Susceptibility of Healed Gastric Ulcers to Chemical Carcinogenesis in Rats and Implications of Cellular Kinetic Changes

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ABSTRACT

The susceptibility of healed, experimental gastric ulcers to chemical carcinogenesis was investigated. Slowly healing gastric ulcers were induced by the acetic acid method in the fundic and pyloric gastric mucosae of inbred male Wistar rats. N-Methyl-N’-nitro-N-nitrosoguanidine (MNNG) was administered in drinking water at a concentration of 50 mg/liter for 360 days after ulcer induction. Twenty-eight adenomatous hyperplasias and six adenocarcinomas developed in the pyloric mucosae of rats, including five cases of adenomatous hyperplasia which developed in the periphery of the healed ulcer. In contrast, only one adenomatous lesion was found in the regenerated mucosa of the healed pyloric ulcer. No neoplasm was observed in the healed fundic ulcer area. The results demonstrated an increased incidence of neoplasms in the peripheral area of the healed pyloric ulcer and a decreased incidence of neoplasms in the regenerated mucosa within the healed pyloric ulcer scar, although these differences were not statistically significant in comparison with the intact pyloric mucosae of the MNNG-treated rats. Histoautoradiographs of the gastric mucosae demonstrated increased labeling indices in the healed ulcer periphery of the pyloric mucosa and decreased labeling indices in the regenerated mucosa within the healed pyloric ulcer scar of MNNG-treated rats, which might be related to the differential susceptibility of the two regions to gastric carcinogenesis. Intestinal metaplasia preferentially developed near the pyloroduodenal junction in MNNG-treated rats but was not localized in control rats. In the fundic ulcer scar area, an unusual squamous cell metaplasia was observed in one rat.

INTRODUCTION

The relation between chronic gastric ulcer and gastric cancer has been debated for many years, and it has long been considered (19) that adenocarcinoma arising from benign ulcer in the stomach occurs uncommonly. Nonetheless, further study of the complex relationship between these 2 diseases is warranted because: (a) recent data on early gastric cancer show that chronic peptic ulcer still constitutes a slight risk factor for gastric carcinoma (18); (b) the malignant change of linear gastric ulcer may occur more frequently than of other types of gastric ulcer (16); (c) both of these diseases are frequently associated with intestinal metaplasia as a precursor or related disease, and all 3 entities are elevated simultaneously in some population groups such as Japanese residents (1, 9, 18, 22); (d) both gastric cancer and gastric ulcer are more common in males and tend to be located most frequently in the pyloric antrum (28); and (e) preliminary experimental animal data have suggested that chemical carcinogenesis in the stomach might be promoted by experimentally induced gastric ulcer (17, 27, 33, 34). Thus, further studies in animal models on the correlated pathogenesis of these diseases might be rewarding, particularly since atypical hyperplasia has been noted in the periphery of semichronic or slowly healing experimental gastric ulcers in rats (21) and since the acute mucosal ulceration and subsequent, regenerative glandular hyperplasia observed after administration of the gastric carcinogen, MNNG, is localized in the region of the stomach where neoplastic changes preferentially develop (24, 31, 32). Accordingly, the present investigation was conducted to assess the relative susceptibility of both the antral and fundic ulcer scar and ulcer periphery as well as the remainder of the gastric mucosa to malignant change induced by p.o. administration of MNNG in rats.

MATERIALS AND METHODS

Induction of Gastric Cancer and Ulcer. Inbred male Wistar rats, bred and maintained under specific-pathogen-free conditions, 7 weeks old, and weighing 200 ± 10 (S.D.) g, were obtained from the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University, Kyoto, Japan. One hundred rats were fed a standard diet ad libitum, but 56 had the carcinogen MNNG added to their drinking water at a concentration of 50 mg/liter. Forty-four rats not exposed to MNNG were controls for this experiment. Every 2 weeks, MNNG was dissolved in distilled water at a concentration of 1 g/liter and kept in a dark cold room. This stock solution was further diluted with tap water 3 times a week and put into black-painted water bottles. Rats were allowed to drink ad libitum. This procedure of MNNG administration was described previously (30). MNNG administration was continued for 51 weeks.

Semichronic gastric ulcers were induced in all the MNNG-treated and control rats 4 days after the beginning of MNNG administration by the topical acetic acid method of Okabe and Pfeiffer (20). After overnight fasting, laparotomy was performed under ether anesthesia. A round metal mold (4 mm inside diameter) was placed upon the gastric serosal surface. Fifty % acetic acid was applied inside the metal mold for 1 min, then the acetic acid was removed, and the serosal surface was rinsed with phosphate buffer. Two ulcers were induced in standardized locations as described in Charts 1 to 3, one in the fundic gland area near the greater curvature and the other in the pyloric gland area near the lesser curvature, and the abdominal wall was subsequently closed.

