Promoting Effects of 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone on Rat Glandular Stomach Carcinogenesis Initiated with N-Methyl-N′-nitro-N-nitrosoguanidine

Akiyoshi Nishikawa, Fumio Furukawa, In-Seon Lee, Ken-ichiro Kasahara, Zen-yo Tanakamaru, Hideaki Nakamura, Makoto Miyauchi, Naohide Kinae, and Masao Hirose

Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan [A. N., F. F., I.-S. L., K.-i. K., Z.-y. T., H. N., M. M., M. H.]; Department of Food Science and Technology, Keimyung University, Taegu 700-200, Korea [I.-S. L.]; and Laboratory of Food Hygiene, School of Food and Nutritional Science, University of Shizuoka, Shizuoka, 422-8526 Japan [N. K.]

Abstract

The modifying effects of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), a mutagenic by-product in chlorinated water, on the development of glandular stomach cancers were investigated in Wistar rats. A total of 120 males, 6 weeks of age, were divided into six groups. After initiation with 100 ppm N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) solution and 5% NaCl diet for 8 weeks, 30 rats each in groups 1–3 were given MX in the drinking water at concentrations of 30, 10, or 0 ppm for the following 57 weeks. Ten animals each in groups 4–6 were administered the MX without prior carcinogen exposure. There were no statistical significant differences in final body weights between the groups. The incidences and multiplicities of adenocarcinomas in the glandular stomachs were significantly higher (P < 0.05) in the initiated 30 ppm MX group than those in the MNNG/NaCl group. The incidences of atypical hyperplasias in the glandular stomachs were also significantly increased (P < 0.05 or 0.01) by the MX treatments. With their multiplicity, the effects were clearly dose dependent. Interestingly, the 30 ppm MX alone itself induced atypical hyperplasias in the pylorus, although the incidences and severity were low. Moreover, MX showed a tendency to enhance the development of intrahepatic cholangiocellular tumors and thyroid follicular cell tumors in the MNNG-treated animals. The results of the present study thus indicate that MX exerts promoting effects when given during the postinitiation phase of two-stage glandular stomach carcinogenesis in rats.

Introduction

It is well documented that chlorinated drinking water contains a variety of mutagens (1). However, 2-year bioassays conducted by the United States National Toxicology Program showed no evidence of carcinogenic activity in male F344/N rats and male or female B6C3F₁ mice, although equivocal evidence for chlorinated water effects in male F344/N rats and male or female B6C3F₁ mice was obtained, with an increase in the incidence of proliferative lesions. Moreover, MX showed a tendency to enhance the development of intrahepatic cholangiocellular tumors and thyroid follicular cell tumors in the MNNG-treated animals. The results of the present study thus indicate that MX exerts promoting effects when given during the postinitiation phase of two-stage glandular stomach carcinogenesis in rats.

Materials and Methods

Animals. Male Wistar rats (Japan SLC Inc., Shizuoka, Japan), 5 weeks of age, were housed five animals per polycarbonate cage and maintained under standard laboratory conditions: room temperature, 23 ± 2°C; relative humidity, 60 ± 5%; a 12 h/12 h light/dark cycle. After 1-week acclimation period, the animals were used.

Chemicals. MX was synthesized from tetrachloroacetone and (carboxyloxyethylene)-triarylphosphorane according to the method of Padmapria et al. (12). Its purity was determined as 97% by high-performance liquid chromatography (R-ODS-5 column; YMS, Osaka, Japan). MNNG was a commercially available preparation from Aldrich Chemicals, Inc. (Milwaukee, WI). NaCl (purity, >99.5%) was purchased from Wako Pure Chemical Ind. (Osaka, Japan).

Experimental Procedure. As shown in Fig. 2, a total of 120 rats were divided into six groups, groups 1–3 consisting of 30 animals and groups 4–6 of 10 animals. Rats in groups 1–3 were given a 100 ppm MNNG solution as their drinking water and simultaneously fed a basal diet, Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan), supplemented with 5% NaCl for 8 weeks as an initiation treatment, and then given MX in their drinking water at concentrations of 30, 10, and 0 ppm for the following 57 weeks. The concentrations of MX were selected on the basis of our previous short-term study findings (11). Simultaneous NaCl feeding was applied as a coinitiating factor (13). Animals in groups 4–6 were given MX in their drinking water for 57 weeks similarly to the groups 1–3 except without the prior MNNG and NaCl treatments. The experimental animals were macroscopically observed for symptoms weekly and weighed once a month. Necropsy was performed on all animals that were found dead or killed on becoming moribund. At the end of the 65th experimental week, all surviving animals were sacrificed and necropsied. At necropsy, the stomach and other major organs were excised and subjected to careful macroscopic examination. The stomach was opened along the greater curvature, put on a filter paper, and then fixed in 10% buffered formalin. The fixed stomachs were cut into longitudinal strips, 3 mm wide, for examination of the entire gastric mucosa. After processing for histology by routine methods, sections were stained with H&E. In addition, other major organs including the liver, thyroid, and kidney were histopathologically examined for the existence of proliferative lesions.

