DOSE-DEPENDENT PROMOTING EFFECT OF PHENOBARBITAL ON RAT HEPATOCARCINOGENESIS

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Abstract: The dose-dependent promoting effect of phenobarbital (PB) on rat 2-stage hepatocarcinogenesis was investigated. Male F344 rats were given 600, 300, 150, 75 or 38 ppm PB solutions *ad libitum* as their drinking water for 39 weeks following initiation with a single intraperitoneal injection of diethylnitrosamine (DEN) (100 mg/kg). At week 40, the incidences of hepatic tumors were increased significantly in the DEN+PB groups given 300 ppm and 600 ppm PB, as compared to that in the DEN group. Linear dose-response curves for numbers and sizes of enzyme altered foci (γ -GTP or GST-P positive foci) were observed in all DEN+PB groups. Numbers and sizes of foci were increased even in the DEN+PB group given the lowest PB, as compared to that in the group given DEN only, while being not significant. Therefore, second experiment was done using a larger range of PB doses. Rats were given 1,200, 300, 75, 16, 4 or 1 ppm PB solutions in the same manner to the first experiment. The incidence of hepatic tumors was increased clearly in the DEN+PB groups given 300 ppm PB or above, as compared to that in the group given DEN only. Linear dose-response curves for numbers and sizes of enzyme-altered hepatic foci were obtained in the dose range 16 ppm–1,200 ppm PB, and numbers and/or sizes of enzyme-altered foci increased significantly in the DEN+PB groups given 300 ppm PB or above. The minimum promoting dose level of PB for enzyme-altered foci, calculated from dose-response curves by the Logit model, was 15–23 ppm. These results indicate that the calculated level was evident at low dose, while dose-dependence was demonstrated over a large range. The incidence of hepatic tumors, and numbers and sizes of foci decreased slightly in animals given the lowest dose (1 ppm) of PB, as compared to those in animals receiving DEN only. (J Toxicol Pathol 5: 47~53, 1992)

Key words: Promotion, Dose-dependent effect, Phenobarbital, Hepatocarcinogenesis, Rats

Introduction

Environmental chemicals have been suspected of being the most important cause of human cancers. For assessment of the risk of suspect agents, it is important that an understanding of quantitative aspects of carcinogenicity estimation is generated, based on dose-response studies in experimen-

tal animals. A number of methods have been used to determine the relative carcinogenic potencies of chemical carcinogens in rodents¹. However, almost all studies for quantitative estimation of chemical carcinogens have been performed using complete genotoxic carcinogens and few have attempted to quantitate the potency of promoters, although there are some dose-response studies of promoters using 2-stage hepatocarcinogenesis model²⁻¹⁰. In contrast to genotoxic carcinogens, promoters have been expected from a priori considerations to demonstrate threshold levels, below which no promoting effect is noted¹¹.

The present study was performed to assess the

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minimum effecting dose level of phenobarbital, a well-known promoter, for promotion on rat 2-stage hepatocarcinogenesis when very low levels of the promoter were given following initiation with diethylnitrosamine. A short report on part of this subject is to be published¹².

Materials and Methods

Animals

Male 4-week-old F344/DuCrj rats, purchased from Charles River Japan Inc. (Kana-gawa), were maintained on basal diet (CRF-1, Oriental Yeast Inc., Tokyo) and tap water until they were 6 weeks old, when the study was started.

Chemicals

N, N-Diethylnitrosamine (DEN) was obtained from Wako Pure Chemical Ind. (Osaka). Sodium phenobarbital (PB) was purchased from Ishiro Pharmaceutical Co. (Tokyo).

Experimental design

Experiment I; Rats were divided into 7 groups, each consisting of 20 animals. Rats of Groups 1 to 6 received intraperitoneally a single dose (100 mg/kg) of DEN. One week after this DEN injection, rats in Groups 1-5 were respectively given 600, 300, 150, 75 or 38 ppm PB solutions *ad libitum* as their drinking water for 39 weeks. In Group 6, tap water without PB supplement was provided *ad libitum* throughout. For Group 7, 600 ppm solution of PB was administered for 39 weeks, without prior DEN injection.

Experiment II; Figure 1 shows the experimental design. Rats were divided into 8 groups, each

consisting of 20 animals. Rats of Groups 1 to 7 were injected intraperitoneally a single dose (100 mg/kg) of DEN. One week after DEN injection, rats in Groups 1 to 6 were given 1,200, 300, 75, 16, 4 or 1 ppm PB solutions in the same manner to Experiment I. In Group 7, tap water without PB supplement was provided. Animals in Group 8 were given 1,200 ppm PB solution, without prior DEN injection.

