J Korean Med Sci 1999; 14: 171-4
ISSN 1011-8934

Correlation of Changes in Natural Killer Cell Activity and Glutathione S-Transferase Placental Form Positive Hepatocytes in Diethylnitrosamine-Induced Rat Hepatocarcinogenesis

To evaluate the induction of preneoplastic hepatic foci in relation to natural killer cell (NK) activity, we sequentially analyzed glutathione S-transferase placental form positive (GST-P$^+$) hepatocytes and NK activity during diethylnitrosamine (DEN) and phenobarbital (PB)-induced hepatocarcinogenesis in Sprague-Dawley rats. Previous studies have shown that NK activity can modulate the carcinogenic process induced by chemical carcinogens. Newborn females were initially given a single intraperitoneal injection of 15 mg DEN/kg and three weeks later, they were treated with 500 ppm phenobarbital (PB). From week 3, PB was administered in drinking water for 9 weeks. Interim and terminal sacrifices were performed at weeks 12, 15 and 30. GST-P$^+$ hepatocytes increased with age in DEN-treated rats, especially in the population of more than two GST-P$^+$ hepatocytes. The NK activity of DEN-treated rats did not significantly differ from that of control rats until week 12, but it progressively decreased from week 15 to 30. These results indicate that changes of NK activity inversely correlated with the induction of preneoplastic hepatic foci. This strong correlation of decreased NK activity with enhanced induction of GST-P$^+$ foci suggests that NK activity is important in the early progression of hepatocarcinogenesis in rats.

Key Words : *Diethylnitrosamine; Glutathione transferase; Carcinogenesis; Killer cell, natural; Rats*

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Received : 24 July 1998
Accepted : 9 December 1998

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INTRODUCTION

Natural killer (NK) cells were initially defined as a cell population capable of spontaneous cytotoxic activity against a range of both autologous and allogenic target cells. This phenomenon was discovered by chance during investigations examining the susceptibility of tumor target cells to lysis by a cell-mediated immune system. Experimental evidence supporting the in vivo anti-tumor activity of NK cells has been derived largely from murine studies of a variety of tumor models: transplanted or spontaneous tumors, and during chemical carcinogenesis (1, 2). NK cells are more likely to be important as a surveillance mechanism early in tumor cell development rather than having significant influence after a tumor has presented clinically (3). We previously reported that NK activity was closely related to the progression of carcinogenesis in rats (4). The role of NK cells during hepatocarcinogenesis, however, especially during the early stage of tumorigenesis, is not known.

The use of a sequential analytical approach has led to a progressive understanding of carcinogenesis in liver and in other tissues. Putatively preneoplastic enzyme-altered foci were considered to play a key role in the process of hepatocarcinogenesis in the rat (5). The placental form of glutathione S-transferase (GST-P) is known to be a useful marker for the different stages of hepatocarcinogenesis, including single initiated hepatocytes and focal nodular lesions (6). In the multistep concept of carcinogenesis, the essence of tumor promotion appears to be the propagation (clonal expansion) of initiated cells and their early progeny. Diethylnitrosamine (DEN) is a complete carcinogen that when administered at subcarcinogenic, non-necrogenic dose exhibits only an initiating action in rat liver. Studies on initiation in weaning and neonatal (7) rodents suggest that they may provide a more sensitive method for the induction of altered hepatic foci than older animals (8). Peraino et al. (9) reported that a single treatment of DEN within one day of birth, with dietary exposure to promoter beginning at weaning and continuing for nine weeks, was sufficient to induce preneoplastic hepatic foci in rats.

The purpose of this study was to investigate whether sequential changes in NK activity correlated with the induction of

preneoplastic GST-P$^+$ hepatocytes, and thus elucidate the role of NK activity during the early stage of hepatic tumorigenesis in rats.

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley rats (Division of Experimental Animal Care and Management, Korea Cancer Center Hospital) were obtained five days before parturition and were caged individually under constant temperature and a 12-hr light and 12-hr dark illumination cycle. All rats were fed solid pellets prescribed by NIH-7 open formula and water ad libitum.

Treatment

Within 24 hrs of birth, total 90 female newborn rats were divided into two groups. Group 1 was given 15 mg/kg body weight of diethylnitrosamine (DEN, Sigma Chemical Co., U.S.A.) intraperitoneally. After weaning, phenobarbital (PB, Luminal, Dae-Won Pharmaceutical Co., Korea) was administered in drinking water at a concentration of 500 ppm for 10 weeks. Group 2 was the untreated control group.

