Flow Cytometric Analysis of the Effect of Sodium Chloride on Gastric Cancer Risk in the Rat

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ABSTRACT

Dietary sodium chloride has been identified, both experimentally and epidemiologically, as a risk factor for gastric cancer. In order to elucidate the manner in which salt increases gastric tumor incidence in N-methyl-N′-nitro-N-nitrosoguanidine-treated animals, flow cytometric cell cycle analyses were performed on rats which had been treated with 1 ml of a solution of saturated NaCl by gavage and sacrificed 0, 1, 6, 12, 24, or 48 h after treatment. The gastric antra were excised, disaggregated, and stained with propidium iodide for cell cycle analysis. Results showed that there is a reduction in cell yield at early time points due to the toxicity of NaCl, followed by a net increase in the number of cells in the S phase of the cell cycle at 24 h. Treatment of rats with NaCl 24 h prior to a dose of 10 µg of 3H-labeled N-methyl-N′-nitro-N-nitrosoguanidine did not lead to an increase in alkylation of DNA isolated from mucosal cells. Therefore, the hypothesis that salt enhances gastric cancer risk from N-methyl-N′-nitro-N-nitrosoguanidine by disruption of the "mucosal barrier" leading to an increased effective dose to target cells is not supported by the results of these experiments. Several studies have shown that cells in S phase are the most susceptible to mutagenesis and that increasing the number of cycling cells in a target organ will increase tumor incidence (e.g., partial hepatectomy). Thus it is possible that NaCl increases gastric cancer risk through the mitogenesis which results from the damage caused to the mucosa by this agent.

INTRODUCTION

Morbidity and mortality rates of gastric cancer in Japan are the highest in the world (1). A dietary component which stands out as an important factor which contributes to Japanese stomach cancer is a high salted food intake (2, 3). In particular, the Japanese eat a lot of dried fish which has been preserved with salt (3 to 20%), soy sauce (19% salt), and preserved pickles (13 to 25% salt). Daily consumption of a very hot rice gruel seasoned with soybean sauce (salt content, 18%) has also been related to high gastric cancer incidence in Japan (1). One group reports that persons excreting as much as 50 g of salt daily in the urine are not uncommon in areas where salted fish are a dietary staple (2, 3). Their geographic comparison within Japan indicates that the incidence of gastric cancer parallels the amount of salted foods consumed. Another study which examined the role of diet in cancer incidence in Hawaii found an association of stomach cancer incidence with the consumption of salted fish (4).

Experimentally, salt has been shown to increase the incidence rate of gastric cancer when administered concurrently with MNNG to rats (5). Interestingly, administration of 1 ml of a saturated NaCl solution by gavage once weekly increased MNNG-induced gastric carcinoma to a greater extent than providing a 6-g/liter NaCl solution ad libitum to drink. Treatment with NaCl also increases the induction of carcinoma of the mouse forestomach by 4-nitroquinoline 1-oxide (6), and hypertonic saline enhances the uptake of 7,12-dimethylbenz(a)anthracene by the guinea pig gastric wall (7).

Mice which were fed a diet containing salted fish and salted vegetables were demonstrated to have bleeding and general damage in their glandular stomach mucosa (2, 3). Salt may act, therefore, by damaging human gastric mucosa and increasing the likelihood of precancerous conditions such as gastritis. Or, salt may facilitate the uptake of chemical carcinogens by gastric cells, for example, by exposure of gastric pits following injury.

Table 1 summarizes what is known about NaCl-modified MNNG-induced gastric carcinogenesis in the rat. When MNNG is administered in the drinking water over the lifetime of rats, gastric adenocarcinomas are observed (8). If administered for a shorter period of time, for example, 2 to 6 mo, fewer tumors are observed (9). If this shortened regimen is followed by once weekly intragastric treatment with 1 ml of saturated NaCl (10) or includes and is followed by 10% dietary NaCl (11), no increase in tumor incidence occurs. If MNNG-drinking water solutions and weekly NaCl treatments are administered concurrently over the lifetime of rats, a large increase in tumor incidence is seen as compared to MNNG alone (5). A single intragastric dose of 450 mg of MNNG per kg is inadequate to cause tumors. If such a single dose is followed by weekly salt treatments over the animal's lifetime, no tumors appear. If, however, the single dose of MNNG is preceded 24 h earlier by a single dose of saturated NaCl, tumors develop (12). It can be concluded from these experiments that NaCl does not act as a tumor promoter, in the classic initiation/promotion sense of the word, as administration of NaCl after initiation by MNNG does not increase tumor incidence. NaCl appears to enhance the initiation of tumorigenesis by MNNG. Whether NaCl does so by increasing the uptake of carcinogen by the gastric mucosa, as postulated by Capaferro and Torgersen (7), or by increasing the susceptibility of the target tissue has not previously been established and is the subject of the present investigation.

