Jpn. J. Cancer Res. 84, 495–500, May 1993

Effect of Experimental Hypochlorhydria on the Histological Differentiation of Adenocarcinomas in the Canine Stomach

Takeshi Sano,[1] Oichiro Kobori, Sho Kuroki, Hirokazu Nagawa and Tetsuichiro Muto

First Department of Surgery, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113

Hypochlorhydria is considered to be a risk factor for gastric cancer, both clinically and experimentally. In humans, the hypo-acidic condition appears to be closely associated with the development of differentiated-type gastric carcinomas. We investigated relationships between antral pH and histological types of gastric cancer using an animal model. A total of 7.65 g of N-ethyl-N′-nitro-N-nitrosoguanidine (ENNG) was orally administered to male beagle dogs. Subsequently, the dogs were divided into four groups and underwent four surgical interventions designed to create various conditions in the antrum. Antral pH was recorded at the time of killing after an observation period ranging from 16 to 33 months. The number of gastrin-secreting cells (G-cells) was counted after immunohistochemical staining. Carcinomas were found in the antrum of 12 of the 21 dogs that had survived. Although limited doses of ENNG, such as those used in this study, have been reported to produce only undifferentiated adenocarcinomas, differentiated adenocarcinomas were found in seven of these 12 dogs. Antral pH and the number of G-cells were significantly higher in these seven dogs than in the other five dogs, which had developed only undifferentiated adenocarcinomas. Bile inflow showed no significant effect on the development of differentiated adenocarcinomas. Neither atrophic gastritis nor intestinal metaplasia was seen in any of the dogs. These results suggest that the hypo-acidic condition itself may play a role in the development of differentiated adenocarcinomas.

Key words: Gastric cancer — Carcinogenesis — Acid — Histological differentiation

Hypochlorhydria is generally accepted to be a risk factor for stomach carcinogenesis in humans and experimental animals. In rats, hypo-acidic states induced by vagotomy[1)] or antrectomy[2)] enhanced the occurrence of carcinoma. In humans, gastric acidity seems to be closely related to the degree of histological differentiation of the stomach cancer: atrophic gastritis and intestinal metaplasia, usually associated with hypochlorhydria, are considered to be precancerous conditions leading to carcinomas of the differentiated type rather than the undifferentiated type. The mechanisms underlying the contribution of hypo-acidity to histological differentiation remain, however, to be elucidated.

Carcinomas induced by ENNG[2] in the canine stomach show various degrees of histological differentiation, as do human gastric cancers.[3)] Sunagawa *et al.*[4)] found that administration of a small dose of ENNG to beagle dogs produces only undifferentiated adenocarcinomas, while high doses of ENNG produce both undifferentiated and differentiated adenocarcinomas.

In order to assess the possible role of hypochlorhydria in the histological differentiation, we administered a small dose of ENNG to beagle dogs and then operated on them to create hypo-acidic conditions in the antra. Relationships among histological types of tumors, antral pH, bile inflow and mucosal gastrin are discussed.

MATERIALS AND METHODS

Animals and carcinogen Thirty 4-month-old male beagle dogs, weighing an average of 7.5 kg, were used. They were purchased from CSK Animal Institute (Nagano) and were housed one dog per cage with free access to water.

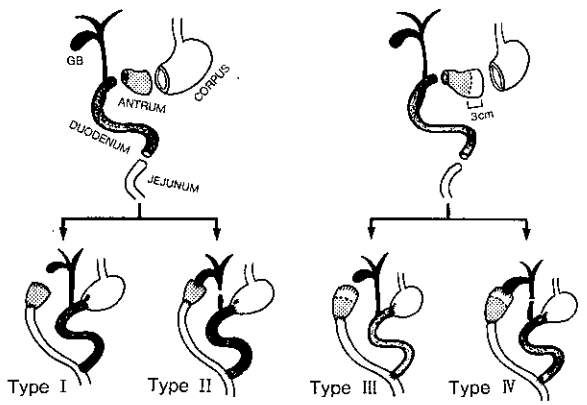
ENNG (Aldrich Chemical Co., Milwaukee, USA) was orally administered according to Kurihara's method.[3)] modifying the duration. Briefly, a stock solution of ENNG (1.5 g/liter) was prepared once a week and a 10-fold diluted solution was used. Pellet diet was soaked with 250 ml of this solution and given twice daily, except on Sunday, for 4 months (75 mg/day, a total of 7.65 g/dog).

