INVASIONS OF STOMACH TUMORS IN HYPOCATALASEMIC MICE (C3H/Cb/Gen) TREATED WITH N-METHYL-N-NITROSOUREA BUT NOT N-METHYL-N'-NITRO-N-NITROSOGUANIDINE

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Abstract: Hypocatalasemic mice were treated with N-methyl-N-nitrosourea (MNU) in their drinking water or gastric intubation for 16 weeks or were given N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in their drinking water for 6 months. With MNU treatment in the drinking water, well differentiated tumors were observed in the glandular stomachs of one male (4%) and 5 female (15%) animals. Sarcomas of the dermis also appeared in 10 males (40%). Adrenal lesions developed in 11 females (35%) along with kidney, ovary, and uterus tumors. In the Group given MNU intubation, squamous cell carcinomas of the forestomach appeared from 122 days after the first treatment, reaching incidences of 81% in males, 94% in females, with invasion of the diaphragm in 3 male cases and the diaphragm, liver, pancreas, or adrenal in 11 female cases. Glandular stomach tumors occurred in 12 males (52%), with 3 being signet ring cell carcinomas, and in 12 females (35%). In a sub-Group undergoing forestomach removal after MNU intubation esophagectasis appeared from 80 days after the operation at incidences of 63% in males and 74% in females. The glandular stomach tumors including 3 signet ring cell carcinomas were found in 17 of 46 (40%) males, 2 of 18 (11%) females demonstrated lesion, one being a signet ring cell carcinoma. In the Group receiving MNNG treatment, anemia appeared from the 5.5 month time point and no gastric tumors were observed. The present results do not support the conclusion that MNU induction of glandular stomach lesions in hypocatalasemic mice may provide a useful model of signet ring cell carcinomas. (J Toxicol Pathol 9: 223–228, 1996)

Key words: Gastric tumor, Squamous cell carcinoma, MNNG, MNU, Hypocatalasemic mice

Introduction

Little is known about poorly differentiated adenocarcinomas of the glandular stomach in mice. Recently we reported that localized X-irradiation of hypocatalasemic mice (C3H/Cb/Gen, a C3H mutant; Cb) induced signet ring cell carcinomas1. Tatematsu et al. also reported that signet ring cell carcinomas appear in BALB2 or C3H mice3 after MNU treatment. There has been a few papers for tumor invasion in mice. However, no good experimental models are yet available for the induction of these lesions in the mouse. An appropriate model for poorly differentiated adenocarcinomas or for invasion of tumors in this animal would provide a welcome new research tool for experimental gastric carcinogenesis. Therefore, we conducted the present study to assess the possibility of inducing signet ring cell carcinomas and to invasive carcinomas in the stomach by MNU or MNNG treatment.

Materials and Methods

Animals

Cb mice have been maintained under a sibling mating regime in our laboratory. They were housed seven or eight per polycarbonate cage, and maintained...
under conditions of constant temperature (24 ± 2°C) and relative humidity (50 ± 10%) with a 12 h light/12 h dark cycle under the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals", established by Hiroshima University. They were fed commercial diet MF (Oriental Yeast, Co. Ltd., Tokyo) and were provided with normal tap water ad libitum except during the treatment with MNU or MNNG in their drinking water.

**Chemicals**

MNU (N-methyl-N-nitrosourea, Sigma Chemical Co., St Louis) was dissolved in distilled water at the concentration of 100 ppm and administered to mice of both sexes from light-opaque bottles as their drinking water for 16 weeks. This MNU solution was exchanged at 3- to 4-day intervals. In a second group the same carcinogen was given at 0.5 mg in 0.5 ml/animal/week by intragastric intubation.

MNNG (N-methyl-N'-nitro-N-nitrosoguanidine, Aldrich Chemical Co. Inc., Milwaukee, WI) was dissolved in distilled water at the concentration of 100 mg/liter just before use. This solution was given to mice of both sexes ad libitum for 6 months from light-opaque bottles with exchange at 3- to 4-day intervals.

**Surgical removal of the forestomach**

In a subgroup of animals receiving MNU by intragastric intubation, the forestomachs were resected by operation under Nembutal anesthesia at the 16 week time point.