In both experiments, animals were maintained in an air-conditioned animal room ($24 \pm 1^\circ\text{C}$, $55 \pm 5\%$ relative humidity). Throughout the experiments, rats in all groups were given basal diet *ad libitum*. They were observed daily and clinical signs were recorded. At experimental week 40, all animals in both experiments were killed and autopsied. The livers and other main organs were removed and fixed with 10% buffered formalin, sectioned and stained with hematoxylin and eosin for histological observation. Part of the liver was also fixed in ice-cold acetone for immunohistochemical staining of γ -glutamyl transpeptidase (γ -GTP) or glutathion S-transferase (placental form) (GST-P), which are known to be suitable enzyme markers for preneoplastic changes in rat hepatocarcinogenesis¹³. In the livers of 5-20 animals in each group (Experiment I) and 5 animals in each group (Experiment II) quantitation of enzyme-altered hepatic foci was performed by counting the numbers and measuring the areas/cm² of γ -GTP or GST-P positive foci greater than 0.2 mm in diameter with the aid of a Video Image Processor (TAS-Plus, Leitz).

Results

Experiment I

All rats in each group survived to the end of the experiment. At week 40, the incidence of hepatic tumors was increased significantly in the DEN+PB groups given 300 ppm (11/20, 55%) and 600 ppm (11/20, 55%) PB, as compared to that in the DEN group (1/20, 5%). It was also increased in the DEN+PB groups given 75 ppm (5/20, 25%) and 150 ppm (6/20, 30%) PB, while being not significant. Figure 2 illustrate data for numbers and areas of enzyme-altered foci/cm² in each group. Numbers and areas of enzyme altered foci

Fig. 1. Experimental design (Experiment II).

Fig. 2. Dose-effect relation of PB to induction of enzyme-altered foci (Experiment I).

(γ -GTP and/or GST-P) increased dose-dependently in all DEN + PB groups, as compared to the DEN alone group.

Experiment II

Growth curve, liver weight and hepatic tumor incidence

All of the rats in each group survived to the end of the experiment. At week 40, significant suppression of body-weight gain was observed in the DEN + 1200 ppm PB group (Group 1), as compared to the DEN group (Group 7). Figure 3 shows data for liver weights and hepatic tumor (hepatocellular adenoma/carcinoma) incidences in each group. The absolute and relative liver

weights were increased dose-dependently in the DEN + PB groups given 75 ppm PB or above (Groups 1-3). As compared to the DEN alone group values, the liver weights were also significantly increased in the 1,200 PB group. The incidence of hepatic tumors was the highest in Group 1 (95%), followed by Group 2 (65%). The incidences in the DEN + 75 or 16 ppm PB (Groups 3 or 4) and in the DEN + 4 or 1 ppm PB (Groups 5 or 6) were 10 and 5%, respectively. In the DEN alone group (Group 7), it was 15%. No hepatic tumors were found in Group 8.

Numbers and areas of enzyme-altered hepatic foci

Figures 4 and 5 illustrate data for numbers and areas of enzyme-altered foci/cm² in each

Fig. 3. Tumor incidence and liver weight (Experiment II).

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Fig. 4. Dose-effect relation of PB to induction of γ-GTP positive foci (Experiment II).

Fig. 5. Dose-effect relation of PB to induction of GST-P positive foci (Experiment II).

group. Numbers of γ-GTP positive foci increased dose-dependently in the DEN+PB groups given 75 ppm PB or above, as compared to the DEN group level. However, there were no significant differences in numbers of foci between the DEN+PB groups given 16 ppm PB or less and the DEN group. A similar tendency was observed for areas of γ-GTP positive foci. With regard to numbers and areas of GST-P positive foci/cm², absolute values were generally larger than those obtained for γ-GTP positive foci, but there were no essential differences in the results for dose-dependency. While numbers and areas of γ-GTP or GST-P positive foci in the DEN+1

ppm group were in both cases slightly lower than those in the DEN group, this was not significant.

In both experiments, hyperplasias/adenomas of the lung were also observed in all DEN-treated groups. No promoting effects of PB on induction of these lung lesions were detected. In other organs, no DEN- and/or PB-related proliferative lesions were observed.

Discussion

For assessment of the risk of genotoxic carcinogens, estimation of a relative potency index such as the so-called virtually safe dose (VSD) based on dose-response data in experimental animals has

been recently proposed¹⁴⁻¹⁶. We therefore have reported dose-response carcinogenicity studies of N-nitroso compounds in terms of VSD values^{17,18}. Until now, however, there have been few attempts at quantitative evaluation of promoters. In contrast to genotoxic carcinogens, promoters have been considered to demonstrate threshold levels below which no promoting effect is noted¹¹.

