COMMENTARY

The role of the stages of initiation and promotion in phenotypic diversity during hepatocarcinogenesis in the rat

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The multistage nature of the development of malignant neoplasia has been demonstrated in several mammalian organ systems, most notably rat liver (1–5) and mouse epidermis (6). Epidemiologic studies in the human have also presented substantial evidence for the multistage nature of the development of malignant neoplasia in a variety of organ systems (7) including the uterus (8–10), the lung (11) and the colon (12). In experimental systems, three distinct stages termed initiation, promotion and progression have been defined (13–18). Although epidermal carcinogenesis in the mouse has been studied longer and in more detail than hepatocarcinogenesis in the rodent, the latter model in the rat affords a more quantitative biochemical approach to studies of neoplastic development. The first two stages of carcinogenesis—initiation and promotion—have been extensively investigated (1–5). Although many of the characteristics of initiation are known, such as its permanent and heritable nature (19–20), the actual mechanism(s) of initiation is not yet completely understood. Specific mutations in protooncogenes have been proposed as one key factor in the initiation process (21). Promotion is less well characterized than initiation and is operationally defined as the reversible, clonal growth of initiated cells, reflecting a reversible alteration of genetic expression (19,20). These clonal outgrowths of initiated hepatocytes can be detected and quantified on the basis of the cytochemical expression of a number of phenotypic enzyme markers (1–4,22–24).

Early studies demonstrated the phenotypic diversity of skin papillomas and individual colonies of altered hepatocellular foci (AHF*) on the basis of differences in gene expression, cell division rate and progression to autonomous malignant growth (3,14,25–27). In apparent contrast to such functional heterogeneity, several groups have described a generalized pattern of change for xenobiotic metabolism in AHF (28–30). These observations have been further substantiated by studies with isolated hepatocytes from AHF (31). However, following administration of inducers of phase I enzymes, a heterogeneous expression of these gene products was observed in the population of AHF (32). Since the phenotypic diversity of AHF is a reflection of alterations in the genome of an initiated cell that developed into a clonal AHF, the diversity in phenotypic expression of genetic markers of AHF may be related to those mutations which are critical for the ultimate development of cancer. In support of this thesis, phenotypic diversity is the rule for both AHF and the hepatocellular carcinomas derived from them (3,19,33).

Although a relatively few ‘master genes’ have been postulated to control the expression of multiple genes within the cell (34), mutations in those genes are not necessary to explain the phenotypic diversity observed in AHF or neoplasms (35,36), since the administration of even low doses of initiating agents produces numerous alterations at multiple sites in the cellular genome (37–39). Our extensive knowledge of the spectrum of DNA adducts that occur upon administration of a single carcinogen as an initiator has not yet led to the identification of those critical lesions that predispose a particular cell to the development of neoplasia. Rather, there has accumulated considerable evidence that, though initiation may set the stage for later cancer development, further genetic changes occurring during the stage of progression (40) are necessary for the malignant transformation. The multitude of structural alterations in cellular DNA induced by the single administration of an initiating agent can readily explain the phenotypic diversity seen in the early stages of cancer development, the imprint of which may carry over into the later stages of carcinogenesis (39). The vast majority of specific genetic alterations that occur in the stage of initiation may not themselves be critical for the ultimate expression of malignancy, but rather may be conducive to or result in the enhancement of the opportunities for the malignant transformation. Therefore, the phenotype imprinted on each individual initiated cell at the time of initiation may play a significant role in the overall development of neoplasia from that particular cell.

Mechanisms and characteristics of the stages of initiation and promotion in hepatocarcinogenesis