MATERIALS AND METHODS

Tissue Disaggregation and Flow Cytometry. This method will be described in detail elsewhere. Briefly, gastric tissue is excised and

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incubated for 30 min at 37°C in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer containing 2% bovine serum albumin and 2 mM EDTA. The mucosa is removed from underlying tissue by scraping and is then incubated in the same buffer, omitting EDTA and including 0.15% diethyrlitol and 20 mM CaCl₂·H₂O₂ with gentle stirring for 40 min at room temperature. Cell counts and viability are determined using trypan blue exclusion and a hemacytometer. Cells are washed twice with phosphate-buffered saline + 2 mM MgCl₂, suspended in that solution at a concentration of 10⁶ cells/ml, and then fixed by the addition of 10 ml of 70% ethanol. Fixed cells are stored until needed for analysis. This method produces a high yield of gastric cells in single cell suspension (>95%) with high viability (>99%).

For flow cytometric analysis, cells are centrifuged and resuspended in 1 ml of 40% ethanol. Two thousand units of Rnase (RASE; Worthington Biochemical Co.) are added, and cells are incubated at 37°C for 1 h. Cells are then centrifuged at 100 × g for 10 min and resuspended in 1 ml of propidium iodide solution (50 µg of propidium iodide per ml of 0.1% sodium citrate). Cells are incubated on ice for 20 min and then filtered through 20-µm nylon mesh (Nytex; Small Parts, Inc.).

Cell cycle analysis is performed on an Ortho Diagnostic Systems Cytoperfograf System 50-H, equipped with an 8 W argon laser, at an emission wavelength of 488 nm, gated for narrow angle forward scatter. Analyses are performed with a Data General/Ortho Diagnostics System 2150 computer using the Constant Method.

Cell cycle analyses were also performed on rats which received a single dose of 1 ml of NaCl by gavage once weekly during this period. Animals from each group were sacrificed at the end of 3 mo, while the rest were sacrificed at the end of 1 yr. Following excision, stomachs were opened and mucosa was spread flat on filter paper, and fixed in buffered formalin. Tissue sections were prepared on microscope slides, developed for autoradiography, and stained with hematoxylin and eosin. For LI determinations, sections were examined microscopically, and well-aligned gastric pits were scored for the total number of cells per pit as well as the number of labeled cells per pit. Cells were considered labeled if they contained at least 12 grains. Ten pits were scored per animal. The LI was calculated by dividing the number of labeled cells by the total number of cells per pit.

Carcinogenesis. In order to verify that sodium chloride-induced hyperplasia is associated with an increased incidence of gastric adenocarcinoma, weanling Sprague-Dawley rats received 75 mg of MNNG per liter of drinking water for 3 mo. Half of the animals received 1 ml of saturated NaCl by gavage once weekly during this period. Animals from each group were sacrificed at the end of 3 mo, while the rest were sacrificed at the end of 1 yr. Following excision, stomachs were opened and spread flat, pinned onto cardboard, and preserved in formalin. Hematoxylin- and eosin-stained sections were examined histologically. Autoradiography was performed for the 3-mo group as described above.

Statistical Methods. Analyses of variance, Student’s t tests, and Wilcoxon rank-sum tests were performed using standard methodology as described in Brown and Hollander (14).

RESULTS

Toxicity. Figs. 1 and 2 show the damaging effects of a single dose of saturated NaCl on the gastric mucosa. The extensive edema which results from the damage is evident as the tissue appears wetter and becomes much easier to spread out. The mucosa appears to lift away from the underlying layers as fluid fills the space. The mucosa is thinner than normal, as about one-third of the outer epithelial cells has been lost.

This experiment was carried out in order to determine whether several doses of NaCl could increase the effect seen with a single dose.

For dose-response experiments, single doses of 1 ml of 1 m or 3 m NaCl were administered to rats by gavage. Twenty-four h later, animals were sacrificed, and their gastric cells were processed for flow cytometry as above.

