Role of Chemically Induced Cell Proliferation in Carcinogenesis and Its Use in Health Risk Assessment

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There is much interest in incorporating knowledge of biological mechanisms of carcinogenesis into assessments of health risks to humans posed by chemicals in the environment. Debate over the soundness of using data from animal bioassays conducted at minimally toxic doses or fractions thereof for predicting cancer risks to humans exposed to much lower doses has stimulated interest in the question of whether genotoxic or mitotic effects predominate in chemical carcinogenesis. Cell division plays a key role at each stage in the evolution of cancer, and it is well documented that increased rates of cell proliferation can escalate the risk of malignancy. This article examines the current understanding of both mechanisms by which chemicals provoke cell proliferation and the contribution of various kinetic patterns of cell proliferation to carcinogenesis.

Introduction

Malignant transformation is a complex process involving multiple genetic changes that result in uncontrolled patterns of cell growth. Dividing cells are at increased risk for both spontaneous genetic damage as well as that induced by genotoxic agents. Thus, chemical or physical agents that cause damage to DNA and/or increase the rate of cell division also increase the probability of the occurrence of important genetic changes leading to cancer.

The circumstances in which genotoxic or mitogenic activities predominate in the cancer process has recently stimulated debate concerning the soundness of using data from high-dose animal bioassays for the purpose of predicting cancer risk to humans. In the absence of mechanistic information, the EPA and other regulatory agencies currently follow the conservative assumption that cancer incidence decreases (or increases) linearly with dose throughout the entire range of possible exposures (1). There are, however, an increasing number of examples of nonlinear dose-response relationships in which the slope of the dose-response curve was found to change dramatically at higher doses (2). Changes in a number of factors including pharmacokinetics, metabolism, and toxic effects have been associated with such dose-related changes in cancer potency. Chemically induced cell proliferation is one such factor that is of particular interest with regard to determination of cancer potency relationships for chemicals that have genotoxic effects, as well as so-called nongenotoxic chemicals (those not directly reactive with DNA) for which a direct cause and effect relationship with genetic change is lacking.

There is conflicting evidence as to whether chemically induced cell proliferation per se can increase the risk of neoplastic transformation. One view contends that an increased rate of cell division is primarily responsible for the carcinogenic effects of many nongenotoxic chemicals. Ames and Gold (3), for example, have suggested that many chemicals observed to cause tumors in rodents act indirectly by stimulating cell proliferation that, in turn, increases both the likelihood that endogenous DNA damage will produce mutations and the number of cells at risk for progressive changes in gene expression that lead to malignancy. On the other hand, it has been pointed out that hematopoietic tissues and the small intestine, both of which have high rates of cell proliferation, have a low incidence of tumors (4). This apparent conflict indicates the fragmentary state of knowledge concerning how different patterns of cell growth and death in various tissues influence the carcinogenic process.

This paper discusses the importance of chemically...
induced cell proliferation in assessing the carcinogenicity of certain chemicals. In examining this broad subject, my objective was not to provide a comprehensive review but rather to highlight areas of uncertainty and indicate where generalizations are not supported by adequate data. A brief review of the basic role of cell proliferation in carcinogenesis is given. I then describe bioassay results for several chemicals in which chemically induced cell proliferation has been clearly associated with a nonlinear relationship between dose and tumor formation. In addition, some examples of molecular mechanisms by which chemicals can produce mitotoxic responses will be discussed. Finally, I will consider what information about cell proliferation would be necessary to enable its prudent use in cancer potency estimates. Where necessary, topics that require further investigation either in the scientific literature or through experimental studies have been indicated.

Role of Cell Proliferation in Carcinogenesis

Chemical carcinogenesis has been empirically divided into sequential steps or stages in which cells progress from normal to premalignant foci to localized tumors and to invasive, malignant growths (5). There is little disagreement that cell proliferation plays a key role in each stage of tumor formation. The first step in the sequence of events that transforms a normal cell to malignancy has been termed initiation. Initiated cells have been permanently altered so as to have the potential to express the malignant phenotype. Additional steps (stages) in tumor formation have been termed "promotion" and "progression." Promotion involves reversible changes in cell populations within a tissue that place initiated cells at increased risk for further genetic changes. Progression occurs when changes in the cell's genetic program produce irreversible characteristics of the malignant phenotype.

More than one genetic change may be required to complete each stage in the carcinogenic process. In human colon carcinogenesis, specific genetic alterations have been related to different stages in the evolution of malignancy (6). Such genetic changes could not occur and would not be expressed in the absence of DNA replication and cell division. It seems likely that a variety of treatments that accelerate the carcinogenic process do so through their ability to increase cell division. Treatments such as partial hepatectomy that induce cell proliferation greatly enhance the appearance of preneoplastic foci and hepatocellular carcinomas in the livers of rats treated with carcinogenic chemicals (7).

The etiology and mechanisms of genetic damage found in malignant cells is unknown. There are several ways in which cell proliferation can in principle aid the diverse processes that increase the amount of genetic damage in cells. Replication of damaged DNA increases the probability that chemically damaged bases in DNA will result in mutations or other genetic fla. Errors in the replication of undamaged DNA can produce various kinds of genetic alterations. Thus chemicals that do not directly damage DNA but are able to induce cell division may also induce genetic changes, some of which may result in mutations important to cancer. In human somatic cells, point mutations, deletions, and insertions occur spontaneously (8,9). Such random errors are thought to account for the incidence of spontaneously initiated cells in the livers and other organs of control animals. It has been suggested that hyperplasia per se can increase the probability of genetic change by increasing the likelihood of mutations arising from normal DNA replication processes or the conversion of DNA lesions produced by oxygen radicals formed endogenously to mutations (3,10). Various estimates have been made of the amounts of spontaneous DNA damage suffered by rats and humans (11,12), but to date, there have been no estimates of the frequency of mutations caused by such DNA damage. Thus, the significance of DNA damage resulting from endogenous reactants such as oxygen radicals remains speculative.