Initiation

Initiation is the first stage in carcinogenesis (14) and involves the irreversible formation of individual cells possessing the potential to expand clonally under the influence of one or more promoting agents. Initiated hepatocytes are indistinguishable morphologically from one another as well as from the surrounding hepatocytes in H&E-stained sections. Recently Moore, Ito and their colleagues (41,42) have proposed that initiated cells may be detected histochemically by virtue of their increased expression of the placental isozyme of glutathione S-transferase (PGST). In these studies, the number of such single, putatively initiated cells varies with time and dose following initiation by diethylnitrosamine (DEN). On the basis of these data, one can estimate that the number of such putatively initiated cells is near zero in livers of young unincluded animals (42), while ~4 x 10⁵ isolated PGST-positive hepatocytes can be detected in livers of animals 1 week after administration of 120 mg DEN/kg body wt (39), on the assumption that the single-edge dimension of a cuboidal hepatocyte is ~25 μm (43). In studies from our laboratory (Y. Dragan, J. Peterson, and H.C. Pitot, unpublished observations), the number of such putatively initiated cells was 3 x 10⁶ in livers of rats initiated as neonates with a single 30 mg/kg dose of dimethylbenzanthracene (DMBA). The calculated number of PGST-positive single cells in this study was much higher than the maximum number of AHF reported in several different studies (44,45) and 20-fold higher than observed.
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in this experiment. For example, rats initiated neonatally with DEN (15 mg/kg) had 1–3 × 10^6 AHF per liver (44), while in rats given a 70% partial hepatectomy and then initiated with DEN (10 mg/kg) as young adults, this number ranged from 2.5 to 5 × 10^5 AHF per liver (45). Pereira has calculated that administration of 10 mg DEN/kg results in 6 × 10^5 AHF per liver under similar conditions (46). These data suggest that if single PGST-positive hepatocytes are initiated cells, then even under the most effective promotion regimens known, only a small fraction of such cells are capable of developing into AHF. This might be expected if the vast majority of mutational events induced by the initiating agent are irrelevant to the ability of the initiated hepatocyte to develop clonally into an AHF in the presence of a promoting agent. Furthermore, the expression of the single marker, PGST, may be insufficient to predict whether a cell that expresses this gene aberrantly will develop into an AHF under the influence of a promoting agent. The lack of significant clonal growth of some PGST-positive hepatocytes may be due to irreversible damage by the initiating agent, which prevents further replication. Furthermore, some genetic changes may not be permissive either for the selective growth or for the selective inhibition of apoptosis by the specific promoting agent (47). Studies in mouse skin have demonstrated that initiated cells have many potential fates that may depend upon the type and extent of the genetic damage sustained (25).

Chemical, biological and physical initiating agents can induce numerous structural and regulatory genetic changes in living cells, any one or combination of which might provoke the potential for neoplastic development in hepatocytes (7,19,48). DEN is an initiating agent often employed in many of the current protocols for the study of hepatocarcinogenesis (1–3,44). A common genetic lesion following DEN administration is O'-ethyguanine (49), but these DNA adducts are not co-localized in the liver lobule with single GSTP-positive cells (putatively initiated cells) (50). In addition, most O'-ethyguanine adducts are repaired within 2 days after administration of an alkylating agent (49,51), whereas some single hepatocytes that are PGST-positive are detectable weeks after DEN administration (42) or months after DMBA administration (Y. Dragan et al., unpublished observations). These observations suggest that, though O'-ethylthymidime lesions are more persistent than are the alkylated purine bases following DEN administration (49,51), it is likely that the formation of no single adduct or its ensuing mutation(s) results in the initiation of a hepatocyte that can clonally expand into AHF in the presence of a promoting agent. Therefore, as Farber (33) and Singer (52) have suggested, adduct formation and the mutagenesis resulting from a single carcinogenic exposure may not be the only molecular change that leads directly to the malignant phenotype.

Just as most single PGST-positive hepatocytes do not increase their rate of replication under the influence of a promoting agent, those initiated cells that do respond to promoting agents by clonal growth do so at different rates. This fact was first noted by Pugh and Goldfarb (53) and later confirmed by Estadella et al. (54), who also showed that those AHF exhibiting the greatest deviation from the normal phenotype had the highest nuclear labeling index. Peraino and his associates (27,44) further substantiated this finding in demonstrating that the greater the degree of deviation or phenotypic ‘complexity’ possessed by an AHF, the greater its growth rate as reflected by its size. Furthermore, the diversity of individual AHF from the normal hepatocyte phenotype increases with the dose of the initiating agent (55), and this greater phenotypic diversity is correlated with a more likely advance-ment to malignant neoplasia (56). These studies suggest that inherent genetic changes of specific initiated hepatocytes may dictate the growth response of these cells to promoting agents. In addition, Shirai et al. (57) and Imaida et al. (58) have also reported differences in the development of γ-glutamyltranspeptidase (GGT)-positive AHF after initiation with DEN, N-hydroxyaminoo-acetylfuorene, or aflatoxin B1, followed by treatment with the same promotion regimen.