After the ENNG administration, the dogs were divided into four groups and underwent surgical interventions.

Operations Four operations were designed to create different conditions in the antrum (Fig. 1).

[1] Present address: Department of Surgical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104. To whom correspondence should be addressed.

[2] Abbreviations: ENNG, N-ethyl-N′-nitro-N-nitrosoguanidine; G-cells, gastrin-secreting cells.



ANTRAL	Type I	Type II	Type III	Type IV
Acid	↓↓	↓↓	↓~→	↓~→
Gastrin	↑	↑↑	↓	↓~↑
Bile	(−)	(+)	(−)	(+)

Fig. 1. The operative models designed to create different antral conditions.

In group I, the antrum was separated from the corpus and drained into the jejunum. In this model, gastric acid does not flow into the antrum, and gastrin secretion is therefore assumed to be maintained at a high level due to lack of negative feedback by acid.

In group II, the antrum was separated in the same way as in group I, and the gallbladder was anastomosed to the separated antrum. The common bile duct was ligated and cut. Antral pH is neutral or slightly alkaline with constant bile (pH 7–8) inflow. Gastrin secretion is higher than in group I due to bile stimulation.

In group III, the antrum was separated in a similar way, along with a portion of the acid-secreting lower corpus (3 cm segment). Antral pH is lower than in groups I and II. Negative feedback from the acid and lack of food passage reduce gastrin secretion.

In group IV, the antrum was separated in the same way as in group III, the gallbladder anastomosed to the separated portion, and the common bile duct ligated and cut. Antral pH fluctuates in a range between those of groups II and III because of the coexistence of acid and bile. Bile inflow stimulates while acid suppresses gastrin secretion.

The dogs were operated on within a week of completion of the ENNG administration. Intravenous thiopental was used for anesthesia. Routine chow feeding started 4 to 5 days postoperatively.

Observations and killing Half of the dogs in each group were killed at 16 months after surgery, as carcinomatous lesions were first recognized in the antrum of a dog in group I that died in the 16th month. The others were killed at 33 months.

Fig. 2. Immunohistochemical staining of the gastrin-secreting cells. Scale bar = 100 μm.

Table I. Antral Conditions and Pathological Data of the 21 Dogs which Survived for the Entire Experimental Period

Dog No.	Post-op period (mo)	No. of G-cells (/4 mm)	pH	No. of carcinomas		Foveolar hyperplasia
				Undif.	Dif.[a]	
Group I						
1	16	118	—[b]	13	15	—
2	16	125	—	0	0	+
3	16	118	4.40	5	0	—
4	33	110	7.77	1	2	—
5	33	94	7.41	0	0	+
Group II						
6	16	145	8.14	0	1	+
7	16	142	7.04	2	1	+
8	33	138	—	0	0	+
9	33	152	7.30	1	1	+
10	33	140	7.47	0	1	+
Group III						
11	16	79	3.10	1	0	+
12	16	98	3.20	2	0	—
13	16	69	3.50	0	0	—
14	33	95	2.53	0	0	—
15	33	105	3.23	0	0	—
16	33	110	2.40	4	0	—
Group IV						
17	16	132	4.70	2	0	+
18	16	130	3.30	0	0	+
19	16	125	4.10	0	0	+
20	33	120	4.28	0	3	+
21	33	106	—	0	0	+

a) Undif., undifferentiated adenocarcinoma; Dif., differentiated adenocarcinoma.
b) The pH was not recorded for technical reasons.

Antral mucosal pH was recorded at the time of killing with the tip of a pH-meter placed on the mucosal surface of the middle antrum.