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2 To whom requests for reprints should be addressed, at Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Phone: 81-3-3700-9819; Fax: 81-3-3700-1425; E-mail: nishikawa@nihs.go.jp.

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The abbreviations used are: MX, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine.
Statistical Analysis. The tumor incidences were analyzed by the Fisher’s exact probability test or the $\chi^2$ test. Data for lesion multiplicities and the body and organ weights were examined by the Student’s $t$ test.

Results

MX/Water Consumption and Body/Organ Weights. As shown in Table 1, the total intakes of MX estimated from water consumption data correlated well with the doses applied. There were no significant differences in final body weight between groups (Table 2), although the MNNG-treated groups showed a temporary growth retardation during the initiation phase (Fig. 3). Relative organ weights of the major organs including the liver, heart, spleen, kidneys, and lungs were not significantly affected by the treatments of MX and/or MNNG only except that of left lungs (Table 2). Four rats in group 3 died before the termination of the experiment, but none of them had any gastric proliferative lesions. Therefore, only the animals that survived until the termination were determined to be effective.

Effects of MX on Glandular Stomach Carcinogenesis. Cancerous and precancerous lesions in the glandular stomach were diagnosed as adenocarcinomas and atypical hyperplasias as described previously (14). Briefly, adenocarcinomas were judged as invasive growths of atypical tubules (Fig. 4). Atypical hyperplasias were defined as non-invasive growths of atypical tubules histologically similar to adenocarcinomas but lacking complete expansion to the whole layer of lamina propria or expansive growths of atypical tubules with moderate to severe dysplasia but involving the whole layer of lamina propria (Fig. 5). Simple and nonatypical hyperplastic lesions were strictly discriminated from atypical hyperplasias. As shown in Table 3, the incidence of adenocarcinomas in rat glandular stomachs of group 1 (29.6%) was significantly higher ($P < 0.05$) than the group 3 value (3.8%). The incidences of atypical hyperplasias in rat stomachs of groups 1 (92.5%) and 2 (96.2%) were significantly higher ($P < 0.05$ and 0.01) than the group 3 value (61.5%). The incidences of atypical hyperplasias in the pyloric mucosa of groups 1 (88.8%) and 2 (62.9%) were significantly increased ($P < 0.01$) as compared with the group 3 value (30.7%). Atypical hyperplasias were also found in the pylorus of group 4, although the incidence was low at 22.2% and the severity was slight (Fig. 6). No other proliferative stomach lesions were detected in groups 4–6. As shown in Table 4, the mean numbers of lesions per animal, i.e., multiplicities of adenocarcinomas and atypical hyperplasias in the glandular stomachs of group 1 (0.33) and groups 1 (2.44) and 2 (2.22) were significantly greater ($P < 0.05$) than the group 3 values (0.03 and 1.11, respectively).

Effects of MX on Tumorigenicity in the Organs Other Than the Stomach. Cholangiocarcinomas, cholangiomas, cholangiofibrosis, and bile duct hyperplasias were found in the livers of groups 1–5. The combined incidences of cholangiocarcinomas and cholangiomas in groups 1 (13%) and 2 (17%) showed a tendency for increase as compared with the group 3 value (4%), although there was no statistical significance. Although C-cell adenomas and C-cell hyperplasias of the thyroid were detected in all of the groups, follicular cell hyperplasias were only noted in groups 1 (8%) and 2 (5%). No other neoplastic lesions were increased by the MX and/or MNNG treatments.

Discussion

In a recent carcinogenicity study using rats, it was shown that MX is definitely carcinogenic when given in drinking water at doses of 5.9, 18.7 and 70.0 ppm for 104 weeks, with evidence of statistically significant increases of thyroid follicular carcinomas, cholangiomas, and some other tumors (8). In addition to such carcinogenicity, the results of the present study clearly indicate that MX enhances the development of gastric cancers in rats exposed to MNNG. The present

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>0</th>
<th>8</th>
<th>65 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td></td>
<td></td>
<td>30 ppm MX</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td></td>
<td></td>
<td>10 ppm MX</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td></td>
<td></td>
<td>30 ppm MX</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td></td>
<td></td>
<td>30 ppm MX</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
<td>10 ppm MX</td>
</tr>
</tbody>
</table>

Animals: Wistar rats (Males, 6-weeks-old)
Test chemical: 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) in drinking water

100 ppm MNNG + 5% NaCl in diet

Fig. 1. Structural formula of MX.

Fig. 2. Effects of MX on glandular stomach carcinogenesis in rats after initiation with MNNG and NaCl.