These studies of putatively initiated hepatocytes and their clonal progeny demonstrate the relatively large number of hepatocytes whose genetic expression is permanently altered following the administration of a single, non-necrogenic dose of a complete carcinogen. This number may be as high as 1 in 10^5 (42 and Y.P. Dragan and H.C. Pitot, unpublished observations). However, relatively few of these altered cells exhibit the potential for clonal expansion under the influence of promoting agents. Thus, although it is possible that initiation, the first stage of hepatocarcinogenesis, may be extremely frequent as evidenced by the altered expression of one or more genes, advancement into the stage of promotion is a property of a subset of these altered cells. While one or more of several mechanisms may account for this phenomenon, this review considers the hypothesis that clonal expansion of an initiated cell may be a function of the specific promoting agent employed.

Promotion
The stage of promotion has been defined as ‘an operationally reversible enhancement of the proliferation and the genetic expression of initiated cells’ (7). During hepatocarcinogenesis, promotion is characterized by an increased focal proliferation of hepatocytes, termed AHF, which are potential precursors for subsequent neoplastic development (59–61). The stage of promotion differs from initiation by virtue of its reversible nature (62–68) and its modulation by environmental factors (7,19). The continued presence of at least a threshold level of the promoting agent has been shown to be necessary for the maintenance of promotion in the skin model of carcinogenesis (69). Cessation of the administration of a promoting agent (66,68) in models of rat hepatocarcinogenesis results in a decrease in the number of histochemically detectable AHF. The dose—response characteristics of promoting agents include the existence of a threshold dose below which there is no promotional effect, a linear portion of the response curve, and a maximal level of effect (70). The maximal effect level of a promoting agent is dependent on the duration (71) and format (72) of its administration (69), as well as the finite number of initiated cells responsive to that specific promoting agent (70). However, this dose-related increase in the number of AHF in response to the administration of a promoting agent is not necessarily evidence of a one-to-one correlation of initiated cell formation with AHF formation for a single promoting agent.

Initiated cells that respond to promoting agents are a heterogeneous population (3,44) that possesses a variety of genetic lesions. Changes in AHF gene expression can involve the enzymes of carbohydrate metabolism (73), DNA synthesis (19) and xenobiotic metabolism (28–32), as well as alterations in cellular structural components such as gap junctions (74) and elements of the cytoskeleton (75). One or more combinations of such changes may confer an enhanced ability to survive and replicate in the biochemical environment created by a promoting agent (76).

Analysis of multiple enzyme markers has demonstrated the heterogeneity of phenotypes in different AHF (3,19,29,44). These
expression of these genes. The alternative to this mechanism, as mentioned above, is discussed below.

Clofibrate (102-104) and butylated hydroxyanisole (105) are specifically responsive to the promoting agent and that express pathways and provide the GSH required for the increased cell replication of their growth rate. Thus, the increase in PGST and in GGT expression in AHF might be the promoter-induced alteration of expression of particular genes within such cells (100). Alternatively, the effects of the promoting agent on altered hepatocytes may allow for the selective expansion of a subset of initiated cells that are specifically responsive to the promoting agent and that express a specific pattern of marker genes (101). Several promoting agents have been found to alter the expression of specific marker enzymes. For example, PGST expression is depressed by clofibrate (102-104) and butylated hydroxyanisole (105) administration. Additionally, PB enhances GGT expression in AHF (99,105), whereas numerous agents including ethanol (106), butylated hydroxyanisole (106-110), butylated hydroxytoluene (106-108), ethoxyquin (106,108,110), acetaminophen (106,110,111), di(2-ethylhexyl)phthalate (DEHP) (112) and nafenopin (80,113,114) inhibit this effect. Thus, there is evidence that the expression of some genes observed in AHF populations is related to the regulatory action of the promoting agent on the expression of these genes. The alternative to this mechanism, as mentioned above, is discussed below.