**Examination of animals**

All animals were regularly observed and necropsies were performed on those, which died, were spontaneously or killed when they became moribund or at the termination of the experiment at 13 months after the beginning of chemical treatment. At the time of necropsy, the body, liver, and other major organs were weighed and prepared for histopathologic studies. Particular attention was paid to the stomach. Each was cut open along the greater curvature, stretched out, pinned on a board with the mucosal surface upward, and washed several times with physiological saline before gross examination and was fixed in 10% neutral formalin. Strips were cut perpendicularly to the mucosal surface, two taken from the lesser curvature and four from the greater curvature. The strips were embedded in paraffin and serially sectioned at 3 μm. Sections were routinely stained with hematoxylin and eosin (HE), and for the alcian–blue–periodic acid–Schiff reaction (AB-PAS). Mucin was also stained with high-iron diamine (HID)-AB. Neoplastic lesions of the glandular stomach were classified into atypical hyperplasia (ATP) and adenocarcinoma categories. Adenocarcinomas of the glandular stomach were classified into well differentiated lesions characterized by tubular structures, poorly-differentiated tumors characterized by little tendency to form glandular structures with severe cellular atypia, and signet ring cell carcinomas characterized by isolated tumor cells containing abundant mucin stained with AB-PAS and HID-AB.

**Immunohistochemistry**

The formalin-fixed paraffin-embedded sections were incubated for 1 hr at room temperature with a polyclonal anti-rat manganese- (Mn-SOD), copper zinc-superoxide dismutase (Cu-SOD) antibody, (Courtesy of Dr. Keiichiro Suzuki, School of Medicine, Osaka University)4,5, and polyclonal anti-mn-23 antibody (Courtesy of Prof. Eiichi Tahara, School of Medicine, Hiroshima University)6. Then the sections were sequentially incubated with a biotin-labeled secondary antibody and an alkaline phosphatase conjugated streptavidin complex using nex fuchsin as the chromogen substrate (DAKO LSAB kit alkaline phosphatase system 40, K0628, DAKO Co.)

**Statistical analysis**

The significance of difference in numerical data was evaluated with the chi-squared test or Student's t-test.

**Results**

**MNU in the drinking water**

Thirty-one male and thirty-three female mice were used in this experiment. Twenty-five male and thirty-three female mice which survived beyond 165 days after the start of MNU treatment were counted in the effective numbers, those dying before this time point being without tumors. Mean survival was 216 ± 36 days in males and 338 ± 36 days in females.
Table 1. Body and Organ Weights for Hypocatalasemic Mice Treated with MNU or MNNG

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Mean survival (days)</th>
<th>Body (g)</th>
<th>Liver (g)</th>
<th>Liver/BW</th>
<th>Kidney (mg)</th>
<th>Kidney/BW</th>
<th>Testis Ovary (mg)</th>
<th>Testis/BW Ovary/BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNU in the drinking water</td>
<td>Male</td>
<td>216±36</td>
<td>32.3±4.3</td>
<td>1.890±0.363</td>
<td>58.1±8.0</td>
<td>617±82</td>
<td>19.4±3.2</td>
<td>140±17</td>
<td>4.58±0.90</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>338±36</td>
<td>29.2±2.6</td>
<td>1.536±0.627</td>
<td>52.5±19.0</td>
<td>394±47</td>
<td>13.6±1.7</td>
<td>38±34</td>
<td>1.13±1.06</td>
</tr>
<tr>
<td>MNU by intubation</td>
<td>Male</td>
<td>285±104</td>
<td>34.8±6.3</td>
<td>1.894±0.427</td>
<td>53.8±8.3</td>
<td>620±260</td>
<td>17.7±7.6</td>
<td>131±39</td>
<td>3.73±1.05</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>253±85</td>
<td>28.1±6.7</td>
<td>1.770±0.414</td>
<td>60.6±10.9</td>
<td>421±58</td>
<td>14.6±2.4</td>
<td>28±30</td>
<td>0.92±0.75</td>
</tr>
<tr>
<td>MNU by intubation with forestomach removal</td>
<td>Male</td>
<td>242±123</td>
<td>30.4±5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.3±3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.02±0.184</td>
<td>50.1±9.6</td>
<td>488±79</td>
<td>21.6±3.1</td>
<td>134±22</td>
</tr>
</tbody>
</table>

<sup>a</sup> Esophagectomy 27 cases (63%)
<sup>b</sup> Esophagectomy 14 cases (74%)

