EFFECTS OF ETHANOL, POTASSIUM METABISULFITE, FORMALDEHYDE AND HYDROGEN PEROXIDE ON GASTRIC CARCINOGENESIS IN RATS AFTER INITIATION WITH N-METHYL-N'-NITRO-N-NITROSOGUANIDINE

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Ethanol, potassium metabisulfite, formaldehyde and hydrogen peroxide were tested for tumor-promoting activity in a two-stage stomach carcinogenesis experiment. Male outbred Wistar rats were given N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in the drinking water (100 mg/liter) and a diet supplemented with 10% sodium chloride for 8 weeks. Thereafter, they were maintained on drinking water containing either 10% ethanol, 1% potassium metabisulfite, 0.5% formalin (formaldehyde) or 1% hydrogen peroxide for 32 weeks and then sacrificed for necropsy and histological examination. In the pylorus of the glandular stomach, potassium metabisulfite and formaldehyde significantly increased the incidence of adenocarcinoma after initiation with MNNG and sodium chloride. Hydrogen peroxide did not enhance the tumor yield, and ethanol showed a tendency to decrease neoplastic development. In the forestomach the incidence of squamous cell papilloma was significantly increased in the groups given hydrogen peroxide or formaldehyde, irrespective of prior initiation. Duodenal adenocarcinoma was induced by the initiation alone (10%) and the incidence was not affected by the subsequent treatments. The results indicate that potassium metabisulfite and formaldehyde both exert tumor-promoting activity in the rat glandular stomach.

Key words: Gastric carcinogenesis — Sulfite salts — Aldehydes — N-Methyl-N'-nitro-N-nitrosoguanidine — Rat stomach

Cancer of the stomach is the commonest cause of cancer death in Japan. Although the etiology of gastric cancer is still unknown, a number of epidemiological studies have suggested that, among possible environmental factors, dietary salt level is strongly related to cancer incidence. In northern Japan, where the consumption of dietary salt is much higher than in southern areas, gastric cancer is the commonest malignancy. However, these studies of human population groups provide very little information relevant to the pathogenesis of gastric cancer. In recent years the discovery of the potent carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) has facilitated investigation of experimental gastric carcinogenesis. This compound, when administered to rodents, induces benign and malignant neoplasms of the stomach which are strikingly similar in most respects to gastric tumors in man, and it has provided the opportunity to study the effects of diet,13) exogenous chemicals,14) pathological conditions in the stomach, such as ulcer4) or partial gastrectomy,15) genetic factors,6) and other factors7) on tumor production under controlled laboratory conditions.

Our previous work8) demonstrated a possible promoter action of sodium chloride on the two-stage process of gastric carcinogenesis initiated by MNNG. To our knowledge, there has been no report concerning the effect of ethanol on gastric carcinogenesis under two-stage (initiation and promotion) experimental conditions. Ethanol appears to be a major etiologic factor for the development of cancers of the upper digestive tract including the esophagus,9) although exactly how cancers in this region are related to excessive alcohol consumption is not known. One mechanism may be related to the tissue damage produced by the high local ethanol concentrations to which some of
these tissues are subjected. Since animal experiments have not shown that ethanol alone has a carcinogenic effect, ethanol may act as a promoter in carcinogenesis. Moreover, ethanol is a possible inducer of gastric epithelial damage.

Sulfite salts, which have been used as preservatives in a variety of foods and drinks, have been the subject of toxicological investigation in several animal species. The most striking finding is the occurrence of hyperplastic changes in the fundic mucosa as well as in the forestomach. The sulfite-induced lesions are characterized by necrosis of epithelial cells and atypical glandular hyperplasia. Aldehydes are also commonly present in the environment, e.g., in tobacco smoke. Among aldehydes, formaldehyde is ubiquitous and is known to cause dermal and mucosal irritation.

Hydrogen peroxide, which is a germicidal and bleaching agent, is widely used for medical purposes. In addition, hydrogen peroxide is endogenously formed in cells as a consequence of free radical formation and is reported to enhance duodenal and upper jejunal tumor development in rats treated with methylazoxymethanol acetate and to cause duodenal tumors in mice.

This range of environmental chemicals is of particular interest because of their potential role in tumor promotion in man. Therefore, we examined the promoting potential of ethanol for gastric carcinogenesis utilizing a two-stage carcinogenesis model with MNNG plus sodium chloride treatment as the initiator, and the effects were compared with those of potassium metabisulfite and formaldehyde, which are mucosal damaging agents, and hydrogen peroxide.