**Phenotypic diversity of altered hepatic foci**

Although alterations in nearly 50 marker gene products have been detected in AHF during promotion in experimental rat hepatocarcinogenesis (19), the consequences of changes in one or more of these markers to the rate and carcinogenic potential of the preneoplastic AHF are not known. The majority of AHF are scored by PGST in many studies (87,88), but PGST does not score 100% of these lesions even after phenobarbital (PB) promotion (88). The generalized pattern of a decrease in phase I xenobiotic metabolizing enzymes (28,30,89,90) and the concomitant increase in phase II enzymes (28,29,91-93) argue for a selective growth advantage over normal hepatocytes of AHF exhibiting such a phenotype in a toxic environment (28,29,94). The enhanced GGT activity of many AHF increases the availability of intracellular glutathione (GSH) (94). Increased levels of PGST may permit a more effective utilization of GSH. Recent studies by Winokur and Lieberman (95) suggest that PGST and GGT are discordantly expressed in hepatocytes of animals treated with DEN in that PGST expression precedes that of GGT in a dose- and time-dependent manner under their experimental conditions. In addition, a decrease in GGT expression in AHF is observed when PB is removed after a period of promotion (66,68). This decrease in GGT activity may occur the availability of GSH to AHF, possibly resulting in an alteration of their growth rate. Thus, the increase in PGST and in GGT may reflect the increased need for GSH for detoxification pathways and provide the GSH required for the increased cell replication rates of the AHF compared with the normal hepatocytes.

The decrease in canalicular adenosine triphosphatase (ATPase) and glucose-6-phosphatase (G6Pase) observed in many AHF (96) may result in an enhanced rate of intracellular carbohydrate metabolism (76,97-99) necessary to sustain the more rapidly dividing cells of AHF compared with the surrounding liver. One explanation for the presence of distinctive phenotypic distributions in AHF might be the promoter-induced alteration of expression of particular genes within such cells (100). Alternatively, the effects of the promoting agent on altered hepatocytes may allow for the selective expansion of a subset of initiated cells that are specifically responsive to the promoting agent and that express a specific pattern of marker genes (101). Several promoting agents have been found to alter the expression of specific marker enzymes. For example, PGST expression is depressed by clofibrate (102-104) and butylated hydroxyanisole (105) administration. Additionally, PB enhances GGT expression in AHF (99,105), whereas numerous agents including ethanol (106), butylated hydroxyanisole (106-110), butylated hydroxytoluene (106-108), ethoxyquin (106,108,110), acetaminophen (106,110,111), di(2-ethylhexyl)phthalate (DEHP) (112) and nafenopin (80,113,114) inhibit this effect. Thus, there is evidence that the expression of some genes observed in AHF populations is related to the regulatory action of the promoting agent on the expression of these genes. The alternative to this mechanism, as mentioned above, is discussed below.

**Evidence for subsets of initiated cells**

The diversity in the expression of numerous enzyme markers observed in Morris hepatomas led to the proposal that a common feature of hepatocellular carcinomas is their marked variation both in phenotypic expression and the regulation of gene expression (115). Examination of early changes in chemically induced carcinogenesis in the liver found two histological types of preneoplastic hepatic lesions, which appeared at different times during the carcinogenic process (116). Studies by Gössner and Friedrich-Freksa (96) reported the occurrence in hepatocarcinogenesis protocols of focal hepatic lesions that exhibited a deficiency of G6Pase, and a description of morphological variation in rat liver lesions detected by routine histological stains was reported by Reuber (117). Further descriptive morphological analyses have been reported by Bannasch (118) and by Hirota and Williams (119). The occurrence of several types of lesions in the liver, both transient and persistent, has also been described during selection-based protocols of hepatocarcinogenesis in the rat (120).