Autoradiography. In order to compare results obtained using flow cytometry to the traditional autoradiographic method of determining proliferative cell fractions, an experiment was performed comparing control (untreated) rats to rats which received 1 ml of saturated NaCl and were sacrificed 24 h later, or to rats which received NaCl once weekly for 3 mo. Three rats were used for each group. Rats received 200 µCi of [³H]thymidine i.p. 1 h prior to sacrifice. Upon sacrifice, the antra were excised, spread flat on filter paper, and fixed in buffered formalin. Tissue sections were prepared on microscope slides, developed for autoradiography, and stained with hematoxylin and eosin. For LI determinations, sections were examined microscopically, and well-aligned gastric pits were scored for the total number of cells per pit as well as the number of labeled cells per pit. Cells were considered labeled if they contained at least 12 grains. Ten pits were scored per animal. The LI was calculated by dividing the number of labeled cells by the total number of cells per pit.

Table 1: Summary of experimental systems in which MNNG and sodium chloride have been used to induce gastric cancer

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Relative tumor incidence</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>MNNG</td>
<td>++</td>
<td>8</td>
</tr>
<tr>
<td>MNNG</td>
<td>NaCl</td>
<td>+</td>
</tr>
<tr>
<td>NaCl</td>
<td>MNNG</td>
<td>+++, 5</td>
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<tr>
<td>MNNG</td>
<td>NaCl</td>
<td>–</td>
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<tr>
<td>NaCl</td>
<td>MNNG</td>
<td>–</td>
</tr>
<tr>
<td>NaCl</td>
<td>MNNG</td>
<td>–</td>
</tr>
</tbody>
</table>

*Compound alone, single dose; compound followed by arrow, continuous administration.

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Chart 1 shows the number of cells recovered per mg of tissue wet weight at various time points after treatment with a single dose of saturated NaCl. There is clearly a toxic effect due to NaCl treatment at the earlier time points. Analysis of variance of the corrected data shows that there is a treatment effect ($P < 0.0005$), and Student's $t$ tests of the sample means showed that the number of cells recovered at 1, 6, and 12 h after treatment are significantly different from control values. At 24 h, however, cell recovery has returned to normal.

Thus a single dose of saturated NaCl causes an acute toxic effect to the gastric mucosa, but the tissue's ability to rapidly renew itself replaces lost and damaged cells within 24 h after treatment.

Methylation of DNA. Administration of a single dose of saturated NaCl 24 h prior to MNNG treatment did not significantly increase the extent to which DNA is methylated. Student's $t$ test of the sample means ($2.8 \times 10^{-6}$ versus $3.6 \times 10^{-6} \pm 1.0 \times 10^{-6}$ µCi/µg of DNA for MNNG- and MNNG + NaCl-treated, respectively) showed there was no difference between the results obtained for the two groups. The contention of other authors that NaCl increases gastric cancer risk by allowing greater access of carcinogens to the target tissue, thus increasing the dose of carcinogens, is not supported by experimental evidence.

Cell Cycle Analysis. Chart 2 shows the effect of a single dose of saturated NaCl on the number of cells in the DNA proliferative, or S, phase of the cell cycle, at various time points after treatment. Chart 3 illustrates the flow histograms obtained using control or NaCl-treated rats. Note the broader S-phase section of the curve which resulted from NaCl treatment. The coefficient of variation for the G_{1}G_{0} peak was less than 10% for all samples. The root mean square error for each sample (error associated with estimating the S-phase population) was 2.0 or less (1.6 is the ideal root mean square error for the Constant Method).

Analysis of variance showed that there was an effect due to NaCl treatment ($P < 0.0005$), and Student's $t$ tests of the sample means showed the number of cells in S phase was significantly different from that of control animals at 6, 12, and 24 h after treatment ($P < 0.005$). Animals have returned to normal at 48 h.
causes a small but detectable increase in the number of cells in S phase as compared to controls (significant at P < 0.025), while 3 M NaCl causes an increase equivalent to that of 6 M NaCl (significant at P < 0.005). Concentrations of NaCl less than that of saturated are apparently capable of maximally stimulating gastric cell proliferation.