**Materials and Methods**

Seven-week-old male Wistar rats (Shizuoka Laboratory Center, Shizuoka) were housed in plastic cages, 5 rats/cage, and maintained under constant conditions of temperature (23±2°C), relative humidity (55±5%) and a 12-hr light-12-hr dark cycle.

One hundred and sixty-two rats were divided into 10 groups. Thirty rats were treated with the initiation procedure alone (group 1), 20 or 21 rats were assigned to each of the other initiated groups (groups 2 to 5) and 10 rats to each of the groups without the prior initiation (groups 6 to 10). The animals in groups 1 to 5 were given MNNG (Aldrich Chemicals, Inc., USA) continuously in their drinking water at a concentration of 100 mg/liter and were simultaneously administered the sodium chloride diet, a stock diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) supplemented with 10% sodium chloride (Wako Pure Chemical Co., Osaka), for the first 8 weeks as the initiation procedure. After this 8-week treatment period, animals were fed the stock diet without any supplementation and were given each test chemical in their drinking water. Groups 6 to 10 received neither MNNG nor sodium chloride diet in the first 8 weeks of the experiment and were maintained on stock diet alone as negative controls. The animals in groups 1 and 6 received drinking water without any supplementation during the promotion stage (weeks 8 to 40). Animals in groups 2 and 7 were given drinking water supplemented with 10% ethanol (Kokusan Chemical Co., Tokyo), groups 3 and 8 were maintained on drinking water supplemented with 1% potassium metabisulfite (Wako Pure Chemical Co.), groups 4 and 9 received 0.5% formalin (formaldehyde) (Wako Pure Chemical Co.), and groups 5 and 10 received 1% hydrogen peroxide (Mitsubishi Gas Chemical Co., Inc., Tokyo) during the promotion stage. All animals had free access to food and water.

The experimental animals were observed daily and weighed once every 4 weeks. Necropsies were performed on all animals which died or were killed when they became moribund, except for a few animals in groups 3 and 4 which died in the early stages of the experiment. At the end of the 40th experimental week, all surviving animals were sacrificed and autopsied. Animals surviving beyond the 30th week of the experiment were included in the effective numbers. The stomach and other organs in the peritoneal cavity were examined carefully and then fixed in 10% buffered formalin. The stomach was inflated with fixative before removal. Tissues were prepared for histopathological examination by routine methods and stained with hematoxylin and eosin.

Histological lesions of the glandular stomach have been classified into two categories in the fundic and pyloric areas: hyperplasia and adenocarcinoma in the fundus, and preneoplastic hyperplasia and adenocarcinoma in the pylorus. Hyperplasia (adenomatous hyperplasia) of the fundus is characterized by proliferation of the foveolar epithelium and deep foveolar pits. Preneoplastic hyperplasia is defined as relatively
small, proliferative, non-invasive mucosal lesion
with slight atypism of the epithelial cells and
usually with tubular formation. Adenocarcinoma
is irregular glandular structures lined by moder-
ately atypical, darkly stained, or mucin-producing
columnar epithelium with enlarged nuclei. The
degree of invasiveness and metastasis of aden-
ocarcinomas were carefully examined.

The tumor incidences were analyzed by means
of Fisher's exact probability test.

RESULTS

During the initiation period the growth
of animals was significantly affected by the
treatment with MNNG and simultaneous
administration of the sodium chloride-sup-
plemented diet (Fig. 1). Animals receiving
MNNG (groups 1 to 5) showed a marked
repression of weight gain when compared
to MNNG-untreated animals (groups 6 to
10). After cessation of MNNG administra-
tion (at 8 weeks), these weight-reduced
animals exhibited a compensatory increase
in growth rate, although their weights at
the termination of the experiment were still
lower than those in the MNNG-untreated
groups. Body weight gains of rats in the
different MNNG-treated groups were sim-
ilar to each other, including the control
(group 1), during the promotion period
with the exception that in rats receiving
the formaldehyde- and hydrogen peroxide-
supplemented water (groups 4 and 5) a
marked retardation of growth as compared
to the other groups (groups 1 to 3) was
observed. A similar response was observed
in groups 9 and 10 when compared with
groups 6 to 8.