Inter-lesion variability in AHF and nodules detected by differences in histochemical staining were first described by Goldfarb and Zak (121) and later by Kitagawa (122). Several studies (3,44,55) attributed these differences in histochemical staining to the presence or absence of a promoting agent in the treatment protocol. Histochemical changes in the liver during carcinogenesis have also been reported by numerous other investigators (23,124-128), and descriptions of the quantitative application of the analysis of multiple phenotypes in the development of carcinogenesis have been described (19,129,130). These types of analyses have led Peraino et al. (44) to argue that AHF and hepatic neoplasms arise from distinct initiation events and, in addition, that individual AHF are phenotypically stable. At least two populations of nodules have been detected histochemically in a commonly used selection procedure (131). One study used a combination of a histochemical marker (GGT) and morphological characteristics to define three distinct populations of AHF (126). Although this (126) and similar studies (57,58) argue that the phenotypic diversity obtained in any one protocol was determined at the time of initiation, the difference in phenotypes of promoted cell populations observed when the initiating agent and dose are held constant suggests that subpopulations of initiated cells are differentially responsive to the growth-stimulating action of promoting agents.

**Phenotypes of AHF promoted by different agents.** Analysis of the expression of a number of marker gene products in individual AHF permits a comparison of determination and the phenotypic distribution of AHF whose growth is enhanced by different promoting agents (132). The phenotypic distribution of AHF scored with four markers resulting from promotion with several types of agents is shown in Figure 1. In PB-treated animals, GGT and PGST score 90% of the focal lesions detected by the four markers utilized (41). Promotion by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent promoting agent known (132-135), is best scored by PGST and by G6Pase (133). Chlorendic acid, a cyclic, chlorinated hydrocarbon that has been found to be carcinogenic in rodents (136,137), promotes AHF that are detected primarily by their increased expression of PGST and their lack of expression of GGT (133). PGST (74,138) and GGT (139,140) are inefficient markers for the class of promoting agents that induce peroxisomes (Figure 2), whereas promotion by the anti-estrogen tamoxifen results in a novel phenotypic distribution with G6Pase as the predominant marker, scoring 70% of the AHF. Thus, the phenotypic distribution of AHF observed
Farber (142) has suggested that at least three factors may be active in the environment produced by that agent.

Closer scrutiny of the phenotypic distributions shown in Figure 1 and also of the total number of AHF detected, as given in the ANY category (45), further suggests that multiple subsets of initiated cells exist. For example, the AHF scored by PGST and G6Pase during TCDD promotion comprise different populations of AHF, since foci expressing these changes are not coincident (Figure 1). In addition, the large number of small lesions scored by PGST and GGT with WY-14,643 promotion (Figure 2) indicates that this promoting agent does not selectively enhance AHF with a PGST/GST-positive phenotype, as has been previously discussed (78–86,141). Furthermore, this result shows that not all initiated cells are equally responsive to a single promoting agent. Kraupp-Grasel et al. (86) observed three distinct morphological phenotypes of AHF that responded differently in the presence of nafenopin. Studies by Peraino et al. (81) that compared promotion by PB and by a coal-derived mixture found a sex difference as well as a phenotype difference in response to each of these agents. Their studies also suggested that the use of multiple phenotypic markers to characterize AHF may result in the identification of phenotypes of AHF that have an increased chance of developing into a cancer. The delineation of multiple phenotypes of AHF promoted by a specific promoting agent may allow the further characterization of the biological properties of promoting agents that are most important in their actions during multistage hepatocarcinogenesis.

**Diversity of mechanisms of action of promoting agents**

Farber (142) has suggested that at least three factors may be active during the stage of promotion: differential inhibition of cell growth (resistant cell hypothesis); differential selection of cell populations (over-response theory); or differential recovery of target cells. At least one of these mechanisms can be identified in most, if not all, of the models of multistage hepatocarcinogenesis in the rat. The demonstration by Haddow (143) that neoplasms are resistant to the cytotoxic effect of many carcinogens led to the development of the selection procedures of hepatocarcinogenesis of Farber (1,144) and Ito (5,145) and their colleagues. The inhibition of division of normal hepatocytes and the concurrent lack of inhibition of cell replication in AHF by each of several carcinogens was demonstrated in these selection regimens (144,146,147). An increase in phase II enzyme activity has also been observed in GGT-positive-enriched cell populations derived from AHF (31,93). The differential inhibition of the growth of normal compared with initiated hepatocytes may be due to an increase of inactivation or detoxification pathways of xenobiotics in those cells, an increase in elimination pathways, a decreased uptake of toxins, and/or an increased rate of repair of genetic lesions in initiated compared with normal hepatocytes (148–152). Differential stimulation or an ‘over-response’ of cells in the promotion stage is seen in the protocols described by Pitot et al. (3) and by Peraino and his associates (4,44) for the chemical induction of multistage hepatocarcinogenesis. In this case, the promoting regimen is non-toxic and not inhibitory to hepatocyte proliferation. However, initiation may result in an impairment of the normal growth control mechanisms in initiated hepatocytes.