Histopathological and immunohistochemical examination

Ten rats from each group were sacrificed at 12, 15 and 30 weeks after birth. Body weights were recorded and livers removed, weighed and examined for grossly visible lesions. Two representative sections were cut from each liver lobe and fixed in ice-cold acetone; they were embedded in paraffin, and stained with hematoxylin and eosin for histopathological observation and GST-P immunohistochemistry. A rabbit polyclonal antibody to rat GST-P (MBL, Japan) was used at a dilution of 1:100, together with the Vectastain Rabbit Elite Kit (Vector Labs., Burlingame, CA).

Morphometric analysis

For GST-P immunohistochemistry, a total of four sections were examined morphometrically. Numbers were quantitated, and areas of each group of GST-P$^+$ hepatocytes were recorded as single, mini foci (2-19 GST-P$^+$ cells) or foci (20 or more GST-P$^+$ cells). Morphometry was performed using a video image analysis system (Kontron, Vidas, Carl Zeiss, Germany).

Assay of NK cell activity

At 12, 15 and 30 wk, five rats per group were sacrificed. The spleens were excised and minced into complete media consisting of RPMI 1640 (Gibco Lab., U.S.A.), 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum (FCS, Gibco Lab., U.S.A.). The lymphocytes were collected by the Ficoll-Hypaque (Sigma Chemical Co., U.S.A.) method, and all cells were resuspended in complete media to the desired concentration. NK cell cytotoxicity was tested by the ^{51}Cr release method, using YAC-1 tumor cells as targets. The effector cells were mixed with Na_2CrO_5-labelled target cells at a ratio 100:1 in triplicate. After 4 hr of incubation the supernatant was run with a gamma counter (Gamma 5500, Beckerman Instruments Inc., U.S.A.). Lytic unit (LU) was calculated from the dose response curve and linear regression analysis, and expressed per 10^6 effector cells. LU_{20} was defined as the number of effector cells which lyse 20% of target cells.

Statistical analysis

The significance of differences between the experimental groups and the corresponding controls was assessed by Student's t test.

RESULTS

Body weight and relative liver weight

No toxicity was found in DEN and PB-treated rats. Mean body weight was the same in control and treated rats. Relative liver weight, however, was significantly higher in the DEN-PB-treated groups than in the untreated control group ($P<0.05$) (Table 1).

Histological examination and quantitation of GST-P$^+$ hepatocytes

For each age of DEN and PB-treated rats, the distribution of GST-P$^+$ hepatocytes was mainly mini GST-P$^+$ foci. Single GST-P$^+$ hepatocytes decreased with age, while mini GST-P$^+$ foci and large foci, on the other hand, gradually increased (Table 1). From 15 weeks, hyperplastic nodules without cellular atypia was detected in the DEN and PB-treated group and at week 30, one case of hepatocellular carcinoma and hyperplastic nodules without cellular atypia were seen.

Activity of NK cells

From week 15, NK activity represented as LU_{20} was slightly less than in the untreated control group. At week 30, however, it was significantly lower in the DEN-treated group than in the untreated control group ($p<0.01$, Fig. 1).

Table 1. Body weight, liver weight and quantitative values of GST-P$^+$ hepatocytes

Groups	Week	Mean body weight (g)	Relative liver weight (mg/100 g b.w.)	GST-P$^+$ hepatocytes/cm^2 of liver section		
				S	M	F
Control	12	447.5±37.4	4.5±1.2	0	0.6±0.6	0
	15	471.2±33.5	3.5±0.7	0	0.8±0.7	0
	30	621.2±94.8	3.2±0.5	0	0.3±0.6	0
DEN + PB	12	496.9±39.9	5.6±0.8*	2.5±4.6	16.8±20.2	2.8±5.2
	15	502.3±47.0	4.5±0.6*	4.2±6.5	25.8±19.2	3.7±3.6
	30	653.0±55.9	4.3±0.5*	3.7±6.1	35.7±9.2	7.9±3.0

Values represent Mean ± SD, S: Single, single cells, M: Mini foci, 2-19 GST-P$^+$ cell, Large foci, 20 or more GST-P$^+$ cells, DEN: Diethylnitrosamine, PB: Phenobarbital, *Significantly different from the untreated control group at $p<0.05$.

