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Decreased Diethylnitrosamine-induced Liver Preneoplastic Lesions by Estradiol-3-benzoate Treatment

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To clarify whether inhibitory effect of estrogen on liver tumor is associated with cell proliferation, we investigated its role in diethylnitrosamine (DEN)-induced rat preneoplastic lesions, with time sequenced manners. F344 male rats (n = 90) were divided into three groups at 5 weeks of age. The mini-osmotic pumps providing a continuous infusion of DEN was implanted into the abdominal cavity of each animal in group 1, 2 and 3 at 6 weeks of age. To see the effect of estrogen, pellet containing 1 or 10 μg of estradiol-3-benzoate (EB) was implanted subcutaneously in the animals of groups 2 or 3, respectively, one week prior to DEN treatment. Ten animals of each group were euthanized at 10, 14 and 18 weeks after DEN treatment. Liver tissues at each time point were fixed in 10% phosphate-buffered formalin and were processed and embedded in paraffin and 5 μm sections mounted on a silanized slide. Glutathione *S*-transferase placental form (GST-P) positive foci and 5-bromo-2-deoxyuridine (BrdU) labeling cells were detected at each time point. Area of GST-P positive foci in DEN+EB 1 or 10 μg group was significantly decreased compared to DEN alone at 14 weeks ($p < 0.01$ or $p < 0.05$, respectively) an at 18 weeks ($p < 0.05$ or $p < 0.01$, respectively). BrdU index in DEN+EB 1 or 10 μg groups was significantly decreased compared to DEN alone at 14 weeks and at 18 weeks ($p < 0.01$). Taken together, we conclude that EB treatment decrease the DEN-induced liver preneoplastic lesions and this may be associated with decrease of cellular proliferation.

***Key words*:** Liver carcinogenesis, Diethylnitrosamine (DEN), Estradiol-3-benzoate (EB), Glutathione *S*-transferase placental form (GST-P) positive foci, 5-bromo-2-deoxyuridine (BrdU)

INTRODUCTION

It has been accepted men show higher incidence of liver tumor than women (Bosch *et al.*, 1999), with men:women ratios usually averaging between 2 : 1 and 4 : 1 (El-Serag and Rudolph, 2007), probably associated with hormone imbalance and altered hormone metabolism (De Maria *et al.*, 2002). In experimental animals, male also show higher incidence of liver tumors than female in carcinogen-induced tumors as well as spontaneous ones (Kemp and Drinkwater, 1989). These evidences suggest that there may be a sex-differentiated difference, associated with sex hormones.

Among sex hormones, estrogens were associated with decreased incidence of hepatocellular carcinoma (Lindhe *et al.*, 1990; Nakatani *et al.*, 2001). Actually, our previous study reported that estrogen treatment inhibited diethylnitrosamine (DEN)-induced hepatic tumors associated alteration of ERα loss (Kang *et al.*, 2005). However, chronic use of estrogens was associated with an increased risk of developing liver tumors in humans and some synthetic estrogens might act as cancer promoting agents (Dragan *et al.*, 1995; Dragan *et al.*, 1991). And several human studies have reported an increased risk of developing malignant liver tumors as well as benign liver ones in women using oral contraceptives (El-Serag and Rudolph, 2007). For example, treatment of ethinyl estradiol induced promotion of hepatocarcinogenesis, possibly associated with liver cell turnover (Mayol *et al.*, 1991). These data indicate that treatment of some kind of estrogens may promote liver carcinogenesis associated with the cellular proliferation. So, in this study, we tried to define whether the action of estrogen was inhibi-

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tory or promotive on preneoplastic lesion, and investigate the effect of estrogen on cellular proliferation.

Diethylnitrosamine (DEN), widely used as a carcinogen in experimental animal model systems (IARC, 1978), is activated by CYP2E1 (Kang *et al.*, 2007; Yamazaki *et al.*, 1992), induces glutathione *S*-transferase placental form (GST-P) positive foci in rodents (Ito *et al.*, 1988). As it has been wildly considered that GST-P positive foci are preneoplastic lesions of the liver (Ito *et al.*, 2000; Sato, 1988; Tsuda *et al.*, 2003), we carried out to clarify the modifying effect of estrogen on rat DEN-induced GST-P positive foci and cell proliferation.