A large number of hepatic promoting agents are now known to exist, including steroid (153) and polypeptide (154) hormones, antioxidants (155), polychlorinated biphenyls (156), naturally occurring metabolites (e.g. orotic acid; 157), a choline-deficient diet (158), and peroxisome-proliferating agents (19,159). In addition, a portacaval anastomosis procedure results in promotion...
proliferation (141). Such agents cause a marked hepatomegaly (179).

Proteins including several of the cytochrome P450 isozymes (29,97,177,178). In addition, PB acts to increase the synthesis, causes the expansion of the undine nucleotide pool (160). Numerous dietary factors may also contribute to promotion (161) including high levels of dietary fat (162–164), high levels of dietary protein (165), amino acids such as tryptophan (166), as well as sucrose (167). Diets deficient in selenium (164) or methyl group donors (168) can also contribute to hepatic promotion. Promoting agents have a vast array of effects, but those critical for stimulation of the growth and phenotype of initiated cells are largely unknown. Two classes of hepatic promoting agents have been widely studied: the barbiturates and the peroxisome proliferators (3,23,169–172). These types of promoting agents have been widely studied: the barbiturates and WY-14,643 (174) have also been shown to enhance the development of neoplasms in previously initiated animals. PB (173) and WY14,643 (174) have also been shown to enhance the development of neoplasia in aged animals. Barbiturates and peroxisomal agents are not directly mutagenic (175,176). An action of PB that may be linked to its promotional activity includes the induction of an increase in the smooth endoplasmic reticulum of the hepatocyte, resulting in increased synthesis of microsomal and other proteins including several of the cytochrome P450 isozymes (29,97,177,178). In addition, PB acts to increase the proliferation rate and inhibit apoptosis of hepatocytes in the liver (179).

In contrast, the peroxisome-proliferating agents may act as promoting agents by increasing cellular oxidant stress (141,160,180) or by eliciting a sustained increase in hepatocyte proliferation (141). Such agents cause a marked hepatomegaly and hypertrophy secondary to the increased number and size of peroxisomes in hepatocytes (160,181). There is evidence for the involvement of a high-affinity binding site in the cellular effects of these agents (182–184).

Other effective promoting agents exhibit quite different actions. For example, a choline-deficient diet dramatically alters certain aspects of lipid metabolism in liver (185) and with continued feeding induces hepatocellular necrosis and regeneration (186). Synthetic estrogens, which are potent promoting agents in hepatocarcinogenesis (187), presumably exert many of their effects through the estrogen receptor. Interaction of the estrogen receptor with its ligand, which then interacts at specific sites on DNA to control the expression of genes within the hepatocyte, may underlie the mechanism(s) for promotion by estrogens (187). The anti-estrogen tamoxifen may also exert its promoting effects as a similar site, but other actions of this chemical have also been described (188). Although ethanol is an effective promoting agent in the human as determined by epidemiology (189), there have been only isolated demonstrations of its promoting effectiveness in experimental hepatocarcinogenesis (190–192). The effects of ethanol on lipid metabolism and cellular oxidant metabolism in hepatocytes are well known (193), but whether any of these effects is involved in promotion by ethanol is unknown. A number of halogenated hydrocarbons, including α-hexachlorocyclohexane (194–197), chlorendic acid (136,137), TCDD (198,199) and polychlorinated biphenyls (200) are members of a general class of promoting agents that exhibit little if any mutagenic or DNA-damaging activity but are hepatocarcinogenic on prolonged administration. The mechanisms of action of some of these agents, such as TCDD and polychlorinated biphenyls, involve an interaction with the Ah receptor and the subsequent derepression of a number of the genes of xenobiotic metabolism (200). Butylated hydroxyanisole may also act as a promoter by increasing cell proliferation (201), while administration of orotic acid, an endogenous metabolic intermediate in pyrimidine biosynthesis, causes the expansion of the uridine nucleotide pool with subsequent imbalance of the deoxynucleotide precursors of DNA synthesis (202). Although increased proliferation is a common effect of many promoting agents, increased cellular proliferation is not sufficient for promotion (203). Thus, the known mechanisms of promoting agents on gene expression vary widely, and their effects on a population of initiated cells exhibiting a dramatic diversity of mutations combine to reflect this inherent heterogeneity.

