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Epigenetic Effects of Liver Tumor Promoters and Implications for Health Effects

by Gary M. Williams*

During chemical carcinogenesis in the liver, a population of abnormal cells in lesions referred to as altered foci precedes the appearance of neoplasms. Most altered foci do not develop further, but a small fraction progress to formation of neoplasms. Liver tumor promoters increase the fraction that progress.

The mechanisms for this action of promoters may involve an effect on the cell membrane. Cells *in vivo* and *in vitro* exchange molecules through specialized membrane organelles known as gap junctions. Intercellular transfer of growth and/or differentiation regulating factors could be involved in suppressing the growth of initiated cells in the altered foci. Several liver tumor promoters have been found to inhibit intercellular communication in an *in vitro* liver culture system. This effect on the cell membrane could, thus, be the basis for the release of cells in foci for further growth into neoplasms. Such an epigenetic action would account for the requirement for high doses and prolonged exposure for certain liver tumor promoters. In addition, it implies a distinct type of health risk analysis for chemicals of this type.

Several chemicals, particularly halogenated hydrocarbons, produce primarily or exclusively an increase in liver tumors in rodent strains that are characterized by a substantial background incidence of such tumors. These chemicals have not been demonstrated to have the DNA damaging capability of genotoxic carcinogens and several enhance the hepatocarcinogenicity of previously administered liver carcinogens. Moreover, they exert an inhibition of intercellular communication. Thus, carcinogens of this type may be epigenetic carcinogens functioning as liver tumor promoters. Accordingly, the health risk analysis for these chemicals is different from that for genotoxic carcinogens.

Introduction

A tumor promoter is an agent that facilitates formation of neoplasms from altered cells that otherwise would remain dormant. Although tumor promoters are usually identified by their enhancement of the tumor yield resulting from a previously administered carcinogen, called an "initiating" agent in the terminology adopted for two-stage carcinogenesis (*1*), they can also be conceived to promote tumor formation by cells altered through effects other than experimentally induced initiation, such as cells with an inherited genetic abnormality or a spontaneous mutation.

Two general schemes shown in Figure 1 have been postulated for the process of promotion: (1) promoters could complete the conversion of partially transformed cells to fully neoplastic cells (scheme A) which would then be capable of progressive growth into tumors; (2) promoters could act on dormant neoplastic cells to enable them to proliferate into overt neoplasms (scheme B) increasing the incidence of tumors over that which would occur in the absence of promotion.

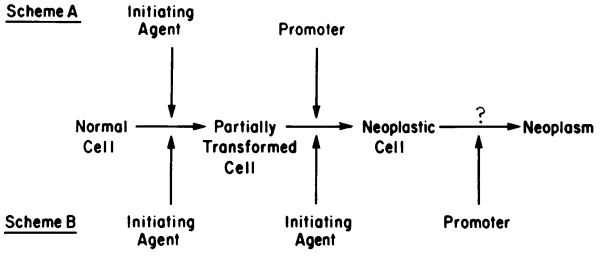

FIGURE 1. Schemes for the action of tumor promoters.

The first hypothesis that promoters complete or

*American Health Foundation, Naylor Dana Institute for Disease Prevention, Valhalla, NY 10595.

somehow render permanent neoplastic conversion has been supported by reports of their effects on cell differentiation (2-4) and genetic organization (5, 6). Such effects, however, thus far have been demonstrated only for skin tumor promoters, phorbol esters and teleocidin B (7). These agents function at very low doses and have almost hormonelike characteristics. In fact, Weinstein et al. (8) have suggested that this class of promoters may act by usurping the action of endogenous hormone or growth regulatory substances. In a somewhat different version of the hypothesis of conversion of initiated cells to neoplastic cells by promoters, Boutwell and associates (9) have proposed that skin tumor promoters induce the neoplastic phenotype in all epidermal cells and that this phenotype cannot be reversed in the defective initiated cells.

Some liver tumor promoters induce enzyme activities, reflecting a modulation of gene expression, but they do not produce the effects on differentiation and chromosomes reported for phorbol esters. Thus, no substantial evidence exists for any action at the level of genetic organization that might lead to completion of neoplastic conversion by agents that function as tumor promoters for this organ.