In the present study, the dose-dependence of promoting activity of phenobarbital was therefore examined with regard to both the incidence of hepatic tumors and the numbers or areas of enzyme-altered foci. In both experiments, development of hepatic tumors, especially hepatocellular carcinomas was increased clearly in the DEN+PB groups given 300 ppm PB or above. Pereira *et al.*⁷ earlier reported that the incidence of DEN-initiated hepatocellular carcinomas was enhanced by administration of 250, 500 and 1000 ppm PB during the promotion stage but not by 62.5 or 125 ppm PB. Our results are generally in good agreement. In Experiment I, the incidence of hepatic tumors increased slightly in the DEN+75 ppm PB group and two of 5 hepatic tumors were hepatocellular carcinomas, although no carcinomas were observed in the DEN+38 ppm PB group (1 adenoma) and the DEN alone group (1 adenoma). In Experiment II also, a shift from benign towards malignant lesions was observed in the DEN+75 ppm PB group (1 adenoma and 1 carcinoma) while no significant difference was found in the incidence of hepatic tumors between the DEN+75 ppm PB and the DEN alone groups, and no carcinomas were found in the DEN+PB groups given 16 ppm PB or less and the DEN group. Therefore, the 75 ppm PB might have exerted a promoting effect in terms of morphology. Previously, Peraino *et al.*² performed a dose-response study of the promoting effects of PB on acetylaminofluorene-initiated rat hepatocarcinogenesis and found an increase in hepatic tumors with 100 ppm PB but not 20 ppm PB. These results may indicate that the minimum effective dose of PB for enhanced development of hepatic tumors is about 75-100 ppm.

In both experiment, clear dose-dependent increases in numbers and areas of γ -GTP or GST-P positive foci were also observed in the DEN+

PB groups given 75 ppm PB or above. In Experiment II, however, there were no significant differences in quantitative data for enzyme-altered foci between the DEN+PB groups given 16 ppm PB or less and the DEN group, while slight increase in numbers and areas of enzyme altered foci was observed in the DEN+38 ppm PB group of Experiment I. These results further indicate that the minimum dose of PB required for promoting effects on hepatocarcinogenesis may be in the range of 16-38 ppm, when numbers and/or areas of enzyme-altered foci are used as indicators. To assess more precisely the minimum promoting dose level we adopted the Logit model for analysis of the present dose-response data for enzyme-altered foci in Experiment II. The estimated minimum promoting dose level of PB was 15-23 ppm. Previously, Kitagawa *et al.*⁴ reported promoting effects of PB on enzyme-altered foci in the range of 10-500 ppm. Goldsworthy *et al.*⁵ noted no promotion for PB below 10 ppm with regard to the numbers of foci. These results may indicate that the dose level calculated in the present study is the critical dose for promoting effect of PB. The use of mathematical models for the calculation of minimum effective dose of promoters may be useful, although the question remains of which model should be selected. Quite recently, however, Appel *et al.*¹⁹ reported that the promoting effects of PB depend on the actual concentration of the compound and the duration of treatment rather than on the total dose administered. In mice, strain differences in hepatic tumor promotion by PB have been pointed out²⁰. Therefore, whether a suitable model is used or not, the minimum effect of PB for promotion may be different in the experimental designs, and further studies will be needed on this point.

On the other hand, Goldsworthy *et al.*⁵ reported a linear increase in the numbers of enzyme-altered foci in rats initiated by a single intragastric administration (10 mg/kg) of DEN, when rats were chronically fed doses of PB ranging from 10 to 500 ppm in the diet, whereas at dietary concentrations of more than 500 ppm no extra increment was observed. The maximal effect of phenobarbital may support the concept that the initiating agent induces a finite number of altered cells and

that the promoting agent do not recruit further clones of altered cells. In our present study (Experiment II), however, such a maximum effect level was not apparent. The reason for this discrepancy is unclear. In the present study, however, the dose of DEN administered was ten times as large as that in Goldsworthy *et al.*'s case. Therefore, numbers of initiated cells might be much in our study than the Goldsworthy *et al.*'s study.

In Experiment II, the finding that both the incidence of hepatic tumors, and also the numbers and areas of enzyme-altered foci decreased slightly in the DEN+1 ppm PB group, as compared to DEN alone group values, is of very interest since similar results were also reported for two-stage rat hepatocarcinogenesis by Pitot *et al.*¹⁰. These results indicate that low doses of PB below the minimum promoting dose level may in fact exert a protective effect. This aspect may be of real importance because exposure of human beings to environmental promoters is practically always at very low levels. Kitagawa *et al.*⁴ described inhibitory effects of promoters on hepatocarcinogenesis when they were given together with carcinogens, and it has been considered that induction of elevated activity of drug metabolizing system by such agents would increase detoxication potential. In the present study, however, PB was given only during the promotion stage following initiation by DEN, and thus the reason for the inhibitory effect on appearance or growth of foci or tumors at doses of PB below the minimum effecting dose level for promotion is not clear.

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