The study of human cancers has provided evidence that malignant progression is associated with genetic change. It has been suggested that some genetic alterations in tumors may be the result of direct or indirect processes related to environmental chemical exposure. This hypothesis has been supported by genetic evidence in liver tumors which has associated aflatoxin B1 exposure with the detection of inactivating DNA mutations within the human p53 tumor suppressor gene. The detection of activating ras oncogene mutations at high frequency in liver tumors of feral fish suggest that the survey of mutations in genes, such as p53 or other genes, might provide a genetic signature for specific chemical exposure in tissues of aquatic animals derived from environmentally damaged sites. — Environ Health Perspect 102(Suppl 12):75–80 (1994)

Key words: chemical carcinogenesis, oncogene, tumor suppressor gene, genetic epidemiology, ras, p53, DNA damage

Environmental Chemical Exposure and Malignant Progression

There is increasing evidence that human cancers contain stable and heritable genetic changes which may signify biologic mechanisms to alleviate normal tissue growth constraints (1). In addition, a substantial body of knowledge indicates that specific cellular proteins may be affected by such changes at the DNA level. Such changes, generally, can take the form of DNA (or gene) loss, gene conversion (or translocation), mutation (inactivating or activating), DNA amplification, or gene overexpression. Some DNA alterations have been shown to result in modified function of normal cellular gene products (2).

The progression of a normal cell to one with malignant features requires the acquisition of multiple and distinct genetic changes resulting in modified growth features that may be unique for specific tumor forms derived from particular tissue-specific progenitor cells. The rate at which such changes occur may be contingent upon the accumulation of rare genetic events that enhance the replicative capacity of the progenitor cell (3). Environmental chemicals favoring the replicative capacity of the progenitor (due to cytotoxicity) may increase the likelihood of such rare and heritable mutagenic events to occur (4). In addition, environmental chemicals that are DNA damaging, may further enhance the likelihood of achieving those genetic changes since they may favor the reduction of growth restraints intrinsic to the cell or imposed by neighboring cells in the tissue.

While the first such events may give only a modest growth advantage and minor changes in cytopathology, the probability of that this cell will steadily accumulate genetic changes increases as the proliferative capacity of the cell is enhanced. In addition, DNA repair processes may be overwhelmed or reduced due to genetic alterations leading to a further escalation of DNA damage. This could result in a further alleviation of normal growth controls. Some of these genetic events may signify successful attempts of the emergent malignent lesion to overcome tissue-specific growth or immunologic constraints. In the case of human cancer, this process is best illustrated in human colorectal cancer where genetic activation of dominant-oncogenes and loss of tumor suppressing genes are features of the tumor. In addition, accumulation of these alterations marks sequential progression to more malignant tumor forms (5).

The homeostasis of normal adult tissues relies upon the ability of tissue-specific cell progenitors to repopulate (mitosis), to give rise to cell populations with defined function (differentiation), or to reduce the cell number as part of tissue modeling and regenerative processes (apoptosis) (Figure 1). Environmental chemicals perturb cellular homeostatic controls resulting in increased mitosis and regenerative differentiation following exposure to cytotoxic agents. This is especially true of the liver, in which many chemical substances are processed for detoxification and in which chronic liver damage and necrosis may occur under conditions of long-term exposure. In addition, programmed cell death or apoptosis may occur in those tissues containing populations of cells which enter into the cell cycle in response to regenerative process following cytotoxic injury. In this case, cells may undergo active death processes when cells are unable to divide or differentiate.

Cancer Genetics and Cellular Growth Control

The mechanistic connections relating alterations of specific cellular oncoproteins and tumor-suppressive proteins with the regula-

