Role of metabolism and viruses in aflatoxin-induced liver cancer

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Abstract

The use of biomarkers in molecular epidemiology studies for identifying stages in the progression of development of the health effects of environmental agents has the potential for providing important information for critical regulatory, clinical and public health problems. Investigations of aflatoxins probably represent one of the most extensive data sets in the field and this work may serve as a template for future studies of other environmental agents. The aflatoxins are naturally occurring mycotoxins found on foods such as corn, peanuts, various other nuts and cottonseed and they have been demonstrated to be carcinogenic in many experimental models. As a result of nearly 30 years of study, experimental data and epidemiological studies in human populations, aflatoxin B1 was classified as carcinogenic to humans by the International Agency for Research on Cancer. The long-term goal of the research described herein is the application of biomarkers to the development of preventative interventions for use in human populations at high-risk for cancer. Several of the aflatoxin-specific biomarkers have been validated in epidemiological studies and are now being used as intermediate biomarkers in prevention studies. The development of these aflatoxin biomarkers has been based upon the knowledge of the biochemistry and toxicology of aflatoxins gleaned from both experimental and human studies. These biomarkers have subsequently been utilized in experimental models to provide data on the modulation of these markers under different situations of disease risk. This systematic approach provides encouragement for preventive interventions and should serve as a template for the development, validation and application of other chemical-specific biomarkers to cancer or other chronic diseases.

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Introduction

The use of biomarkers for identifying stages in the progression of development of the health effects of environmental agents has the potential for providing important information for critical regulatory, clinical and public health problems (Anonymous, 1987; Wogan, 1992). Since the development of a paradigm for molecular biomarkers by a committee of the National Research Council over a decade ago, some progress has been made in applying such chemical biomarkers to specific environmental situations that may be hazardous to humans, as exemplified by the study of aflatoxins. The major goals of environmental chemical-specific biomarker research are to develop and validate biomarkers that reflect specific exposures and predict disease risk in individuals. Presumably after an environmental exposure each person has a unique response to both dose and time to disease onset. These responses will be affected both by intrinsic (genetic) and by extrinsic (such as dietary) modifiers. It is assumed that biomarkers that reflect the mechanism of action of an environmental chemical will be strong predictors of an individual’s risk of disease. It is also expected that these biomarkers can more clearly classify the status of exposure of individuals, local communities and larger populations. These studies should also help to elucidate the molecular processes of chemically induced human disease and underlying susceptibility factors. A conceptual model for this work is shown in Fig. 1.
The molecular epidemiology investigations of aflatoxins probably represent one of the most extensive data sets in the field and this work may serve as a template for future studies of other environmental agents. The aflatoxins are naturally occurring mycotoxins found on foods such as corn, peanuts, various other nuts and cottonseed. They have been demonstrated to be carcinogenic in many animal species including rodents, non-human primates and fish. They were also initially suspected to contribute to human hepatocellular carcinoma (HCC) (Busby and Wogan, 1984). As a result of 40 years of study, experimental data and epidemiological studies in human populations, aflatoxin B₁ (AFB₁) was classified as carcinogenic to humans by the International Agency for Research on Cancer (Aflatoxins, 1993).

Molecular epidemiological studies of aflatoxin and human liver cancer

HCC is one of the most common cancers worldwide and there is a striking geographic variation in incidence. For example, in the People’s Republic of China (PRC), HCC accounts for over 300,000 deaths annually and this malignancy is the third leading cause of cancer mortality (National Cancer Office of the Ministry of Public Health, 1980). During the 1960s and 1970s several epidemiological studies were conducted in Asia and Africa that showed there was an association between high aflatoxin exposure, estimated by sampling foodstuffs or by dietary questionnaires, and increased incidence of HCC (reviewed in Groopman et al., 1996). These early studies could not account for additional factors such as hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, this information provided a strong motivation to further investigate the circumstantial relationship between aflatoxin ingestion and liver cancer incidence.

In general, the most rigorous test of an association between an agent and disease outcome is found in prospective epidemiological studies, in which healthy people are recruited, questionnaires and biological samples taken and the cohort followed until significant numbers of cases are obtained. A nested study within the cohort can then be designed to match cases and controls. Since the controls were recruited at the same time and with the same health status as the cases, they are better matched than in traditional case–control studies.