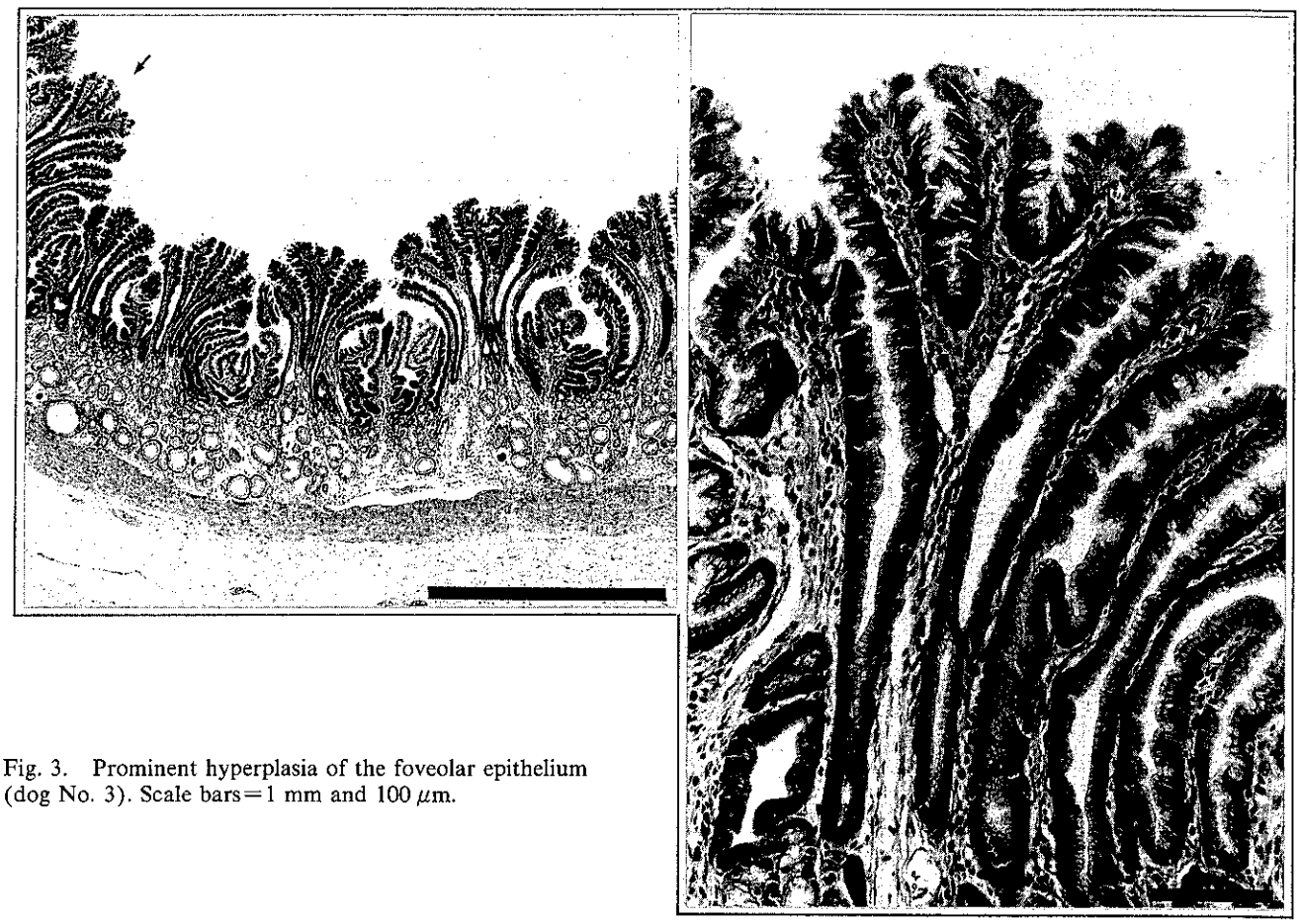


Fig. 3. Prominent hyperplasia of the foveolar epithelium (dog No. 3). Scale bars=1 mm and 100 μm.

Histological analysis The stomach was opened along the greater curvature, fixed on a flat board in formalin, cut into 5 mm-wide longitudinal strips and embedded in paraffin. The sections were stained with hematoxylin and eosin.

The carcinomas were classified histologically as either undifferentiated or differentiated adenocarcinomas; poorly differentiated and signet ring cell carcinomas were included in the former category, and papillary and tubular adenocarcinomas in the latter.

Two additional slices were made from the blocks of the lesser curvature of the antrum for immunohistochemical staining of the G-cells (Fig. 2). Anti-gastrin rabbit antisera was kindly provided by the First Department of Pathology, Hiroshima University. The numbers of G-cells per 4 mm length of the antral mucosa were counted in three different regions of the stomach, and the average was recorded as the number of G-cells in the animal.