For many nongenotoxic or weakly genotoxic chemicals, the ability to induce cell proliferation is believed to be a critical factor for the development of tumors in experimental animals (13,14). Some evidence suggests, however, that chemicals that accelerate the promotion and progression stages of carcinogenesis do more than simply increase the rate of cell proliferation. Various phorbol esters, for example, reportedly block differentiation of keratinocytes (15) and can indirectly produce physical damage to cellular DNA (16,17). In the rat liver, phenobarbital prevents the death of cells in altered foci of hepatocytes (18). Thus, although the ability to induce cell proliferation appears to be necessary for tumor development, it may not be sufficient. There are, in fact, several reports (discussed below) of animal experiments in which increased cell proliferation inhibited or had no observable effect on tumor formation.

Despite these exceptions, the ability of chemicals to increase the rate of cell proliferation in tissues has emerged as an important factor that can influence estimates of carcinogenic potencies. Furthermore, accumulating epidemiological evidence points to increased cell division as an important factor in the pathogenesis of many human cancers (19). We can infer from this evidence that consideration of genotoxic effects alone will not provide an accurate assessment of cancer risk to humans from chemical exposure. At present, however, results of rodent bioassay experiments provide the best evidence against the existence of simple linear dose-response relationships for chemical carcinogens. The following section briefly discusses several experiments in rodents in which proliferation of cells in target tissues has dramatically affected the cancer dose-response relationship.
Nonlinear Dose–Response Relationships for Carcinogens

Several lines of investigation provide persuasive evidence that chemically induced cell proliferation can determine the shape of the dose–response curve for chemical carcinogens. Because more than one independent mutation is believed to be required for most cancers, genotoxic effects alone could produce a nonlinear dose–response relationship. In several cases, however, such nonlinear relationships for genotoxic chemicals lead to the inference that effects other than direct damage to DNA can govern cancer potency. The inference has been substantiated in several cases by determining both the concentration of promutagenic DNA adducts and the extent of cell proliferation in the target organ as a function of dose. Investigators of nongenotoxic chemicals found several dose–response relationships in rodents that display apparent no-effect levels. These agents apparently act through diverse means to stimulate cell proliferation. In some cases cytotoxicity has been implicated in the tumorigenic abilities of these chemicals, and their effect on target tissues is obvious. The actions of other chemicals may be more subtle and have been attributed to their ability to subvert specific receptors that regulate cell growth. Such is apparently the case for the tumor-promoting phorbol esters that activate protein kinase C.

A limited number of dose-response relationships for carcinogens have been investigated using doses much lower than those customarily used in rodent bioassays. Both linear and nonlinear relationships between dose and rate of tumor formation have been observed for different chemicals. In some studies, these distinct types of relationships have been observed simultaneously in different tissues of the same species. Figure 1 shows that different dose relationships were observed for liver and bladder tumors in female BALB/c mice fed 2-acetylaminofluorene (AAF) in the diet for 24 months (20,21). The incidence of liver cancer showed a linear relationship with dietary concentration of AAF, whereas a sharp increase occurred in the slope of the dose-response curve for bladder tumors at approximately 60 ppm. AAF is a potent genotoxin, which would be expected to be the primary basis for its tumorigenic effects. But, as discussed below, in some circumstances the likelihood of bladder cancer from AAF appears to be primarily determined by its cytotoxic effects.

Experimental bladder carcinogenesis has provided several examples for which hyperplasia was associated with nonlinear dose-response relationships. Chronic exposures to the genotoxic chemicals 2-acetylaminofluorene (AAF) and N-[4-(5-nitro-2-furyl)-thiazoyl]formamide (FANFT) have been shown to produce bladder cancer in mice (22,23). In the case of AAF, a sharp increase in bladder tumors (see Fig. 1) was observed in animals receiving 60 ppm AAF in the diet. However, the concentration of AAF adducts in bladder DNA was found to be a linear function of dose over a range of 5-150 ppm AAF in the diet (24). The amount of promutagenic DNA damage was related linearly with dose, but tumor formation was not. With both AAF and FANFT, the sharp increase in the dose response for tumor formation occurred at doses that resulted in toxic and proliferative effects on the bladder epithelium. Littlefield et al. (22) reported that although a dietary level of 30 ppm AAF did not produce hyperplasia in the bladder epithelium, levels of 60 ppm did. Thus, the observed bladder-tumor response was more

![Figure 1. Relationship between dose of 2-acetylaminofluorene fed to female BALB/c mice and prevalence of liver and bladder carcinomas after 24 months (20,21).](image-url)
clearly correlated with the ability of AAF to cause tissue hyperplasia than with the risk of genetic damage. As a consequence, dietary levels below those that cause hyperplasia in the bladder epithelium would be much less likely to cause bladder tumors than would be predicted by simple extrapolation of the dose response at levels of AAF >60 ppm (25).

In contrast to the bladder's response to AAF, as shown in Figure 1, AAF-induced liver tumors in mice demonstrated a linear relationship with dietary concentrations between 30 and 150 ppm (21). A number of explanations have been proposed for divergence in tumor responses to AAF between the liver and bladder. These include pharmacokinetic and metabolic differences as well as the number of genetic alterations necessary for the malignant transformation of the different cell types. Biochemical evidence and mathematical models, however, have indicated that tissue dissimilarity in toxic and proliferative responses to AAF provide the best explanation for AAF's distinct dose-response relationships in the liver and bladder. Based on the absence of liver enlargement, Cohen and Ellwein (26) have suggested that 30-150 ppm AAF in the diet did not increase the rate of liver cell proliferation above background. On this evidence, they have inferred that AAF's only effect on the liver was an increase in the number of initiated cells. In the absence of cytotoxic effects, additional genetic alterations needed for the malignant transformation of initiated cells depended on normal rates of proliferation, which, although low in the liver, were effective because a large number of such cells produced by AAF were at risk. In contrast, similar doses produced few initiated cells in the bladder where endogenous rates of cell division were too low to increase the probability of tumor formation.