Table 1 Intake of drinking water and MX

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Mean water consumption (g/animal/day)</th>
<th>Total MX intake (g/animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNNG/30 ppm MX</td>
<td>21.8</td>
<td>0.238</td>
</tr>
<tr>
<td>MNNG/10 ppm MX</td>
<td>22.0</td>
<td>0.080</td>
</tr>
<tr>
<td>MNNG</td>
<td>21.2</td>
<td>0.078</td>
</tr>
<tr>
<td>30 ppm MX</td>
<td>22.7</td>
<td>0.248</td>
</tr>
<tr>
<td>10 ppm MX</td>
<td>21.5</td>
<td>0.078</td>
</tr>
<tr>
<td>Control</td>
<td>21.7</td>
<td></td>
</tr>
</tbody>
</table>
results are thus consistent with our previous finding that MX induces cell proliferation in the gastric mucosa of rats even at low, nontoxic doses such as 25 ppm (11) as well as data for higher doses (10). In our previous study (11), gastric erosions were mainly found in the fundic mucosa, whereas gastric cancers were predominantly detected in the pyloric mucosa in the present study. Thus, mitogenic potential in addition to possible regenerative response might be involved in the mechanism underlying the stomach tumor-promoting activity observed in the present study.

The mutagenicity of MX has been reported to be in the range of 2,800–13,000 induced revertants/nmol in the Ames S. typhimurium TA100 assay without metabolic activation (3, 15), and MX is therefore a most potent TA100 mutagen. The contribution ratios of MX to the whole mutagenicity of chlorine-treated tap water were estimated as 15–57% in Finland (16), 15–34% in the United States (5), and 7–21% in Japan (7). It has also been shown that MX is a mammalian cell clastogen (17), which gives rise to peculiar DNA adducts (18–20). Despite this strong in vitro mutagenicity, however, MX was found to be only marginally active at inducing nuclear anomalies in the small intestines of mice when given as a single oral dose of 0.37 mmol/kg (21). It has thus been hypothesized that mammalian cells may effectively detoxify chlorohydroxyfuranones, including MX (21, 22). It has in fact been shown that MX is efficiently inactivated by endogenous defensive systems such as that involving glutathione and

### Table 2 Relative organ weights of rats treated with MX and/or MNNG

<table>
<thead>
<tr>
<th></th>
<th>MNNG + 30 ppm MX</th>
<th>MNNG + 10 ppm MX</th>
<th>MNNG 30 ppm MX</th>
<th>10 ppm MX</th>
<th>Nontreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>472.10 ± 36.72a</td>
<td>473.84 ± 22.01</td>
<td>479.40 ± 32.06</td>
<td>500.16 ± 36.46</td>
<td>476.53 ± 36.08</td>
</tr>
<tr>
<td>Liver (g%)</td>
<td>2.63 ± 0.29</td>
<td>2.58 ± 0.21</td>
<td>2.54 ± 0.21</td>
<td>2.43 ± 0.24</td>
<td>2.45 ± 0.13</td>
</tr>
<tr>
<td>Heart (g%)</td>
<td>0.24 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.23 ± 0.01</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>Spleen (g%)</td>
<td>0.19 ± 0.04</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.07</td>
<td>0.17 ± 0.02</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>Kidney Right (g%)</td>
<td>0.28 ± 0.03</td>
<td>0.29 ± 0.02</td>
<td>0.29 ± 0.02</td>
<td>0.28 ± 0.02</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>Kidney Left (g%)</td>
<td>0.28 ± 0.03</td>
<td>0.29 ± 0.03</td>
<td>0.29 ± 0.02</td>
<td>0.28 ± 0.01</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>Lung Right (g%)</td>
<td>0.20 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>0.20 ± 0.03</td>
<td>0.20 ± 0.04</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Lung Left (g%)</td>
<td>0.11 ± 0.01b</td>
<td>0.10 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
</tbody>
</table>

* Mean ± SD.
* Significantly different from the MNNG-alone group at *P* < 0.05.

Fig. 3. Body weight curves for rats treated with MX.

Fig. 4. Photomicrograph illustrating an adenocarcinoma found in a group 1 rat (×66, H&E stain).

Fig. 5. Photomicrograph illustrating an atypical hyperplasia found in a group 2 rat (×66, H&E stain).
cysteine (23, 24). However, several equivocal results regarding in vivo increases even by the low dose (10 ppm) treatment of MX. Additional studies are now necessary to determine any no-effect level, and the carcinogenicity (8) and teratogenicity (30).

It has been suggested that MX might primarily affect the mucosa of the alimentary tract, if exposure levels are sufficient to overwhelm the defense capacity due to glutathione or other factors, or when defense systems are impaired (11, 22). Moreover, because MX is stable under acidic conditions (9), the stomach could be the major target for MX toxicity. In two-stage glandular stomach carcinogenesis models using rats, several stomach tumor promoters have been identified in our laboratory (14, 31). Regarding the mechanisms underlying the stomach tumor-promoting activity, it was shown that NaCl, a typical stomach tumor promoter, increases cell proliferative activity in the gastric mucosal epithelium of rats like other tumor promoters targeting the liver and skin (31). In the present study, atypical hyperplasias were increased even by the low dose (10 ppm) treatment of MX. Additional studies are now necessary to determine any no-effect level, amounts of MX in drinking water being estimated to range from 3 to 67 ppt (32).

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