There have been two major cohort studies to address the relationship of aflatoxin exposure to HCC incidence reported to date. The first comprises over 18,000 people in Shanghai from whom urine and blood samples were collected (Qian et al., 1994; Ross et al., 1992). After a 7-year follow-up, cases and controls were age and residence matched to examine the association between markers of aflatoxin exposure and HBV infection and the development of HCC. The data revealed a highly significant increase in the relative risk (RR = 3.4) for those liver cancer cases where urinary aflatoxin biomarkers were detected. The relative risk for people who tested positive for hepatitis B surface antigen (HBsAg) was 7.3, but individuals with both urinary aflatoxins and positive HBsAg status had a relative risk for developing HCC of about 59. These results strongly supported a causal relationship between markers of aflatoxin exposure and HBV infection and the development of HCC. The data revealed a highly significant increase in the relative risk (RR = 3.4) for those liver cancer cases where urinary aflatoxin biomarkers were detected. The relative risk for people who tested positive for hepatitis B surface antigen (HBsAg) was 7.3, but individuals with both urinary aflatoxins and positive HBsAg status had a relative risk for developing HCC of about 59. These results strongly supported a causal relationship between two major HCC risk factors, HBV and AFB₁ exposure. Finally, when individual aflatoxin metabolites were stratified for liver cancer outcome, the presence of the aflatoxin-nucleic acid adduct (AFB-N7-gua) in urine always resulted in a two- to threefold elevation in risk of developing HCC.

Subsequent cohort studies carried out in Taiwan have also examined the relationship of HBV status, AFB₁ exposure
and incidence of HCC and have confirmed the results of the Shanghai investigation (Wang et al., 1996; Yu et al., 1997). A nested case–control study from a cohort of over 15,000 people in Taiwan found that in HBV-infected males there was an adjusted odds ratio of 2.8 for detectable compared with non-detectable aflatoxin-albumin adducts and 5.5 for high compared with low levels of aflatoxin metabolites in urine (Wang et al., 1996). A second cohort study in Taiwan observed a dose–response relationship between urinary AFM1 levels and HCC in chronic HBV carriers (Yu et al., 1997). Similar to the Shanghai data, the HCC risk associated with AFB1 exposure was more striking among the HBV carriers with detectable AFB-N7-gua in urine.

The use of biomarkers in cohort studies has clearly shown the chemical–viral interaction in the induction of HCC. However, aflatoxin exposure in the absence of chronic hepatitis B infection is also etiologically associated with liver cancer. These findings provide the compelling basis to increase efforts both in HBV immunization programs and in the development of concerted programs to lower dietary aflatoxin exposure as means of lowering human cancer risk. A model for the overall process of hepatocellular carcinoma was developed by Kensler et al. (2003) and is shown in Fig. 2.

**Aflatoxin exposure and mutations in the p53 tumor suppressor gene**

In addition to the evidence from epidemiological studies and the use of biomarkers of biologically effective dose, further support for the involvement of aflatoxin in HCC incidence in certain parts of the world has come from investigations of mutations in the p53 tumor suppressor gene. The p53 gene is found mutated in a majority of human cancers and there is a large variation in number and type of mutations between cancers of different tissues (Greenblatt et al., 1994; Hollstein et al., 1991). Such diversity lends itself to the analysis of the mutational spectrum within the gene with a view to determining information about the etiology of the tumor and potential risk assessment (Harris, 1996; Vogelstein and Kinzler, 1992). One of the most striking examples of a ‘molecular fingerprint’ in the p53 gene is a characteristic G → T transversion at the third base of codon 249 observed in liver cancer patients from regions of high aflatoxin exposure.

Initial reports of a specific mutation in the p53 gene of HCCs in populations exposed to high levels of aflatoxin came from two independent studies in southern Africa and Qidong, China, in which a G → T transversion in codon 249 of the p53 was observed in approximately 50% of HCCs (Bressac et al., 1991; Hsu et al., 1991). In contrast, no codon 249 mutations were detected in areas with low aflatoxin exposure such as Japan, Europe and the United States (Challen et al., 1992).