The second possible means by which promoters may operate, i.e., by assisting dormant neoplastic cells to form tumors corresponds to the original concept of Berenblum (1). According to this hypothesis, a tumor promoter enables the growth into tumors of carcinogen-altered cells that otherwise would remain latent. This effect could be achieved through a variety of mechanisms resulting either in selective stimulation of proliferation in dormant neoplastic cells or their release from controlling elements. Recently, through the work of Trosko and associates (10-12) and Murray and Fitzgerald (13), a cellular effect of promoters which could produce a liberation of initiated cells from tissue regulatory controls has been identified.

Inhibition of Intercellular Communication as a Mechanism of Tumor Promotion

For some years, interest has focused on the effects of promoters on cell membranes (14-17), and a variety of hypothesis have been advanced to account for tumor promotion on the basis of such effects. Yotti et al. (10) and Murray and Fitzgerald (13) discovered the ability of tumor promoters to inhibit cell membrane-mediated intercellular communication in fibroblast cell cultures. Williams and colleagues subsequently documented this action in liver cell cultures for the liver tumor promoters, phenobarbital (18) and DDT (19). Inhibition of cell-cell communication represents an action that could account for the characteristics of tumor promotion in the liver.

Intercellular communication involving transmission of molecules between cells occurs through membrane organelles known as gap junctions (20-22). This process can be measured in cell culture as the phenomenon of metabolic cooperation (cross feeding) in which a lethal metabolite generated from a precursor by one cell type is transferred to a mutant that cannot produce it; as a result, the mutant, which otherwise would be resistant to the effects of addition of the precursor, is passively killed. For example, hepatocytes possess the purine salvage pathway enzyme, hypoxanthine-guanine phosphoribosyl transferase, which is required to metabolize the synthetic purine analog 6-thioguanine (TG) to a toxic metabolite. Consequently, TG is lethal to hepatocytes. In contrast, a strain of 6-thioguanine resistant TG^r mutant adult rat liver cells selected by its resistance to TG lacks HGPRT and is not affected by TG (23) unless the metabolite is transferred to it through metabolic cooperation. In a mixed culture system, the lethal phosphoribosylated metabolite of TG can be transferred from freshly isolated hepatocytes to the TG^r mutant cells thereby killing them. In this system, the degree of metabolic cooperation is evidenced by the reduction in colony formation of TG^r cells cocultivated with hepatocytes in primary culture. As Trosko and associates have described in their cell system (12), inhibition of metabolic cooperation by promoters can then be measured by restoration of colony formation of the mutants. The use of isolated hepatocytes in the present system is advantageous because they presumably retain elements of in vivo cell characteristics which might be essential for a response to promoters and, of practical value, they are incapable of colony formation in primary culture and thus do not interfere with the determination of colony formation by the target TG^r cells.

The inhibition of metabolic cooperaton between hepatocytes and adult rat liver epithelial- TG^r cells by the liver tumor promoter DDT is shown in Table 1. A similar effect is displayed by phenobarbital (18) and heptachlor and chlordane (Table 2). The latter two organochlorine pesticides have not yet been demonstrated to be promoters, but have characteristics which suggest that they would act as such (24).

Inhibition of cell-cell communication *in vitro*, although not perfectly correlated with promoting activity (25), is the only property that has been demonstrated for a variety of tumor promoters (10-12, 18, 19, 25, 26).

The inhibition of intercellular communication between cultured liver cells by promoters could correspond to an in vivo effect that would account for liver tumor promotion. Cell proliferation in all tissues is a precisely controlled process in which the production of new cells balances those lost through differentiation or death. The regulation of this process is incompletely understood, but could involve cell to cell transfer of factors that regulate growth, including growth factors, chalones and other substances that induce differentiation and thereby, loss of proliferative capability. Several lines of evidence indicate that differentiation/growth regulation is exerted on neoplastic cells. Most significantly, an interval of up to one year can be allowed between initiation and the beginning of promotion with essentially the same incidence of tumors occurring as when promotion immediately follows initiation (*27*). It seems certain that during the lengthy interval between initiation and promotion, neoplastic cells persist in the tissue along with normal cells and are kept under control by the regulatory factors that operate on normal cells.

