Original

Modifying Effects of 2,6-Dimethylaniline on Nasal Carcinogenesis in RasH2 Mice Initiated with 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone

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Abstract: In order to investigate the nasal carcinogenic potential and nasal tumor modifying effects of 2,6-dimethylaniline (DMA), a metabolite of xylazine which is used for food-producing animals, male transgenic mice carrying the human prototype c-Ha-ras gene (rasH2 mice) were given diet containing 0 or 2000 ppm DMA for 51 weeks after initiation with/without 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Their non-transgenic CB6F1 littermates (non-Tg mice) were also treated in the same manner. Histopathologically, in rasH2 mice, the incidences of inflammations, respiratory metaplasias of the olfactory epithelium, and hyperplasias of the respiratory epithelium in the NNK+DMA group significantly increased as compared with those in the NNK alone group. In both rasH2 mice and non-Tg mice, proliferative Bowman’s glands were induced in the NNK alone, DMA alone, and NNK+DMA groups. The value for PCNA-positive index of proliferative Bowman’s glands in the NNK+DMA group of rasH2 mice significantly increased as compared with the NNK alone case. In rasH2 mice, the incidence of adenomas (33%) in the NNK+DMA group that might be derived from proliferative Bowman’s glands was slightly, but not significantly, higher than that in the NNK alone group. These results suggest that DMA promotes the development of nasal proliferative lesions in rasH2 mice. (J Toxicol Pathol 2003; 16: 41–47)

Key words: 2,6-dimethylaniline, nasal carcinogenesis, rasH2 mice, tumor promotion

Introduction

2,6-Dimethylaniline (DMA) is one of the major metabolites of xylazine, an α₂-adrenergic receptor agonist with sedative, muscle relaxant and analgesic properties that is commonly used as a veterinary medicine to reduce aggressiveness in food-producing animals associated with livestock bleeding¹⁻³. It is also found in tobacco smoke and as a major metabolite of the local anesthetic lidocaine⁴. In mutagenicity tests, DMA has been reported to be positive in mouse lymphoma cells, sister chromatid exchange, and chromosomal aberrations, although not in the Ame’s tests⁵⁻⁸. DMA produced carcinomas and papillary adenomas in the nasal cavity of both male and female rats fed diet containing 3000 ppm DMA for 2 years⁹. In addition, our previous two-stage nasal carcinogenesis study of DMA in rats using N-bis(2-hydroxypropyl)nitrosamine (DHPN) as an initiator demonstrated that DMA has a tumor-promoting effect on the olfactory mucosa in the nasal cavity¹⁰. However, there is no report demonstrating that nasal tumors are also induced in mice by the long-term administration of DMA.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the tobacco-specific nitrosamines and a potent procarcinogen in laboratory animals¹¹. The activation of NNK may occur via α-hydroxylation, resulting in the formation of the promutagenic adduct O⁶-methylguanine. This methylguanine is known to have miscoding properties, causes for point mutations, and is hypothesized to be important in carcinogenesis of NNK¹²⁻¹⁷. NNK induces tumors of the nasal cavity, lung, liver, and pancreas in rats, but only the lung in mice¹⁸⁻²². However, in the nasal tissue, since O⁶-methylguanine-specific protein was found in the nuclei of sustentacular cells, Bowman’s glands, respiratory epithelial cells, and serous glands of mice²³, it can be considered that NNK also acts as an initiator in the nasal cavity of mice.

Mice are usually less susceptible to the induction of epithelial tumors of the nasal cavity than rats²⁴⁻²⁵. On the other hand, it has been indicated that transgenic (Tg) mice
carrying the human prototype c-Ha-ras gene (rasH2 mice) are much more susceptible to genotoxic carcinogens than their wild type littermates (non-Tg mice)\textsuperscript{26,27}. In addition, as described above, NNK was predicted to act as an initiator in the nasal cavity in rasH2 mice. Therefore, in the present study, the nasal carcinogenic potential and nasal tumor modifying effects of DMA were investigated using a unique two-stage nasal carcinogenesis model initiated with NNK in rasH2 mice.