The implication from these studies in populations of varying exposure to aflatoxin that the G → T mutation at the third base of codon 249 is aflatoxin specific has been supported by studies in bacteria which have shown that aflatoxin exposure causes almost exclusively G → T transversions (Foster et al., 1983). It has also been shown that the aflatoxin-epoxide can bind to codon 249 of p53 in a plasmid in vitro, providing further indirect evidence for a
putative role of aflatoxin exposure in p53 mutagenesis (Puisieux et al., 1991). A recent study has mapped AFB$_1$ adduct formation to codon 249 (Denissenko et al., 1998).

While the detection of specific p53 mutations in liver tumors has provided insight into the etiology of certain liver cancers, the application of these specific mutations to the early detection of cancer offers great promise for prevention (Sidransky and Hollstein, 1996). In a seminal report by Kirk et al. (2000) reported for the first time the detection of codon 249 p53 mutations in the plasma of liver tumor patients from the Gambia; however, the mutational status of the tumors were not known. These authors also reported a small number of cirrhosis patients having this mutation and given the strong relation between cirrhosis and future development of HCC, the possibility of this mutation being an early detection marker needs to be explored.

In a paper by Jackson et al. (2001), Short Oligonucleotide Mass Analysis (SOMA) was compared with DNA sequencing in 25 HCC samples for specific p53 mutations. Mutations were detected in 10 samples by SOMA; in agreement with DNA sequencing. Analysis of another 20 plasma and tumor pairs showed 11 tumors containing the specific mutation and this change was detected in six of the paired plasma samples. Four of the plasma samples had detectable levels of the mutation; however, the tumors were negative suggesting possible multiple independent HCCs. Ten plasma samples from healthy individuals were all negative. This molecular diagnostic technique has implications for prevention trials and the early diagnosis of HCC.

In a recent investigation, Jackson et al. (2003) explored the temporality of the detection of this mutation in plasma before and after the clinical diagnosis of HCC in the same patient. This study was facilitated by the availability of longitudinally collected plasma samples from a cohort of 1638 high-risk individuals in Qidong, P.R.C., that have been followed since 1992. Sixteen liver cancer cases diagnosed between 1997 and 2001 were selected for study on the basis of having available plasma samples that spanned the years before and after HCC diagnosis. The results showed that in samples collected prior to liver cancer diagnosis, 21.7% of the plasma samples had detectable levels of the codon 249 mutation, with a 95% confidence interval of 9.7% to 41.9%. The persistence of this pre-diagnosis marker was borderline statistically significant ($P = 0.066$, two tailed). The codon 249 mutation in p53 was detected in 44.6% of all plasma samples following the diagnosis of liver cancer with 95% confidence intervals from 21.6% to 70.2%. This level of positive samples following liver cancer diagnosis compares with about 50% of all liver tumors in Qidong, suggesting a nearly 90% concordance between plasma and tumor p53 codon 249 mutation outcome. Further the persistence of this mutation for detection in plasma once it became measurable was statistically significant ($P = 0.024$, two tailed) in repetitive samples following diagnosis. Collectively these data suggest that nearly one-half of the potential patients with this marker can be detected at least 1 year and in one case 5 years prior to diagnosis.

In summary, studies of the prevalence of codon 249 mutations in HCC cases from patients in areas of high or low exposure to aflatoxin suggest that a G→T transition at the third base is associated with aflatoxin exposure and in vitro data would seem to support this hypothesis. A majority of codon 249 mutations are found in patients with an HBV infection implicating an association. However, in comparisons of codon 249 mutations in regions of high HBV infection but varying levels of AFB$_1$ exposure, the mutation only occurs in areas of high AFB$_1$ exposure. HBV evidently plays an important role in mutagenesis, perhaps by causing preferential selection of cells harboring the mutation. The use of the codon 249 mutation as a marker of exposure to aflatoxin must be done with caution until evidence has been obtained from studies measuring both AFB$_1$ adducts and mutations in the same individual.