AAF does, in fact, produce a nonlinear tumor response in the liver at doses greater than used in the study described above. At higher dietary concentrations, AAF produces overtly cytotoxic effects in the liver, resulting in compensatory proliferation and a sharp increase in the rate of tumor formation (27). Perhaps other chemicals would show the same pattern of tumor response if tested over such a large range of doses.

Peto et al. (28) examined the incidence of liver cancer in rats exposed to drinking water containing 0.4-40 ppm diethylnitrosamine (DEN) and found a nonlinear response. A sharp increase in the cancer potency of DEN for liver tumors occurred at approximately 4 ppm DEN. The increased cancer potency of DEN concentrations greater than 4 ppm was not due to enhanced genotoxic effects. Investigations by Boucheron et al. (29) found that continuous exposure to DEN resulted in the concentration-dependent accumulation of promutagenic adducts in DNA over a similar range of drinking water concentrations. Further investigations by Deal et al. (30) found that exposure to drinking water containing 4 ppm or greater DEN resulted in cytotoxic effects and increased cell proliferation in the target tissue. At 4 ppm DEN there was a 3-fold increase in hepatocyte replication after 10 weeks; 100 ppm DEN led to a 15-fold increase. They suggested that the increased rate of hepatocyte proliferation was a major factor relating the nonlinear tumor response to the linear accumulation of DNA adducts.

These experimental findings indicate that genotoxic carcinogens are likely to display their most potent effects in the range of doses for which both genotoxic and proliferative effects coincide. Outside this range tumor response will be diminished. At high doses, lethal effects can result in the destruction of initiated cells and a reduction in the predicted incidence of tumors. Low doses that do not increase normal rates of cell proliferation will not provide greater opportunity for genetic changes required to advance initiated cells through the later stages of neoplasia. In this latter case, disturbances in the control of cell proliferation may have a crucial role in tumor development.

Nonlinear dose–response relationships have also been observed for carcinogenic substances that do not directly damage DNA. Unleaded gasoline produced kidney tumors in male rats at doses that cause epithelial cell death and regenerative proliferation in the proximal tubule—doses that did not cause cell injury did not cause tumors (31). Bladder tumorigenesis in rats by sodium saccharin requires doses sufficiently high to form urinary calculi that result in focal hyperplasia (32). Dose-related effects have also been documented for tumor promoters on mouse skin. Verma and Boutwell (33) reported that repeated application of less than 1.0 nmole of 12-O-tetradecanoylphorbol-13-acetate (TPA) did not elicit tumors on mice previously initiated with 7,12-dimethylbenz[a]anthracene (DMBA). The available evidence has been interpreted by some to support the inference that practical thresholds exist for carcinogenesis by nongenotoxic chemicals, but this proposition is not universally accepted.

Sharp changes are likely to exist in the slope of the dose–response relationships for many chemical carcinogens. If such is the case, there is considerable uncertainty in the use of methods that employ linear extrapolations to predict health effects well beyond the range of experimental data. At present, only descriptive animal studies can answer the question of how the carcinogenic potency of a chemical may change as a function of dose. This approach is, however, an impractical one, since the expense of rodent bioassays that use large numbers of animals and a wide range of doses is prohibitive. A more rational strategy is to acquire an understanding of the basic mechanisms that can alter dose–response relationships. It should then be possible to determine how and under what circumstances chemicals that affect particular biochemical pathways involved in growth regulation influence tumor response. Such knowledge also opens the door for the development of practical biochemical tests to identify chemicals that have such activities.
Mechanisms of Chemically Induced Cell Proliferation

Progressive changes in the regulation of cell growth are fundamental to the evolution of cancer. Up till now, molecular studies of the cancer process have focused primarily on irreversible genetic changes to key genes that regulate growth and development. It has become increasingly clear, however, that abnormalities in tissue growth and development induced by chemical injury may augment the rate of genetic damage and the development of malignancies. Unfortunately, although there are many descriptive studies of the toxic effects of chemicals on certain tissues such as the liver, there is only sketchy information at the molecular level concerning the mechanisms by which cell growth and development are affected by acute or chronic cell injury.

Figure 2 shows a paradigm indicating various mechanisms by which chemicals can increase the rate of cell proliferation in tissues. The means by which chemicals affect growth regulatory pathways can be divided into two general categories: those that act indirectly as a consequence of cytotoxic and/or inflammatory effects and those that act directly by interaction with cellular receptors that produce biochemical changes triggering growth.

The generality of the schemes presented in Figure 2 has not been fully explored. It is unlikely that a single pathway is responsible for a chemical’s proliferative effects. Evidence in vitro and in vivo indicates that cell growth and behavior are determined by combinations of interacting stimuli. Cytotoxic damage and tissue responses vary with dose and frequency of exposure.

The molecular basis for proliferative responses to acute or chronic cell injury are also likely to vary with dose. For example, inflammatory mediators are likely to be incriminated in the regenerative proliferation resulting from necrotic doses of \( \text{CCl}_4 \), that produce an intense inflammatory response, whereas local production of cell-specific factors may be primarily responsible for cell division at much lower doses not resulting in inflammation. How some chemicals produce their proliferative effects is discussed below.

Proliferative Responses to Cytotoxic Damage

Rodent liver has received the most attention with respect to the identity of tissue-specific factors involved in proliferative response to injury. In the adult liver, hepatocytes rarely divide except in response to injury or xenobiotics. The identity of polypeptide factors that mediate liver regeneration is an active area of research. Several groups of investigators have identified related polypeptide growth factors that may be involved in regenerative growth of the liver after partial hepatectomy or after injury from toxic chemicals. This factor, which has been referred to as hepatocyte growth factor (34), hepatopoietin A (35), or hepatotropin (36) promotes DNA synthesis and cell division in primary cultures of rat hepatocytes.

