RESEARCH COMMUNICATION

Lack of Promoting Effects of Phenobarbital at Low Dose on Diethylnitrosamine-induced Hepatocarcinogenesis in TGF-α Transgenic Mice

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Abstract

Phenobarbital (PB), a rodent non-genotoxic carcinogen, showed hormesis, biphasic effects on rat liver carcinogenesis. To test the hypothesis that the hormesis earlier observed for PB induced hepatocarcinogenesis might also exist in the TGF-α transgenic mice model, one which is highly susceptible to carcinogenesis, the carcinogenic or promotion effects of a wide range of phenobarbital (PB) concentrations were investigated. Two weeks after a single i.p. dose of 5 mg /kg bw of diethylnitrosamine (DEN) to 15 day old mice, animals were treated with diet containing PB at doses of 0, 2, 15 or 500 ppm. The incidence and multiplicity of tumors, including hepatocellular adenomas and carcinomas, were significantly increased by the high dose of PB, but no significant difference among the groups receiving 2 and 15 ppm for liver tumors when compared to DEN alone group. The proliferating cell nuclear antigen indices for liver tumors and surrounding hepatocytes in high dose PB treated mice were significantly increased, but no change was noted at the lower doses. The total cytochrome P450 content in the liver was also elevated by 500 ppm of PB, while hepatic 8-OHdG levels demonstrated no significant change. In conclusion, PB at high dose enhances DEN-induced hepatocarcinogenesis in TGF-α transgenic mice, but low doses lack any significant effects. One possible mechanism of phenobarbital carcinogenicity might be influenced by cytochrome P450 system exhibiting a strong promoting activity for liver of mice.

Key Words: Cancer risk assessment - hepatocarcinogenesis - phenobarbital - TGFα - transgenic mouse model

Asian Pacific J Cancer Prev, **7**, 274-278

Introduction

Carcinogenicity of chemicals, whether genotoxic or non-genotoxic, is determined after chronic exposure to rats or mice. Phenobarbital (PB), a sedative and anti-epileptic drug widely used in the clinical treatment, is a non-genotoxic carcinogen in rodents that lacks mutagenicity in a variety of tests. Long-term treatment with a high dose of PB results in hepatocarcinogenicity in rats and mice (Kaufmann et al., 1988; Feldman et al., 1981) and PB has been used frequently as a model liver tumor promoter in rodents. The carcinogenic effects of PB are generally considered to depend on enhancing growth of spontaneously initiated hepatocytes, with reduction of the apoptotic rate (Ward and Henneman, 1990; Schulte-Herman et al., 1983). Different from genotoxic carcinogens, non-genotoxic agents like PB do not direct interact with DNA, so that a no-observed effect level may exist regarding their carcinogenic activity, with non-linear dose-response curves at low doses. A number of dose-response studies of promoting activity of PB have been performed (Kolaja et al., 1996; Goldworthy et al., 1984; Kitagawa et al., 1984) and our own data indicate that doses of PB in the range from 60-500 ppm significantly increase numbers and areas of glutathione *S*-transferase placental form (GST-P) positive foci, considered as preneoplastic lesions of rat liver, but that doses of 15-30 ppm do not show any effects in a well-established liver medium-term carcinogenicity bioassay (Ito test) (Kitano et al., 1998). Indeed 1-2 ppm of PB even significantly suppressed the development of GST-P positive foci and chronic exposure to 2 ppm of PB significantly inhibited liver tumor formation in male F344 rats initiated with a liver carcinogen, diethylnitrosamine (DEN), while 500 ppm caused significant promotion, in line with hormesis (Kinoshita et al., 2003).

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Mitogenic signals might be a crucial mechanism for development of cancer and transforming growth factor alpha (TGF-α), a potent mitogen for hepatocytes which plays a role in cell cycle progression and is transiently increased in regenerative processes (Webber et al., 1994), might be important in the liver. Transgenic technology allowed TGF-α MT42 transgenic mice to be produced by Jhappan and his group (1990), and these have proved to be extremely sensitive to DEN and PB, with overexpressed TGF-α acting as a co-carcinogen (Takagi et al., 1993). To test the hypothesis that the hormesis earlier observed for PB hepatocarcinogenesis (Fukushima et al., 2005; Kinoshita et al., 2003) might also exist in this highly susceptible TGF-α transgenic mice model, the present investigation was performed, focusing on tumor yield, proliferation status, P450 expression and 8-OHdG formation.