MATERIALS AND METHODS

Animals. Male F344 rats were supplied by the Department of Laboratory Animal Resources, National Institute of Food and Drug Safety Evaluation, Korea Food and Drug Administration. The animals were housed in polycarbonated cages with hardwood chips in a room with 12/12 h light/dark cycles and controlled humidity and temperature. They were allowed free access to normal water and pellet chow diets (CRF-1, Charles River Japan, Tokyo, Japan).

Experimental design and treatment. Rats (n = 90) were divided into three groups. To examine the role of estrogen on hepatocarcinogenesis, a tube containing 1 μg or 10 μg of estradiol-3-benzoate (EB) was implanted subcutaneously in all animals of groups 2 and 3, respectively, one week prior to DEN treatment under ether anesthesia. The tubes were replaced with new ones every 4 weeks. EB was pelleted in medical grade silastic tube (silicon medical tube no. 2, 100-2N; Kaneka Medix Corporation, Japan). The administration dose was prepared by mixing 0.132 or 1.32 mg of EB with 2 g of cholesterol, and 1.7 m*l* of olive oil. The total content of EB in each 1 cm tube was approximately estimated as 1 or 10 μg.

For the induction of liver tumors, mini-osmotic pumps (Alzet 2002; Durect, Cupertino, CA) providing a continuous infusion (0.5 μl/hour for 2 weeks) of DEN (Sigma, St Louis, MO) dissolved in dimethyl sulphoxide were used. The mini-osmotic pumps were inserted into the abdominal cavity of each animal to provide a total dose of 47.5 mg to each rat under ether anesthesia at 6 weeks of age.

Rats of group 1 were administered DEN alone, and animals of group 2 and 3 were received DEN and EB 1 μg or EB 10 μg, respectively. Ten animals of each group were sacrificed at 10, 14 and 18 weeks after DEN treatment, respectively. For examination of proliferation, BrdU (Sigma, St. Louis, MO) was injected at 1 h before sacrifice.

Body weights and organ weights. At necropsy, final body weights and organ weights of liver and testis were measured to observe the effects of EB treatment.

Immunohistochemical examination of GST-P and BrdU. Liver tissues were prepared for imminohistochemical staining. Briefly, samples were fixed in 10% phosphate-buffered formalin for 24 h and were routinely processed and embedded in paraffin and 5 μm sections mounted on a silanized slide (S3003; Dako, Denmark). The avidin-biotin complex method was used to demonstrate GST-P and BrdU in sections of liver tissue dewaxed with xylene and hydrated through a graded ethanol series. Sections were treated sequentially with 0.3% hydrogen peroxide, normal goat serum, rabbit anti-GST-P antibody (MBL, Japan) or rabbit anti-BrdU antibody (SC-2018, SantaCruz Biotechnology, USA), biotin-labeled goat anti-rabbit IgG and avidin-biotin-peroxidase compelx (ABC kit; Vector Laboratories, Burlingame, CA, USA). Immune complexes were visualized with 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. As a negative control, normal serum was used instead of primary antibodies. The sections were counterstained with Mayer's hematoxylin to facilitate examination under a light microscope.

Quantification of GST-P positive foci. Total areas of GST-P positive foci and of the entire liver sections were measured using a color image processor to allow calculation of the area (mm^2) per cm^2 of liver section. Data were the mean ± SD values for all samples per group.

Quantification of BrdU. Sections were analyzed blinded for counts of BrdU positive cells. Quantification of BrdU positive hepatocytes was performed by scoring over 1,000 cells from 10 random different fields from each animal at 400X magnification. The results were expressed as BrdU index relative to the total hepatocytes. Data were the mean ± SD values for all samples per group.

Statistical analysis. Statistical analysis of data for GST-P and BrdU were performed using Student's *t*-test. All analyses were performed using JMP program (SAS Institute, Cary, NC). For all comparisons, $p < 0.05$ was considered to be statistically significant.

RESULTS

Body weight and organ weights. Comparing with DEN alone, the body weight in DEN+EB 1 or 10 μg group was significantly decreased compared to DEN alone at 10, 14 and 18 weeks ($p < 0.05$). The liver weight in DEN+EB 1 mg group was significantly increased compared to DEN alone at 10, 14 and 18 weeks ($p < 0.05$), however, the liver weight in DEN+EB 10 μg group was significantly decreased compared to DEN alone at 14 weeks ($p < 0.05$). The testis weight in DEN+EB 10 μg group was significantly decreased compared to DEN alone at 10, 14 and 18 weeks ($p < 0.05$). These were presented in Table 1.