In rodent liver during carcinogenesis by chemicals, an abnormal population of cells in lesions referred to altered foci appears early in the process, preceding the development of neoplasms. These foci (Fig. 2) can be identified by a variety of phenotypic abnormalities (*28*), including a reduced ability accumulate cellular iron (Fig. 3), and thereby can be readily studied quantitatively (*29*). Under some conditions, in the absence of promotion less than one in a hundred of these altered foci will progress to tumor formation (*30*); thus these foci appear to be under strong regulatory control. Other examples of the control of neoplastic cells are the induction of differentiation in cells receiving a transplanted nucleus from a neoplastic cell (*31*), in neoplastic cells associated with normal embryonic cells *in utero* (*32*, *33*) and in neoplastic cells exposed to embryo extracts (*34*, *35*). If the control of dormant neoplastic cells can be mediated by intercellular communication, as seems likely, then inhibition of communica-

Table 1. Inhibition of metabolic cooperation between hepatocytes and an ARL TG^r strain by a liver tumor promoter DDT.[a]

Condition	TG^r colonies per flask[b]	
	No HPC	0.75×10^6 HPC
ARL 14-TG^r	126 ± 7	-
+ TG	110 ± 3	63 ± 10
+ TG + DDT 10^{-7}	103 ± 9	86 ± 4
+ TG + DDT 10^{-6}	101 ± 13	112 ± 10
+ TG + DDT 10^{-5}	105 ± 11	117 ± 6

[a]From Williams et al. (19).

[b]500 TG^r cells were cocultured with 0.75×10^6 HPC. Values are the average of three flasks. Results are means ± SD for triplicate determinations.

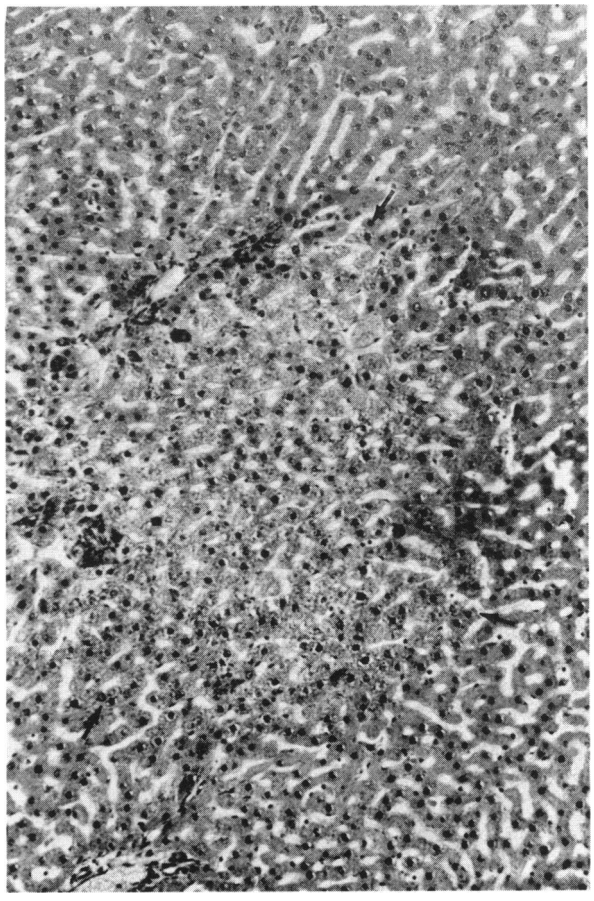

FIGURE 2. Altered focus (arrows) in rat liver induced by *N*-2-fluorenylacetamide. Hematoxylin and eosin, × 125.

Table 2. Inhibition of metabolic cooperation between hepatocytes and an ARL TG^r strain by Chlordane and Heptachlor.

Condition	ARL 14 – TG^r colonies per flask[a]	
	No hepatocytes	1.25×10^6 Hepatocytes
TG	186[a]	87
TG + 5×10^{-6} Chlordane	181	155
TG	146	55
TG + 1×10^{-6} Heptachlor	156	105

[a]Results are means of triplicate determinations.

tion could release the neoplastic cells for the progressive growth that results in the formation of neoplasms. In this manner, the inhibition of intercellular communication could be one basis for tumor promotion.

The liver-altered foci induced by carcinogens are stimulated for further development by liver tumor promoters (*30*, *36*, *37*). The enchancement of development of these foci, as shown in Figure 4 for phenobarbital (*30*), may result from the inhibition by tumor promoters of transmission of regulatory factors from normal cells to the altered cells.