Table 2. Induction of Tumors or in Hypocatalasemic Mice by MNU and MNNG

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Initial animal number</th>
<th>Effective animal number</th>
<th>Total</th>
<th>Squamous cell carcinoma</th>
<th>Sarcoma</th>
<th>Lung tumor</th>
<th>Adrenal tumor</th>
<th>Other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNU in the drinking water</td>
<td>Male</td>
<td>31</td>
<td>25</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>10 (40)</td>
<td>0</td>
<td>1 (4)</td>
<td>0 (39) Ovary 3, Uterus 4, Kidney 4, Leukemia 2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>33</td>
<td>33</td>
<td>3 (9)</td>
<td>2 (6)</td>
<td>5 (15)</td>
<td>2 (6)</td>
<td>4 (12)</td>
<td>11 (33)</td>
</tr>
<tr>
<td>MNU by intubation</td>
<td>Male</td>
<td>29</td>
<td>26</td>
<td>9 (39)</td>
<td>3 (13)</td>
<td>12 (52)</td>
<td>21 (81)</td>
<td>3 (11)</td>
<td>0 (11) Uterus 1, Liver 1, Plasmacytoma 1, Leukemia 1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>37</td>
<td>34</td>
<td>11 (32)</td>
<td>1 (3)</td>
<td>12 (35)</td>
<td>32 (94)</td>
<td>0 (13)</td>
<td>0 (11) Leukemia 1, Lymphoma 1</td>
</tr>
<tr>
<td>MNU intubation of the forestomach removal</td>
<td>Male</td>
<td>50</td>
<td>46</td>
<td>11 (26)</td>
<td>3 (7)</td>
<td>17 (40)</td>
<td>4 (9)</td>
<td>4 (9)</td>
<td>3 (7) Liver 2, Duodenum 1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>24</td>
<td>19</td>
<td>1 (5)</td>
<td>0 (5)</td>
<td>2 (11)</td>
<td>4 (21)</td>
<td>0 (5)</td>
<td>2 (11) Leukemia 2, Lymphoma 1</td>
</tr>
<tr>
<td>MNNG in the drinking water</td>
<td>Male</td>
<td>38</td>
<td>31</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0) Leukemia 1</td>
</tr>
</tbody>
</table>

* Atypical hyperplasia
Body and organ weights are shown in Table 1.

Glandular stomach tumors appeared in 1 of 25 (4%) males and 5 of 33 (16%) females. All gastric tumors were of well differentiated type, and there were no poorly-differentiated or signet ring cell lesions. Sarcomas in the dermis were observed in 10 of 25 (40%) males and 2 of 33 (6%) females. Adrenal tumors were found in 1 male and 11 females. Four lung, 4 kidney, and 4 uterus and 3 ovary tumors as well as 2 leukemias also appeared in females. Tumors at different sites were found in 1 male and 7 females (Table 2).

**MNU intubation**

Body and organ weights are shown in Table 1. Twenty-six males and 34 females which survived beyond 122 days after the start of MNU treatment were counted in the effective numbers. The first squamous cell carcinoma was observed 122 days after the start of MNU treatment. Mean survival was 285±104 days in males and 253±85 days in females. Squamous cell carcinomas in the forestomach were found in 21 of 26 (40%) males and 32 of 34 (94%) females (Table 2). Invasion to the diaphragm was evident in 5 male animals (19%) and to the diaphragm, liver (Fig. 1), pancreas and/or adrenals in 11 female animals (32%) (Table 3). The invading tumors were not stained by Mn-SOD or Cu-SOD and showed a reduction in mn23 immunoreactivity. Total incidences of glandular stomach tumors were 52% in males and 35% in females. Most of the tumors were well differentiated but 3 of 26 (12%) in males were of signet ring cell type (Fig. 2). One lung tumor, 1 leukemia, 1 plasmacytoma, and a uterus and a liver tumor were found in females (Table 2).

