Original

Susceptibility of Heterozygous p53 Deficient CBA Mice to Induction of Liver Proliferative Lesions by Phenobarbital after Dimethylnitrosamine Initiation

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Abstract: To investigate the susceptibility of heterozygous p53-deficient CBA mice [p53 (+/-) mice] to promotion of liver proliferative lesions in a two stage hepatocarcinogenesis model, 30 p53 (+/-) mice and 30 wild-type littermates [p53 (+/+) mice] received an i.p injection of 5 mg/kg of N-nitrosodimethylamine (DMN), and from one week later, each group was given free access to drinking water containing 0.05 or 0 % of phenobarbital (PB) for 26 weeks. The final body weights were significantly decreased in both p53 (+/-) and p53 (+/+) mice of the DMN+PB compared to the DMN alone groups and the liver weights were significantly increased. The survival rate was 67 and 73% in p53 (+/-) and p53 (+/+) mice of the DMN+PB group, respectively, and there were no deaths in any of the mice receiving DMN alone. The incidences of eosinophilic foci in the liver (90 and 54.6% in p53 (+/-) and p53 (+/+) mice, respectively) in the DMN+PB groups were significantly higher than those with DMN alone (6.7% in p53 (+/-) mice, 0% in p53 (+/+) mice). The incidences of clear cell foci and hepatocellular adenomas were 10.0 and 9.1%, and 60.0 and 27.3%, respectively, in p53 (+/-) and p53 (+/+) mice receiving DMN+PB. These lesions were not seen in the DMN alone group. The PCNA labeling indices for eosinophilic foci and hepatocellular adenomas in the DMN+PB group were significantly higher in p53 (+/-) than in p53 (+/+) mice. The present results suggest that p53 (+/-) CBA mice are very susceptible to promotion of the development of liver proliferative lesions by PB after DMN initiation. (*J Toxicol Pathol* 2001; **14**: 273–278)

Key words: hepatocarcinogenesis, susceptibility, p53 knockout mice, N-nitrosodimethylamine, phenobarbital

Introduction

Recently, in worldwide trials to investigate the utility of two stage carcinogenesis models, newborn mice and genetically altered mice have been utilized for short-term testing alternatives to the current mouse carcinogenicity studies.

In particular, since loss and mutations of p53 gene have been associated with various human malignant tumors¹, p53 knockout mice in which the p53 suppressor gene is inactivated have received much attention for detection of carcinogenicity with high sensitivity². Spontaneous tumors such as thymic lymphomas or sarcomas are reported to develop at an early age in homozygous p53 knockout mice [p53 (-/-) mice] with bilateral p53 alleles inactivated³, so that

it is difficult to perform evaluation studies because of the short survival. On the other hand, spontaneous tumors generally only develop after more than one year in p53 (+/-) mice in which only one allele of the p53 gene is inactivated. A great number of validation studies of p53 (+/-) mice have been conducted, demonstrating that these animals exhibit high susceptibility to genotoxic carcinogens compared with the current mouse models³. N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) has been documented to induce urinary bladder carcinomas in p53 (+/-) mice earlier and with greater malignancy, the number of the p53 alleles playing an important role in bladder tumorigenesis^{4,5}.

Currently, two different p53 knockout mice are available in Japan. One is the p53 (+/-) TSG mouse provided by Taconic Farms (Germantown, NY), derived from the C57BL6 strain with inactivation of exon 5 of one p53 allele, and the other is the p53 (+/-) CBA mouse, the F1 offspring of heterozygous p53 deficient C57BL/6J male mice with inactivation of exon 2 of one p53 allele, back-crossed with CBA females⁶. Administration of N-methyl-N-nitrosourea (MNU) at 30 ppm in drinking water to p53 (+/-) TSG mice produces tumors of the glandular stomach at high incidence⁷,

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while at 50 ppm in p53 (+/-) CBA mice no gastric but rather tumors of the liver and lung were the result⁸. In p53 (+/-) TSG mice, treatment with MNU or PB induced no liver tumors⁹.

Furthermore, N-nitrosodimethylamine (DMN) or N-nitrosodiethylamine (DEN) plus phenobarbital (PB) caused no liver tumors in a two stage hepatocarcinogenesis model using p53 (+/-) TSG male mice^{10,11}, and DMN treatment of newly born p53 (+/-) mice produced liver tumors at lower incidences than in p53 (+/+) mice¹². These observations indicate low susceptibility of p53 (+/-) TSG mice to chemical hepatocarcinogenesis. However, little is known regarding the susceptibility of p53 (+/-) CBA mice in this respect. Therefore, the present two stage hepatocarcinogenesis study was performed to investigate whether numbers of liver proliferative lesions are increased by treatment of female p53 (+/-) CBA mice with DMN+PB.

