Mechanism of the Paradoxical, Inhibitory Effect of Phenobarbital on Hepatocarcinogenesis Initiated in Infant B6C3F1 Mice with Diethylnitrosamine

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ABSTRACT

Phenobarbital (PB), a classical rodent hepatopromoter, remarkably enhances hepatocarcinogenesis initiated by diethylnitrosamine (DEN) in adult B6C3F1 mice. However, it is also known to strongly inhibit liver tumor development in the B6C3F1, mice initiated with DEN in their infancy. The present study aimed to elucidate the unknown biological mechanisms for this paradoxical, inhibitory effect of PB on B6C3F1 mouse hepatocarcinogenesis. Male 12-day-old infant B6C3F1 mice were injected Lp. with DEN and, at 6 weeks of age, divided into PB-treated (PB+ group) and untreated (PB— group) animals. At 24 weeks, PB treatment was ceased for half of the PB+ group (PB+/- group) and started for half of the PB— group (PB—/+ group). Finally, all mice were sacrificed at 36 weeks and examined for the development of liver tumors. The mean multiplicity of gross tumors in the PB+ group was only one-fifteenth of that in the PB— group. PB—/+ animals developed fewer than half of the tumors found in the PB— mice, indicating that the PB effect depends solely on the treatment duration, rather than the animal age. The effect was proven to be reversible, because the mean tumor multiplicity for the PB+/- group was seven times larger than that for the PB+ group. Stereological analysis revealed the mean volume of hepatocellular proliferative lesions in the PB— animals to be 7.7- and 4.1-fold the values for the PB+ and PB—/+ groups, respectively. The mean proliferating cell nuclear antigen-labeling indices for hepatocellular adenomas in PB+ and PB—/+ animals were also one-third of that for tumors in PB— animals, whereas no significant differences were observed with regard to the mean apoptotic index. In conclusion, the inhibitory effect of PB seemed to be primarily caused by the suppression of tumor cell proliferation. Irrespective of the group, most lesions observed were basophilic hepatocellular adenomas or foci, positive for Bcl-2 oncoprotein. They were thus distinct from the eosinophilic Bcl-2— lesions that predominate with PB promotion after the initiation of adult B6C3F1 mice. This age-dependent nature of initiation, together with the differential responses of Bcl-2+ and Bcl-2— lesions, may be responsible for the apparently contradictory outcomes of PB treatment in infant and adult B6C3F1 mice.

INTRODUCTION

PB is well known to promote rat hepatocarcinogenesis after initiation with a genotoxic chemical carcinogen (1). It is itself nongenotoxic and lacks initiating activity (2). PB can also promote the development of chemically initiated liver tumors in mice (3-5); for example, PB has been found to substantially increase the final yields of liver tumors in B6C3F1 mice induced by DEN alone (3-6). In B6C3F1 mice, on the other hand, only a promoting effect is exerted, which PB unexpectedly suppresses cell proliferation. In the mouse, PB is known to inhibit chemical hepatocarcinogenesis under certain conditions (12-14). Despite its promoting effect on B6C3F1 mice initiated with DEN as young adults, a single exposure of 15-day-old infant B6C3F1 mice to the carcinogen, followed by chronic administration of PB shortly after weaning at 3 weeks of age, results in a dramatic decrease in liver tumor multiplicity as compared to that of the mice treated with DEN alone (12, 13). In BALB/c mice, on the other hand, only a promoting effect is exerted, independent of the timing of the DEN injection (13). Thus, the modulatory effects of PB seem to be determined by the timing of the carcinogenic treatment and the genetic background. Some investigators have considered that the paradoxical decrease of liver tumors in B6C3F1 mice may depend on the timing of the PB administration rather than that of the DEN injection (12, 13).

The biological effects of PB on rodent hepatocytes in culture are also complicated. We recently provided evidence that PB induces apoptosis in mouse hepatocytes, depending on deregulated c-myc expression, possibly through up-regulation of the apoptosis inducer, Bax (15). It was further demonstrated that activation of c-H-ras could completely suppress this apoptotic effect, accompanied by de novo induction of the Bax antagonist, Bcl-2 (15). These findings are somewhat surprising, given that previous studies have suggested that PB promotes rat hepatocarcinogenesis through the suppression of apoptosis in chemically initiated hepatocytes (8). In fact, it has also been reported that PB suppresses UV- or 2-acetylaminofluorene-induced apoptosis of primary rat hepatocytes in culture (16).