The concentration of hepatocyte growth factor (HGF) has been found to increase dramatically in the liver after physical injury and hepatitis caused by infectious agents or chemicals. HGF has been identified in the plasma of patients with fulminant hepatic failure (37). It has also been found to be markedly increased in the livers of rats that suffered necrotic injury resulting from treatment with \( \text{CCl}_4 \) (38). After \( \text{CCl}_4 \) injury, there is a rapid rise in the level of HGF transcript in nonparenchymal cells (principally Kupffer cells), but not in hepatocytes (39). Further evidence of the role of HGF in liver regeneration has been provided by Higuchi et al. (40), who recently identified a receptor on the surface of rat hepatocytes that binds HGF with high affinity. Rapid disappearance (down-regulation) of HGF receptors from the cell membrane as a result of internalization of HGF-receptor complexes occurred in response to partial hepatectomy or \( \text{CCl}_4 \)-induced hepatitis. This suggests that Kupffer cells are crucial to the liver’s ability to replace cells that are damaged or lost.

The discovery of HGF stimulated speculation about the existence of a variety of tissue-specific growth factors. Further investigations, however, have evidenced a more general role for HGF in proliferative responses in extrahepatic tissues. Gherardi and Stoker (41) have reported that the receptor for HGF is the product of the protooncogene \( \text{c-met} \). Studies on the expression of \( \text{c-met} \) have identified its transcript in a number of human tissues including kidney, thyroid, liver, and stomach (42). High concentrations of HGF have been

![Figure 2](image-url)
also been observed in extrahepatic tissues (43). These findings indicated a role of HGF and its receptor in the regulation of growth and regeneration of the liver and possibly several other tissues. HGF, however, is not the only growth regulator for the liver or other organs. A variety of evidence indicates that tissue homeostasis is maintained by the collaborative effects of polypeptide hormones.

Neither does growth regulation hinge exclusively on growth factors. Growth inhibitors play an important role, especially in vivo where cells rarely divide at maximum rates. These findings suggest several ways in which chemicals can affect the rate of cell proliferation: (a) activation of positive factors such as epidermal growth factor (EGF) or HGF or (b) inactivation of negative growth factors such as transforming growth factor-β (TGF-β). It can be inferred that the proliferative response to cytotoxic injury is likely determined not by a single growth factor but by the combined effects of positive and negative regulators. This inference is supported by the presence of both types of regulators in various tissues and their effects on cells in vitro.

Combinations of polypeptide growth factors have been observed to either stimulate or inhibit the proliferation of hepatocytes in culture (44). EGF and TGF-α have been demonstrated to be potent mitogens for hepatocytes (45,46). TGF-β reportedly inhibits the growth of hepatocytes and other cell types both in vivo and in vitro (47). Combinations of growth factors are also implicated in the regenerative growth of the skin after injury by toxic chemicals. Akhurst et al. (48) have reported evidence of localized production of TGF-β in stimulated mouse epidermis. Keratinocytes also both produce and respond to a number of growth factors including the cytokines interleukin 6 (IL-6) and interleukin 1(IL-1) as well as transforming growth factor α(TGF-α). High levels of both IL-6 and TGF-α have been found in activation of epidermal cell growth in psoriatic skin (49,50).

The repeated observation of increased expression of several polypeptide hormones in injured tissues suggests that most may have a broad range of target tissues. Cytokines released from immune cells are likely to be important mediators of tissue response to acute or chronic injury of many tissues. Increased levels of IL-6 have been found in a variety of tissues in response to infection or injury (51). Likewise because of their wide tissue distribution, TGF-β may be significant in the coordination of growth responses in many tissues. Unfortunately, there is a general absence of information about the mechanisms by which cellular injury induces the synthesis or triggers the release of TGF-β or other polypeptide factors that mediate inflammatory and hyperplastic responses.

Continued diversification of the list of polypeptide growth factors seems likely. A recent review by Cross and Dexter (32) discusses the influence of stimulatory and inhibitory factors in the growth and development of various tissues. This broadening spectrum makes understanding the molecular bases of chemically induced cell proliferation difficult. Although the number of these factors that stimulate cells in vitro is increasing, there may be only a limited number that make varied contributions to regenerative cell growth in vivo. Given adequate information about these key factors, we should be able to predict cellular responses in vivo.

### Proliferative Response in the Absence of Cytotoxic Damage

Not all chemicals that increase the rate of cell division in tissues do so through responses to cell injury. Chemicals can also subvert the biochemical circuitry that regulates cell growth. They can do so by activation of cellular molecules that provoke biochemical changes within the cell. In some cases, this can be accomplished by low levels of a chemical with a high affinity for its cellular target. In others, relatively large doses are required, perhaps to displace an endogenous ligand that has greater affinity. Regardless, prolonged exposure to such chemicals is frequently required, presumably to override the cell’s fail-safe mechanism that prevents accidental triggering of its mitotic program. Consequently, evidence suggests that chemicals that are rapidly metabolized and eliminated are generally ineffective mitogens.

Pharmacologic concentrations of chemicals that are members of a diverse group that includes hypolipidemic drugs, such as clofibrate, stimulate peroxisome proliferation and hyperplasia in the liver. Evidence suggests that cellular responses to these chemicals are mediated through their interaction with an intracellular receptor structurally related to those of the steroid hormone family (53). Interestingly, the proliferative response to some of these chemicals is transient, while others produce a sustained elevation of cell proliferation. Carcinogenic potency shows a positive association with the ability of several peroxisome proliferators to produce a sustained proliferative response (54). However, for other chemicals in this group, including clofibrate acid and nafenopin, a sustained proliferative response did not appear to be a factor in hepatocarcinogenesis (55), suggesting the involvement of other factors. If receptor activation is the key to proliferative responses to peroxisome proliferators, further studies at the molecular level will provide the opportunity to determine how sustained or transient patterns of liver cell hyperplasia are determined for these chemicals and their role in the malignant process.