Statistics Fisher's exact probability test was used for comparing carcinoma incidence among groups. Mann-Whitney and Scheffe's tests were used for comparing the values of pH and the number of G-cells. The relationships between pH and the number of G-cells were assessed in terms of the Pearson correlation coefficient. Multiple regression analysis was used for multivariate assessment.

RESULTS

Two dogs died of viral infection during the period of ENNG administration and seven other dogs died during the observation period due to intestinal invagination or anastomotic leakage. Twenty-one dogs survived and were analyzed (Table I).

Antral conditions The number of G-cells in group II was significantly higher than in group III (Scheffe, $P<0.01$), and that in the bile-positive groups (II and IV) was significantly higher than in the bile-negative groups (I and III) (Scheffe, $P<0.01$). Antral pH in groups I and II was significantly higher than in groups III and IV. There was a significant positive correlation between antral pH and the number of G-cells ($r=0.564$, $P<0.05$).

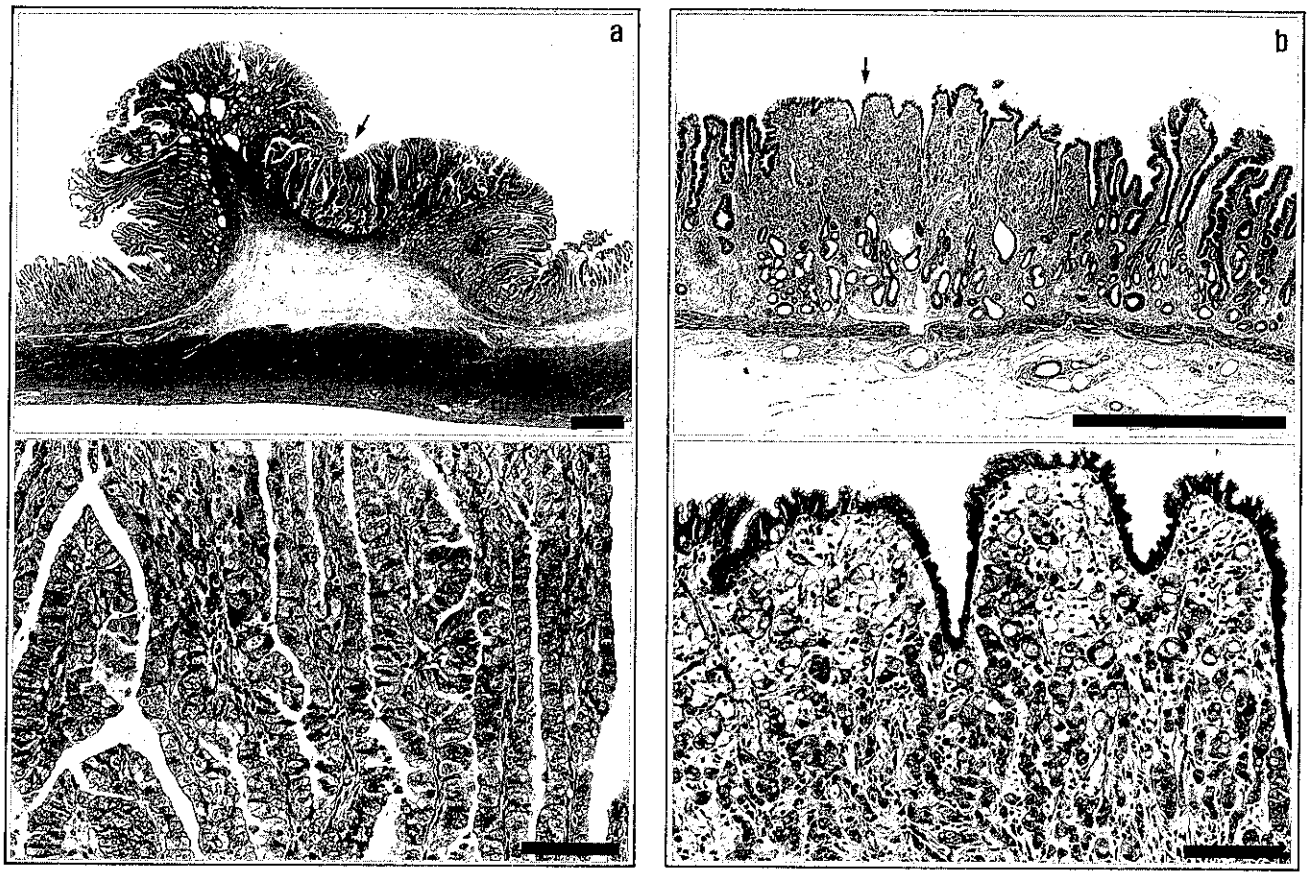


Fig. 4. Microscopic features of the carcinomas. (a) A polypoid lesion of well differentiated adenocarcinoma (scale bars=1 mm and 100 μm). (b) A flat lesion of poorly differentiated adenocarcinoma (scale bars=1 mm and 100 μm).

Carcinoma incidence There were no differences in carcinoma incidence or the distribution of histological types between the dogs killed at 16 months and those killed at 33 months. Thus, the dogs in these two categories were evaluated together in the following analyses.

Carcinomas had developed in 12 (57%) of 21 dogs. Carcinomatous foci were detected exclusively in the antrum and were multiple in nine of the 12 dogs. Macroscopically no tumors were found in other organs.

