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Association between Responsiveness to Phenobarbital Induction of CYP2B1/2 and 3A1 in Rat Hepatic Hyperplastic Nodules and Their Zonal Origin

by Zhi-Ying Chen[1] and David L. Eaton[1]

To explore further the mechanism underlying the alteration in expression of P450 enzymes in hepatic preneoplastic lesions, expression of CYP2B1/2 and 3A1 in individual hepatic hyperplastic nodules induced by an aflatoxin B_1 (AFB) administration protocol and the Solt-Farber resistance protocol in male F344 rats was examined via immunohistology. In nodules induced by the resistance protocol, expression of both CYP2B1/2 and 3A1 proteins was highly variable among different nodules, whereas these P450 proteins were expressed in all nodules induced by the AFB protocol. Nodules induced by the resistance protocol have been shown previously to arise from throughout the acinar lobule. In contrast to the resistance protocol, the AFB protocol causes extensive periportal necrosis, potentially resulting in a heavy selection pressure for clonal expansion of initiated cells arising from the centrilobular area. As phenobarbital-induced expression of both CYP2B1/2 and 3A1 in normal liver is heavily localized to the centrilobular zone, these observations suggest that the ability of preneoplastic nodules to respond to induction of these P450 proteins is determined primarily from the zonal origin of the precursor hepatocytes. Thus, the nodules from the resistance protocol that express little or no CYP2B1/2 and 3A1 may have been derived from the periportal hepatocytes, whereas all the nodules in the AFB group and some of the nodules from the resistance protocol which expressed these P450 proteins in response to phenobarbital induction may have been derived from centrilobular hepatocytes.

Introduction

Alternation in expression of cytochromes P450 in preneoplastic and neoplastic lesions is a common phenomenon during chemical hepatocarcinogenesis (*1–5*). There is increasing evidence to suggest that altered expression of P450 enzymes in the preneoplastic and neoplastic lesions may play a functional role in liver tumor promotion by nongenotoxic carcinogens. Numerous studies have demonstrated an association between induction of P450 enzymes and the potential for liver tumor promotion by nongenotoxic carcinogens such as barbiturates, phenytoin, and DDT (*6–10*). In recent years, expression of several forms of P450 proteins, e.g., CYP1A1, 1A2, 2B1/2, 2C6, 2C11, and 3A1, has been examined in individual preneoplastic and neoplastic lesions via immunohistological studies (*11–17*). Results of these studies suggest that individual forms of P450 protein are expressed differently in different lesions. Furthermore, some studies (*13,14*) have demonstrated that larger or more advanced lesions underexpress P450 enzymes relative to smaller or less advanced lesions, suggesting that decreased expression of P450 enzymes may be an important factor in the growth and development of the preneoplastic lesions in the absence of exogenous promoter treatment.

Recently, a series of studies has been conducted in our laboratory to further explore the mechanism underlying the alteration of P450 enzyme expression in preneoplastic lesions in rat livers. We present here evidence to suggest that the ability of preneoplastic nodules to respond to induction of CYP2B1/2 and 3A1 by phenobarbital is determined primarily from the zonal origin of the precursor hepatocytes.

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[1] Department of Environmental Health, SC-34, University of Washington, Seattle, WA 98195.

Address reprint requests to Z.-Y. Chen, Department of Environmental Health, SC-34, University of Washington, Seattle, WA 98195.

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Induction of Rat Hepatic Nodules

Hepatic hyperplastic nodules were induced in male F344 rats (Simenson Co., Gilroy, CA) either by a modified Solt-Farber resistance protocol or an aflatoxin B_1 (AFB) administration protocol. Details of these two protocols have been reported elsewhere (*16–18*). The expression pattern of GGT (gamma-glutamyltranspeptidase) was determined histochemically, and express of GST-P (glutathione S-transferase) was determined immunohistochemically, as described previously (*16,17*). The expression of CYP1A1, 1A2, 2B1, 2B2, 3A1, 2C6, and 2C11 in individual lesions was determined immunohistochemically, and by Northern or slot-blot analysis of mRNA preparations using specific P450 oligomers.