Phenobarbital (PB) is another example of a nontoxic chemical that stimulates liver cell proliferation and is an effective promoter of initiated cells. As is the case with some of the peroxisome proliferators, PB produces a transient increase in mitosis in liver cells. However, persistent effects of PB have been reported on mitotic rates of preneoplastic cells. Analysis of PB’s effects on proliferation of normal hepatocytes and
those in preneoplastic foci revealed an increased mitotic rate in these foci (56). The results of further experiments suggested that PB's ability to decrease the rate of cell death (apoptosis) was primarily responsible for increased cell proliferation in foci (18,57). Thus, PB's effects on the rates of birth and death of normal and preneoplastic liver cells result in the preferential growth and accumulation of cells that are at greater risk for tumorigenic transformation. There is genetic evidence that PB interacts with an intracellular receptor, but it has been difficult to obtain biochemical evidence to confirm the receptor's existence.

Interaction with intracellular molecules underlies the ability of several chemicals to stimulate cell division and fuel the cancer process. Activation of the estrogen receptor seems to mediate the liver's mitotic response to estrogenic compounds. Tumor promoters such as 17 α-ethynylestradiol interact with intracellular receptors resulting in responses similar to those triggered by endogenous hormones (58,59). The basis of dioxin's ability to induce epidermal hyperplasia, among other effects, is its interaction with the Ah receptor (60). Similarly, TPA and related phorbol esters are effective tumor promoters bind to and activate protein kinase C (PKC), resulting in the stimulation of signal transduction pathways (61).

How can we develop a better understanding of the ways in which chemicals stimulate cell division? The paradigm that both toxic and nontoxic doses of chemicals cause cell proliferation through diverse receptor-mediated processes suggests a strategy for developing an understanding of both the qualitative and quantitative aspects of this response. For cytotoxic chemicals, focus should be on identification of growth factors that mediate tissue reactions such as hyperplasia. Understanding the regulation of their synthesis, release, and degradation, as well as the regulation of their receptors, is key to predicting cellular response. This knowledge could provide a means of identifying the types of chemical damage to cells that are capable of causing proliferative responses. For example, the identification and isolation of the gene for HGF, discussed above, opens the way to investigate the types of cellular injury that provoke increased levels of HGF and/or its receptor on target cells.

One means by which cells recognize and respond to injury is through the synthesis of a small group of proteins that have been called heat shock proteins (HSP). The synthesis of members of this family of proteins is activated under conditions of stress including that induced by elevated temperature or damage to DNA or proteins (62). There are a number of reasons to suspect a link between the induction of HSP and stress-induced cell proliferation. The evidence for this connection has recently been reviewed by Pechan (63). An intriguing finding is that one of the two prominent 70 kD HSPs is induced just before S phase when quiescent serum-starved cells are fed again (64). The HSP-70 protein has also been implicated in cellular responses to peroxisome proliferators (65). In relation to cytotoxic chemicals, it has been reported that the antitumor drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), which carbamoylates proteins and nucleic acids, selectively induced the synthesis of the human heat shock and stress-induced genes HSP-90 and HSP-70 (66).

The biochemical activities of HSPs are well suited for diverse roles in the assembly and function of cellular molecules. The ability of various HSPs to recognize denatured or abnormally folded proteins is believed to be essential for induction of both stress responses and a number of essential roles of HSP proteins in cellular processes that occur during normal growth and development (67). Although a number of specific roles for HSPs in cell proliferation have been proposed, none is certain as yet. It is certain, however, that continued investigation of the HSPs will uncover more diverse roles for these proteins in normal and injured cells. Even without knowing the precise mechanisms, any understanding that we gain will contribute to our ability to evaluate the proliferative potential of unknown chemicals, given the types of cytotoxic damage they can inflict.

Patterns of Chemically Induced Cell Proliferation Determine Carcinogenic Response

Increased rates of cell division do not always enhance tumor formation. One should remember that while hematopoietic cells have a high rate of division, cancers of these cells are relatively rare in humans. This may be because the majority of cell divisions do not take place in hematopoietic stem cells, but in cells with relatively brief life spans and are eventually lost to the host. In experimental chemical carcinogenesis, there are a number of examples where cell proliferation ultimately either suppressed tumor development or had no effect. The following discussion reviews several such studies that point to the need for a better understanding of the dynamic interactions between cell populations and their influences on carcinogenesis.

Numerous descriptive studies have documented different kinetic patterns of cell growth and death in tissues exposed to toxic substances. The term "hyperplasia" has been used to describe a wide range of regenerative responses to toxic and physical agents as well as reversible changes that result directly from mitogenic substances. Hyperplasia can result in an increase in both the cell number and size of the tissue or, if the tissue has suffered loss of cells, restoration of the normal cell number and tissue architecture. Both types of response produced by chemical and physical agents can enhance the carcinogenic process—to do so, however, a sustained increase in cell number is necessary. The dynamics of hyperplasia depend on the biological activities of the chemical as well as the fre-
frequency of exposure and dose. In some circumstances, withdrawal of mitotic agents results in elimination of cells and return of the tissue to its normal size. Elimination of excess cells takes place by a process of controlled cell death termed "apoptosis". The process of apoptosis can have an adverse influence on tumor formation.

In the liver, various treatments that induce regenerative hyperplasia are effective in enhancing the neoplastic process, whereas others that induce a temporary increase in cell number are not. Partial hepatectomy or treatments with hepatotoxic chemicals that induce strong proliferative responses result in accelerated growth and clonal expansion of initiated cells, placing them at increased risk of further genetic change. In contrast, as the results of several experiments described below indicate, treatments that produce repeated patterns of cell growth and death are typically ineffective in promoting tumor formation.