Gastric tumors were predominantly locat-
ed in the pyloric region and were grossly
observed as plaque-like or nodular lesions
with ulceration. Preneoplastic hyperplasias
in the pyloric mucosa were defined as pro-
iferative, noninvasive mucosal lesions.
Grossly and histologically, these tumors
were similar to those described in our pre-
vious paper8) and corresponded to the
lesions classified as adenomatous hyper-
plasia or adenoma by other investigators.13)
However, since they are considered to be
the earliest manifestation of carcinogen-
induced growth abnormalities in the stom-
ach, the term “preneoplastic” seems ap-
propriate. On the other hand, adenomatous

Fig. 1. Mean body weight gain of rats given various chemicals beginning after the 8th
experimental week. Animals in groups 1 to 5 were treated with both MNNG and sodium
chloride, whereas groups 6 to 10 were maintained on control diet as negative controls.
hyperplasia occurring in the fundic region has not been clearly established as being of preneoplastic nature, and since no adenocarcinoma developed in this experiment we assigned it to a separate category. Most adenocarcinomas were relatively well-differentiated and composed of typical glandular structures demonstrating a tubular pattern and cellular or structural atypism. No metastasis was evident.

Table I summarizes the occurrence of gastroduodenal tumors. The incidence of adenocarcinoma was significantly increased in the groups given potassium metabisulfite and formaldehyde after the initiation procedure (P<0.05). Neither ethanol nor hydrogen peroxide showed any enhancement of tumor development in the glandular stomach, although adenomatous hyperplasias in the fundic region were frequently found after the latter treatment and in formaldehyde-treated animals.

Papillomas in the forestomach were observed in formaldehyde- and hydrogen peroxide-treated rats. Although relatively higher incidences were observed in the groups with initiation treatment, the differences between the groups with and without prior initiation were not significant. Of the animals treated with potassium metabisulfite after initiation, 2 rats (10.5%) developed papillomas in the forestomach.

Pyloric metaplasias, which are focal abnormalities of fundic mucosa and represent a type of regenerative lesion after carcinogen-induced damage, were found in the stomachs of many MNNG-treated rats and were unrelated to cancers. Therefore, the incidences of this lesion are not tabulated.

Table I: Effects of Various Chemicals on Gastroduodenal Carcinogenesis Initiated by MNNG and NaCl in Male Wistar Rats

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Chemical</th>
<th>Total No. of rats</th>
<th>No. of carcinoma-bearing animals (%)</th>
<th>Forestromach Papilloma</th>
<th>Number of animals (%) with tumor in Glandular stomach Fundus</th>
<th>Pylorus</th>
<th>Pneoplastic hyperplasia</th>
<th>Duodenum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After initiation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>30</td>
<td>4 (13.3)</td>
<td>0</td>
<td>1 (3.3)</td>
<td>7 (23.3)</td>
<td>3 (10.0)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Ethanol</td>
<td>21</td>
<td>2 (9.5)</td>
<td>0</td>
<td>0 (4.8)</td>
<td>0 (4.8)</td>
<td>2 (9.5)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Potassium metabisulfite</td>
<td>19</td>
<td>6 (31.6)</td>
<td>2 (10.5)</td>
<td>0</td>
<td>1 (5.3)</td>
<td>5 (26.3)*</td>
<td>4 (21.1)</td>
</tr>
<tr>
<td>4</td>
<td>Formaldehyde</td>
<td>17</td>
<td>5 (29.4)</td>
<td>15 (88.2)**</td>
<td>0</td>
<td>18 (88.2)**</td>
<td>4 (23.5)*</td>
<td>7 (41.2)</td>
</tr>
<tr>
<td>5</td>
<td>Hydrogen peroxide</td>
<td>21</td>
<td>2 (9.5)</td>
<td>21 (100)**</td>
<td>0</td>
<td>8 (38.1)**</td>
<td>2 (9.5)</td>
<td>6 (28.6)</td>
</tr>
<tr>
<td><strong>Without initiation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Ethanol</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Potassium metabisulfite</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Formaldehyde</td>
<td>10</td>
<td>0</td>
<td>8 (80.0)**</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Hydrogen peroxide</td>
<td>10</td>
<td>0</td>
<td>0 (50.0)*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Significantly different from group 1 at *P<0.05  **P<0.01.
a) Not different from group 9.
b) Significantly different from group 10 at P<0.01.
While the incidences of adenomatous hyperplasias in this area were increased in the treated groups after initiation (groups 4 and 5), only regenerative mucosa was evident in the groups without initiation (groups 9 and 10).

As compared to normal gastric mucosa, diffuse deep gastric pits with clearly increased numbers of mucous neck cells in the fundic mucosa were the major finding in the potassium metabisulfite- and formaldehyde-treated rats (groups 3, 4, 8 and 9).

In the ethanol-treated rats (groups 2 and 7), the glandular stomach mucosae were intact.