**MNU intubation with forestomach removal**

Fifty male and twenty-four female mice were used in this experiment. Forty-six males and nineteen females which survived beyond 73 days after the start of MNU treatment were counted in the effective numbers, those dying before this time point being without tumors. Mean survival was 242±123 days in males and 179±95 days in females. Body weights are shown in Table 1. Glandular stomach tumors appeared from 73 days after the beginning of MNU treatment in 17 of 46 (40%) males and 2 of 24 (11%) in females. Most were well differentiated but 3 of 46 (7%) in males and 1 of 19 (5%) in females were signet ring cell carcinomas. Squamous cell carcinomas appeared in 4 animals of both sexes. Two liver tumors and 1 duodenum tumor were also found in males and 1 leukemia and 1 lymphoma in females. Esophagectasis first appeared from 80 days after the

![Fig. 1. Squamous cell carcinoma in the forestomach invading to the liver, ×100, HE.](https://example.com/figure1.png)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Total</th>
<th>Diaphragm</th>
<th>Liver</th>
<th>Pancreas</th>
<th>Adrenal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
operation in males, and reached incidences of 63% (27 of 46) in males and 74% (14 of 19) in females.

**MNNG in the drinking water**

Animals began to die 5.5 months after the start of MNNG, mean survival being 227±42 days. All animals were anemic but no tumors developed (Table 2).

**Discussion**

In the present study a relatively small incidence of gastric tumors was induced by MNU administration in the drinking water in hypocatalasemic mice. Hirota et al. earlier reported that this treatment approach selectively induces glandular stomach carcinomas at high incidence in F344 rats. This was confirmed by Fujita et al. and also reported that MNU in the drinking water caused gastric tumors in Wistar and F344 rats with differences in the pathological findings between the two cases. Recently Tatematsu et al. described selective induction of neoplastic lesions in the glandular stomach epithelium of C3H male mice with this carcinogen. In the present experiment, however, the incidence of glandular stomach tumors was lower than that with 30 ppm in the results of Tatematsu et al. and incidences of other tumors provided higher in the C3H case with a different tumor spectrum. Mutant C3H hypocatalasemic mice have lower catalase and higher superoxide dismutase activities than the original C3H mouse and therefore the metabolism of MNU may be different between the two strains.

In the present experiment, MNU intubation caused tumors in the glandular stomach and squamous cell carcinomas with invasion to other organs. Tatematsu et al. also reported that 6-week-old male BALB/c mice which received intragastric inbution of MNU, developed squamous cell carcinomas in the forestomach as well as well differentiated adenocarcinomas, poorly differentiated adenocarcinomas, and signet ring cell carcinomas in the gastric portion. The problem with MNU intubation was that the squamous cell carcinomas arose earlier, resulting in invasion of various organs and death before glandular lesions could became evident. In rats administration of MNNG by gastric intubation similarly induced multiple tumors in the forestomach but when administrated in the drinking water the same carcinogen was found primary to cause carcinomas in the glandular stomach, especially in the pyloric mucosa. Thus there is a link between intubation of MNNG or MNU and induction of squamous cell lesions in the forestomach. The fact that, in our experiment, the squamous cell carcinomas invaded the liver and diaphragm is unusual. C57 mice spontaneously develop tumors of the liver and mammary gland where metastases occur to the lung in aged mouse. (Watanabe et al. unpublished data) Recently Satomi et al. reported that SOD activity in human colorectal cancer tissue increased with the stage progression and changed with the depth of invasion, suggesting that venous invasion was the most significant factor influencing SOD activity.

![Fig. 2. Signet ring cell carcinoma, tumor cells, invading to the serosa, ×40. AB-PAS.](image-url)
However, in this experiment, there was no association between immunohistochemical results for SOD and invasion, while nm23 was decreased. The nm23 gene has been proposed to be a metastasis suppressor gene and introduction of an nm23 expression vector into highly metastatic cells resulted in a reduction in the incidence of primary tumor formation and of metastatic potential. Nakayama et al. also reported that decrease in expression of nm23 may participate in metastasis of gastric carcinoma.

No tumors were caused by MNNG treatment in the present experiment. Since 1967, when a high rate of adenocarcinoma development in the glandular stomach of rats with MNNG was first described by Sugimura and Fujimura, this model has provided a powerful tool for experimental studies of gastric carcinoma with the carcinogen also inducing stomach adenocarcinomas in hamsters and dogs. Attempts using mice have been unsuccessful, however, for example administration of MNNG in the drinking water to BRSUNT/NJms mice over the life span resulted only in adenomatous hyperplasia of the gastric epithelium. Catechol, which is a strong carcinogen in the rat glandular stomach, also failed to cause adenocarcinomas in the mouse organ. Thus the glandular stomach of mice has generally been found to be resistant to MNNG action.

References