Materials and Methods

Thirty female p53 (+/-) CBA mice and the same number of wild-type female littermates p53 (+/+) mice were obtained from Oriental Yeast Inc. (Tokyo, Japan). Three to five mice were housed in a polycarbonated cage with white wood chips for bedding under standard conditions (room temperature, $23 \pm 2^\circ\text{C}$; relative humidity, $55 \pm 5\%$; 12 hr light and dark cycle) and given basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. N-Nitrosodimethylamine (DMN) and phenobarbital (PB) were obtained from Nacalai Tesque, Inc (Kyoto, Japan) and Iwaki Seiyaku, Inc. (Tokyo, Japan), respectively. All animals were initiated with a single intraperitoneal injection of DMN (5 mg/kg); one week later, the p53 (+/-) and p53 (+/+) mice were both divided into two groups of fifteen animals each and given drinking water containing 0.05 or 0% PB for 26 weeks. Basal diet was given to all animals during the experiment period. Mice that died were subjected to a complete necropsy as soon as they were found. All surviving animals were killed under ether anesthesia at week 26. Body weights were measured once a week during the experiment period. At sacrifice, after measuring the liver weights, a wide variety of organs and tissues including the liver were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sectioned at 4–5 μm , and stained with hematoxylin and eosin (H-E) for microscopic examination.

Immunohistochemical staining using a monoclonal antibody to proliferating cellular nuclear antigen (PCNA) (DAKO, Glostrup, Denmark) was performed at a dilution of 1: 100. Avidin-biotin peroxidase complex kits (DAKO) were applied for the performance of immunohistochemistry with 3,3'-diaminobenzidine as the chromogen and hematoxylin counterstaining. The numbers of PCNA positive cells per 100 cells in each proliferative lesion were counted from five different areas to give the PCNA labeling index (PCNA LI).

The incidences of liver proliferative lesions observed

were analyzed by the Fisher's exact test. Significant differences in particular lesion types were also assessed between the p53 (+/-) and p53 (+/+) mice of DMN+PB and DMN groups. Data for the PCNA LI and multiplicity were used to generate mean and standard deviation, significant differences between groups being analyzed by the Student's *t* test.

Results

Dead or moribund animals appeared from around weeks 3 and 7 of treatment with PB in the p53 (+/-) and p53 (+/+) mice receiving DMN+PB, their final survival rates being 67 and 73% (Fig. 1). However, there was no mortality with DMN alone. Most of the animals which died had thymic lymphomas characterized by solid white to white-yellow nodules. The body weight of p53 (+/-) and p53 (+/+) mice of the DMN+PB group tended to decrease through the study (Fig. 2), final values being 28.6 ± 2.0 and 29.3 ± 2.9 g in p53 (+/-) and p53 (+/+) mice of the DMN+PB group, respectively, significantly lower as compared than in the counterpart DMN alone group (32.3 ± 4.6 and 32.7 ± 3.8 g in p53 (+/-) and p53 (+/+) mice). The absolute and relative liver weights in p53 (+/-) and p53 (+/+) mice of the DMN+PB group were 1.82 ± 0.17 and 1.88 ± 0.17 g, and 0.639 ± 0.062 and 0.644 ± 0.066 g/10 g body weight, respectively, and they were significantly increased as compared with the relevant DMN alone cases (1.36 ± 0.17 and 1.31 ± 0.13 g, 0.424 ± 0.037 and 0.401 ± 0.029 g/10 g body weight, respectively) in p53 (+/-) and p53 (+/+) mice. Hepatocellular proliferative lesions induced were diagnosed as altered foci, consisting of enlarged eosinophilic cells (EF), small basophilic cells (BF) or glycogen-storing clear cells (CF), and hepatocellular adenomas (Ads) (Fig. 3). The latter were solid proliferative lesions consisting of hepatocytes with slight cellular atypia, associated with a sharp demarcation from the surrounding parenchyma and loss of the normal lobular architecture. The incidences

Fig. 1. Survival curves for p53 (+/-) or p53 (+/+) CBA mice treated with or without phenobarbital after dimethylnitrosamine initiation.

Fig. 2. Body weight curves for p53 (+/-) or p53 (+/+) CBA mice treated with or without phenobarbital after dimethylnitrosamine initiation.

Fig. 3. Photomicrographs of a hepatocellular eosinophilic cell focus (EF), and a hepatocellular adenoma (Ad) in p53 (+/-) CBA mice treated with phenobarbital after dimethylnitrosamine initiation.

(multiplicity) of EF were 90.0% ($2.1 \pm 2.4/\text{animal}$) and 54.6% (0.6 ± 0.7) in p53 (+/-) and p53 (+/+) mice of the DMN+PB group, respectively, with significant differences on the incidence between the two (Fig. 4, Table 1). With DMN alone EF were confined to p53 (+/-) mice, with an incidence of 6.7% (0.1 ± 0.3). BF were seen at 6.7% (0.1 ± 0.3) only in p53 (+/-) mice of the DMN alone group. CF were present only in the DMN+PB group, at incidences (multiplicity) of 10.0% ($0.1 \pm 0.3/\text{animal}$) and 9.1% (0.2 ± 0.5) in p53 (+/-) and p53 (+/+) mice, respectively. Ads were seen in the DMN+PB group, with incidences (multiplicity) of 60.0% (2.7 ± 4.5) and 27.3% (0.2 ± 0.4) in p53 (+/-) and p53 (+/+) mice, respectively, the differences being significant.