Zonal Differences in Responsiveness

A polyclonal antibody reacting to both CYP2B1 and 2B2 was used to study the expression pattern of CY2B1/2 in rat liver via immunohistochemical staining. The proteins demonstrated by this antibody are therefore designed as CYP2B1/2 proteins. Data from these studies demonstrated that CYP2B1/2 proteins were positive only in acinar zones 2 and 3 (centrilobular) and negative in zone 1 (periportal) in normal, surrounding tissue in the livers of phenobarbital-treated rats in both AFB and Solt-Farber resistance protocol groups (Fig. 1*D*, 2*D*, and *E*). These data are in agreement with previous studies of the distribution of mRNA and protein of these two P450 enzymes (*19–21*). Using an antibody specific to CYP3A1, CYP3A1 protein was demonstrable only in zone 3 following phenobarbital induction (Figs. 1*C* and 2*C*). Very weak reaction of CYP2B1/2 proteins and no detectable CYP3A1 protein was observed in the nonphenobarbital treated control groups. These observations indicate that the ability of hepatocytes to respond to phenobarbital induction of these P450 proteins is determined, at least in part, by the acinar zone location of the cells within the liver lobule.

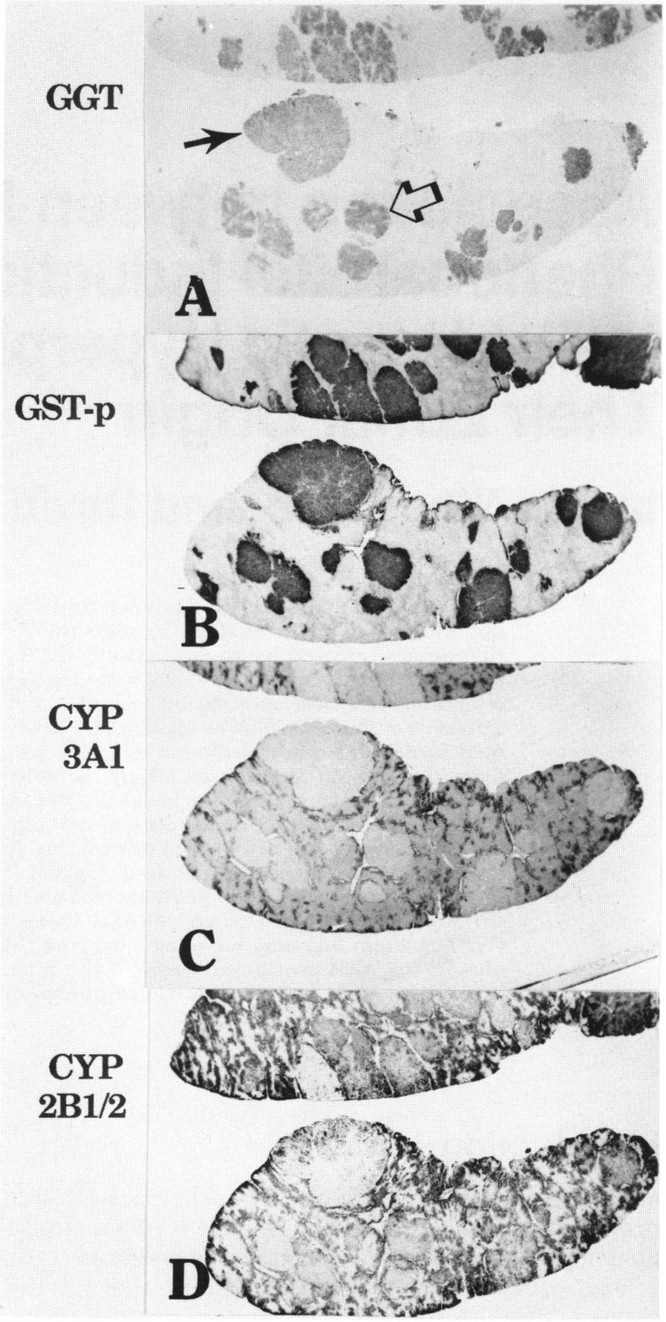

Figure 1. Expression of γ-glutamyltranspeptidase (GGT), glutathione S-transferase-p(GST-P), CYP2B1/2, and 3A1 in persistent GGT nodules and remodeling nodules induced by the Solt-Farber resistance protocol in semi-serial sections (*17*). The black arrow indicates a persistent GGT nodule, and the open arrow indicates a remodeling nodule 12.5×.