Table 1. Body weight and liver and testis weight

Weeks after DEN	Groups	No. of rats	Body weight (g)	Liver weight (g)	Testis weight (g)
10	DEN alone	10	266.97 ± 9.57[a]	2.86 ± 0.08	1.15 ± 0.06
	DEN + 1 μg EB	10	237.72 ± 13.60*	3.27 ± 0.26*	1.19 ± 0.05
	DEN + 10 μg EB	10	248.18 ± 6.34*	2.91 ± 0.08	1.08 ± 0.04*
14	DEN alone	10	289.16 ± 12.46	2.97 ± 0.09	1.10 ± 0.04
	DEN + 1 μg EB	10	257.67 ± 13.33*	3.10 ± 0.13*	1.13 ± 0.07
	DEN + 10 μg EB	10	257.16 ± 9.85*	2.80 ± 0.15*	0.51 ± 0.06*
18	DEN alone	10	319.69 ± 11.49	3.09 ± 0.03	1.07 ± 0.08
	DEN + 1 μg EB	10	269.99 ± 12.32*	3.43 ± 0.27*	1.16 ± 0.03
	DEN + 10 μg EB	10	273.47 ± 14.74*	2.99 ± 0.17	0.53 ± 0.07*

+Data represent mean ± SD.
*Significantly different from DEN alone at $p < 0.05$.

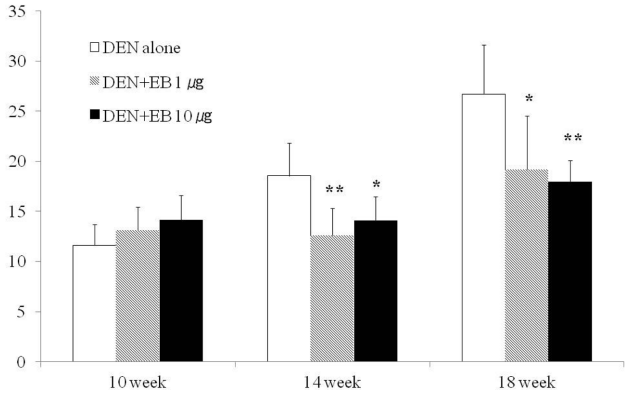

Fig. 1. Analysis of the area of glutathione *S*-transferase placental form (GST-P) positive foci. Note the significant decrease of area of GST-P positive foci in DEN+EB 1 or 10 mg groups compared to DEN alone at 14 weeks ($p < 0.01$ or $p < 0.05$, respectively) and at 18 weeks ($p < 0.05$ or $p < 0.01$, respectively).

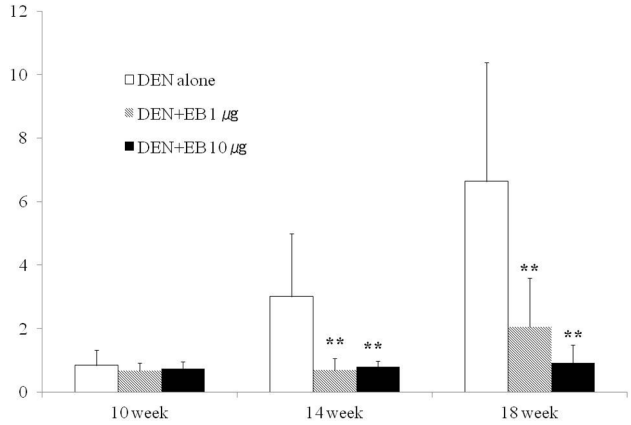

Fig. 2. Analysis of the area of 5-bromo-2-deoxyuridine (BrdU) index. Note the significant decrease of BrdU index in DEN+EB 1 or 10 μg groups compared to DEN alone at 14 weeks and at 18 weeks ($p < 0.01$).

Quantitative data for GST-P positive foci. Area of GST-P positive foci in DEN+EB 1 or 10 μg groups was not different from DEN alone group at 10 weeks after DEN treatment. However, Area of GST-P positive foci in DEN+EB 1 or 10 μg groups was significantly decreased compared to DEN alone at 14 weeks ($p < 0.01$ or $p < 0.05$, respectively) and at 18 weeks ($p < 0.05$ or $p < 0.01$, respectively) (Fig. 1).

