chemically induced somatic mutations in biologically relevant genetic loci may be present in the emergent tumor cell in Fischer rats. It is therefore of interest to compare these molecular events in the experimental model with information concerning genetic alterations in human primary liver tumors that has become available through the application of analytical approaches similar to those used in the analysis of experimentally induced rat liver tumors. Gu et al. (14) reported that human N-ras oncogene sequences were present in the DNA from a high proportion of human liver tumors. Expression of N-ras was also markedly enhanced, as was the level of the gene product. Furthermore, c-myc was also highly expressed in most tumors, implying that the cooperating activities of the two oncogenes might be responsible for the malignant phenotypic alterations in some cases of human PLC. Identification of mutations in the N-ras oncogene associated with its activation was not reported. Activated (mutated) N-ras oncogene sequences were subsequently found in human PLC tissue (15), but they were present in only a small fraction of tumor cells, suggesting that activation of this gene may have been a manifestation of tumor heterogeneity rather than a major initiating event in formation of the tumor. However, expression of ras and myc oncogenes was found to be greatly elevated in human PLC tissue, suggesting that overexpression of the two oncoproteins may be important for the malignant phenotypic alterations in the human disease (16). Similarly, c-erbA was found to be overexpressed in human liver tumors (17), suggesting that this oncogene may also be involved in establishing or maintaining malignancy in human PLC.

Analysis of DNA from liver tumors for loss of heterozygosity by the restriction fragment length polymorphism method revealed frequent losses at chromosomes 16 and 4, changes in loci not previously observed in tumors of other tissues (18, 19). These results were interpreted as indicating that alterations of these regions may be specifically associated with development and/or progression of human PLC. These results further suggest that an antioncogene (tumor suppressor gene) may be located on chromosome 4 and that liver cancer caused by HBV or other environmental agents could be linked through genetic events responsible for the loss of a tumor suppressor locus located on chromosome 4. Additional evidence of loss of tumor suppressor gene activity in PLC was provided by observations of loss of expression of the p53 gene located on chromosome 17 and the presence of abnormal forms of the p53 gene product in the majority of cell lines derived from human PLC tumors (20). Recent reports of analysis of mutations in the p53 tumor suppressor gene are relevant in this context. In two studies involving analysis of DNA from liver tumors of patients in China (21) and South Africa (22), mutations in codon 249 of the p53 gene were found in a substantial majority of patients. Virtually all the mutations resulted from G-C to T-A transversions and were localized at a single site in the codon (AGG to AGT). The striking uniformity of the nature of the observed base changes and their similarity to the mutational spectrum induced by AFB₁ in various experimental systems support the interpretation that they may have resulted from exposure to aflatoxins. Further characterization of genetic alterations in human liver tumors and comparisons with those occurring in tumors induced in experimental animals by aflatoxin and other carcinogens may reveal mechanistic bases through which HBV, aflatoxins, and other etiological agents exert their effects. Such information will be essential for the design and implementation of strategies for reduction of incidence of this highly fatal form of cancer through intervention and preventive measures.

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References

AFLATOXIN AND PRIMARY LIVER CANCER


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