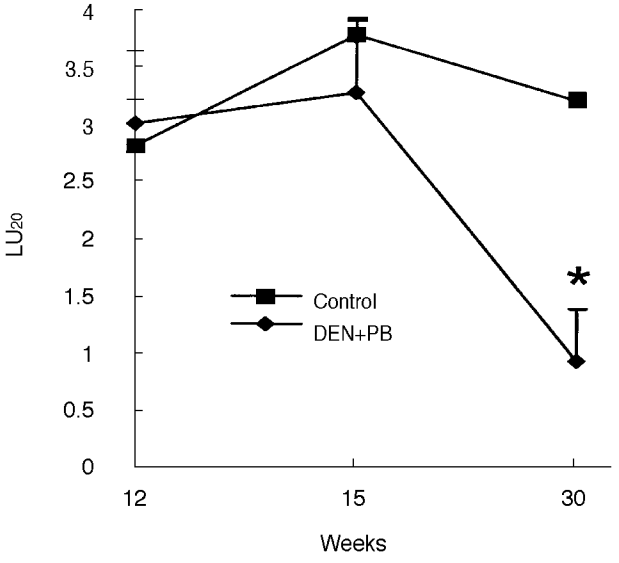

Fig. 1. Changes of NK activity during DEN-PB-induced hepatocarcinogenesis in rats. LU_{20}: Lytic unit was calculated from the dose response curve and linear regression analysis, and was expressed per 10^6 effector cells. LU_{20} was defined as the number of effector cells which lysed 20% of target cells. *Significantly different from the untreated control group at $p<0.01$.

DISCUSSION

The present data demonstrated that in DEN and PB-induced hepatocarcinogenesis, changes in NK activity inversely correlated with the development of preneoplastic hepatic foci.

NK cell activity was reported to vary according to the kinds and dosages of carcinogen treatment and the general tendency being for it to decrease (10). Our previous study showed that NK cell activity was greatly modulated by the progression of chemically induced carcinogenesis. In this study, NK cell activity slightly decreased during the initial stage after DEN treatment, and tended to correlate inversely with GST-P$^+$ foci induction. At week 30, NK cell activity was significantly lower than in the untreated control group, suggesting that immune survaillence was dramatically decreased at 30 wk after DEN treatment, while before 15 wk, host immune system could overcome. These phenomena suggest that NK activity may play a role in the development of early-stage hepatic tumors; the result could be explained by the fact that carcinogenesis inhibits the immune system such as NK cell activity.

Due to the nonspecific suppressive effects of large tumor burdens, a definitive role for NK cells in human malignancy has been more difficult to prove. Depressed NK activity has been reported in various malignant diseases. Patients with advanced stomach or breast cancer have a lower NK function than those in whom the disease remains confined to a local site (11, 12). NK defects encountered in malignant disease may directly influence the tumor itself or, alternatively, may represent a risk factor for the development of cancer.

Several rat liver foci bioassays have been developed, at first for the investigation of early steps in hepatocarcinogenesis, and later for the detection of chemical. According to current knowledge of liver carcinogenesis, DNA reactive hepatocarcinogens rapidly induce foci of altered liver cells with specific phenotypic characteristics, including abnormalities in enzymes, adenosine triphosphatase, glucose 6-phosphatase, gamma glutamyltranspeptidase, glutathione S-transferase placental form, glycogen accumulation, iron storage and other histochemical features. (13). The rat liver model system is diverse; a sensitive model may be obtained by using newborn rats (9). In immature rats the incidence of diploid nuclei in hepatocytes is higher than in adults (14). Diploid hepatocytes are preferentially a critical target for the action of an initiating agent (15) and these findings

could partially explain the high sensitivity observed in these age groups.

With regard to the time course of induction of positive cells over 24 wk, it has been reported that after a single dose of DEN, single GST-P$^+$ cells were induced after one week and developed into mini-foci and large lesions (16). Single cells also disappeared because of cell death; this induced irreversible damage, reversion to normal cells, or redifferentiation. These results show that the disappearance of single cells may be due to the development of mini foci because mini foci or large foci were increased when single cells disappeared, and this effect might be attributed to PB. Accordingly, the ability of PB to enhance the development of liver foci and neoplasms has been used to facilitate the study of neoplastic liver lesions (17).

In general, the number of foci induced by carcinogen exposure is predictive of the numbers of neoplasms with the same phenotype that evolve (18). Our results showed that by week 30, only one case of hepatocellular carcinoma had been induced. Dragan et al. (7) reported that at doses of above 30 mg DEN/kg, a complete carcinogenic effect observed, an initiating dose of 10 mg DEN/kg should therefore be the maximum dose used in the neonatal rat model of hepatocarcinogenesis. If the duration is extended or the dose of DEN is increased, the incidence of liver cancer will be higher.

In conclusion, strong correlation between decreased NK activity and enhanced induction of GST-P$^+$ foci suggests that in rats, NK activity is important for the development of early-stage hepatic tumorigenesis.

ACKNOWLEDGMENT

This study was supported in part by grants No. 02-96-342 from the Seoul National University Hospital Research Fund, and by Basic Medical Science Grant from the Ministry of Education, Republic of Korea. The authors thank Mr. Won-Suk Jang, Miss Sun-Ju Lee and Ms. Mi-Sook Lee for their excellent technical support.

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