Experimental Course. The physical condition of the rats was exam-
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ned daily, every fourth week rats were weighed, and the abdomen was palpated. From 12 to 30 weeks, 3 or 4 healthy or sick rats were sacrificed at 4-week intervals. At the end of 30 weeks, 10 rats in each group were sacrificed for histautoradiography. Thereafter, only moribund rats were killed, and all the remaining rats were sacrificed at the end of 60 weeks.

Tissue Processing for Histological Examination. After sacrifice, the stomach was excised and opened along the greater curvature, washed with 0.9% NaCl solution, pinned flat on cardboard without tension, and fixed in Bouin’s fluid. A photo of each fixed rat stomach was taken under water immersion, as shown in Fig. 1. Gross examination of the gastric mucosa was done with a dissecting microscope. All areas of the glandular stomach were cut into 2- to 3-mm-wide longitudinal strips. An additional photograph of the stomach was taken after cutting to record the correct alignment of strips. The specimens were then processed for hematoxylin-eosin staining. Histoautoradiography was carried out at 30 and 60 weeks after MNNG administration. [6-3H]Thymidine (specific activity, 5 Ci/μmol) was purchased from the Radiochemical Centre, Amersham, England. The MNNG-treated and control animals were fasted for 24 hr, and [6-3H]thymidine was injected at a standardized time of day (1 p.m.). In each group, 10 rats at weeks 3 and 5 weeks at 60 weeks were given a single s.c. injection of [6-3H]thymidine (1 μCi/g body weight) and were sacrificed 60 min later. The stomach was processed as described above. Paraffin sections (4 μm thick) were mounted on glass slides, dipped in Sakura NR-M2 nuclear emulsion (Sakura Photo Co., Ltd., Japan), and developed with Sakura FD 111 after 5 weeks exposure. Sections were then stained with hematoxylin-eosin.

Histological Examination and Calculation of Labeling Indices. The healed ulcer region was easily recognized by finding the area of disrupted muscle in the gastric wall, and the regenerated mucosal area of the healed ulcer (ulcer scar area) was defined as the mucosal area above the ulcer scar area as determined by the use of the histological criteria for adenocarcinoma and adenomatous hyperplasia were defined as described previously (24). Intestinal metaplasia was identified by the presence of goblet cells. As the photos of fixed stomachs were taken before and after strip cutting, the exact location of any visible or microscopic lesion was ascertained by the combination of these photos and microscopic slides. When the neoplasia was large, the center of the tumor was considered as the original location of the neoplasm; when the neoplasia was relatively small and with a down-growth type of growth, the position where the neoplastic cells invaded the submucosa was considered as the original location. For measuring length in the microscopic specimens, a round thin glass, in which 100 squares were marked, was placed near the ocular lens, and the length of the square was calibrated with an objective micrometer (Nikon). All the rats sacrificed at 45 weeks or later were used to calculate the surface area of the glandular stomach and pyloric mucosa and the body weights of MNNG-treated and control rats. The boundary between fundic gland and pyloric gland area was traced on the photo of rat stomach using microscopic specimens. Outlines of the 2 glandular areas were then traced on thin waxed weighing paper, cut out, and converted to the surface area from the obtained weight. Weight variation of the paper was monitored before use and was found to be ±2%. When autoradiographic specimens were evaluated, only normally appearing mucosa was selected, and the area near erosions or neoplasms was not used. A comparable part of the posterior wall of the rat stomach was used for comparison with the ulcer scar and its peripheral area. The labeling index of the gastric mucosa was determined as follows. Proliferative zones of gastric glands were randomly selected using a round thin glass placed near the ocular lens as described above. The proliferative zone was considered as the part of the glandular neck between the uppermost labeled cell and lowermost labeled cell in each gland. Five hundred cells in the proliferative zone of each of the 3 areas (ulcer scar area, peripheral area of the ulcer scar, and intact mucosal area) of the fundic or pyloric mucosal surface of each rat stomach were counted. Cell nuclei were identified as labeled when they were covered with more than 10 grains. The percentage of labeled cells among the 500 cells of the proliferative zone was calculated for the labeling index for each of the 3 areas of the fundic or pyloric mucosa of the stomach.

