Tumor Promoting Effect of Phenolphthalein on Development of Lung Tumors Induced by N-ethyl-N-nitrosourea in Transgenic Mice Carrying Human Prototype c-Ha-ras Gene

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ABSTRACT. In order to examine tumor modifying effects of phenolphthalein (PhP), female transgenic mice carrying human prototype c-Ha-ras gene (rasH2 mice) were given a single intraperitoneal injection of 60 mg/kg body weight of N-ethyl-N-nitrosourea (ENU), followed by the diet containing 12,000 ppm PhP for 26-week. Histopathologically, alveolar hyperplasias, adenomas and adenocarcinomas were observed in the ENU + PhP group, but only hyperplasias and adenomas were observed in the ENU alone group. The incidence and multiplicity of adenocarcinomas in the ENU + PhP group was significantly increased as compared to that in the ENU alone group. The combined multiplicity of adenomas and adenocarcinomas in this group was also significantly higher than that of the ENU alone group. In addition, the ratio of area of adenomas in the ENU + PhP group was significantly higher than that in the ENU alone group. The result of our study suggests that PhP has a clear tumor promoting effect in the lung of rasH2 mice.

KEY WORDS: lung tumor, phenolphthalein, rasH2 mouse, tumor promotion.

RasH2 mice were originally established by Saitoh et al. at the Central Institute for Experimental Animals (CIEA, Kawasaki, Japan) [11]. They carry the human prototype c-Ha-ras gene with its own promoter region, which encodes a prototype c-Ha-ras gene product, p21 that has no capacity for transforming NIH3T3 cells [11]. Recently, it has been shown that rasH2 mice are much more susceptible to genotoxic carcigenes than their wild type littermates, thus providing a promising animal model for the detection of the carcinogenic potential of various genotoxic and non-genotoxic chemicals [6, 13–16]. Especially, it has been pointed out that rasH2 mice are extremely sensitive to genotoxic lung carcigenes such as dimethylbenzanthracene [1], urethane [10] and vinyl carbamate [6]. In addition, rasH2 mice initiated with urethane have been used as a two-stage lung carcigenesis model for detecting lung tumor promoters [12]. In our previous 6-month carcigenicity study using rasH2 mice, lung tumors were induced by a single intraperitoneal injection of 60 mg/kg body weight of N-ethyl-N-nitrosourea (ENU) [5]. Therefore, a medium-term study for pulmonary carcigenesis using rasH2 mice initiated with ENU is considered to be useful for the evaluation of modifying effects of various chemicals.

Phenolphthalein (PhP) has been used as a laboratory reagent, acid-base indicator and in over-the-counter laxative preparations [8]. It has carcigenic activity to rats and mice in 2-year carcigenicity studies, causing neoplasms of the kidney and adrenal medulla in rats and hematopoietic and ovary tumors in mice [2]. In addition, malignant lymphomas of thymic origin were also induced in heterozyous p53 deficient TSG mice [3]. However, it is unknown whether the lung is the tumor target organ of PhP. In the present study, we investigated the changes on the incidence and multiplicity of lung proliferative lesions by ENU/PhP treatment in rasH2 mice to evaluate the modifying effect of PhP on lung carcigenesis in rasH2 mice.

MATERIALS AND METHODS

Animals and chemicals: A total of 30 female rasH2 mice were obtained from the CIEA (Kawasaki, Japan). They used at 6-8 weeks of age for this study. The randomization continued until the body-weight and age distribution was approximately equal between two groups. The mice were housed at five per cage. Animal rooms were on a 12-h light/12-h dark cycle with continuous temperature and humidity monitoring. Basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo) and tap water was provided ad libitum. All mouse husbandry and handling were consistent with the guideline of the National Institutes of Health [9]. ENU (purity, 99%) and PhP (>98%) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Experimental design: After 1-week acclimatization period, the mice were divided into two groups (10 mice for Group 1 and 20 mice for Group 2). All mice received an intraperitoneal injection of 60 mg/kg body weight of ENU. After one week of the ENU initiation, the animals were given pulverized basal diet alone (Group 1) or the same diet containing 12,000 ppm PhP (Group 2) for 26 weeks. PhP was directly admixed with the basal diet. The test diet was prepared every week and stored in a refrigerator (tempera-
ture, 4°C) before use, being changed once a week during the experimental period. Body weight and food consumption were measured every week. The mean actual intakes of chemicals were calculated from the mean body weight and mean food consumption.

*Necropsy and light microscopic examination:* After the end of the 26-week experimental period, all surviving animals were killed under ether anesthesia by exsanguination and necropsied. All of the gross findings were recorded. All major organs and tissues from all animals were fixed with 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for light microscopic examination. Animals that died during the experimental period were subjected to a complete necropsy as soon as they were found. Moribund animals were subjected to the same processes. Multiplicities of lung proliferative lesions were expressed as the average numbers of lesions per 5 sections of different lobes. The areas of adenomas and the whole area of the lung sections examined were measured with the aid of a computer-assisted image morphometrical analyzer (ICD-740, OLYMPUS, Tokyo), and the data were expressed as the ratio of tumor area to total area examined.