There were no significant differences in carcinoma incidence among the four groups. Antral pH and the number of G-cells in the carcinoma-positive dogs were slightly higher than in the carcinoma-negative dogs, but the differences were not statistically significant.

Pathology Neither intestinal metaplasia nor atrophic gastritis was seen in the non-cancerous area of the antra. Prominent hyperplasia of the foveolar epithelium was observed in 13 dogs (Fig. 3). Ten of them were in bile-positive groups. No relation was found between cancer development and foveolar hyperplasia. The tumors showed various degrees of histological differentiation (Fig. 4). Most of the differentiated adenocarcinomas were macroscopically of the elevated or polypoid type, while the undifferentiated carcinomas were flat or slightly depressed.

The depth of carcinoma invasion was confined to the mucosal layer except for two tumors in dog No. 1 and one in dog No. 16 that had penetrated into the muscularis propria.

Histological types and antral conditions Seven of the 12 cancer-positive dogs developed differentiated adenocarcinomas (D-dogs) with or without synchronous occurrence of undifferentiated adenocarcinomas. In the other 5 dogs, only undifferentiated adenocarcinomas were found (U-dogs). Antral pH and the number of G-cells were significantly higher in D-dogs than in U-dogs (Fig. 5). Bile inflow appeared to increase the risk of developing differentiated adenocarcinomas, but the difference was not statistically significant. Fig. 6 shows the distribution of D- and U-dogs demonstrating that differentiated adenocarcinomas developed in the achlorhydric, hypergastrin mucosa created in this study. A multivariate analysis

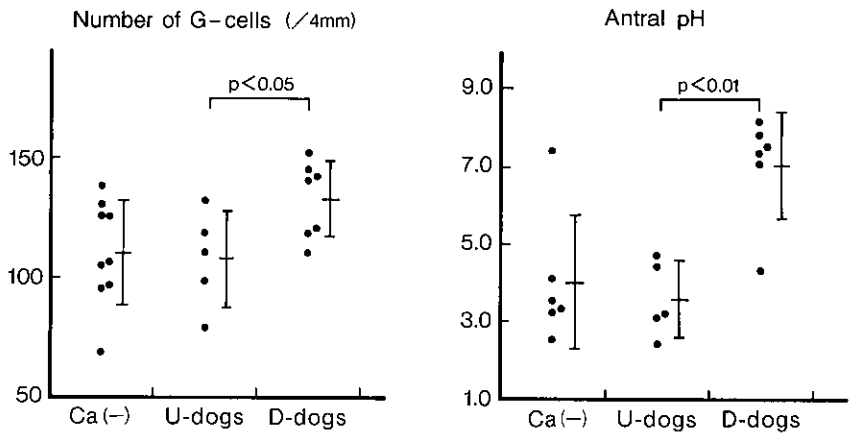


Fig. 5. Comparison between the dogs that had developed only undifferentiated adenocarcinomas (U-dogs) and the dogs with differentiated adenocarcinomas (D-dogs). *P*-values, Mann-Whitney test; bars, mean ± SD.