RESULTS

Time Course for Development of Neoplasia and Metaplasia. Six MNNG-treated rats and 4 control rats were removed from the study; these rats had died soon after gastric ulcer induction due to complications from anesthesia or ulcer perforation. Neoplastic lesions were not seen in control rats throughout the experiment. No neoplastic lesion was seen in the 10 MNNG-treated animals sacrificed at 30 weeks, but a lesion characterized by squamous cell metaplasia was observed in the regenerated mucosa (ulcer scar area) of the healed fundic ulcer. The earliest development of adenomatous growth was noted in the stomach of a moribund rat sacrificed at 45 weeks. Adenocarcinoma first appeared at 51 weeks. All of the 16 rats sacrificed at 60 weeks had adenocarcinoma, adenomatous hyperplasia, or fibrosarcoma. In total, 28 adenomatous lesions were found in the stomachs of 18 rats. Six adenocarcinomas in 6 rats and 2 fibrosarcomas in 2 rats were also noted. Development of intestinal metaplasia was correlated with the development of neoplastic lesions. Intestinal metaplastic glands were observed in 16 MNNG-treated animals sacrificed between 45 and 60 weeks, during which time a total of 25 rats were sacrificed. In 15 control rats sacrificed at 60 weeks, 9 intestinal metaplastic glands were found in 5 rats. In the fundic ulcer scar area, no evidence of intestinal metaplasia was observed. Regenerated pseudopyloric glands were already partly replaced with fundic glands at 12 weeks, and the pseudopyloric glands were seen in the central part of ulcer scar area. When 10 MNNG-treated and control rats were sacrificed and processed for autoradiography at 30 weeks, pseudopyloric glands were seen only in the small portion of the fundic ulcer scar area of one control rat.

Distribution of Neoplastic Lesions. At sacrifice, all ulcer scars were found in the standardized locations as described in Chart 1. The area of the healed ulcer (ulcer scar) did not contract due to tight adhesion between the gastric ulcer scar region and the liver surface. Two adenocarcinomas were excluded from the
distribution study, as these tumors were too large to locate the position in relation to the ulcer. Two fibrosarcomas were also excluded. As shown in Chart 1, tumors were found diffusely throughout the pyloric gland area in rat stomachs except in the healed ulcer area. In the peripheral area of the pyloric ulcer scar, 5 adenomatous lesions (Fig. 2) developed in contrast to only one lesion in the pyloric ulcer scar area. Three adenocarcinomas developed in the lesser curvature (Chart 1; Fig. 3), and one adenocarcinoma developed in the posterior wall near the lesser curvature. None was spatially related to the ulcer scar area or the lesser curvature. None was spatially related to the ulcer scar area or the lesser curvature.

None of the 23 with intestinal metaplasia were observed within 2 mm of the pyloric ulcer scar area, and the scar peripheral area, was about one-half or double that of the other areas of the pyloric mucosa, although the actual incidence of neoplasms in the ulcer scar area, and the scar peripheral area, was not significantly different from the incidence in the other area of the pyloric mucosa, although the actual incidence of neoplasms in the ulcer scar area, and the scar peripheral area, was not significantly different from the incidence in the other area of the pyloric mucosa.

Distribution of Intestinal Metaplasia and Squamous Cell Metaplasia. Intestinal metaplasia was found only in the pyloric gland area. In contrast to neoplasms, this variant of gastritis was not diffusely distributed. As shown in Chart 2, 19 instances of the 23 with intestinal metaplasia were observed within 2 mm from the pyloroduodenal junction. Of the remaining 4 instances, 2 cases of intestinal metaplasia were observed in the peripheral area of the pyloric ulcer scar. On the other hand, only 3 observations of intestinal metaplasia were made within 2 mm proximal to the pyloroduodenal junction in control rats, and a total of 9 metaplastic gland sites were apparently distributed randomly within the pyloric mucosa (Chart 3). One intestinal metaplastic gland locus of a control rat developed in the peripheral area of the pyloric ulcer scar. Intestinal metaplasia was not found in either the fundic gland area or in the fundic ulcer scar area, where squamous cell metaplasia developed, as depicted in Figs. 4 and 5. Fig. 4 illustrates 3 gastric glands that are in the process of transformation to the squamous cell metaplasia. In the central gland, the upper half is composed of squamous cells, whereas the lower half is composed of a dilated gastric gland. An autoradiograph of another section of this lesion (Fig. 5) indicates that this junction of squamous cells and gastric cells corresponds to the proliferative zone of the gastric, fundic gland. On the right side of the gland shown in Fig. 4, the entire gastric gland is replaced with the squamous cells, and the lower part of the transformed gland is atrophic. Because the squamous cell metaplasia was sectioned obliquely in the autoradiographic specimens, it was impossible to count the labeled cells in the basal cell layer. However, it appeared similar to that of the normal squamous layer of the rat forestomach.