Figure 1. Environmental chemicals and cell division. Cells enter the cell cycle from a resting state (G0) and are fated to divide (mitosis), differentiate, or die (apoptosis). Environmental chemicals may enhance the likelihood of mutation by stimulating cell division in response as part of regenerative processes following tissue injury. Genotoxic chemicals may modify the DNA directly, leading to genetic alteration following DNA synthesis (S) and mitosis. Cellular growth is regulated by the number of cells entering the cell cycle (G0 to G1) and by cellular proteins which arrest cells between G1 and S phases. Genetic alterations associated with cancer contain oncogene mutations and loss of tumor suppressor gene function which may contribute to a loss of cell growth control at the G1 to S transition.
tion of cellular growth control is just emerging. Genetic alterations in cellular protooncogenes may result in modified proteins which impart transformation to normal cells. For example, transformation of normal rat fibroblasts in tissue culture involves at least two oncproteins (6). Studies such as these have classified oncogene products according to their relative ability to transform normal cells. These studies have indicated that multiple oncogene products are required to transform primary cultures of normal cells (for example, ras and myc). In addition, transforming viruses have been shown to be specific to particular tissue types and encode specific proteins which perturb normal growth control (for example, adenovirus Ela and Elb, polyoma large T antigen, and human papillomavirus E6 and E7 proteins). Moreover, it has been shown that both viral oncoproteins and the loss of normal growth gene function (tumor suppressor genes) may play similar roles in the escalation of the cancer process. In the case of tumor suppressor genes, genetic alterations in the form mutation or deletion have been shown to result in the loss of function of the gene (7).

Oncogenes and tumor suppressor genes have been shown to encode proteins of varied function in the cell. They have included receptor tyrosine kinases (for example, EGFR, PDGFR, HER-2, met, and trkb), nonreceptor tyrosine kinases (for example, bcr-abl and src), serine-threonine kinases (for example, raf-1), GTP-binding proteins and effectors (H-, K-, and N-ras, Gs, NF-1), transcription factors (for example, c-, N-, and L-myc, fos, jun, bcl-3, WT-1, p53), cell cycle regulators (for example, p53, Rb, mdm2, and bcl-1), cell surface proteins (for example, DCC, NF-2), and death suppressors (for example, bcl-2) (Figure 2). Much effort has been directed towards cataloguing these alterations to determine whether common genetic features predominate in particular tumors. In this regard, an extensive survey of activating ras mutations (8) and loss-of-function p53 mutations (9) has been conducted using samples of human primary tumors of varied tissue origin.

Chemical Exposure, Mutation, and Carcinogenesis

As in the case of human tumors, the systemic tabulation of genetic alterations in rodent protooncogenes (ras) and tumor suppressor genes (p53) has provided some rationale to associate particular target genes and specific genetic alterations with cancer type and specific chemical etiology (10–12). However, these have been confounded by findings which implicate different target genes and genetic changes when tumor samples of different animal models yet similar pathology and chemical etiology are compared.

For example, primary liver tumors have been analyzed from rainbow trout, mice, and rats induced with a single etiologic agent, aflatoxin B1. Genetic analysis has revealed that mice contain H-ras mutations in most or all of the tumors examined (13). Trout contain K-ras mutations in a majority of the tumors (14) and rats contain K-ras or N-ras mutations in a small subset of liver tumors (15,16). Using more sensitive detection methods, K-ras mutations have been detected in the majority of samples representing early and late-stage hepatocellular adenomas and carcinomas obtained after treatment of rats with aflatoxin B1 (17). In contrast, other hepatocarcinogen-induced rat liver tumors (18) and primary hepatocellular carcinoma samples obtained from humans contain few ras mutations even in those samples derived from individuals at geographic risk for mycotoxin exposure (19,20).

In human liver tumors, the lack of ras mutations was found to be in sharp contrast to findings which have indicated that the majority of human liver tumors contain inactivating p53 mutations (9). This finding is consistent with the findings that associate p53 mutations with the vast majority of human tumors of varied tissue origin. However, the frequent detection of inactivating p53 mutations in rodent tumors of specific chemical etiology has been variable. p53 Genetic alterations have been detected at high frequency in radiation-induced mouse skin tumors (21,22) and formaldehyde-induced rat squamous cell nasal carcinomas (23) whereas at low frequency in rodent tumors of liver (24–27), colon (28), or lung (29) tissue origin.