Immunohistochemical examination and quantification of BrdU. BrdU index in DEN+EB 1 or 10 μg groups were not different from DEN alone group at 10 weeks after DEN treatment. However, BrdU index in DEN+EB 1 or 10 μg groups significantly decreased compared to DEN alone at 14 weeks and at 18 weeks ($p < 0.01$) (Fig. 2).

DISCUSSION

In this study, we used slow release model of DEN in rat for evaluating of EB effect on liver preneoplastic lesions. The slow release of a small amount of DEN through mini-osmotic pump for 2 weeks could induce liver tumors, without the use of tumor promoter, within 26 weeks after DEN treatment (Kang *et al.*, 2005). It suggested that DEN treatment induced mutated cells, which might not differentiate normal cells and proliferate in clonal expansion, resulting in tumors (Pitot *et al.*, 1996). The model we used in this study did not need promoters, therefore has an advantage to screening material having promotion potential, mainly focus on EB effect as tumor inhibitor or promoter.

Area of GST-P positive foci in DEN+EB 1 or 10 μg groups was significantly decreased at 14 weeks and at 18 weeks compared to DEN alone, however, not different at 10 weeks. These data showed that formation of liver preneoplastic lesions was increased by time sequence manner, and it was inhibited by estrogen treatment at 14 and 18 weeks after DEN treatment, confirming that this inhibition was started at 14 weeks after DEN treatment. And EB treatment inhibited hepatocytes proliferation at 14 and 18 weeks, not at 10 weeks after DEN treatment. It was coin-

cided with time points of inhibition of GST-P. So, our study strongly suggested that inhibition of GST-P was associated with inhibited of hepatocytes proliferation by EB treatment.

In some cases, treatment of some kind of estrogens may promote liver carcinogenesis. Firstly, it seems that this contrary effect may be related to types of estrogens. Treatment of ethynyl estradiol or tamoxifen induced hyperplastic nodules in liver (Shimomura *et al.*, 1992) and tamoxifen treatment was associated with a progressive increase in the number of GST-P positive foci in the livers of animals (Styles *et al.*, 2001). Secondary, this effect may be related to time points of estrogens treatment before or after carcinogen exposure. For example, indole-3-carbinol, one of phytoestrogens, showed inhibition of liver tumor when it was applied before or concurrent exposure of carcinogen (Dashwood *et al.*, 1989), associated with pathways of cell cycle arrest (Cover *et al.*, 1999; Cover *et al.*, 1998). However, in the medium-term liver bioassay, it exerted a promoting effect on post-initiation stage (Kim *et al.*, 1994). As EB treatment showed inhibition of GST-P positive foci at 14 and 18 weeks after carcinogen treatment in this study, we could not guarantee that EB treatment after carcinogen exposure may have same effect or not. Further studies will be required to investigate whether the post-initiation treatment of EB induce inhibition of preneoplastic lesions as well as liver tumors.

Comparing with DEN alone, the animals treated with estrogen had alterations of body weight and testis weight. The body weight and testis weight of animals treated with estrogen was decreased compared to DEN alone at each time point, implying that there might be hormonal modulation. Our previous report showed that there was a decrease of total testosterone and increase of estradiol in estrogen treatment groups (Kang *et al.*, 2005). As the serum testosterone/estradiol ratio and testosterone levels were important predictors for hepatocellular carcinoma development (Tanaka *et al.*, 2000), a decrease of testosterone and an increase of estradiol might be associated with liver preneoplastic lesions. Testis weight in animals treated with high dose EB was decreased compared to DEN alone at each time point. But, testis weight in animals treated with low dose EB was not different at each time point. Further studies will be required to investigate the relationship of GST-P with hormone modulation and testis weight. About liver weight, it was increased at low dose treatment of EB at all time points, and decreased at high dose treatment of EB at 14 weeks. As it did not show any dose or time dependency, it is thought that it had not biological meaning.

Taken together, we conclude that EB treatment has an inhibitory effect in DEN-induced hepatocarcinogenesis in F344 rats and this may be associated with decrease of cellular proliferation.

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