Cell Kinetic Changes in Fundic and Pyloric Mucosa. Labeling indices of the proliferative cell zone in the normal-appearing pyloric and fundic mucosa of the ulcer scar area, peripheral area of the ulcer scar, and the remaining gastric area in MNNG-treated and control rats were compared by the split-plot type analysis of variance and Scheffe's test. Pseudopyloric glands, observed in the fundic ulcer scar area of one control rat killed at 30 weeks, were not used to count the labeled cells. As shown in Table 3, there was no significant difference between these labeling indices in the fundic gland area of the stomach at 30 and 60 weeks, although the effect of location on the labeling indices of fundic mucosa at 60 weeks was very close to significant level (p = 0.052). The interaction between MNNG treatment and location was also not significant. The results suggest that the labeling indices in the ulcer scar area and peripheral area of ulcer scar of the fundic mucosa were not altered significantly compared with that of the intact fundic mucosal area of control and MNNG-treated rats.

In the pyloric gland area of the stomach, there was a significant effect of location and a significant interaction between MNNG treatment and location at 30 and at 60 weeks (Table 4). Comparison of labeling indices between MNNG-treated and control rats, for each location, showed that the labeling index in the scar peripheral area of MNNG-treated rats increased at 30 weeks. This difference became further prominent at 60 weeks. In contrast, the labeling index of the ulcer scar area decreased signifi-
The relationship of gastric ulcer to cancer has long been a controversial subject of considerable interest. It is now believed that the incidence of gastric ulcers which undergo malignant changes is very low (3, 16, 19, 37), although recent study of early gastric cancers still suggests that chronic gastric ulcer is a risk factor for gastric cancer even though it is a less likely precursor than intestinal metaplasia or adenomatous gastric polyps (16, 18, 37). Of particular importance, however, are the facts that (a) recent data from animal experiments suggest that sites of induced experimental ulcer may respond with altered sensitivity to malignant transformations induced by site-specific gastric carcinogens, and (b) clinical and animal findings suggest that there may be a common denominator for gastric ulceration and gastric cancer, i.e., chronic gastritis or intestinal metaplasia. Thus, detailed study of these interactions may clarify the early pathogenesis of these diseases. This study showed that the decreased incidence of neoplasia in the reepithelialized pyloric glands over the pyloric ulcer itself (ulcer scar area) and the increased incidence in the pyloric glands at the healed pyloric ulcer margin were observed, although these differences were not statistically significant from the incidence of the neoplasms in the intact pyloric mucosa of the MNNG-treated rats. These findings suggest that the general effect of this ulcer model was not conducive toward experimental gastric carcinogenesis. The data suggest, however, that the healing acetic acid ulcer may exert differential effects; i.e., the reepithelialized pyloric glands over the ulcer are not at high risk to induced cancer when compared to other areas of pyloric glands, but the pyloric glands at the ulcer scar periphery may be at high risk. This finding supports recent data reported by Wong and Ong (36) on ulcers induced at the fundoantral junction in rats. Further, this differential effect was precisely predicted from autoradiographic findings in vitro with tissue culture and with in vivo experimental ulcer data (29). Other workers have reported that MNNG induced early superficial mucosal erosions in both dogs and rats and that cancers were preferentially formed in the same regions where such erosions appeared (25, 31, 32). Also, Takahashi et al. (33, 34) demonstrated increased cancer susceptibility, attributable to a greater number of regenerating cells during mucosal regeneration, to MNNG in rats at the site of experimental ulcer. Earlier findings prior to the development of site-specific carcinogenic nitrosamines also had demonstrated that the damaged gastric mucosa elicited increased susceptibility to carcinogenesis. Neoplastic lesions tended to form at gastrostomy sites in the rat stomach after administration of methylcholanthrene or dimethylbenzanthracene (27), and gastric adenocarcinomas were more prevalent in 2,7-diacetylaminofluorene-treated rats with experimental thermocautery ulcers than in rats without such gastric ulcers (17).