Materials and Methods

Animals and chemicals

A total of 50 male CB6F1-TgHras2 (rasH2) mice and 50 male non-transgenic CB6F1 (non-Tg) littermates were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan), at 6 weeks of age. All rasH2 and non-Tg mice were housed in a polycarbonate cage with white wood chips under barrier system conditions (room temperature, 23 ± 2°C; relative humidity, 55 ± 5%; and 12 hr light and dark cycle). This animal experiment was carried out following the guideline set out by the Guide for the Care and Use of Laboratory Animals in the National Institute of Health Sciences.

NNK and DMA were obtained from Toronto Research Chemicals (Toronto, Canada) and Wako Pure Chemical Industries (Osaka, Japan), respectively.

Experimental design

After 2 weeks acclimatization period, 30 rasH2 and non-Tg male mice received an intraperitoneal injection of 3 mg/mouse of NNK in physiological saline once a week for two weeks and another 20 rasH2 and non-Tg mice were given an intraperitoneal injection of physiological saline in the same manner at 8 weeks of age. Among the 20 rasH2 and non-Tg mice given physiological saline, eight rasH2 and non-Tg mice were allocated to Group 1 (Control) and the remainder to Group 2 (DMA alone). Twelve out of 30 NNK-initiated rasH2 and non-Tg mice were allocated to Group 3 (NNK alone) and the remainder to Group 4 (NNK+DMA). The rasH2 and non-Tg mice in Groups 1 and 3 were fed pulverized basal diet alone and those in Groups 2 and 4 the diet containing 2000 ppm DMA for 51 weeks. All animals were given tap water ad libitum.

Necropsy and light microscopic examination

Dead or moribund animals during the experimental period were subjected to a complete necropsy as soon as they were found. All surviving animals were euthanized by exsanguination under ether anesthesia at week 53. Nasal cavities were fixed with 10% neutral buffered formalin and decalcified in 5% formic acid solution. Using the upper incisor teeth, incisive papilla, and molar teeth as guides for trimming, three transverse sections were made through the nasal cavity, and the parts obtained were processed for histology after paraffin embedding. They were sectioned at 4–5 µm thick and stained with hematoxylin and eosin (HE).

Table 1. Survival Ratio and Final Body Weights of rasH2 and non-Tg mice Fed Diet Containing DMA for 51 Weeks with or without NNK Initiation

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival ratio</th>
<th>Body weight (g) ± SD</th>
<th>*Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>rasH2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cont.</td>
<td>5/8 (63)</td>
<td>45.16 ± 3.38</td>
<td></td>
</tr>
<tr>
<td>DMA alone</td>
<td>10/12 (83)</td>
<td>27.99 ± 0.79</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>NNK alone</td>
<td>9/12 (75)</td>
<td>40.30 ± 4.09</td>
<td></td>
</tr>
<tr>
<td>NNK+DMA</td>
<td>15/18 (83)</td>
<td>28.02 ± 2.12</td>
<td></td>
</tr>
<tr>
<td>non-Tg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cont.</td>
<td>8/8 (100)</td>
<td>50.45 ± 2.95</td>
<td></td>
</tr>
<tr>
<td>DMA alone</td>
<td>12/12 (100)</td>
<td>31.69 ± 1.93</td>
<td></td>
</tr>
<tr>
<td>NNK alone</td>
<td>12/12 (100)</td>
<td>46.11 ± 4.90</td>
<td></td>
</tr>
<tr>
<td>NNK+DMA</td>
<td>15/17 (88)</td>
<td>31.93 ± 1.58</td>
<td></td>
</tr>
</tbody>
</table>

a: No. of surviving mice / No. of mice examined (survival rate, %), b: Mean ± SD (g), *: Significantly different from the non-treated group at p<0.05 (Student’s t test), †: Significantly different from the non-treated and NNK alone group at p<0.01 (Student’s t test).