Autoradiography. Results of the [3H]thymidine autoradiography experiments are shown in Table 2. The LI values are given for control animals as well as for animals treated with a single dose of saturated NaCl, or with weekly doses of saturated NaCl for 3 mo. The control LI value of 9% agrees with the proportion of S-phase cells determined using flow cytometry (10%). The LI values of 17% at 24 h and 18% at 3 mo are also in agreement with flow cytometric results (16% at 24 h). Fig. 3 shows what is observed microscopically following autoradiography using the gastric mucosa. The results of these experiments indicate that about 17% is the maximum percentage of the gastric cell population which can be found in S phase after tissue damage, whether one or many doses of the damaging agent are used.

Cell Sorting. The data from the cell-sorting experiment, which was undertaken in order to determine how the gastric carcinogen MNNG is distributed among mucosal cells in different locations in the gastric pits as well as in different states of proliferation, are as follows. For the MNNG group, there were 661 dpm/10^6 cells for the G_1-G_0 population and 569 dpm/10^6 cells for the S + G_2-M population. The MNNG + NaCl group had 413 dpm/10^6 cells and 345 dpm/10^6 cells for the G_1-G_0 and S + G_2-M populations, respectively. In each case, the dose of MNNG to S + G_2-M cells was about 85% of that to G_1-G_0 cells, possibly due to the location of S-phase cells at the bottom of the gastric pits in the antrum. The MNNG + NaCl group had twice as many cells in S + G_2-M as the MNNG group. Thus NaCl does not appear to increase the dose of MNNG per S-phase cell but increases the number of S-phase cells.

Carcinogenesis. Table 3 shows the results of the carcinogenesis study, with the labeling index at 3 mo and the incidence of gastric adenocarcinomas detected at 12 mo. Most of the adenocarcinomas occurred at the union of the antrum and the corpus. The labeling index is somewhat increased by MNNG treatment,
and to a greater extent by NaCl and MNNG, correlating well with ultimate tumor development.

**DISCUSSION**

Dietary sodium chloride has been identified both epidemiologically (2, 3) and experimentally (5) to be capable of increasing gastric cancer risk. Sodium chloride is known to damage the gastric mucosa (2, 3). Tissue damage may be involved in cocarcinogenesis because tissues respond to damage by increasing replicative cell activity in order to replace the damaged tissue. For example, patients with chronic atrophic gastritis, a condition that has been identified as part of the same etiological entity as gastric cancer (15), have been shown using [3H]thymidine to have expanded generative cell regions in their gastric mucosae (16). Expansion of the compartment of proliferating cells as well as incorporation of [3H]thymidine at or near the luminal surface of the mucosa continues during intestinalization (17). Furihata et al. (18) have recently demonstrated increases in both DNA synthesis and ornithine decarboxylase activity following single p.o. doses of saturated sodium chloride to rats.

Cells which are replicating are known to have increased susceptibility to mutagenesis and carcinogenesis. Synchronized rat liver epithelial cells were shown to be most sensitive to mutation at the phosphoribosyltransferase locus by methyl methane sulfonate or the gastric carcinogen, MNNG, during the period of DNA synthesis (19). Rats which were given a single injection of dimethylaminoiline 24 h after undergoing partial hepatectomy (the peak time of DNA synthesis in the regenerating liver) developed hepatocellular carcinomas, while nonhepatectomized rats did not (20).

Thus cells which are exposed to a potential carcinogen which is capable of adducting DNA (such as MNNG) while actively replicating their DNA are more likely to experience a mutagenic event than are cells which are not proliferating. Cells which are not actively proliferating have time to repair DNA damage before entering S phase. The gastric mucosa contains a larger proportion of nonproliferating epithelial cells which are continuously sloughed off into the gastric lumen. Modification of DNA by a potential carcinogen in these cells will probably have no mutational consequence, as these cells live only a few days and will never enter S phase and so will never replicate their DNA. Compounds which increase the number of S-phase cells in a population are therefore likely to increase the possibility that a mutagenic and potentially carcinogenic event may occur, should a carcinogen also be present.

The present work has shown that a single intragastric dose of concentrated sodium chloride can cause extensive toxicity to the rat gastric mucosa. Toxicity is reflected as edema and a reduction in the number of cells which can be recovered following tissue disaggregation. Other authors have suggested that sodium chloride-induced toxicity is responsible for increased gastric cancer risk because any potential carcinogens present in the gastric lumen would then have greater access to the proliferative cell population, located deeper in the mucosa (12). Examination of the extent of methylation of gastric mucosal DNA by tritium-labeled MNNG in the present work demonstrated that methylation occurs to the same extent whether or not animals have been pretreated with sodium chloride. Increasing the tissue dose of carcinogen is apparently not the manner in which sodium chloride increases gastric cancer risk.