Materials and Methods

Chemicals

PB sodium salt (CAS no.57-30-7)(purity > 98%) was purchased from Wako Pure Chemical Industries (Osaka, Japan) and DEN from Tokyo Kasei Kogyo (Tokyo, Japan).

Animals and experimental protocol

Transgenic mice harboring the mouse metallothionein promoter-human TGF-α fusion gene (MT42) were developed using outbred CD-1 mice (Jhappen et al, 1990) and kindly provided by Dr. Glenn Merlino (Laboratory of Molecular Biology, National Cancer Institute, NIH, USA). All mice were housed in plastic cages with paper chips for bedding and had free access to water and MF pellet diet (Oriental Yeast, Co. Tokyo, Japan) under environmental conditions controlled for humidity (55 ± 5%), lighting (12 hour light/dark cycle), and temperature (25 ± 10°C). The animals were observed daily for abnormalities, and body weights were recorded once a week.

Male TGF-α transgenic mice line MT42 were intraperitoneally injected with 5 mg/kg bw of DEN at 15 days of age and then were randomly divided into 4 groups (20, 20, 6 and 6 mice for groups 1, 2, 3 and 4, respectively). Starting one week after DEN initiation, diet containing various concentrations of PB, 0 (group 1), 2 (group 2), 15 (group 3), and 500 (group 4) ppm, was fed for 20 weeks. Mice were then sacrificed under diethylether anesthesia and whole livers were immediately removed and weighed. Each lobe was fixed in 10% buffered formalin and embedded in paraffin for routine histological and immunohistochemical analyses. The other part was frozen in liquid nitrogen and kept in –80°C until used.

Histological Examination and PCNA Immunohistochemistry

For routine histological analysis, 4 mm sections were prepared from routinely prepared paraffin blocks and stained with hematoxylin and eosin. All neoplastic changes were determined at the end of the experiment at 22 weeks of age. The incidences of histopathological changes including hepatocellular adenoma and hepatocellular carcinoma, were recorded. Multiplicity of lesions in each mouse was analyzed. For proliferating cell nuclear antigen (PCNA) staining, liver sections were deparaffinized and rehydrated, and then were microwaved at 98°C for 20 min for antigen retrieval. To quench endogenous peroxidase, tissue sections were placed in 3% hydrogen peroxide for 5 min. Sections were stained for PCNA with a mouse anti-PCNA monoclonal antibody (at 1:500 dilution) (DAKO, Glostrup, Denmark) and the avidin-biotin peroxidase complex (ABC) technique, using a mouse Vectastain ABC-PO kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine was applied for final color development. The number of PCNA positive cells per 3000 hepatocytes was randomly counted in neoplastic and surrounding areas showing normal histology to allow calculation of PCNA indices (%) (Kinoshita et al., 2003).

8-OHdG measurement

The level of 8-OHdG formation was measured by the method described previously by Nakae et al. (1995). Briefly, DNA was extracted from liver using a DNA Extractor WB kit (Wako Pure Chemical Industries Ltd., Osaka, Japan), containing NaI, deferoxamine mesylate (Sigma Chemical Co., St. Louis, MO) and RNase (Wako Pure Chemical Industries Ltd., Osaka, Japan). After DNA was digested to nucleosides by combined treatment with nuclease P1 (Yamasa Shoyu Co. Ltd., Chiba, Japan) and alkaline phosphatase (Sigma Chemical Co., St. Louis, MO), samples were filtered using an Ultrafree-MC filter unit 100000 (Millipore Co., Bedford, MA). The levels of 8-OHdG of each sample were quantified by high-performance liquid chromatography (HPLC) with electrochemical detection by a modification of the method of Kasai et al. (1997).

Total P450 content

Liver homogenates were prepared in ice-cold 1.15 % KCl buffer pH 7.4 containing 1 mM EDTA and 0.25 mM phenylmethylsulfonyl fluoride using a Teflon-glass homogenizer. Microsomal fractions were prepared by differential centrifugation and P450 contents were measured by the method of Omura and Sato (Omura T. and Sato R., 1964). Protein concentrations were determined by Lowry method using BSA as the standard.