### Biomarkers and HBV infection in liver cancer

HBV is a significant risk factor for HCC in the developing world where there are over 400 million viral carriers (Lee, 1997). The biology, mode of transmission and epidemiology of this virus continues to be actively investigated and has been recently reviewed (Lee, 1997). The HBV genome encodes its essential genes with overlapping open-reading frames; therefore, a mutation in the HBV genome can alter the expression of multiple proteins. In many cases of HCC in China and Africa a double mutation in the HBV genome, an adenine to thymine transversion at nucleotide 1762 and a guanine to adenine transition at nucleotide 1764 (1762T/1764A), has been found in tumors (Arbuthnot and Kew, 2001; Hou et al., 1999; Kensler et al., 1996). This segment of the HBV genome contains an overlapping sequence for the base core promoter and the HBV X gene; therefore, the double mutation in codon 130 and 131 of the HBV X gene reported in human HCC is identical to the 1762 and 1764 nucleotide changes (Lee, 1997).

Kuang et al. (2004) examined, with mass spectrometry, the temporality of an HBV 1762T/1764A double mutation in plasma and tumors. Initial studies found 52 of 70 (74.3%) tumors from Qidong P.R.C. contained this HBV mutation. Paired plasma samples were available for six of the tumor specimens; four tumors had the HBV 1762T/1764A mutation while three of the paired plasma samples were also positive. The potential predictive value of this biomarker was explored using stored plasma samples from a study of 120 residents of Qidong who had been monitored for aflatoxin exposure and HBV infection. After 10 years passive follow-up there were six cases of major liver disease including HCC (4 cases), hepatitis (1 case) and cirrhosis (1 case). All six cases had detectable levels of the HBV 1762T/1764A mutation up to 8 years prior to diagnosis. Finally, 15 liver cancers were selected from a prospective cohort of 1638
high-risk individuals in Qidong on the basis of available plasma samples spanning the years before and after diagnosis. The HBV 1762\textsuperscript{A}/1764\textsuperscript{G} mutation was detected in 8 of the 15 cases (53.3\%) prior to cancer. The persistence of detection of this mutation was statistically significant (\( P = 0.022, \) two tailed). We have therefore found that a pre-diagnosis biomarker of specific HBV mutations can be measured in plasma and suggest this marker for use as an intermediate endpoint in prevention and intervention trials.

**Biomarkers and liver cancer prevention**

Several approaches can be considered for the prevention of liver cancer. A first approach is vaccination against HBV. Unfortunately, many people living in high-risk areas for liver cancer acquire the HBV infection before age three. Thus, an immunization program for total population protection would have to occur over several generations, provided that mutant strains of HBV do not arise, thereby eliminating the utility of current vaccines. Despite these problems, vaccination programs for HBV have been implemented in many areas of Africa and Asia. A second approach for cancer prevention would be the elimination of aflatoxin exposures. Primary prevention of aflatoxin exposures could be accomplished through large expenditures of resources for proper crop storage and handling; however, this approach is not economically feasible in many areas of the world. Secondary prevention measures using chemopreventive agents which block the activation and enhance the detoxification of AFB\textsubscript{1} are being investigated in high-risk populations.

Cancer prevention trials that use biomarkers as intermediate endpoints provide the ability to assess the efficacy of promising chemopreventive agents in an efficient manner by reducing sample size requirements as well as the time required to conduct the studies compared to trials that have cancer incidence or mortality as endpoints (Baptista et al., 1999). These intermediate markers are particularly valuable when investigating chemopreventive agents such as oltipraz that may have an effect at early, preclinical stages of carcinogenesis. The key issue in trials that use biomarkers as the outcome of interest is to have a marker that is sensitive and directly associated with the evolution or development of neoplasia. These biomarkers are typically organ site-specific genomic, proliferation and differentiation markers that reflect different intermedial stages of the neoplastic process. As an adjunct to these process-dependent markers it may also be possible to devise markers specific to interventions in selected groups at high risk for carcinogen exposure. These agent-dependent approaches would be based upon knowledge of the etiologic agent(s) in the study population.