Because B6C3F1 mice have been extensively used as experimental animals to assess the human risk potential of suspected carcinogens (17), these apparently conflicting actions of PB raise a serious issue regarding tumor promotion and inhibition. Thus, the biological effects of PB require detailed elucidation for better evaluation of cancer risk.

In the present study, we therefore characterized the inhibitory effects of PB on liver tumor development in B6C3F1 mice initiated with DEN in their infancy. We found that DEN treatment of infant B6C3F1 mice selectively induces the precursors of Bcl-2+ tumors, for which PB unexpectedly suppresses cell proliferation.

MATERIALS AND METHODS

Animals. B6C3F1 mice were produced in our laboratory by mating female C57BL/6J mice with male C3H/HeJ mice. The parental strains were purchased from CLEA Japan, Inc. (Tokyo, Japan). Only male B6C3F1 mice were used in this study.

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3. The abbreviations used are: PB, phenobarbital; DEN, diethylnitrosamine; PCNA, proliferating cell nuclear antigen; PBST, PBS with 0.5% Tween 20.
Treatment of the Animals. At 12 days of age, all male B6C3F1 mice were injected i.p. with DEN (WAKO, Osaka, Japan) dissolved in physiological saline at a dose level of 5 mg/g body weight and, after weaning at 3 weeks, fed the basal diet (CEll; CLEA Japan, Inc.; Ref. 18). Starting at 6 weeks, half the mice received 500 ppm of PB (WAKO) in the diet (PB+ group), and the other half were maintained on the basal diet (PB— group). At 24 weeks, half of the animals in the PB— group were switched to the PB-containing diet (PB—/+ group), whereas this was replaced with the basal diet for half of the animals in the PB+ group (PB+/— group). Some selected mice in the PB— and PB+ groups were sacrificed at 24 weeks for the examination of gross liver tumors. At 36 weeks, all surviving mice in the four groups (i.e., PB—, PB+, PB—/+ and PB+/— groups) were sacrificed for histological examination of the liver. Portions of representative liver tumors were freshly frozen for Western blot studies.

Histological and Immunohistochemical Analysis. From each liver specimen embedded in paraffin, 4-μm thick serial sections were cut. One section was routinely stained with H&E, and the remainder were used for immunohistochemical studies. Hepatocellular proliferative lesions were classified as basophilic and eosinophilic lesions by histological examination of the H&E-stained sections, as described previously (6, 19). Immunohistochemical detection of the PCNA or Bcl-2 protein was performed by the avidin-biotin complex method using mouse monoclonal antihuman PCNA antibodies (PC10; DAKO Japan Co., Ltd., Tokyo, Japan; 1:200 dilution) and rabbit antimouse Bcl-2 (D-18) antibodies (1:200 dilution), respectively, and an appropriate HISTOFINE kit (Nichirei, Tokyo, Japan). For both the proteins, the deparaffinized sections were heated at 100°C for 30 min in acidic citrate buffer (pH 6) before the application of primary antibodies (6).

Western Blot Analysis. For Western blot analysis of Bcl-2 protein, total tissue lysates containing 50 μg of denatured proteins were run on 12.5% polyacrylamide gels containing SDS and electroblotted onto nitrocellulose filters. The filters were then blocked with 5% nonfat dry milk in PBST and incubated with rabbit antimouse Bcl-2 serum (1:3000 dilution) and anti-rabbit IgG conjugated to horseradish peroxidase (Amersham, Buckinghamshire, United Kingdom) in PBST and washed again. Finally, bound antibodies were visualized using the enhanced chemiluminescence system (Amersham).

Stereological Analysis. Focal hepatocellular proliferative lesions, including foci, adenomas, and carcinomas, were detected by examination of H&E-stained sections, and their numbers and diameters were recorded. Only lesions larger than 160 μm in diameter were counted to assure reproducible identification. The total number of lesions/cm³ liver, volume percentage of lesions/liver, and the mean volume of lesions were calculated by the stereological method of Enzmann et al. (20), as described previously (5). The number of lesions/liver was determined by multiplying the value per cm³ by the liver weight in grams.