Statistical Analysis

The statistical significance of differences between groups for each parameter was analyzed by one-factor ANOVA test using Super ANOVA software (Abacus Concepts, Berkeley,

Table 1. Final Body and Liver Weights

Group	Treatment	No of mice	Final body weight (g)	Liver weight Absolute (g)	Liver weight Relative (%)
1	DEN alone	15	36.5±5.8	1.7±0.6	4.7±0.6
2	DENPB 2 ppm	17	39.0±2.5	1.9±0.4	4.9±0.3
3	DENPB 15 ppm	4	40.3±0.4	1.9±0.3	4.7±0.4
4	DENPB 500 ppm	5	39.2±1.1	3.1±1.1*	8.0±1.1*

*: P < 0.01 compared with the DEN alone group

Table 2. Incidence and Multiplicity Data for Liver Tumors in Human TGF-α Transgenic Mice

Treatment	No of mice	Total tumors			Hepatocellular Adenomas			Hepatocellular Carcinomas		
		Incidence (%)	Multiplicity (no/mouse)	Multiplicity (no/ cm2)	Incidence (%)	Multiplicity (no/mouse)	Multiplicity (no/ cm2)	Incidence (%)	Multiplicity (no/mouse)	Multiplicity (no/ cm2)
DEN+PB 0	15	6/15 (40)	0.53±0.74	0.75±1.01	6/15 (40)	0.53±0.74	0.75±1.01	0/15 (0)	0±0	0±0
DEN+PB 2	17	5/17 (29)	0.53±0.87	0.57±0.94	2/17 (12)	0.18±0.53	0.22±0.64	3/17 (18)	0.35±0.79	0.35±0.80
DEN+PB 15	4	1/4 (25)	0.25±0.50	0.26±0.93	1/4 (25)	0.25±0.50	0.26±0.83	0/4 (0)	0±0	0±0
DEN+PB 500	5	4/5 (80)*	4.00±3.16*	1.94±1.58*	4/5 (80)*	3.20±3.27*	1.59±1.67*	2/5 (40)*	0.80±1.30*	0.34±0.55*

*: P < 0.01 compared with DEN alone group

CA). The Dunnett test was applied as a post-hoc test. Significance was concluded at p<0.05.

Results

The body weights, food consumption and water intake did not differ among the groups throughout the experimental period (data not shown), and in turn there were no inter-group differences in terms of final body weights (Table 1). Absolute and relative liver weights were significantly elevated in the 500 ppm of PB treated group (group 4) compared with the control values (group 1) (Table1), due to large numbers of coalescing liver tumors and also centrilobular hypertrophy of hepatocytes.

Hepatocellular adenomas and/or carcinomas were observed in all groups of DEN initiated TGF-α transgenic mice (see Table 2 for incidence and multiplicity data). Quantitative data were significantly increased in TGF-α transgenic mice treated with 500 ppm of PB, while 2 and 15 ppm PB treatment tended to reduce lesion development when compared to the DEN alone group. No statistically significant differences were seen because of limited animal number in 15 ppm treated group.

PCNA labeling indices of hepatocytes in background parenchyma and liver tumors in TGF-α transgenic mice are given in Table 3. Values were consistently higher in the liver tumors than in normal appearing hepatocytes. The PCNA indices in high dose PB treated mice were significantly increased, but no change was evident at low doses. Results for total P450 content and 8-OHdG in livers are shown in Table 4. While values for total P450 content were significantly increased in the high dose PB treated group, there was no effect at low doses, and 8-OHdG formation did not differ among the groups.

Table 3. PCNA Labeling Indices for Tumorous and Non-tumorous Liver in Human TGF-α Transgenic Mice

Treatment	Number of mice	PCNA labeling Index (%) Non-tumor site	Tumor site
DEN alone	14	0.07±0.06 (14)	3.39±2.56 (6)
DEN+PB 2 ppm	17	0.10±0.09 (17)	6.57±3.10 (5)
DEN+PB 15 ppm	4	0.14±0.13 (4)	3.20 (1)
DEN+PB500 ppm	5	1.17±0.65 (5)*	10.60±3.18 (4)*

( ) number of mice; *P < 0.01 compared with DEN alone

Table 4. P450 Content and 8-OHdG formation in Livers of Human TGF-α transgenic Mice

Treatment	Number of mice	P450 content (nmol/mg protein)	8-OHdG (adducts/105dG)
DEN alone	14	0.159±0.03	0.524±0.03
DEN+PB 2 ppm	17	0.151±0.03	0.511±0.04
DEN+PB 15 ppm	4	0.159±0.01	0.511±0.03
DEN+PB500 ppm	5	0.266±0.05*	0.527±0.07