Immunohistochemical staining was applied for nasal proliferative lesions in the NNK alone and NNK+DMA groups using antibody against proliferative cell nuclear antigen (PCNA). The sections were pretreated with a microwave for 10 min in citrate buffer and allowed to cool slowly to room temperature. The sections were treated with ortho-periodic acid solution for 30 min to block endogenous peroxidase activity, and non-specific binding proteins were blocked with casein. The sections were incubated with monoclonal antibody against PCNA (DAKO, Glostrup, Denmark) and then incubated with mouse immunoglobulin (DAKO). The dilutions of primary and secondary antibodies were 1:50 and 1:100, respectively. 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-positive index of nasal proliferative lesions was calculated as the ratios of positive cells per 100 cells.

Statistical analysis

The incidences of nasal proliferative lesions observed were analyzed with Fisher’s exact test. The data of PCNA-positive index of these lesions were given as mean and standard deviation, and differences were analyzed by Student’s t test.

Results

The final body weights of rasH2 and non-Tg mice in the NNK, DMA alone, and NNK+DMA groups were significantly lower than those in non-treated groups, those of rasH2 and non-Tg mice in the NNK+DMA groups being...
significantly lowered as compared with the NNK alone groups (Table 1). After 23 weeks, 3 rasH2 mice treated with NNK alone or without any treatment, 2 rasH2 mice treated with DMA alone, 3 rasH2 mice treated with NNK+DMA and 2 non-Tg mice treated with NNK+DMA were found dead or became moribund (Table 1).

Histopathologically, inflammations consisting of neutrophilic infiltrations in the dorsal olfactory epithelium (Fig. 1) were found in all of the NNK+DMA groups of rasH2 and non-Tg mice and in 4/12 non-Tg mice treated with DMA alone (Table 2). All of the DMA alone and NNK+DMA groups in rasH2 and non-Tg mice disclosed respiratory metaplasias of the dorsal olfactory epithelium (Fig. 2). All of the NNK, DMA alone and NNK+DMA groups had respiratory metaplasias of Bowman’s glands (Fig. 3).

Proliferative Bowman’s glands (Fig. 4) were observed in all of the treated rasH2 mice except for the non-treated mice and all of the non-Tg mice of the DMA alone and NNK+DMA groups. Nine of 12 non-Tg mice in the NNK alone group had the same lesion. The incidence of proliferative Bowman’s glands in the DMA alone group of non-Tg mice significantly increased as compared with that in the non-treated group. Proliferative Bowman’s glands were characterized by increased numbers of Bowman’s glands consisting of cuboidal cells with luminal dilatation in the lamina propria of the olfactory mucosa. The incidence of hyperplasias of the respiratory epithelium (Fig. 5) in the NNK+DMA group significantly increased as compared with that in the NNK alone group. Hyperplasias of the respiratory epithelium were composed of focal small nests of glandular

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**Table 2.** Incidences of Nasal Lesions in the Olfactory Mucosa of RasH2 and Non-Tg Mice Fed Diet Containing DMA for 51 Weeks with or without NNK Initiation

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals examined</th>
<th>Inflammation of olfactory epithelium</th>
<th>Respiratory metaplasia of Bowman’s glands</th>
<th>Proliferative Bowman’s glands</th>
<th>Hyperplasia of respiratory epithelium</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>rasH2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cont.</td>
<td>5</td>
<td>0 (0)†</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>DMA alone</td>
<td>10</td>
<td>0 (0)</td>
<td>10 (100)</td>
<td>10 (100)</td>
<td>1 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NNK alone</td>
<td>9</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>9 (100)</td>
<td>9 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NNK+DMA</td>
<td>15</td>
<td>15 (100)*</td>
<td>15 (100)*</td>
<td>15 (100)</td>
<td>13 (87)*</td>
<td>5 (33)</td>
</tr>
<tr>
<td>non-Tg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cont.</td>
<td>8</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>DMA alone</td>
<td>12</td>
<td>4 (33)</td>
<td>12 (100)†</td>
<td>12 (100)†</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NNK alone</td>
<td>12</td>
<td>0 (0)</td>
<td>1 (8)</td>
<td>9 (75)</td>
<td>9 (75)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NNK+DMA</td>
<td>15</td>
<td>15 (100)*</td>
<td>15 (100)*</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*a: Number of animals bearing lesions (%). †: Significantly different from the NNK alone group at \( p<0.01 \) (Fisher’s exact test). ‡: Significantly different from the non-treated group at \( p<0.05 \) (Fisher’s exact test).
Fig. 2. Respiratory metaplasia of the dorsal olfactory epithelium in a rasH2 mouse of the NNK+DMA group. HE ×300.