The inhibition of intercellular communication by promoters can conceivably be produced in several ways. Because of the lipophilicity of DDT and other organochlorine compounds, they may accumulate in the lipid layer of the liver cell membranes and directly interfere with the function of gap junctions. The means of inhibition by phenobarbital may be more complex. Phenobarbital affects the hepatic activity of several membrane-associated enzymes (*38*, *39*) and recently, was shown to affect membrane functions in cultured liver cells (*40*). It is possible, therefore, that the action of phenobarbital may be mediated both by its interaction with cell membranes and its effect on gene expression giving rise to alterations in the composition of liver cell membranes. Relatively little is known about the factors that regulate permeability of gap junctions. It has been shown by Rose et al. (*41*) that ionic calcium produces a graded decrease in permeability. Several studies (*42*, *43*) have now shown that phorbol ester promoters alter the permeability of membranes to calcium. The effect of liver tumor promoters such as DDT and phenobarbital on intracellular calcium levels has not been studied, but it is possible that their inhibition of intercellular communication could be mediated through such an action. Other effects by surface active substances which would inhibit intercellular communication can also be conceived.

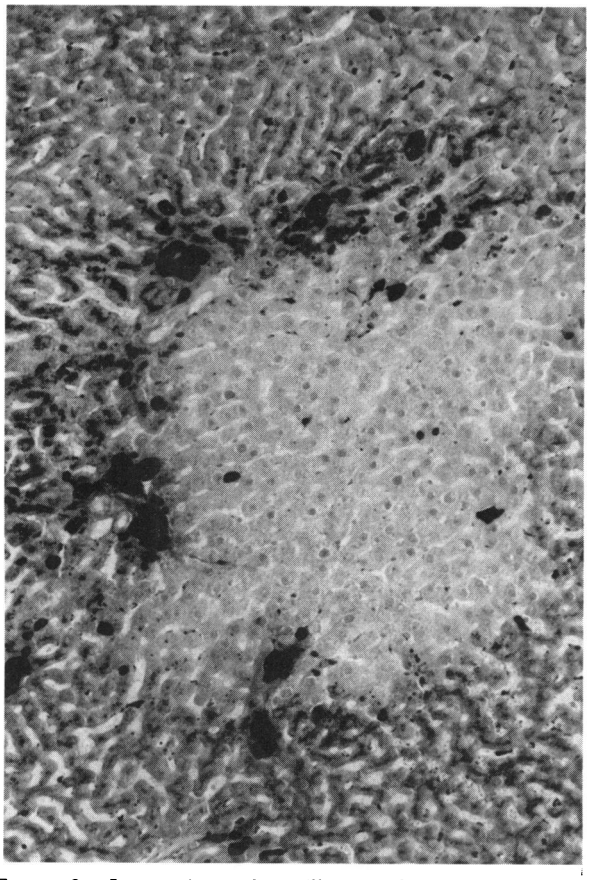

FIGURE 3. Iron-resistant focus illustrated in Fig. 2. Iron reaction, × 125.

Promotion as the Mechanism of Carcinogenicity of Certain Epigenetic Hepatocarcinogens

Several organochlorine compounds and drugs produce primarily or exclusively liver tumors in rodents under conditions of prolonged administration at high levels (Table 3). The properties of these substances differ significantly from those of the more thoroughly studied carcinogens described by Miller and Miller (*44*) as giving rise to reactive electrophiles; the latter are sometimes effective with a single exposure, are often active at low doses, and generally produce neoplasms in high yield, at multiple sites, and usually after short or moderate latent periods. In part because of such differences in the carcinogenic effects between diverse carcinogens, the distinction between two major categories of car-

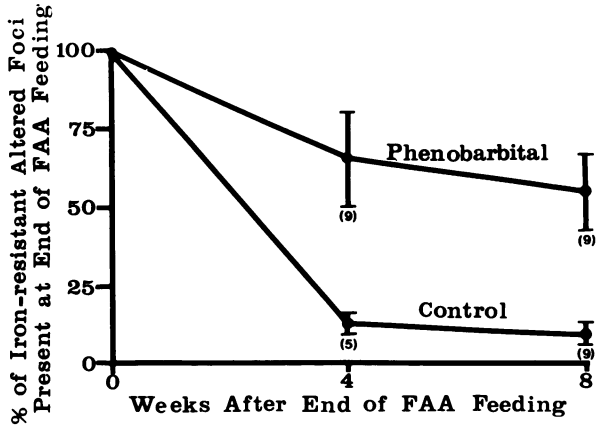

FIGURE 4. Effect of phenobarbital on persistence of liver altered foci induced by *N*-2-fluorenylacetamide.

Table 3. Chemicals that are primarily hepatocarcinogenic after prolonged exposure.