Adenocarcinomas of the duodenum were found mostly within 10 cm of the pyloro-duodenal junction as well circumscribed nodules. The luminal surfaces of the tumors were papillary, nodular, or ulcerative. All duodenal adenocarcinomas were well differentiated and of tubular structure. No malignant tumors were found outside of the gastroduodenal tract.

**DISCUSSION**

Although our understanding of experimental gastric carcinogenesis has recently progressed as a result of the utilization of different animal models, the results require detailed discussion and evaluation before they can be applied to the control of the human disease. For example, the availability of experimental models has permitted assessment of risk factors, such as high salt consumption, and consideration of activity in both initiation and promotion stages of multi-step carcinogenesis.8) Tumor promotion has been widely investigated in several organs such as the skin,14) liver,15) kidney,16) urinary bladder,17) mammary gland,18) and thyroid.19) Recently, the rat MNNG model has provided a suitable system for evaluation of the modifying effects of chemicals on rat gastric carcinogenesis.

In the mouse skin model, a single administration of a very small amount of a carcinogen such as DMBA is sufficient for initiation. In contrast, with the glandular epithelium of the stomach a relatively longer treatment with MNNG over 12 weeks is necessary for induction of a comparable basal level of preneoplastic lesions.20) However, our previous experiment demonstrated a significant modifying effect of sodium chloride on the initiation stage of MNNG-carcinogenesis in rats.1) When combined with administration of a high concentration of sodium chloride, an 8-week treatment with MNNG results in development of gastric tumors in rats. Using this initiation treatment, we subsequently demonstrated possible promoting potential of sodium chloride in two-stage gastric carcinogenesis.8)

In this experiment, it was also demonstrated that subsequent administration of potassium metabisulfite or formaldehyde raises the incidence of neoplastic and preneoplastic lesions of the glandular stomach in rats, suggesting a possible promoting effect of these chemicals.

The histological findings for the MNNG-induced lesions listed in Table I were similar to those reported previously.1,8) Although the range of tumor types occurring in each group treated with MNNG was unrelated to subsequent treatment with chemicals, the development of adenocarcinomas in the pyloric area was significantly promoted in rats given potassium metabisulfite or formaldehyde.

As in our previous experiments using MNNG, duodenal adenocarcinomas were also induced, but none of the chemicals tested, including hydrogen peroxide, affected the tumor yield when given after MNNG initiation. Hirota and Yokoyama11) reported an enhanced incidence of duodenal carcinoma in animals continuously given hydrogen peroxide following intraperitoneal administration of methylazoxymethanol acetate. In the present experiment, while we could not observe any comparable enhancing effect of hydrogen peroxide on duodenal tumor development, characteristic diffuse lesions showing fusion of the duodenal villi were found throughout the canal.

The mechanisms underlying the promoting effects of potassium metabisulfite and formaldehyde on gastric carcinogenesis remain unclear. One possibility is that growth of the initiated population is indirectly enhanced by gastric irritation and damage to the mucous membrane. Foveolar hyperplasia was a common finding after treatment with these two chemicals. It has previously
been observed that diffuse mucosal thickening in the fundic region is a finding common to both sodium chloride- and saccharin-treatment regimens associated with promotion of gastric tumor development.\textsuperscript{8}) Although the exact mechanism of action of many organospecific promoters is uncertain, one common observation during the promoting stage is hyperplasia or increased DNA synthesis in target sites.\textsuperscript{21,22}) In this study it was also shown that the enhancing treatments with potassium metabisulfite and formaldehyde induced diffuse proliferative changes in the superficial epithelium of the glandular stomach, whereas the nonenhancers, ethanol and hydrogen peroxide, showed neither effect. While such a proliferation-stimulating mechanism operating diffusely in the gastric mucosa should be important for the enhancing effect of potassium metabisulfite or formaldehyde on gastric carcinogenesis, elucidation of the exact biochemical alterations underlying the effects of these chemicals will require further investigation. Formaldehyde has also been shown to be a weak promoter in C3H/10T1/2 cells\textsuperscript{23}) and to cause nasal passage cancer in rats.\textsuperscript{24)}

In this study, ethanol did not cause any increase of tumor incidence. On the contrary, it even appeared to exert inhibitory activity, although because of the relatively low incidence of gastric tumors observed in the control group in this experiment, no statistical significance could be established.

Extrapolation of conclusions drawn from experimental data to the human situation is very difficult. However, since there are no epidemiological data indicative of a positive relation between alcohol consumption and gastric cancer, the present negative result suggests that alcohol also lacks promoting potential in this organ in man.

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