This study also demonstrated by historadioautography a differential interaction between ulceration and carcinogenesis with respect to gastric region. An increased labeling index of the peripheral zone of the pyloric ulcer scar and a decreased labeling index of the ulcer scar itself were demonstrated in MNNG-treated rats. These effects apparently became prominent as late seque-
than 600 μm from the ulcer edge. Our method for calculating the cells in response to ulceration over a width of 20 glands, or more explain why fundic glands are resistant to carcinogenesis. A combination of ulceration and carcinogenesis. This may partly explain why the increased labeling index was not observed in the ulcer scar lae. The result may explain the observed low incidence of neoplasm development in pyloric ulcer scar area and the higher incidence in the peripheral area of pyloric ulcer scar. Moreover, this increased labeling index was not observed in the ulcer scar periphery of the fundic gland area, which suggests the differential behavior of the pyloric and fundic glands of rat stomach to the combination of ulceration and carcinogenesis. This may partly explain why fundic glands are resistant to carcinogenesis.

In the present investigation, the periphery of the healed ulcer was selected at 1 mm for cellular kinetic assessment, since earlier studies (8, 38) indicated enhanced regeneration of gastric cells in response to ulceration over a width of 20 glands, or more than 600 μm from the ulcer edge. Our method for calculating the labeling index involved counting nuclei in the regenerating cell zone of gastric glands, and our results were similar to some (12) but not all (2, 23) previous reports. Differences in these reports may be attributable to differences in methods of calculating the index; in the present instance, it was not possible to count labeled nuclei in the entire gastric gland (2) since regenerated glands in the ulcer scar were often distorted and at times buried in granulation tissue.

The role of atrophic gastritis and intestinal metaplasia as an associated lesion with gastric cancer and ulcer is well documented both in humans (1, 9, 11, 28) and in animals with experimental gastric cancer (4), and populations with an elevated incidence of gastric cancer have a higher mucosal background of intestinal metaplasia (1, 15, 28). However, in rats treated with MNNG (35), intestinal metaplasia frequently appeared as a partially independent phenomenon from gastric cancer. In humans with cancer of the cardiac region of the stomach, intestinal metaplasia was unlikely to be an essential premalignant change in Canadian and Japanese patients (13), and it appeared to be a nonessential change for cancer of the noncardiac regions of the stomach (14). The present data showed that, in this experimental ulcer-cancer model, neoplasms developed diffusely over the mucosa although intestinal metaplasia tended to develop mainly close to the pylorus, but in control rats the intestinal metaplasia was more proximally spread throughout the antrum.

Although squamous cell metaplasia of the gastric mucosa had been experimentally induced previously (5, 10, 26), the present report is the first demonstration of induction of glandular mucosal squamous cell metaplasia by a carcinogen. Moreover, histological and autoradiographic study of the squamous cell metaplasia demonstrated the sequential process of the transformation. These metaplastic cells appeared to derive from fundic gland cells, initially from the cells within the proliferative cell zone, and spread at first to the upper part of the gland and then subsequently to the lower part of the gland. After atrophy of the lower part of the glands, regenerative cells in the proliferative cell zone were transformed to make up the basal cell layer of squamous cell metaplasia. This scheme of transformation is also supported by the kinetic study on morphogenesis of intestinal metaplasia in human stomach (7) and by gastric cell kinetic studies in the hamster (6). Because of rarity of the squamous cell metaplasia, it is conceivable that this metaplasia may be induced from the combined effects of MNNG and the gastric mucosal regeneration following the induction of acetic acid ulcer. In the pyloric gland area, 3 intestinal metaplasias developed in the peripheral area of...
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the healed pyloric ulcer. These results may suggest that accelerated proliferation of the gastric epithelial cells may be related not only to carcinogenesis but also to metaplastic transformation.

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Fig. 1. Gross appearance of stomach of MNNG-treated rat sacrificed at 60 weeks. Note adenocarcinoma in the posterior wall of the pyloric mucosa near the lesser curvature.

Fig. 2. Autoradiography of the rat glandular stomach, depicting downgrowth of adenomatous lesion in the peripheral area of the pyloric ulcer scar. The ulcer scar is at the right half. Note relatively few nuclei are labeled with tritium in the ulcer scar area. Bar, 500 µm.

Fig. 3. Early adenocarcinoma at the lesser curvature of the pyloric mucosa after MNNG treatment. Neoplastic glands have invaded the submucosa through the muscularis mucosae. Bar, 500 µm.
**Fig. 4.** Localized squamous cell metaplasia in the ulcer scar area of a MNNG-treated rat sacrificed at Week 30. Note that 3 gastric glands are in the process of replacement with squamous epithelium. Bar, 100 μm.

**Fig. 5.** Autoradiograph of squamous cell metaplasia. Note regenerative cells at base of squamous cell metaplasia and surrounding fundic glands. Bar, 100 μm.
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