	Activity[a]	
	Mouse	Rat
Phenobarbital	+ +	+ / −
Barital	+ +	NT
Clofibrate	NT	+
Nafenopin	+ +	+
DDT	+	+
Dieldrin	+ +	−
Lindane	+ +	+ / −
Mirex	+ +	+
Polychlorinated biphenyls	+ +	+

[a] + + indicates more active than +; − is negative; + / − mostly negative studies; NT not tested.

cinogens, genotoxic and epigenetic was proposed (*45*, *46*). In this classification, genotoxic carcinogens are defined as those that are capable of producing DNA damage through formation of covalent bonds and thus, correspond to carcinogens that act as electrophilic reactants. Epigenetic carcinogens are defined as those that do not damage DNA but rather, act by indirect mechanisms. The genotoxicity of carcinogens can be evaluated by determinaton of their ability to chemically modify DNA or produce genetic damage in short-term tests. Substantial evidence from the evaluation in tests for genetic damage by the hepatocarcinogenic chemicals in Table 3 indicates a lack of genotoxic action (*18*). Therefore, such chemicals have been suggested to be epigenetic carcinogens, probably of the promoter class (*18*, *47*). Consistent with this concept, several of these carcinogens such as phenobarbital and DDT (*48*) have been shown to enhance the carcinogenic effect of previously administered genotoxic carcinogens under conditions in which the proposed epigenetic carcinogens are not carcinogenic by themselves. This effect in sequential administration is usually interpreted as evidence of promoting activity, although in the absence of other information, it could equally well represent a summation of multiple genotoxic effects (*49*). Nevertheless, the lack of genotoxicity combined with the ability to inhibit intercellular communication indicates that the enhancing effect of these chemicals on hepatocarcinogenicity is due to a promoting action.

The ability of chemicals of this type to produce tumors in the livers of certain rodent strains may be due to their action on altered liver cells that arise spontaneously, through inherited genetic defects or through the action of occult viruses or carcinogens. The presence of such cells is indicated by the development of altered foci in nontreated animals (*50*, *51*) and the development of noninduced liver tumors (*52-55*). Such abnormal cells could be released for progressive growth as neoplasms by interference with the transmission of regulatory factors from normal cells as result of inhibition of intercellular communication (*24*). This concept would explain certain of the dose-response characteristics of carcinogens of this type. Such carcinogens would have to be administered in a sufficiently high dose to extensively alter the cell membrane, either through accumulation in the lipid layer as with DDT or through alteration of the membrane composition as with phenobarbital, in order to inhibit intercellular exhange at the many sites of communication between cells. In addition, such chemicals would have to be administered for a sufficient duration in order to enable the altered cells either to achieve a sufficient mass to insulate themselves from regulatory signals transmitted by the normal tissue or to acquire additional abnormalities during proliferation that would enable them to become resistant to intercellular communication.

Implications for Health Risk Analysis

The evidence is growing to implicate promoting agents in the etiology of human cancer. Indeed, the main cancers in the U.S., i.e., cancers of the lung, colon and breast, each appear to be determined in a distinct way by the tumor-promoting effects produced by either cigarette smoking or consumption of a diet high in fat (*56*, *57*). Importantly, however, such agents with promoting action are self-imposed at high levels and for prolonged periods. In contrast, most uses of the synthetic chemicals of the promoting class of epigenetic hepatocarcinogens discussed above do not result in high levels of human exposure. Moreover, exposure to these agents, even where it has been substantial as in the case of phenobarbital, has not been found to lead to an increased incidence of cancer (*58*, *59*). Several explanations may account for the lack of an effect in humans. Perhaps most likely is the possibility that human exposures are too low or of insufficient duration to achieve promotion according to the concepts presented here. Additionally, if human cells were more competent than rodent cells in intercellular communication, then exposures would have to be even greater than in the animal studies to effect promotion. Finally, the low incidence of liver neoplasms in most population groups suggests that humans, in contrast to animals, such as certain mouse strains in particular, do not have a background of initiated cells in the liver upon which promoters can operate. Thus, the evidence supports the conclusion that liver tumor promoters, like certain other epige-

netic carcinogens, such as hormones, are not hazardous at low levels of exposure.

The concepts on mechanism of action of promoters described herein led to the suggestion by Weisburger and Williams (*60*) that the risk analysis for epigenetic carcinogens of this type should be different from that of genotoxic carcinogens. The latter, because of their capacity to directly modify genetic material, should be considered a hazard at any significant level of exposure, although even with these carcinogens metabolic detoxification and DNA repair processes together with the low probability of producing the critical alteration in DNA at a low level of exposure, indicate that thresholds should exist. With epigenetic agents, because of the nature of the underlying mechanisms, thresholds probably occur even at significant levels of exposure.

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