In a general sense, these contrasting observations may be due, in part, to the complexities of the transformation process itself. That is, while the ability of a given chemical to induce DNA alterations (for example, mutation or strand-break) may be an important index of its potential cancer-causing effect, many and different target genes may be affected in a given tissue in a given species by even a single etiologic agent. This observation has been supported by studies which have examined genetic alterations associated with liver tumors of human (HBV), woodchuck (WHV), and ground squirrel (GSHV) hepatitis virus etiology (30). In these cases, c-myc or N-myc genes have been shown to be amplified or activated by viral insertion in liver tumors of GSHV or WHV etiology, respectively, while such insertion events have been absent in HBV-associated human liver disease. These findings have suggested that different mechanisms of transformation may be operative in different species in response to generalized hepatic neoproliferative disease. In rodent tumors of chemical etiology, it has been shown that different strains of mice exhibit differences in the type and frequency of ras mutations which result in response to exposure to the same chemical agents. Some of these differences may be explained, in part, on spontaneous processes which may be operative in particular in-bred strain backgrounds (11).

**Ecological Genetics and the Cancer Process**

The value of ecologic genetics relies upon the founding principle that genetic alteration in a given organism is enhanced by exposure to harmful environmental agents. It assumes that exposure to environmental agents which do not lead to genetic change are, by definition, less harmful. These principles have been espoused for many years by those who study the cancer process and for which genetic change has been shown to accompany the ability of a tumor cell to override those normal processes which limit or regulate growth control. This has been very clearly shown in strains of inbred rodents using tumor models where organ-specific chemical exposure and tissue-specific transformation events which occur subsequent to exposure can be measured. Moreover, there is a good correlation

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**Figure 2.** Genetic changes associated with human cancer. Several DNA alterations have been shown to be associated with human cancers resulting in proteins localized to the membrane and nucleus of the cell. The alterations have included single-base mutations (p53, p21, NF-1, and G0), increased transcription (myc), DNA amplification (myc, mdm2, EGFR, PDGFR, and HER-2), translocation (bcr-abl, trg-met, bcl-1, bcl-2), and gene loss (p53, Rb, WT-1, NF-2, DCC).
between those organ sites (such as the liver or skin) that are physiologic sites for chemical exposure or detoxification (in the case of the liver and kidney) and those tissue types which become malignant.

p53 Protein as a Short-term and Long-term Sensor for DNA Damage

Loss of p53 and Rb (retinoblastoma tumor suppressor protein) function may be the two most common functional changes which occur in human cancer. Unlike Rb, p53 inactivation is most often accompanied by gene mutation, overexpression of the mutant p53 form, and loss of the normal allele. In contrast, Rb function is lost by gene deletion, occasional mutation leading to low levels of Rb, or indirect mechanisms to reduce normal Rb function. Moreover, inactivating p53 mutations can occur at many sites and mutation "hot spots" may correlate to particular tumor cell types (9). Studies have shown that normal p53 protein levels are elevated in response to DNA damaging agents that arrest cells in the G1 phase of the cell cycle (31). This finding supports many studies which have indicated that activation of oncogenes and inactivation of tumor suppressor genes (such as p53) may lead to the alleviation of growth constraints which correlate with cyclin and cyclin-dependent kinase activity in the cell (Figure 3). In this regard, tumor mutations in p53 have been shown to override DNA damage-induced cell arrest. Moreover, p53 protein in combination with DNA damaging chemicals may contribute to apoptotic cell death (32).

All of these findings suggest that inactivating p53 mutations may serve to enhance genetic instability by allowing chemicals to induce mutations that would normally be repaired prior to S phase. This model is strengthened by the finding that p53 may influence the function of the GADD45 DNA repair gene (33) or the G1P1/WAF1/SDII/CAP20 gene (34) which have been found to be activated following exposure of cells to DNA-damaging agents. These and other findings are consistent with a model where p53 may act to suppress growth and may influence the regulation of processes of cellular stress or mechanisms involving DNA replication and repair (35). The role of p53 in limiting genetic instability is further strengthened by the finding that mice which are null for the p53 gene are morphologically normal but develop tumors at high frequency in tissues of varied origin (36).