Fig. 3. Respiratory metaplasia of Bowman’s glands in a rasH2 mouse of the NNK+DMA group. HE ×300.

Fig. 4. Proliferative Bowman’s glands in the lamina propria of olfactory mucosae in a rasH2 mouse of the NNK+DMA group, characterized by increased numbers of Bowman’s glands consisting of cuboidal cells with luminal dilatation. HE ×150.
and/or papillary structures consisting of strong acidophilic cuboidal cells. In rasH2 mice, 2/9 of the NNK alone group and 5/15 of the NNK+DMA group had adenomas consisting of glandular and/or papillary growth patterns of cuboidal cells, projecting into the nasal lumen (Fig. 6), and there were no significant differences in their incidence between them. In PAS staining, small to large numbers of PAS-positive granules in the cytoplasm were observed in proliferating cells of Bowman’s glands and adenomas in the NNK+DMA group in rasH2 mice. Carcinomas were not found in any groups.

Immunohistochemically, the values of PCNA-positive indices of proliferative Bowman’s glands in rasH2 mice were 2.20 ± 1.79 in the NNK alone group and 9.60 ± 3.44 in the NNK+DMA group. The PCNA-positive index of Bowman’s glands in the NNK+DMA group was significantly higher than that in the NNK alone group. In adenomas, the values of PCNA-positive indices were 7.00 ± 5.66 in the NNK alone group and 5.50 ± 6.56 in the NNK+DMA group, there being no significant differences between these groups (Table 3).

Discussion

It has been reported that DMA induced carcinomas and papillary adenomas in the nasal cavity of rats fed diet containing 3000 ppm DMA for 2 years. In our two-stage nasal carcinogenesis study of DMA in rats initiated with

Fig. 5. Hyperplasia of the respiratory epithelium in the respiratory mucosae in a rasH2 mouse of the NNK+DMA group, composed of focal small nests of papillary structures consisting of strong acidophilic cuboidal cells. HE ×150.

Fig. 6. Adenoma of the olfactory mucosae in a rasH2 mouse of the NNK+DMA group, characterized by papillary growth of cuboidal cells projecting into the nasal lumen. HE ×150.
promoting effects of DMA and inflammations may be required.

It has been reported that rats are usually more susceptible to the induction of epithelial tumors of the nasal cavity than mice\textsuperscript{23,25}. This may be the major reason why DMA did not show any clear tumor-promoting effects on nasal carcinogenesis in this study. On the contrary, rasH2 mice seem to be a promising candidate as an animal model for the development of a rapid carcinogenicity testing system, but the data on the nasal carcinogenesis in rasH2 mice are limited in the number of references and it is still premature to make a final conclusion that rasH2 mice are less sensitive to the nasal carcinogenicity. In fact, in the present study, nasal adenomas were induced in treated rasH2 mice but not in non-Tg mice. Further studies are absolutely necessary to clarify the susceptibility of nasal carcinogenicity in rasH2 mice.

Based on the present study and our previous studies, it is suggested that DMA has a tumor promoting effect in the nasal cavity resulting from the enhanced cell proliferation of Bowman’s glands in both rats and mice\textsuperscript{10}. However, the induction of nasal tumors was not observed in the DMA alone groups in the present study and our previous studies\textsuperscript{10,20,29}. In addition, DMA levels in the blood of rats subjected to continuous administration of high doses of the parent compound of DMA, xylazine, remained under the detection limit\textsuperscript{12}. Therefore, the likelihood of nasal carcinogenic effects of DMA in consumers via the ingestion of edible tissues in the food-producing animals treated with xylazine is extremely low.

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