**Agents with different mechanisms of action promote distinct as well as overlapping subsets of initiated hepatocytes**

As discussed earlier, promotion by PB and peroxisome proliferators occurs through distinctly different mechanisms; hence the commonalities and differences of AHF resulting from these two promotion regimens have often been compared. In studies with mice, comparing PB and DEHP promotion, Ward et al. (79) found differences in the morphology and the phenotypic expression of the resulting AHF. Specifically, PB increased the growth of eosinophilic AHF, whereas DEHP increased basophilic lesions. Comparing promotion with PB and WY-14,643 in rat hepatocarcinogenesis, Cattley and Popp (180) reported that resultant AHF from each of these agents express different phenotypic markers. Specifically, WY-14,643 stimulated an increase in size, whereas PB induced an increase in the number of AHF (180). Additionally, comparisons of the promoting action of PB and WY-14,643 by Préat et al. (80), Schulte-Hermann et al. (82), and Kraupp-Grasl et al. (86) have provided evidence that each agent enhances the growth of distinct populations of initiated cells, and...
Fig. 3. Schematic representation of differential responsiveness of subpopulations of initiated cells to different types of promoting agents. A selective increase in growth of subpopulations of initiated cells may be observed when different promoting agents are administered. Initiated cells may contain mutations a, b or c, rendering them able to grow selectively in the permissive conditions established by promoter classes A, B or C respectively.

Based on differences in the histochemical and morphological phenotypes of AHF and on the rate of induction of hepatic malignancy. Studies by Yokota et al. (204), in which spontaneously initiated cells expressing PGST can be promoted to grow with a choline-deficient diet but not with a peroxisome-proliferating agent, further suggest that subpopulations of initiated cells exist, each of which is differentially responsive to specific promoting agents. The effects of two classes of promoting agents acting on different and overlapping populations of initiated cells are shown schematically in Figure 3.

Depicted in Figure 3 are five subsets of initiated cells, each expressing a different complement of potential phenotypes. Changes in clonal growth of these phenotypically distinct subsets can occur when the initiated cell population is exposed to promoting agents. The subpopulation that expands in response to a specific promoting agent is dependent upon the changes wrought in that cell during initiation and the requirements for growth necessary in the environment created by the specific promoting agent of interest. The phenotypic distribution of the cells promoted is a characteristic 'fingerprint' of the promoting agent employed. This fingerprint may be characteristic of classes of promoting agents that act through a common mechanism. Characteristics that allow responsiveness to a given class of promoting agents (e.g. barbiturates) may be different for a second class of promoting agents (e.g. peroxisome proliferators). These differences in subpopulations of initiated cells responsive to specific promoting agents may be reflected in the differential phenotypic expression of the clonal progeny (AHF) of these hepatocytes.

Fig. 4. Promotion of cells with mutation by an agent in class A results in a clonal expansion of cell with mutation a. After a period of promotion, substitution for the class A promoting agent has variable results depending upon whether the agents are from the same or different classes of promoting agents.