Columbano et al. (68–70) found that intermittent administration of any of the three liver mitogens ethylene dibromide (EDB), lead nitrate, and nafenopin did not promote DEN-initiated hepatocytes. They observed no increase in the number of preneoplastic foci in animals that had received four consecutive treatments of these chemicals in which four daily intragastric doses were given every 20 days (70). The same chemicals were also found to be ineffective during the initiation stage of the carcinogenic process when given just before a small dose of a genotoxic chemical (69). In contrast, intermittent treatment with CCl₄ or partial hepatectomy, which induced comparable levels of DNA replication and mitosis, produced numerous preneoplastic foci in the livers of DEN-treated rats (69).

In explaining their negative findings for the three mitogens, Columbano et al. (69,70) suggested that any initiated cells formed during the mitotic response were eliminated when the chemical was withdrawn. Instead of a sustained increase in cell number, the chemical treatments resulted in cycles of cell growth and death in the liver. An increase in liver size (increased liver/body weight ratio) occurred during and shortly after administration of the four daily intragastric doses. In the interim periods between these treatments, liver size returned to normal through apoptosis (68,71). Thus, initiated or preneoplastic cells formed during the liver's mitotic response were likely eliminated. In contrast, initiated cells produced during tissue regeneration in response to acute or chronic injury (CCl₄, partial hepatectomy) were not eliminated because these initiated cells replaced lost cells. In this case, initiated cells were at risk for further genetic changes that can result in cancerous growth.

Xu et al. (72), in a series of experiments with PB, have reported results similar to those found by Columbano et al. (68–70). If administered intermittently, PB was unable to increase the number of preneoplastic foci in livers of animals previously treated with DEN. Continuous administration of PB, however, resulted in a large increase in the number of preneoplastic foci. These findings suggest that the ability of a chemical to function as a promoter in liver carcinogenesis is highly dependent on the dosing regimen used and the kinetic pattern of cell proliferation.

Different patterns of hyperplasia also have distinct effects on tumor formation in mouse skin and the rat forestomach. According to Argyris (73), the ability of a treatment to elicit a sustained regenerative hyperplasia is decisive in determining its effectiveness as a tumor promoter. Efficient tumor-promoting substances such as TPA produce a persistent hyperplastic response with continued treatment. In contrast, acetic acid and mezerine, which are ineffective promoters, initially produce an epidermal hyperplasia that is comparable to that seen with TPA, but repeated treatment results in a diminution of response and reduced mitotic activity. These results indicate that proliferative response alone may give a false indication of a chemical's potential to act as a tumor promoter in mouse skin.

The contrasting effects of the two phorbol esters TPA and retinoic acid (RPA) on mouse skin tumorigenesis provide another example of the complex relationship between cell proliferation and carcinogenesis in mouse skin (74). Both TPA and RPA induce hyperplasia, but RPA is ineffective in promoting the early stages of malignant transformation in NMRI and CD-1 strains of mice. In Sencar mice, however, RPA was found to be an effective tumor promoter of skin tumors (75). Thus, there are strain-specific differences in response to these compounds, the basis of which is not understood.

In the rat forestomach, the ability of chemicals to cause irritation and resultant hyperplasia of the epithelium has been clearly associated with the tissue's potential tumorigenic response. A number of chemicals without demonstrable genotoxic activity, including butylated hydroxyanisole (BHA), propionic acid, sodium saccharin, diallyl phthalate, and possibly ethyl acrylate have been shown to induce cancers in the forestomach of the rat. Experimental studies with BHA have shown a correlation between levels in the diet that induce inflammation and hyperplasia of the forestomach epithelium and those that cause tumors (76). At high levels in the diet (2%), BHA induced both papillomas and carcinomas of the forestomach. Lower levels (0.5%) were noncarcinogenic by themselves, but were found to induce hyperplasia and promote forestomach carcinomas initiated with N,N'-nitrosoguanidine (NMNG) (77).

Other data, however, raise questions concerning the mechanism(s) by which BHA and other chemicals act as promoters in the rat forestomach. Wada et al. (78) have reported preliminary evidence suggesting that all chemicals that produce forestomach hyperplasia are not capable of promoting neoplasia. They reported that p-methoxyphenol (PMP) administered in the diet induced a strong hyperplastic response in the rat.
forestomach, but failed to promote NMNG-initiated tumors. The authors suggested that PMP may adversely affect the carcinogenic process through its cytotoxic effects, induce the “wrong type” of hyperplasia, or that PMP metabolites may be anticarcinogens (78).

Another interpretation of the results of Wada et al. (78) is suggested by the observations of Rodriguez et al. (79), who found diverse responses of the rat forestomach to various phenols and acids. It was observed that while BHA affected the prefundic area of the forestomach, PMP had its principle effect on the cells in the midregion. Thus, PMP's proliferative effects may not be directed at the DEN-initiated cells in the prefundic area that were most at risk for neoplastic transformation. The independence of genotoxic and cytotoxic effects has been evidenced with other chemical carcinogens.