Use of both flow cytometry and autoradiography showed that sodium chloride treatment increases the proportion of proliferating, or S-phase, cells by about 70%. This increase occurs after single intragastric doses of either 3 or 6 m (saturated) NaCl and cannot be extended beyond 70% by multiple doses. Seventy % is apparently the maximum extent by which the proliferative cell fraction can be expanded in the gastric mucosa. This expansion was correlated with the ultimate development of gastric adenocarcinomas when NaCl and MNNG were administered together.

It would appear, then, that in the stomach, sodium chloride causes a mitogenic response which results in an increase in the number of cells which would be susceptible to a potential carcinogen such as MNNG.

The relevance of proliferative cell number to cancer incidence has been emphasized recently by Greenfield et al. (21) in their study of sodium saccharin and FANFT-induced bladder cancer. These authors proposed a probabilistic model which includes variables, such as the populations of normal, initiated, and transformed cells, mitotic rates of these cells, hyperplasia, and the probabilities of cell initiation and transformation during replication, to elucidate the role these chemicals play in bladder carcinogenesis. Data from carcinogenesis studies using rats were used to estimate each parameter. Results indicated that both saccharin and FANFT increase bladder tumor rates by increasing the number of proliferating cells available for initiation (while FANFT also increases the probability of initiation). This study was able to utilize a large data base of dose-response data for sodium saccharin and FANFT-induced bladder tumor incidences. Unfortunately, such a data base does not exist for sodium chloride and MNNG-induced gastric tumor incidences. Thus there is not enough information available to quantitatively evaluate the effects of increased gastric proliferative cell number on tumor incidence.

The fact that the maximal increase in S-phase cells occurs at 24 h following NaCl administration may provide an explanation for the observations of Shirai et al. (12), who observed gastric tumors when a single dose of MNNG was given 24 h after a single dose of NaCl. Shirai observed a 15% incidence of gastric adenocarcinomas following a dose of MNNG (450 mg/kg) 24 h after sodium chloride. MNNG alone elicited no tumors. Since the threshold dose of MNNG is not known, it is not clear what the relationship between these parameters may exist. Further research using varied doses of MNNG would be required to determine whether this relationship might be linear.

Saturated (6 m) sodium chloride was used for most of the present studies. Three m sodium chloride, however, was also demonstrated to increase the proliferative cell fraction in the...
gastrointestinal mucosa. Three m sodium chloride is a concentration which is not inconceivable in terms of human consumption. Three m NaCl is about 17% NaCl. The Japanese, who have the highest rate of gastric cancer in the world, commonly consume soy sauce (18% NaCl), dried fish (20% NaCl), and preserved pickles (13 to 25% NaCl). Frequent consumption of such foods is likely to lead to increased gastric cell proliferation.

Thus concentrations of sodium chloride which can be encountered in the human diet can increase the proliferative fraction of cells in the gastric mucosa and therefore increase the population of cells which is susceptible to mutagenic and potentially carcinogenic events. Sodium chloride does not increase the dose of carcinogen to the mucosa but increases the target size for mutagenesis. The increase in cellular proliferation is a response to sodium chloride’s toxicity. Concentrated sodium chloride overwhelms the ability of mucosal extracellular fluid to osmotically regulate individual cells (22) and may also overwhelm the hydrogen pump that keeps hydrogen ions in the gastric lumen and out of the mucosa. Increased cellular proliferation has been shown, using several models, to increase tumor incidence in the presence of a carcinogen. This model may prove useful for the detection of as yet unidentified risk factors for gastric cancer, as well as of compounds which may provide protection of the gastric mucosa against such risk factors.

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REFERENCES

Fig. 1. A, normal stomach, opened along the greater curvature; B, stomach of rat 6 h after receiving 1 ml of saturated NaCl by gavage. Note extensive edema.
Fig. 2. A, normal rat gastric mucosa. H & E, × 12. B, gastric mucosa of rat 6 h after receiving 1 ml of saturated NaCl by gavage. H & E, × 12. The extent of edema can be seen by comparison with the normal gastric mucosa shown in Fig. 2A.
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