Recently, it was shown that the p53 null mice supported a more rapid malignant progression of chemically initiated skin tumors with little effect on the number, size and growth rate of premalignant papillomas (37). All of these studies suggest that p53 may function as a sensor of DNA damage and inactivating mutations may limit this function and lead to increased proliferative capacity.

Aflatoxin B1 and Human Liver Cancer: A Paradigm for p53 Gene Mutation and Environmental Chemical Exposure

There are few well-characterized genotoxic chemicals which have a clear role in the development of human disease. Mycotoxins, of which aflatoxin B1 is the best studied, have been studied in rodents and implicated in the etiology of primary human hepatocellular carcinoma (38). The correlations with human cancer have been best illustrated using studies which have shown that specific p53 mutations occur in liver tumors of humans at risk for exposure to aflatoxin B1. That is, hepatocellular carcinomas derived from individuals at relatively low risk of exposure to aflatoxin B1 possess few inactivating p53 mutations (39–41). However, p53 inactivation by mutation and gene loss was found to be associated with the majority of human hepatocellular carcinoma samples derived from individuals residing in geographic regions at risk for exposure to aflatoxins (42,43). More important, a signature mutation at codon 249 resulting in G to T base transversions correlated with tumors of individuals at risk to aflatoxin B1 exposure. These findings were significant since a wide variety of single-base mutations have
been shown to inactivate the p53 protein and have been tabulated from human tumors of varied tissue origins (9). The signature mutations at codon 249 were found to be consistent with a substantial number of studies in rodents and tissue culture systems that have studied mutations following AFB1 exposure. These studies have determined aflatoxin B1 DNA adduct forms that occur exclusively with guanine residues and preferred mutations resulting in G to T base transitions (38). Recently, it has been shown that aflatoxin B1 was found to enhance the likelihood of mutation of the p53 gene by a G to T base transition at codon 249 in cultured human HepG2 cells (44). In contrast, mutations were not detected in the region of codon 249 or other exons within p53 genes derived from rat liver tumors induced by exposure to aflatoxin B1 (25,26).

Genetic Epidemiology Using Cancer Markers

Due to the substantial advances made in human cancer genetics and analysis of ras mutations in chemically-induced rodent tumor models, several studies have been performed as an attempt to correlate mutation with genotoxic risk associated with environmentally damaged sites. In these cases, mutation leading to the activation of oncogenes have been identified in tissues of diseased aquatic animals (45). Specifically, K-ras mutations have been detected in the majority of diseased livers derived from winter flounder associated with contaminated sediments of Boston Harbor (46). In addition, K-ras mutations have been detected in the majority of hepatic tumors derived from Hudson River tomcod with environmental risk to the development of hepatocellular carcinoma (47). More recently, transforming genes have been detected in gonadal tumors derived from bivalve mollusks (48).

In a broader sense, the basic principle of these studies conforms to tenets applied to genetic epidemiologic studies of any sentinel species including humans. This principle suggests that specific genetic damage as a result of local environmental chemical hazards should be lacking when genetic damage is assessed in individuals derived from relatively “hazard-free” environments. Ordinarily, cancer or its malignant progeny are not features of young or middle-aged organisms in any healthy ecosystem. Therefore, activating oncogene mutations or inactivating tumor suppressor gene mutations associated with precancerous abnormalities in young or middle-aged individuals can unequivocally be classified as harmful. Moreover, those tissues which are exposed to chemical contaminants (for example, skin or liver) or which detoxify chemicals (for example, liver or kidney) may represent primary organs of tissue and genetic change.

p53 Protein as a Potential Biomarker for Chemically Induced DNA Damage

The level of p53 protein in tissues of animals exposed to environmental chemicals may be a good indicator of DNA damage and cellular stress. This prediction follows recent and increasing evidence to indicate that p53 protein levels may become elevated in response to genotoxic or other cellular stresses associated with premalignancy in human cancer (49–51). Measurement of p53 protein levels in rodent models are few (52). Analysis of p53 protein levels following DNA damage in rodent models is lacking. Studies in rodents that would measure levels of p53 protein in varied target organs following chemical exposure would provide the basis for validating the use of p53 levels as a short-term biomarker of genotoxic stress.