Immunohistochemical staining was applied for lung adenomas using antibody against proliferative cell nuclear antigen (PCNA). The sections were pretreated with microwaving twice for 5 min. Endogenous peroxidase was inactivated using 5% ortho-periodic acid solution, and non-specific binding proteins were blocked with 5% casein. Inactivated sections were preincubated with monoclonal antibody against PCNA (DAKO, Glostrup, Denmark) at a dilution of 1:50. Immunolocalization was visualized using the PAP complex method with 3,3'-diaminobenzidine (DAB, Wako Pure Chemical Industries, Osaka, Japan) as the chromogen and hematoxylin for counterstaining. The cells in which nuclei were positive for PCNA immunostaining were counted as PCNA positive cells. The PCNA labeling index (PCNA LI) of adenomas were calculated as the ratios of positive cells per 100 tumor cells.

*Statistical analysis:* The incidences of lung proliferative lesions observed were analyzed by Fisher’s exact test. The multiplicity of the lesions and the ratio of area of adenomas were analyzed by the F test followed by Welch’s t-test. Data for the PCNA LI were used to generate mean and standard deviation values, and significant differences were analyzed by Student’s t-test. Results were considered as significant where the p value was less than 0.05.

**RESULTS**

There were no significant differences in body weight gain and food consumption between the ENU alone and ENU + PhP group. One mouse of the ENU or ENU + PhP group died of squamous cell carcinomas in the forestomach. Two, 2 and 1 mice in the ENU + PhP group died of osteosarcomas in the mandible, hemangiosarcomas in the spleen, and lung adenocarcinomas, respectively (Fig. 1). One mouse of the ENU alone or ENU + PhP group could not be subjected to histopathological examinations because of advanced autolysis.

Histopathologically, hemangiosarcomas in the spleen and liver, adenocarcinomas or hemangiomas in the uterus, hemangiomas in the ovary, squamous cell papillomas and carcinomas in the forestomach were observed in these two groups that died and were killed, but there were no significant differences in the incidence between these groups.

In the lung, alveolar/bronchiolar epithelial hyperplasias, adenomas, or adenocarcinomas were observed in these groups (Table 1). Hyperplasias were focal lesions consisting of cuboidal alveolar epithelial cells lining the alveolar septa, and their architecture was well maintained (Fig. 2A). Adenomas were generally well demarcated lesions, associated with slight compression of adjacent parenchyma. Adenocarcinomas frequently showed papillary and invasive growth (Fig. 2B). Carcinomas were characterized by foci/nodules consisting of bronchiolar/alveolar epithelial cells with solid/papillary growth pattern (Fig. 2B). Carcinomas originating from bronchiolar/alveolar epithelial cells were pleomorphic. Some adenocarcinomas were sometimes disseminated to whole lobes of the lung. Hyperplasias and adenomas were observed in ENU alone group, while adenocarcinomas as well as these proliferative lesions were found in the ENU + PhP group. The incidence of adenocarcinomas in the ENU + PhP group was significantly increased as compared to that in the ENU alone group. The multiplicity of adenocarcinomas was also significantly increased in the ENU + PhP group. In addition, the combined multiplicity of adenomas and adenocarcinomas in this group was significantly higher than that of the ENU alone group (Table 1). The ratio of area of adenomas to total lung area in the ENU + PhP group was significantly higher than that in the ENU alone group (Table 2).

Immunohistochemically, the PCNA LIs in adenomas were 2.01 ± 2.20 in the ENU alone group and 7.14 ± 6.63 in the ENU + PhP group (Table 2). There was an increasing tendency in the ENU + PhP group, but no significant differences in the PCNA LIs were observed in the ENU + PhP group compared to the ENU alone group.

**DISCUSSION**

PhP has been reported to lack mutagenicity in four strains
TUMOR PROMOTING EFFECT OF PHENOLPHTHALEIN IN RASH2 MICE

Table 1. Incidence and multiplicity of lung proliferative lesions in rash2 mice given PhP for 26 weeks after ENU initiation

<table>
<thead>
<tr>
<th>Group</th>
<th>N0</th>
<th>Alveolar hyperplasia</th>
<th>Adenoma (Ad)</th>
<th>Adenocarcinoma (Ca)</th>
<th>Ad + Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENU alone</td>
<td>9</td>
<td>0.56 ± 1.01</td>
<td>4</td>
<td>0.56 ± 0.73</td>
<td>0</td>
</tr>
<tr>
<td>ENU + PhP</td>
<td>18</td>
<td>1.39 ± 1.88</td>
<td>6</td>
<td>0.78 ± 1.31</td>
<td>7*</td>
</tr>
</tbody>
</table>

PhP: Phenolphthalein, ENU: N-ethyl-N-nitrosourea, a) Number of mice examined, b) Number of mice with lesions, c) Number of lesions/mouse, d): Mean ± SD.
*: Significantly different from the ENU alone group at p<0.05.