Responsiveness to Phenobarbital Induction and Zonal Origin in Hepatic Hyperplastic Nodules

Examination of the expression of CYP2B1/2 and 3A1 in preneoplastic lesions induced by the Solt-Farber protocol revealed that some, but not all, of these nodules respond to induction by phenobarbital [Fig. 1*C*,*D*; (*16*)]. In contrast, all of the nodules induced by the AFB administration protocol positively expressed CYP2B1/2 (*16*) and 3A1 (Fig. 2*C-E*). CYP2B1/2 proteins were uniformly expressed in all of the AFB-induced nodules, although small focal areas of negatively stained hepatocytes could be seen in some of the nodules. In contrast, a wide spectrum of staining patterns for CYP2B1/2, from completely negative staining (similar to that in the periportal area) to highly positive staining (similar to that of the centrilobule area), was observed in the nodules induced by the Solt-Farber resistance protocol. CYP3A1 protein was demonstrable in all of the AFB-induced nodules, with

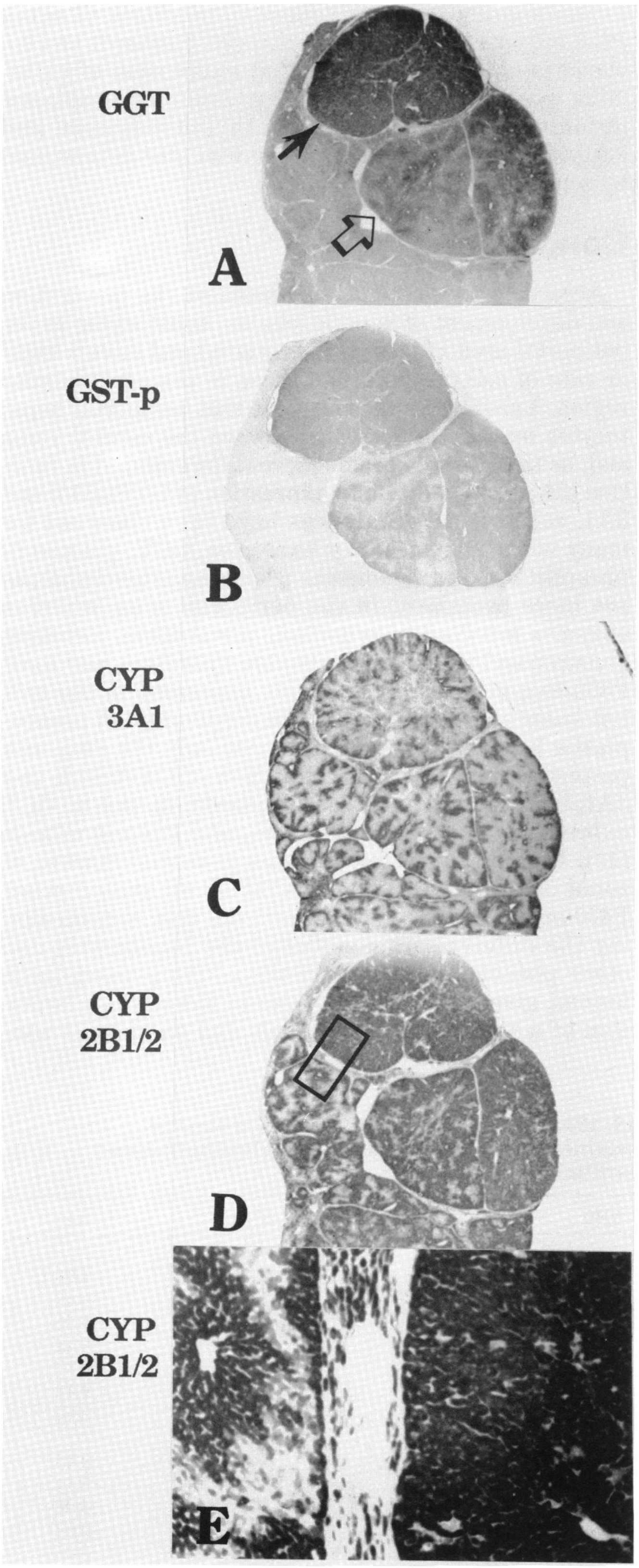

Figure 2. Expression of GGT, GST-P, CYP2B1/2, and 3A1 in hepatic hyperplastic nodules induced by the aflatoxin B_1 administration protocol in semi-serial sections (16) (12.5×) (E) enlargement of the boxed area indicated in *D* (225×).

many small areas of darkly stained cells dispersed among the majority of negatively stained cells, yielding a staining pattern mimicking that of the surrounding tissue. CYP3A1 was expressed only in a small fraction of the nodules in the Solt-Farber resistance group; the majority of nodules had no expression at all.