Determination of PCNA Labeling and Apoptotic Indices. The PCNA labeling index has been widely used for the assessment of cell proliferation (21). In this study, the index was defined as the number of hepatocytes with a PCNA-positive nucleus (or nuclei)/100 hepatocytes (%). Immunohistochemical detection of PCNA was performed as mentioned above, and only densely immunoreacted nuclei were regarded as positive. The apoptotic index was expressed as number of apoptotic figures/100 hepatocytes (%). Because histological examination of H&E-stained sections is considered to be the most reliable way of detecting apoptotic hepatocytes, the histological criteria described by Bursch et al. (22) were applied for detection. For each tumor or control liver, at least 1000 hepatocytes were examined for both indices.

Statistical Analysis. Statistical comparison of mean values was performed using the two-sided Mann-Whitney U test. Ps less than 0.05 were considered significant.

RESULTS

Development of Gross Liver Tumors. Data for the mean multiplicities of tumors (>1 mm in diameter) observed on the liver surfaces are summarized in Table 1. The remarkable inhibitory effects of PB on the development of lesions were already evident at 24 weeks, when the PB-treated animals had less than one-twentieth of the tumors found in untreated mice. At 36 weeks, the PB+ group had a mean tumor multiplicity of 4.2 ± 0.7 (mean ± SE), as compared with 62 ± 5 for the PB— group (P < 0.01). The mean value for the PB+/— group (27 ± 6) was also less than half that for the PB— group (P < 0.01), indicating a duration-dependent rather than an age-dependent effect. On the other hand, the value was reversibly increased for PB+/— (29 ± 4) group relative to the PB+ group (P < 0.01).

The Number and Size of Hepatocellular Proliferative Lesions. For 10 animals randomly selected from each PB—, PB—/+, PB+/, or PB— group, a three-dimensional evaluation of the number and size of hepatocellular proliferative lesions (i.e., foci, adenomas, and carcinomas) was performed. The results are shown in Table 2. Although there was only a 1.5-fold difference in the mean number of lesions/liver between the PB— and PB+ groups (530 ± 60 versus 350 ± 70), the volume percentage for the PB— group (70 ± 4%; P < 0.01) was 11-fold the PB+ group value (6.2 ± 1.0 %). This reflected mean volumes of 5900 ± 1200 (10⁶ μm³) as compared to 760 ± 250 (P < 0.01). Thus, PB primarily affected the size of the lesions. Both the volume percentage and mean volume of lesions for the PB—/— and PB+/— groups were intermediate to the corresponding values for PB— and PB+ groups, confirming the duration dependence of the effect.

Qualitative Characterization of Hepatocellular Proliferative Lesions. Hepatocellular proliferative lesions observed on H&E-stained sections were qualitatively characterized. Although we first tried to differentially diagnose the lesions as hepatocellular foci,
Table 3  Histological characterization of hepatocellular proliferative lesions initiated with DEN in male B6C3F1 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (wk)</th>
<th>No. of mice</th>
<th>No. of lesions examined</th>
<th>Adenomas or foci</th>
<th>Carcinomas</th>
<th>Basophilic (Bcl-2+)</th>
<th>Eosinophilic (Bcl-2+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB−</td>
<td>6–24</td>
<td>10</td>
<td>601</td>
<td>98.2</td>
<td>1.8</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>PB−/+</td>
<td>25–36</td>
<td>10</td>
<td>476</td>
<td>99.4</td>
<td>0.6</td>
<td>98.9</td>
<td>1.1</td>
</tr>
<tr>
<td>PB+/−</td>
<td></td>
<td>10</td>
<td>440</td>
<td>98.4</td>
<td>1.6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>PB+</td>
<td></td>
<td>10</td>
<td>184</td>
<td>100</td>
<td>0</td>
<td>96.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