Proliferative and genotoxic effects in different cell populations in the same tissue have emerged as an important factor determining tumorigenic responses to 4-(N-methyl-N-nitrosamino)-1(2-pyridyl)-1-butane (NNK) in rats. NNK is a major carcinogen found in tobacco products and has produced a high incidence of tumors in the nasal cavity, liver, and lungs of rats (80). In both the liver and nasal cavity, marked increases in cell proliferation due to cytotoxic effects of NNK dramatically affect tumor formation (81). In the nasal cavity, susceptibility to the genotoxic and cytotoxic effects of NNK varies considerably between cells located in the respiratory and olfactory mucosa. Low doses of NNK do not produce toxic effects in the nasal cavity preferentially damage DNA in cells located in the respiratory mucosa. Thus, at low doses one would predict these cells to be at greatest risk of cancer. At high doses the amount of genotoxic damage produced by NNK was similar in both the olfactory and respiratory mucosa, but the olfactory region suffered the greatest cytotoxic effect. Proliferative changes with cellular transformation and progression to neoplasia were most prevalent in the olfactory region coincident with cytotoxic damage. Therefore, in the nasal cavity, toxic effects that produced a marked increased in cell proliferation dramatically affected tumor incidence in cells at similar risk for genetic damage. Belinsky et al. (80) speculated that the steep dose-response curve for induction of tumors by NNK as well as the localization of tumors can be explained by the difference in sensitivity to its cytotoxicity. At low doses the greatest relative risk of cancer was shifted from cells that were most sensitive to NNK's cytotoxic effects to those most sensitive to its genotoxic effects. Such a relationship would not be predicted if only the genotoxic effect of NNK on the respiratory and olfactory mucosa was considered.

In the rat lung also, the cell type at highest risk for malignancy from NNK exposure is apparently not that at greatest risk for genetic damage. The highest level of DNA adducts in the lungs of NNK-treated rats occurred in the nonciliated bronchiolar epithelial (Claracell) cells (82). The efficiency of DNA alkylation in Clara cells by low doses of NNK was 20- to 30-fold greater than that in type II cells, which are located in the alveoli. This would seem to make the Clara cell a sensitive target for neoplastic transformation. Histologic evidence indicates, however, that benign and malignant tumors develop from type II cells that undergo hyperplasia in response to NNK. Interestingly, both hyperplasia and neoplasia in lung tissue occurred in the absence of any apparent cytotoxic effects (83), so routine histologic studies would not have detected any toxic effects in the target cells that would signal concern. The biological basis for the proliferative response of type II cells is unknown. The authors speculated that growth signals for type II cells may originate from the Clara cells that suffer the greatest damage but are unable to proliferate.

There is at least one reported case in which increased cell division does not appear to account for enhanced tumor formation. Administration of butylated hydroxytoluene (BHT) promotes 3-methylcholanthrene-initiated lung tumors in A/J strain mice (84). It was originally assumed that BHT's ability to cause repeated rounds of cell proliferation in lung alveolar cell was responsible for its promoting effect. Witschi (85), however, provided evidence to the contrary. He demonstrated that BHT's proliferative effect, but not its ability to enhance lung tumorigenesis, was dependent on its metabolism by mixed-function oxidases. Inhibitors of these enzymes or other treatments that prevented BHT's metabolism reportedly eliminated its proliferative effects on lung tissue, but had no effect on its ability to promote previously initiated cells in the lung (85). The exact basis of BHT's promoting effect is unknown. As in the case of PB, it is possible that BHT affects apoptosis of initiated lung cells or has other effects on specific cell types that were not apparent in Witschi's experiments.

The studies just described provide several examples of the complex relationships between genotoxic and cytotoxic effects in different cell types within the same tissues. These findings lend some support to the views by Ames and Gold (3,10) that mitogenesis can dominate chemical carcinogenesis at high doses. At the same time, however, they caution against making mechanistic assumptions solely on the basis of cellular dynamics. For the moment, there is little evidence that mitogenesis can be exclusively responsible for malignancy. Tumors most likely arise from the collaboration of genotoxic and non-genotoxic effects. Histopathological analyses of rodent bioassays have not uncovered any general relationships between cytotoxicity, resultant cell proliferation, and carcinogenic effects (86). These findings are limited by the absence of a direct measure of cell division and the restriction of histopathological data to the end of the bioassay studies. Nonetheless, most investigators have not been able to conclude that increased cell proliferation invari-
ably leads to increased cancer risk. It seems likely that although additional histopathological studies will continue to uncover such relationships, they will not be sufficient to justify mechanistic assumptions that can be pragmatically used for assessing human health risks.

**Risk Assessment**

Assessment of cancer risk to humans from chemical exposure should include consideration of the multiple biological factors that influence the carcinogenic process. In various circumstances, either a chemical's genotoxic effects or its ability to cause cell proliferation may limit the rate of neoplastic development. In order to integrate these concepts and formulate more biologically based health risk assessments, more than descriptive studies are required. We need to develop a better understanding of the molecular mechanisms of proliferative responses and their consequences for carcinogenesis. Without such information it is doubtful that a coherent understanding of the carcinogenic process will emerge.

What data will be needed to formulate models that better reflect the collaboration between genetic damage and cell proliferation in chemical carcinogenesis? First of all, knowledge of the mechanisms governing the regulation of growth factors, their receptors, and how growth factor-receptor interactions determine proliferative responses to cytotoxicity will be essential. Findings from *in vitro* studies point the way to important parameters that determine the intensity of proliferative responses. These include the duration of interaction between a growth factor and its receptor, and the number of such interactions that occur on the cell's surface—the latter depending both on the affinity of the growth factor for the receptor and the factor's concentration in the cell's environment (87). *In vivo*, these limiting conditions may be determined by the amount of cell damage and/or the intensity of inflammatory response. The potential of chemically induced cellular damage to orchestrate these responses is not well understood. The fact that proliferative responses are receptor-mediated suggests that dose-response relationships for proliferative responses are different than those for cytotoxicity. This raises the prospect that the cytotoxic effects of many chemicals are irrelevant for tumor formation at low doses—a prospect for which NNK provides a good example.

Second, we need a better understanding of how the dynamics of cell proliferation and death affect the carcinogenic process *in vivo*. The process of apoptosis remains mysterious, with only fragmentary information on how chemicals influence the normal rate of cell death in various tissues. Nonetheless, there are reasons to believe that apoptosis has a major role in the evolution of preneoplastic foci in rodent liver. For the moment, most rapid progress can be made studying proliferative responses because many clues about their mechanisms are already available. We should not, however, neglect these other complexities that are likely to provide explanations for more provocative results.