Table 2. Ratio of area of lung adenomas to total lung area and PCNA LIs [(Number of PCNA positive cells/100 cells adenomas) × 100.] of adenomas in rash2 mice given PhP for 26 weeks after ENU initiation

<table>
<thead>
<tr>
<th>Group</th>
<th>N0</th>
<th>Area (%)</th>
<th>N0</th>
<th>PCNA LI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENU alone</td>
<td>4</td>
<td>0.0032 ± 0.0023</td>
<td>4</td>
<td>2.01 ± 2.20*</td>
</tr>
<tr>
<td>ENU + PhP</td>
<td>6</td>
<td>0.0492 ± 0.0393*</td>
<td>4</td>
<td>7.14 ± 6.63</td>
</tr>
</tbody>
</table>

PhP: Phenolphthalein, ENU: N-ethyl-N-nitrosourea, a) Number of animal examined, b) Ratio of area of lung adenomas to total lung area, c) Mean ± SD, *: Significantly different from the ENU alone group at p<0.05.

of salmonella typhimurium, and no induction of sister chromatid exchanges was observed in cultured Chinese hamster ovary cells [8]. However, significant increases in chromosomal aberrations have been observed in cultured Chinese hamster’s ovary cells after treatment with PhP, and the frequencies of micronucleated erythrocytes were found to be increased in peripheral blood samples from mice that were administered PhP in the food for 13 weeks [8]. PhP has carcinogenic activity, causing pheochromocytomas of the adrenal medulla and renal tubular adenomas/carcinomas in F344 rats as well as histiocytic sarcomas, malignant lymphomas and sex-cord stromal tumors of the ovary in B6C3F1 mice [2]. In addition, malignant thymic lymphomas were also induced in heterozygous p53-deficient female mice given PhP for 6 months [3]. From these studies, it has been recognized that the tumor target organs of PhP were the hematopoietic system, ovary, kidney, and adrenal gland [2]. On the other hand, the organs where spontaneous tumors are observed in rash2 mice are the lung, spleen, stomach, skin and subcutaneous tissue [7]. However, in our previous study in which rash2 mice were given diet containing up to
12,000 ppm PhP for 6 months, there was no increase in the incidence of lung tumors in these treated groups [4]. In the present study, the ratio of the lung adenoma area to the whole lung area and the incidence of adenocarcinomas in the ENU + PhP group were significantly increased as compared to those in the ENU alone group. These results strongly indicate that PhP has a tumor promoting effect on ENU-induced pulmonary carcinogenesis in rasH2 mice.

Previously, it has been reported that epithelial tumors in the lung, liver, forestomach and kidney, and malignant lymphomas were induced in B6C3F1 mice given ENU [17]. On the other hand, in our study, hemangiosarcomas of the spleen, adenocarcinomas of the uterus, hemangiomats of the ovary, and squamous cell papillomas and carcinomas in the forestomach were induced in rasH2 mice by the treatment of ENU. The tumor target organs of the ENU-treated rasH2 mice are almost the same as those of B6C3F1 mice, except for the liver and kidney tumors. Furthermore, since tumors of these organs were observed in 6-month short term studies of rasH2 mice, our results emphasize the high carcinogenic susceptibility of the rasH2 mice to genotoxic carcinogens. However, there was no significant difference in the incidences of the tumors observed between ENU alone and PhP treated groups except for lung tumors. Based on these results, it can be concluded that PhP does not have any tumor promoting effect to the organs described above other than the lung.

As mentioned above, it has been generally accepted that the tumor target organs of PhP were the hematopoietic system, ovary, kidney, and adrenal gland in rats and mice given PhP [2]. However, in our present study, there were no tumors in the hematopoietic system, kidney and adrenal gland. With respect to the hematopoietic tumors, it has been reported that the sensitivity to lymphoma induction by PhP in rasH2 mice is much lower than in heterozygous p53-deficient mice or B6C3F1 mice [4, 5]. Molecular analyses of thymic lymphomas, that were induced in heterozygous p53-deficient female mice by PhP, have shown complete loss of the wild type p53 allele but not the null allele in all cases [4]. In addition, the accumulation of p53 protein in PhP-induced thymic lymphomas has been demonstrated in B6C3F1 mice by immunohistochemical staining [2]. Therefore, mutation and/or loss of the p53 gene are considered to bear important relations to the mechanism of induction of thymic lymphomas in mice by PhP treatment [4]. Since rasH2 mice possess homozygous p53 wild type alleles and have a genetic strain background with only rare spontaneous lymphomas, it might be expected that they would be resistant to the induction of malignant lymphomas by PhP [3]. On the contrary, the mechanism of induction of tumors in the kidney, adrenal gland and ovary in F344 rats or B6C3F1 mice is not still completely clarified. In addition, in the present study, there were no such tumors in rasH2 mice that are considered to be extremely sensitive to genotoxic carcinogens. From these findings, the possibility that non-genotoxic rather than genotoxic mechanism is involved in the induction of these tumors by the treatment of PhP except for malignant lymphomas can not be denied.

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