The PCNA LI for EF and Ads in p53 (+/-) mice of the DMN+PB group were significantly higher than in their counterparts in p53 (+/+) mice (Fig. 5).

Discussion

It has been reported that carcinogenesis and malignancy of proliferative lesions in humans is intimately linked to loss, mutation or instability of the p53 gene which plays a key role in control of the cell cycle and apoptosis^{1,13}. Recently, new testing approaches with newborn, two stage carcinogenesis and genetically altered animal models have been proposed at the International Conference on Harmonization (ICH) as alternatives to the current mouse carcinogenicity studies. With rapid progress in gene technology, specifically altered animals have attracted great attention with introduction of human oncogenes or inactivated suppressor oncogenes. At the present moment, the most widely available transgenic mice (Tg-mice) in Japan are rasH2, Tg/AC, and p53 (+/-) strains. Among these, p53 (+/-) mice have been validated extensively by the U.S. National Institute of Environmental Health Sciences, so that basic data have accumulated and further application to carcinogen research is to be expected. Responding to the validation studies in the USA, we are now also performing studies on p53 (+/-) CBA mice¹⁴. The life span spontaneous incidences of hepatocellular tumors in CBA mice are reported to be low¹⁵ and historically p53 (+/-) CBA mice reared in our laboratory demonstrated extremely few lesions at 6 months of age. In the present study, hepatocellular adenomas occurred not only at an incidence of 60% in p53 (+/-) mice, but eosinophilic foci considered as precursors were induced at the incidences of 90 and 54.6% in p53 (+/-) and p53 (+/+) mice, respectively. These data suggest high sensitivity to development of hepatocellular proliferative lesions with PB treatment after DMN initiation in CBA mice. DMN is known to induce mutations of hepatocytes^{3,16} and hepatocellular tumors¹⁷. Another hepatocarcinogen, flumequine, has been previously reported to induce hepatocellular foci/tumors in p53 (+/-) CBA mice²⁰, whereas other ones, diethylnitrosamine and MeIQx, do not affect hepatocarcinogenesis in p53 (+/-) TSG mice^{11,21}. Treatment of PB alone has also been described to induce liver tumors in

Fig. 4. Incidences of liver proliferative lesions in p53 (+/-) or p53 (+/+) CBA mice treated with or without phenobarbital after dimethylnitrosamine initiation.

Table 1. Multiplicity of Liver Proliferative Lesions in p53 (+/-) or p53 (+/+) CBA Mice Treated with or without Phenobarbital after Initiation of Dimethylnitrosamine

		p53 (+/-)	p53 (+/+)
Eosinophilic foci (EF)	DMN alone	0.07 ± 0.26 ^a	0 ^a
	DMN+PB	2.10 ± 2.42 ^b	0.64 ± 0.67 ^c
Basophilic foci (BF)	DMN alone	0.07 ± 0.26	0
	DMN+PB	0	0
Clear cell foci (CF)	DMN alone	0	0
	DMN+PB	0.10 ± 0.32	0.15 ± 0.52
Hepatocellular adenoma (Ad)	DMN alone	0	0
	DMN+PB	2.70 ± 4.52	0.15 ± 0.38

a: n=15, b: n=10, c: n=11

YellowAvy/A mice¹⁸, but at 500 or 1000 ppm for 26 weeks produced only hypertrophy of hepatocytes p53 (+/-) TSG mice⁹. Moreover, no liver tumors were noted in a two stage hepatocarcinogenesis experiment using DMN and PB in the same animals¹⁰. These data thus suggest low susceptibility of p53 (+/-) TSG mice and relatively high susceptibility of p53 (+/-) CBA mice to hepatocarcinogenesis, though the reasons remain unclear.

Cell proliferative activity in targeting organs was not found to be necessarily high when a variety of carcinogens were administered to p53 (+/-) mice, but chemicals targeting the urinary bladder or skin caused high cell proliferation^{5,19}. In the present study, the PCNA indices for eosinophilic cell foci and hepatocellular adenomas were higher in p53 (+/-) than in p53 (+/+) CBA mice. Thus the results of the present study indicate that p53 (+/-) CBA mice have high

Fig. 5. Frequencies of PCNA positive cells in eosinophilic foci and hepatocellular adenomas in p53 (+/-) or p53 (+/+) CBA mice treated with phenobarbital after dimethylnitrosamine initiation.

susceptibility to liver tumor induction under DMN-initiated condition and consequently utility for evaluating hepatocarcinogenic potential. Differences in sensitivity between p53 (+/-) knockout strains is thought to depend on where and how the p53 gene was altered genetically, and the strains of animals used for backcross. Further studies are needed to evaluate the tumorigenicity in p53 (+/-) CBA mice of other carcinogens which induce tumors in other organs, i.e the urinary bladder in p53 (+/-) TSG mice^{4,5}. When p53 (+/-) mice are scheduled for use in evaluation of the carcinogenic potential of newly developed chemicals, strain differences in p53 (+/-) mice sensitivity should be taken into account.

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