Single large or smaller repeated doses of AFB typically result in periportal necrosis in rat liver (*22–24*). We also observed extensive periportal necrosis in the livers of rats given the AFB protocol. The dose level of AFB used in our experiments (10 doses of 150 μg/kg each in two successive weeks, *IP*) was considerably lower than that used by other investigators to induce liver necrosis (*22,23*). However, using the same dosing regimen, we observed a marked difference in toxicity between animals maintained on a standard rodent chow diet and those maintained on a purified (AIN 76-A) diet. The 150 μg/kg/day dose of AFB produced minimal necrosis and only a few, tiny preneoplastic foci in animals maintained on the chow diet, but resulted in widespread liver necrosis and numerous large nodules in rats maintained on the AIN-76A diet (data not shown).

These data suggest that AFB-nodules produced in our studies might be derived solely from clonal expansion of initiated cells originating in the centrilobular region. Close observation of the distribution of early foci 1 or 2 weeks after the last dose in AFB-treated animals revealed numerous small GST-*P*-positive hepatocyte foci in the centrilobular region, suggesting that these foci originate from this region. Thus, we suggest that the ability of AFB-induced foci/nodules to respond to phenobarbital induction of CYP2B1/2 and 3A1 may be an inherited characteristic reflecting the zonal origin of the precursor hepatocyte. Similarly, the inconsistent expression of the P450 enzymes among nodules induced by the Solt-Farber resistance protocol might result from the fact that these lesions arise from throughout the lobule, without zonal preference (*25,26*). This hypothesis is schematically explained in Figure 3.

Messenger RNA levels of six P450s in 10 surgically isolated large nodules (induced by the Solt-Farber resistance protocol and several additional doses of acetylaminofluorene) are shown in Table 1. In every instance, the mRNA levels for all P450s examined in individual nodules was greatly decreased relative to normal liver or surrounding tissue. These data are consistent with the hypothesis that both constitutive expression and the responsiveness to induction of specific P450s is determined, at least in part, by the zonal origin of the lesions. It is unlikely that the decrease in expression of six different P450 genes resulted from specific "damage" to all 6 genes in all 10 nodules. Similarly, it is unlikely that all the nodules produced by the AFB protocol had damage in all three genes of CYP2B1, 2B2, and 3A1. Roomi and colleagues (*27*) suggested that the uniform underexpression of numerous P450 enzymes in preneoplastic lesions was not a result of alterations in the structural genes or regulatory ele-

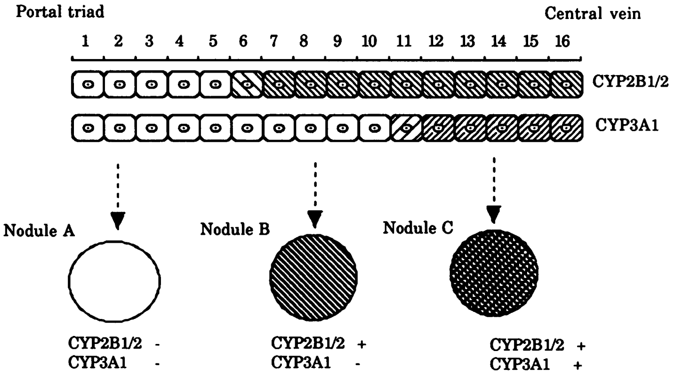

Figure 3. Proposed relationship between zonal origin of the preneoplastic lesions and expression of CYP2B1/2 and 3A1 in preneoplastic lesions. In phenobarbital-treated surrounding tissue, CYP2B1/2 proteins are demonstrable in the zones 2–3, corresponding to hepatocytes in the positions 6-16, whereas CYP3A1 protein is expressed only in zone 3, corresponding to hepatocytes in positions 11–16. The nodules (e.g., nodule A) expressing neither CYP2B1/2 nor CYP3A1 may be derived from a hepatocyte in zone 1, whereas nodules (e.g., nodule C) expressing both CYP2B1/2 and 3A1 may have arisen from a hepatocyte in zone 3. Similarly, nodules (e.g., nodule C) expressing CYP2B1/2 but not CYP3A1 may be derived from a hepatocyte in zone 2.

ments of specific P450s, but rather resulted from an alteration in a more fundamental aspect of P450 function, such as an alteration in a gene(s) related to heme biosynthesis. This hypothesis, however, does not explain the observation that the combination of altered expression of P450 proteins in the lesions can be different among different nodules when expression of multiple P450 genes in the lesions are examined (*13–15,17*). We therefore suggest that the zonal origin of the cell from which each nodule was initially derived may play an important role in determining both constitutive expression and responsiveness to induction of many P450 enzymes (Fig. 3). Interestingly, Gebhardt and coworkers (*28*) also reported that expression of glutamine synthetase in the preneoplastic and neoplastic lesions induced by AFB, dimethylnitrosamine, and acetylaminofluorene in rat liver was also determined by zonal origin.