adenomas, and carcinomas, exact discrimination of foci from adenomas was virtually impossible. Therefore, these benign lesions were combined for the analysis. As shown in Table 3, more than 98% of the lesions were adenomas or foci for all four groups. There was a tendency for the development of carcinomas to be suppressed by PB application. More than 96% of the lesions were basophilic for all of the groups (Table 3). Eosinophilic lesions (all adenomas or foci) were limited to the PB+ and PB−/+ groups (3.8 and 1.1%, respectively). All of the 12 eosinophilic lesions detected and at least 25 basophilic lesions for each group were immunohistochemically examined for Bcl-2 staining. Whereas all of the normal livers surrounding the lesions and all of the eosinophilic lesions proved to be negative for Bcl-2, all of the basophilic lesions showed cytoplasmic staining for this protein (Fig. 1). Western blot studies of randomly obtained tumor tissues from the PB−, PB−/+ , and PB+ groups confirmed a predominance of Bcl-2+ tumors (Fig. 2). Apparently, PB slightly increased the amount of Bcl-2 proteins expressed in the tumors (Fig. 2).

PCNA Labeling and Apoptosis. PCNA labeling and apoptotic indices were determined for two or three of the largest basophilic adenomas of each animal from the PB−, PB+, PB−/+ , and PB+−/− groups. Carcinomas and eosinophilic lesions were excluded because of their limited numbers. For each group, 25 tumors and 5 normal livers were analyzed. As shown in Fig. 3, the mean PCNA labeling indices of tumors in PB+ and PB−/+ animals (3.4 and 3.5%, respectively) were approximately one-third of the values for tumors in PB− animals (11%; P < 0.01 with either PB+ or PB−/+). The labeling index for the PB+/− group (7.9%) was slightly lower than that for the PB− group (P < 0.01) but was more than two times higher than those for the PB+ and PB−/+ group (P < 0.01). Conversely, the PCNA indices of normal livers for the PB+ and
PB−/+ groups (0.95 and 0.77%, respectively) were 2.5–3 times higher than those for the PB− and PB+/− groups. The mean apoptotic indices for tumors were generally very low (less than 0.14% for all groups), and there were no significant intergroup differences (Fig. 3). The apoptotic values for tumors, however, tended to be higher than the values for normal livers (approximately 0.03% for all groups).

**DISCUSSION**

The inhibitory effect of PB on hepatocarcinogenesis in B6C3F1 mice was first described by Diwan et al. (12) and was soon confirmed by Pereira et al. (13). In both cases, male B6C3F1 mice were initiated with DEN on the 15th day after birth, and the PB treatment was started only 1 week after weaning. To explain the inhibitory effect of PB, Diwan et al. (12) pointed to the testicular atrophy that can be caused by this agent in sexually immature animals (12). Because androgens are known hepatopromoters in mice (23), Diwan et al. (12) proposed that a reduction in serum levels due to testicular dysfunction could result in the inhibition of hepatocarcinogenesis. On the other hand, Pereira et al. (13) suggested that an age-dependent influence of PB on hepatic hypertrophy could be of relevance. In their experiment, pronounced hepatic hypertrophy was observed in DEN-untreated B6C3F1 mice continuously fed with PB starting at 11 weeks of age, whereas the hypertrophic change was only marginal in mice given PB from 4 weeks of age. Pereira et al. (13) considered that the earlier application of PB might imprint resistance to its biological effects on the juvenile mice. In any event, both Diwan et al. (12) and Pereira et al. (13) considered the inhibitory effect to depend on the timing of the PB application.

Our main purpose in the present study was to test their hypothesis. In fact, the data provide clear confounding evidence, because the inhibitory effect was observed even when PB administration was from 24 weeks of age. Rather, our data indicate that the timing of initiation with DEN is a critical determinant for the paradoxical effect of PB.

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Mean PCNA labeling (PCNA) and apoptotic (Apo) indices for 5 normal livers and 25 basophilic hepatocellular adenomas for each group. * significantly different from the corresponding value for the PB− group ($P < 0.01$). ** significantly different from the corresponding values for the PB+ and PB+/− group ($P < 0.01$). Error bars, SE.