It has been shown that several agents can provide some level of protection against aflatoxin-induced liver cancer in experimental systems (Kensler et al., 1998a,b). One of the most promising of these agents is oltipraz (4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione) which has been shown to inhibit AFB\textsubscript{1} hepatocarcinogenesis in rats when administered 1 week prior to and throughout carcinogen exposure (Roebuck et al., 1991). This and subsequent studies have also shown that hepatic AFB-DNA adducts, urinary AFB-N\textsuperscript{7}-gua and serum albumin adducts are all reduced in animals given oltipraz during aflatoxin administration indicating the utility of these biomarkers in intervention studies (Egner et al., 1995).

A double-blind Phase IIa clinical chemoprevention trial with oltipraz was conducted in Qidong, PRC (Jacobson et al., 1997). Healthy individuals were randomized into groups receiving 125 mg of oltipraz daily, 500 mg of oltipraz weekly or placebo. Blood and urine specimens were collected biweekly over the 8-week intervention period and an 8-week follow-up period. Levels of aflatoxin-albumin adducts in serum and AFM1 and AFB-NAC excreted in the urine were examined as primary biomarker endpoints in the study. There were no consistent changes observed in levels of aflatoxin-albumin adducts in the placebo arm or the arm receiving 125 mg of oltipraz daily. However, there was a significant decline in aflatoxin-albumin levels beginning 1 month into intervention with 500 mg of oltipraz weekly which continued for 1 month after treatment was stopped (Kensler et al., 1998a,b).

Urinary levels of AFM\textsubscript{1}, the primary oxidative metabolite of AFB\textsubscript{1}, were found to be reduced by 51\% in individuals receiving weekly doses of 500 mg oltipraz as compared to the placebo controls (Jacobson et al., 1997). No significant differences were observed in the levels of AFM\textsubscript{1} in the arm receiving 125 mg of oltipraz daily. An increase in AFB-NAC in the 125-mg group confirms the ability of oltipraz to induce phase 2 enzymes to increase aflatoxin conjugation. The lack of an effect of 500 mg of oltipraz on AFB-NAC probably reflects masking due to diminished substrate formation through the inhibition of cytochrome p450 activities seen in this group (measured as a reduction in AFM\textsubscript{1} levels).

Chlorophyllin is most effective as an anticarcinogen in experimental models when given in large molar excess relative to the carcinogen at or around the time of carcinogen exposure. The efficacy of chlorophyllin as an anticarcinogen in several models; its potential simple mechanism of molecular complexation; its widespread, low-cost availability; and its lack of any known toxicities led to conduct a randomized, double-blind, placebo-controlled, chemoprevention trial in residents of Qidong, PRC. One hundred eighty healthy adults from Qidong were randomly assigned to ingest 100 mg chlorophyllin or a placebo three times a day for 4 months. The primary endpoint was modulation of levels of aflatoxin-N\textsuperscript{7}-guanine adducts in urine samples collected 3 months into the intervention measured using sequential immunoaffinity chromatography and liquid chromatography-electrospray mass spectrometry. Aflatoxin-DNA adduct excretion product serves as a biomarker of the biologically
effective dose of aflatoxin and elevated levels are associated with increased risk of liver cancer. Chlorophyllin consumption at each meal led to an overall 55% reduction in median urinary levels of this aflatoxin biomarker compared to those taking placebo (Egner et al., 2001). Prophylactic interventions with chlorophyllin or supplementation of diets with foods rich in chlorophylls may represent practical means to prevent the development of liver cancer or other environmentally induced cancers.

Overall, these initial oltipraz and chlorophyllin results highlight the use of biomarkers in chemoprevention studies to determine the efficacy of such agents.

Summary

The long-term goal of the research described herein is the application of biomarkers to the development of preventative interventions for use in human populations at high-risk for cancer. Several of the aflatoxin-specific biomarkers have been validated in epidemiological studies and are now being used as intermediate biomarkers in prevention studies. The development of these aflatoxin biomarkers has been based upon the knowledge of the biochemistry and toxicity of aflatoxins gleaned from both experimental and human studies. These biomarkers have subsequently been utilized in experimental models to provide data on the modulation of these markers under different situations of disease risk. This systematic approach provides encouragement for preventive interventions and should serve as a template for the development, validation and application of other chemical-specific biomarkers to cancer or other chronic diseases.

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