The ultimate goal of research should be to establish biologically based models for tumor development at the molecular level. This goal is a distant one because it requires much information not currently available. A reasonable intermediate goal is to develop cellular models that incorporate a better understanding of the circumstances under which various patterns of cell proliferation can influence tumor formation. Biologically based mathematical models of the cancer process have been developed that incorporate both genotoxic effects and cell proliferation. The Moolgavkar-Venzon-Knudson (MVK) two-stage growth model of cancer (and various modifications of it) has been able to describe nonlinear tumorigenic responses observed in rodent bioassays for several chemicals (88–91). The cell kinetic parameters required for these models are not easily obtained. Current models require information on the rates of growth and death of normal and premalignant cells, and the influence of chemicals on these rates. It may prove difficult, however, to identify the cell populations most relevant to the tumorigenic process. In the liver various chemical carcinogens produce several types of histologically distinct foci. There is a general consensus that some of these foci are preneoplastic, although it is not precisely clear which ones. Consequently, there is confusion as to what growth parameters should be used in mathematical models of liver carcinogenesis because various foci display different growth rates. The puzzle of which cells are most at risk for progression to malignancy is not unique to the liver. At present, we can only infer which phenotypically altered cells will be the first to complete the protracted sequence of events that lead to cancer.

There are several important ways that these mathematical models can help us understand various aspects of the cancer process. Refinement of current mathematical models of carcinogenesis that incorporate chemically induced cell proliferation will point to key areas for further molecular investigations. In addition, pursuit of the molecular basis of a cell's proliferative response to chemicals will contribute to a better understanding of the bases of species sensitivity and tissue specificity in chemical carcinogenesis. Cohen and Ellwein (26), in their recent article on the consequences of chemically induced cell proliferation in cancer, summarized their views on the role of mathematical models as follows:

It should be obvious that the real contribution of modeling is enhanced insight, not numbers. In terms of human risk assessment, the existence of a non-effect threshold, for example, cannot be ruled in or out on the basis of model analyses. Only experimental mechanism studies can provide the information base necessary to predict biological response discontinuities between high- and low-dose response.

This is a good perspective to keep in mind. More sophisticated mathematical models are unlikely to provide certain evidence on which to base regulatory deci-
sions. They can, however, be used to distinguish the plausible from the implausible and in that way provide valuable guidance for regulatory decisions and setting research priorities.

Experimental results for several chemicals provide strong support for regulatory decisions based on evidence of their ability to stimulate cell proliferation. For example, kidney tumors induced by chemicals that cause α-2µ-globulin nephropathy, bladder tumors induced by melamine and saccharin, and thyroid-stimulating hormone-mediated thyroid tumors. Although there are currently a small number of such cases, they illustrate the use of such information to craft a more rational basis for regulatory decisions. In the absence of sound data, however, it is reasonable to continue to use conservative assumptions in estimating health risks.

Summary and Recommendations

I have briefly discussed the role of cell proliferation in various stages of the cancer process and some of the evidence that chemicals that cause a sustained increase in the rate of cell division in tissues can increase the risk of cancer. Subjects touched on in this review have received more authoritative treatments elsewhere. The objective of this review has been to point out the complexity of the proliferative responses in various tissues exposed to chemicals and that uncertainties exist as to their exact relation to tumor formation.

The risk of neoplastic disease can be magnified by increases in genotoxicity or the rate of cell division. While there is general agreement among scientists that both cell proliferation and genetic damage are key factors that affect cancer risk, there is disagreement over their relative importance in assessing the risk to human health from exposure to various chemicals shown to cause cancer in rodents. This is particularly true for non-DNA-reactive chemicals as to whether they exhibit a no-effect level if they act exclusively through their cytotoxic and/or proliferative effects.

Renewed appreciation that chemically induced cell proliferation can contribute substantially to the risk of malignant transformation presents an opportunity for a more complete and accurate understanding of the carcinogenic process and the health risks to humans from chemical exposures. Clearly, a chemical's potential to produce cytotoxic effects and induce sustained cell proliferation are factors that can significantly affect the shape of the dose-response curve for malignant transformation. The potential consequence of increased cell division in tissues exposed to high doses of chemical carcinogens is an increased estimate of cancer potency that would seem inappropriate for assessing cancer risk from low-level exposures where increased cell proliferation does not occur.

The absence of cell proliferation at low doses, however, should not be equated with the absence of health risk. Chemicals with genotoxic effects may still cause genetic damage even at low doses and non-genotoxic chemicals can affect cell populations in subtle ways such as by preventing apoptosis. Furthermore, because of the complexities of human chemical exposure, it is unrealistic to presume that the consequences of genetic damage from low doses of a chemical are irrelevant because they are insufficient to produce other effects.

It may be difficult, however, to resolve when and how different patterns of chemically induced cell proliferation influence tumor formation. Few dose-response relationships for both cell proliferation and tumorigenesis have been examined for chemical carcinogens at doses much lower than those customarily used in rodent bioassays. Thus, most views concerning the contribution of chemically induced cell proliferation to tumor formation are based on results of a few studies for which contradictory evidence exists. In the absence of knowledge at the molecular level of how chemicals influence cell growth and how distinct growth patterns affect various stages of the cancer process, it will be difficult to accurately assess the relative contributions of genotoxic and proliferative effects on the cancer process and predict how they will vary with dose.

Although descriptive rodent bioassays could conceivably solve this problem, the expense of such studies is prohibitive. Furthermore, descriptive studies would not resolve the uncertainties inherent in interspecies extrapolation, a major reason for uncertainty when human risk assessments are based on rodent bioassay data. As we gain a better understanding of many fundamentals of the cancer process, we can envision key pieces of the puzzle that will enable us to better assess cancer risk to humans posed by chemicals in the environment. Future research that concentrates on understanding the basic molecular mechanisms by which tissues control their size and shape and how they respond and adapt to chemical injury will provide this essential information.

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