Our recent study revealed that initiated mouse hepatocellular lesions are qualitatively heterogeneous in terms of expression of the Bcl-2 oncoprotein (6). When male B6C3F1 mice were treated with DEN alone at 12 days or 5 weeks of age, more than 90% of the lesions were histologically positive for Bcl-2 with cytoplasmic basophilia, independent of the timing of the carcinogen injection. In contrast, in mice injected with DEN at 5 weeks of age and then continuously fed PB, nearly 90% of the lesions were Bcl-2− with cytoplasmic eosinophilia. Concomitant with this PB-dependent phenomenon, tumor multiplicity was increased by about 5-fold relative to that of the PB-untreated controls. Thus, PB may confer a selective growth advantage on the Bcl-2− lesions, which leads to the gross promotion of hepatocarcinogenesis. It is unlikely that PB converts Bcl-2+ lesions into Bcl-2− ones, because Pedrick et al. (24) reported that the PB-promoted eosinophilic tumor cells exhibit a distinct phenotype from their basophilic counterparts, even in tissue culture. The basophilic tumors were also demonstrated to contain an activated H-ras oncogene that the eosinophilic tumors lack (25).

In the present case of mice initiated with DEN at 12 days of age, we found that only less than 4% of the lesions were Bcl-2−, even with chronic PB administration. This implies that DEN almost exclusively induced Bcl-2+ initiated hepatocytes in infant B6C3F1 mice, unlike in the adult. It was also revealed that PB suppressed the proliferation of Bcl-2+ tumor cells. Thus, the decrease in tumor multiplicity is best explained by the paucity of Bcl-2+ initiated lesions and the unexpected growth-inhibitory effect of PB on the Bcl-2+ lesions. Occasional growth-inhibitory effects of PB on initiated hepatocytes have been also reported for rats (26–28). Intriguingly, the few Bcl-2− lesions observed in this study were indeed promoted by PB, suggesting a qualitative equivalence to those induced in adults. It is also conceivable that the Bcl-2+ basophilic lesions initiated in adult B6C3F1 mice respond negatively to PB, because they are histologically indistinguishable from the basophilic lesions initiated in the infant mice (6).

Hepatocytes in perinatal rodent livers are actively proliferating, but after weaning, the proliferation markedly slows down and is negligible within a few weeks (29). Thus, infant hepatocytes as targets for initiation are less differentiated than adult hepatocytes. We propose that this qualitative difference between infant and adult hepatocytes may be responsible for the age-dependent nature of initiation.

Using an *in vitro* model of mouse hepatocarcinogenesis, we recently demonstrated that PB induces apoptosis in mouse hepatocytes, depending on deregulated c-myc expression (15). We speculated that the inhibitory effect of PB on *in vivo* mouse hepatocarcinogenesis might also involve the induction of apoptosis in c-myc-expressing hepatocytes, because overexpression of this oncogene is common in mouse liver tumors (30). However, in the present study, we observed that PB did not significantly affect the apoptosis of tumor cells. Consequently, PB induction of apoptosis might have no relevance to *in vivo* hepatocarcinogenesis. Even if PB had *in vivo* potential as an apoptosis inducer, its effect might be masked by the Bcl-2 protein, an antiapoptotic agent (9–11), expressed in most tumors.

Our results, in conjunction with those from earlier studies, indicate...
that the classical two-stage concept of carcinogenesis (31) regarding DEN initiation and PB promotion of B6C3F1 mouse livers may only apply to Bcl-2-initiated lesions, because at least some Bcl-2+ lesions were growth-inhibited by PB. In contrast, Ward et al. (3) found that di(2-ethylhexyl)phthalate selectively promotes basophilic lesions in B6C3F1 mice. Therefore, the promoting function of a compound seemed to depend largely on the type of initiated cell. A similar phenomenon is also known for rat hepatocarcinogenesis. Thus, preneoplastic rat hepatocytes induced by X-ray irradiation are reported not to respond to PB promotion (32), in contrast to their chemical carcinogen-initiated counterparts (1, 2). In addition, rat liver tumors promoted by peroxisome proliferators have been shown to be qualitatively different from those promoted by PB (33). Such cell-type dependence should be taken into account, particularly when using B6C3F1 mouse livers as materials for the in vivo assessment of hazard potential, because the results may be extrapolatable to human cancer risk (17).

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