Columbano et al. (78) have suggested that different selection or promotion regimens may promote different subpopulations of initiated cells whose unique properties provide a selective growth advantage in the distinct environments created by different promoting agents. These conclusions were based on the higher efficiency of selection by a choline/methionine-deficient diet compared with that of acetylaminofluorene when a short period separates initiation and the selection regimen. The inability of the former treatment to cause promotion when a long duration separates initiation and selection, in contrast to the undiminished ability for selection by acetylaminofluorene under the same conditions, further argues for the presence of subsets of initiated cells that are differentially sensitive to these promotion regimens. The presence of subsets of initiated cells would indicate that not all initiated cells exhibit an increase in growth in response to every promoting agent, as depicted in Figure 3. This point has been recently explored by Chen and Eaton (205) in a study of the promotion and P450 induction by PB of DEN- or aflatoxin-induced hepatic nodules.

Agents that differ in their mechanism of promotion and/or in the phenotypes of AHF that they promote can also be identified by determining whether they can substitute for one another as selecting or promoting agents. Studies by Yeldandi et al. (140) suggest that the phenotypic expression of AHF whose growth is induced by promotion with a peroxisome proliferator is not altered when 2-acetylaminofluorene is administered in place of
and subsequent to the peroxisomal agent. Laconi et al. (206) have found that switching from one selection agent to another can result in some degree of promotion maintenance. This concept has been extended by Gerbracht et al. (207) in studies in which GGT expression after PB withdrawal was maintained by administration of hexachlorocyclohexane or cyproterone acetate. In the same studies, nafenopin and clofibrate failed to maintain AHF promotion when their administration followed a period of promotion with PB. The inability of PB and WY-14,643 to substitute for each other and maintain promotion further supports the argument that these agents act through different mechanisms to increase proliferation of hepatocytes in AHF (86, 180). Thus, agents that act by a similar mechanism to increase AHF proliferation or that act on the same population of initiated cells may be able to substitute for one another, whereas those having different mechanisms may not, as depicted schematically in Figure 4. Thus, in the presence of promoting agent A, an initiated cell with characteristic a can lie dormant, grow, end-stage differentiate or die. If administration of the promoting agent is later discontinued, then the clonally expanded, initiated cell population may lie dormant, grow or regress through an apoptotic process of single-cell death. Replacement of the promoting agent with one that acts in the same manner (agent 2) can result in growth of the AHF or maintenance of size without further growth, while substitution with a promoting agent of a separate class may result in no change in AHF status or in its regression. This contention is further supported by the study of Hendrich et al. (68) in which a period of promotion with PB was followed by its withdrawal and subsequent readministration. In this study, withdrawal of PB resulted in a diminution of detectable AHF, and subsequent readministration of this promoting agent resulted in a rapid reappearance of the AHF. In both instances the phenotypes of the AHF remained constant. An increased understanding of the mechanisms of promotion should assist in classifying the different types of promoting agents and in assigning the relative risk which these agents may pose to the development of human cancer. A study of multiple markers of AHF and of promoting agents with different mechanisms of action may serve to elucidate the numerous pathways to cancer development.

Summary

Differences in the distribution of phenotypic alterations in initiated and promoted cell populations reflect both the dose and the action of the specific initiating agent, as well as the mechanism of action of the promoting agent. The use of multiple phenotypic markers to characterize AHF should allow further experimentation on the characteristics of promoting agents in hepatocarcinogenesis, especially their ability to promote separate populations of initiated cells, and on those characteristics of initiated cells that enable certain ones to survive and multiply in the specific environment provided by a promoting agent. Studies of promoting agents with differing mechanisms of action should further allow questions of reversibility, substitution and additivity of the actions of promoting agents to be addressed. The possibility that individual promoting agents do not enhance the growth of the entire population of initiated cells indicates that study of combinations of promoting agents is an important future direction of research. Therefore, information on the characteristics of promoting agents, singly and in combination, is necessary to assess more accurately the contribution of promoting agents to the carcinogenic risk for humans distinct from the effects of initiating agents and complete carcinogens.

Acknowledgements

The authors are indebted to Dr Ilse Riegel for constructive and critical editorial comments, and to Mrs Mary Jo Markham for expert technical typing of the manuscript. Many of the studies from this laboratory were supported by grants from the National Cancer Institute (CA-07175, CA-22484 and CA-45700) and by a contract from the National Institute of Environmental Health Sciences (ES-82-12).

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