( ) number of mice; *P < 0.01 compared with DEN alone

Discussion

Our study showed that only a high dose of PB significantly increases liver tumor development in DEN initiated TGF-α transgenic, with no promoting effects at low doses and even a tendency for inhibitory influence, in line with earlier findings in non-transgenic animals (Kitano et al., 1998). TGF-α positively regulates cell proliferation via binding to epidermal growth factor (EGF) receptors on the cell surface and is frequently expressed in human tumors, including hepatocellular carcinomas. It has also been found in glutathione S-transferase placental form positive foci in rats, with evidence of a role in progression of foci to neoplastic lesions (Kitano et al., 1998). Combination of TGF-α overexpression with carcinogen exposure yielded significantly higher incidences of hepatocellular carcinomas than did nitrosamine initiation (DEN or DMN) plus promoter (PB) treatment in CD-1 male mice (Takagi et al., 1993, Tamano et al., 1994). Transgenic mouse in which TGF-α is under the transcriptional control of the metallothionein promoter and is expressed at high levels in the liver and other tissues, spontaneously develop an approximately 75% incidence of liver tumors by 12-15 months of age (Jhappen et al., 1990; Takagi et al., 1993).

Various hypotheses exist as to the mechanism whereby nongenotoxic hepatocarcinogens induce hepatomegaly and/or histopathological changes and finally tumor formation (Butterworth et al., 1995; Kinoshita et al., 2003; Kushida et al., 2005; Puatanachokchai et al., 2005). Upon subchronic administration, many of these compounds either stimulate hepatocyte proliferation and/or act as liver enzyme inducers. Tumor formation in the human TGF-α transgenic mouse promoted by PB is presumably related to liver cell proliferation, and possibly xenobiotic metabolizing enzymes, and in the present study the PCNA labeling indices were only significantly increased with the 500 ppm dose of PB. From our results, TGF-α may act synergistically with PB to selectively stimulate cell proliferation within hepatic lesions.

We also demonstrated that the total P450 content displayed a similar dose-response pattern, in line with the suggestion of Diwans and his colleagues (1996) that induction of phase I and II metabolizing enzymes tightly correlates with hepatocellular carcinoma induction in rats. Thus, one possible mechanism of phenobarbital carcinogenicity in these transgenic mice might be influenced by cytochrome P450 system exhibiting a strong promoting activity in the liver.

Overproduction of reactive oxygen species (ROS) and oxidative DNA damage are thought to play major roles in cancer development (Valko et al., 2004). Burdon (1995) has suggested that ligand binding to cell surface receptors linked to tyrosine kinase activity can trigger signal transduction pathways leading to intracellular ROS generation. ROS can act as intra- and intercellular second messengers and thus modulate various aspects of cellular functions including proliferation, apoptosis and gene expression (Suzuki et al., 1997; Nakamura et al., 1997). Overexpression of TGF-α and c-myc genes in a mouse line was promoted by ROS production (Factor et al., 1998) and thus it might have been expected that high oxidative stress would be observed in the TGF-α transgenic mouse model. However, there was no remarkable increase of 8-OHdG levels with PB promotion in the present study.

In considering the relevance of rodent carcinogenicity to human risk assessment of carcinogenic hazard potential, major questions have been raised regarding the relevance of mouse liver tumors. PB is a rodent non-genotoxic hepatocarcinogen inducing hepatocellular carcinomas in rats and mice with high dose long-term administration, but long-term clinical exposure is not associated with any increase in liver tumors in humans (Oslen et al., 1995). Whether this is due to lower doses than can be given experimentally is unclear, but this and previous reports have shown that 1-15 ppm of phenobarbital does not increase liver preneoplastic lesions and tumor formation in rodents (Kinoshita et al., 2003; Kitano et al., 1998), providing evidence of a dose-threshold and even hormesis, with reduction in tumor development at low doses.

In conclusion, the present study revealed the administration of low doses of PB also lacks promoting effects on DEN induced hepatocarcinogenesis in a very sensitive human TGF-α transgenic mouse model.

Acknowledgements

The authors would like to thank Kaori Touma and Masayo Imanaka for technical support, and Mari Dokoh and Yuko Ohnishi for secretarial assistance. This study was supported by a Grant-in-Aid for Cancer Research from Ministry of Health, Labor and Welfare, Japan.

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