Conclusion

According to a recent hypothesis on the maturation and development of hepatic lobules, hepatocytes in the periportal area are less differentiated and have a higher rate of cell division than those in the centrilobular region. Labeling studies suggest that periportal hepatocytes migrate or "stream" toward the central veins and, as they do so, become more differentiated in function (*29,30*). In addition to expression of CYP2B1/2 and 3A1, acinar zonal differences have been observed for many other enzymes. For examples, GGT, glucose 6-phosphatase, and canalicular adenosine triphosphatase are more prominent in the periportal area, whereas enzymes involved in xenobiotic metabolism, including glucuronosyl transferase, epoxide hydrolase, and most P450 enzymes, are more highly expressed in the centrilobular area (*31,32*). Thus the zonal origin of preneoplastic lesions may determine not only the response pattern to phenobarbital induction of CYP2B1/2 and 3A1, but other phenotypic characteristics such as GGT expression pattern, growth rate, and DNA synthesis (*17*). In addition to serving as a potential marker of zonal origin, expression of CYP2B1/2 and/or other P450 enzymes may play a functional role in determining the differentiation status of the hepatocytes and other phenotypic characteristics of the preneoplastic lesions, given their central roles in the biotransformation of a variety of endogenous and exogenous sub-

Table 1. Slot-blot measurement of mRNA of CYP1A2, 2B1, 2B2, 3A1, 2C6, and 2C11 in rat hepatic hyperplastic nodules (HHN) induced by dimethylnitrosamine and repeated doses of acetylaminofluorene with or without phenobarbital treatment.[a]

Samples	1A2	2B1	2B2	2C6	2C11	3A1
Phenobarbital group						
Control	1.00	1.00	1.00	1.00	1.00	1.00
Surrounding tissue	0.62	0.72	0.62	0.71	0.58	0.46
HHN 1	0.33	0.22	0.31	0.34	0.08	0.08
HHN 2	0.50	0.21	0.30	0.33	0.06	0.12
HHN 3	0.25	0.09	0.13	0.17	0.05	0.07
HHN 4	0.24	0.15	0.17	0.60	0.18	0.16
HHN 5	0.35	0.23	0.31	0.40	0.06	0.10
Nonphenobarbital group						
Control	1.48	0.00	0.04	0.17	0.56	0.15
Control surrounding tissue	0.93	0.00	0.06	0.15	0.42	0.11
HHN 1	0.32	0.00	0.04	0.00	0.03	0.02
HHN 2	0.45	0.00	0.03	0.00	0.04	0.08
HHN 3	0.27	0.00	0.03	0.14	0.04	0.08
HHN 4	0.40	0.00	0.04	0.07	0.04	0.08
HHN 5	0.40	0.00	0.05	0.26	0.03	0.12

[a] Data are expressed as the ratio of mRNA from phenobarbital-treated control liver, which is set as 1.00.

[b] Control animals given acetylaminofluorene and partial hepatectomy but no diethylnitrosamine, such that no HHN were formed.

stances such as steroid hormones, eicosanoid hormones, and phenobarbital, which are modulators of DNA and RNA synthesis (*17*).

The mechanisms of induction and regulation of CYP2B1/2 and 3A1 in hepatocytes remains poorly understood, and the influence of the cell environment on P450 expression remains uncertain. It does appear that the "genotype" of a nodule, which is presumably monoclonal in origin, does not fully determine the expression of P450s. For example, although some AFB-induced nodules are uniformly stained for CYP2B1/2, patches of negatively stained hepatocytes can be seen in other nodules, irrespective of their monoclonal origin. This is most evident for induction of CYP3A1 in AFB-induced nodules, where marked heterogeneity in induction/expression of this P450 was evident in all nodules. More studies are required to answer these questions and to test this hypothesis further.

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