For the reasons outlined previously, the primary DNA sequence of the p53 genetic locus may provide a monitor of genetic change in tissues exposed to potential DNA damaging agents. The lack of p53 mutations in tumors of inbred rodents exposed to specific hepatocarcinogens has suggested that the liver may not be the best target organ for such analyses. In addition, p53 mutations have been detected in high frequency in skin tumors resulting from radiation exposure, suggesting that skin lesions may provide a better source of genetic material for analysis. Moreover, there may be substantial differences in the frequency of detection of p53 mutations between feral animals and inbred rodent strains. This has already been illustrated in the case of activating ras mutations where a high frequency have been detected in liver tumors of feral fish (46,47) compared to the low frequency of ras mutations detected in liver tumors of rats exposed to hepatocarcinogens (18).

To perform such studies, relevant p53 genes would need to be cloned and sequenced. This would then be followed by exon-specific PCR (polymerase chain reaction DNA amplification) using high-fidelity polymerases as it has been done for the human locus. The amplified DNA would be subjected to analysis for mutations using PCR-SSCP (single-strand conformation polymorphism) (53), PCR-mismatch-cleavage analysis (54), primer-mediated-RFLP (restriction fragment length polymorphism) (55), or PCR-direct DNA sequencing methods (46). Alternatively, PCR DNA can be subjected to nested-PCR to enable cloning into phage or alternative vectors followed by plaque screening (16) or conventional DNA sequencing methods. Once a limited database of sequences has been obtained, more rapid methods can be developed to survey for particular single-base mutations. Some complicating features of this analysis include the presence of normal genes from nonmalignant tissues in the sample, especially polyploid hepatocytes, which can reduce the sensitivity of methods to detect mutant p53 forms. In addition, p53 mutations may occur late in the transformation process, suggesting that early premalignant lesions may not contain p53 mutations. However, the ability to detect mutations using PCR-based methods would be enhanced by the feature of cells containing mutant p53 forms to eliminate normal p53 alleles.

Environmental Chemicals and Genetic Toxicology: A Case for Laboratory Studies

With regard to genetic epidemiology, the conclusions that associate particular chemical biohazards to particular genetic mutations will be, at best, correlative until laboratory experiments are performed. The testing of sentinel species under controlled environmental conditions would serve to ascribe particular genetic changes resulting from specific chemical exposure. This association may either be a direct consequence (DNA adduct formation followed by mutation) or indirect consequence of DNA damage (hepatotoxicity, regeneration, and spontaneous mutation).

The complexities and diversity of genetic changes which can overcome cellular growth control for a given cell type may provide an overwhelming technical challenge as an attempt to localize ecosystem damage or ascribe limits on allowed levels of specific hazardous chemicals in a given ecosystem. This limitation is, in part, driven by the flexible capacity of normal cells to achieve growth deregulation by selecting varied species-specific subsets of chemically damaged genes which work to alleviate growth constraint. Moreover, many genes are modified at the level of transcription (as in the case of p53 and c-myc) and would not be measurable using DNA sample
analysis. In this latter case, the potential utility of generic genotoxic markers (such as elevated p53 protein levels) may prove to be more feasible since this may be a central feature of DNA damage. Moreover, immunohistochemical reagents may have reactivities between aquatic species of fish which would enable their broad application.

In the long-term, the identification of signature mutations for specific classes of environmentally damaging agents may have some utility in ascribing impact of specific biohazardous agents on the individual. The analysis of a few strategic surveys using single genetic loci (p53 or K-ras, for instance) in a few tissues (liver or skin) in a limited number of sentinel species (fish) would help to provide the first steps in this area. The surveys should comprise animals derived from ecosystems where highly divergent yet potentially hazardous agents (chemicals or radiation) can be measured (water or sediment). This study would provide a foundation for the genetic and epidemiologic principles needed to ascribe ecotoxicologic risk for particular classes of chemicals. The analysis of such a study would validate whether biomarker-genome could be used as indicators of ecosystem damage. In addition, this would provide a foundation for further studies in the laboratory to associate specific signature mutations with specific and chronic chemical biohazard exposures.

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