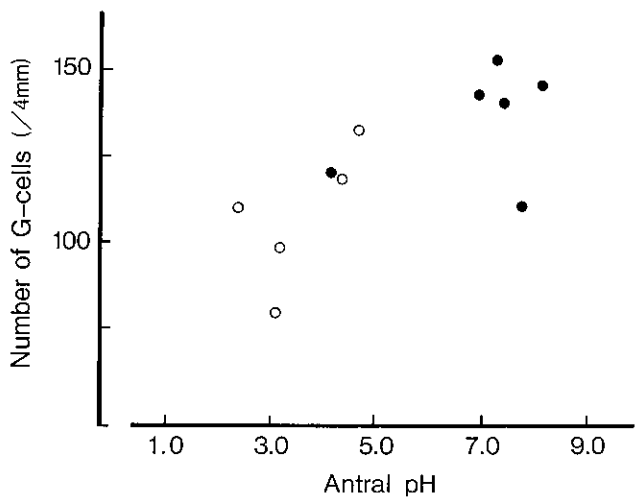


Fig. 6. D-dogs (●) and U-dogs (○) plotted according to the number of G-cells and antral pH. Differentiated adenocarcinomas developed in the high-pH, high-gastrin zone.

revealed that antral pH was the single most important factor associated with the development of differentiated adenocarcinoma in the canine stomach.

DISCUSSION

Gastrin has been demonstrated to enhance the *in vitro* proliferation of cells derived from rat[5] and human[6] stomach cancers. Bile has been reported to be a promoter of gastric carcinogenesis in rats.[7,8] In this study, however, carcinoma incidence was not significantly influenced by these factors. Achlorhydria is considered to be a risk factor for stomach carcinogenesis, but we could not accurately evaluate this risk in our study because all of the antra were in normo- or hypo-acidic states. Within the pH range created in this model, carcinoma incidence had no significant association with antral pH.

The notable finding in the present study was the occurrence of differentiated adenocarcinomas in the hypochlorhydric antra. Kurihara *et al.*[3] established a stomach carcinogenesis model by orally administering 75 mg/day of ENNG for 8 months, and found both differentiated and undifferentiated carcinomas arising in the same stomach. Sunagawa *et al.*[4] investigated the effects of the duration of ENNG administration on histological tumor differentiation, and found that a small dose of ENNG (3 months, total 5.85 g) produced only undifferentiated adenocarcinomas in beagle dogs, while differentiated adenocarcinomas also developed with high doses (6 months, 11.7 g and 9 months, 17.55 g). In the present study, a small dose (4 months, 7.65 g) was utilized and seven of the 21 dogs developed differentiated adenocarcinomas. This strongly suggests that the antral conditions created in our study were favorable for the occurrence of differentiated adenocarcinomas.

Antral pH, the number of G-cells and bile inflow were assessed, and high values of the first two factors, i.e. achlorhydria and hypergastrin, were significantly associated with the development of differentiated adenocarcinomas. A multivariate analysis revealed that antral pH was the single most important factor.

Kurihara *et al.*[9] reported that combined use of gastrin and ENNG produced a scirrhous gastric carcinoma in a beagle dog. Tatsuta *et al.*[10] found that carcinomas induced by combined use of gastrin and MNNG (N-methyl-N′-nitro-N-nitrosoguanidine) in the rat stomach tended to show a low grade of histological differentiation. These results appear to contrast with those of our study, which demonstrate a close relationship between a hypergastrin state and well differentiated adenocarcinoma. However, these results are not inconsistent with

regard to acidity; the gastrin administration in their studies induced a hyper-acidic state which might be favorable for the development of undifferentiated adenocarcinomas, while differentiated adenocarcinomas developed in the hypo-acidic conditions created in our study.

Tani *et al.*[11] studied acid secretion in 110 patients with gastric cancers, and found that, with differentiated adenocarcinomas, acid outputs were significantly lower than with undifferentiated adenocarcinomas. These findings suggest a close association between hypo-acidity and the occurrence of differentiated adenocarcinomas in the human stomach.

Differentiated adenocarcinomas are known to develop in stomachs with atrophic gastritis and intestinal metaplasia in relatively elderly persons and these mucosal changes have been implicated as important precancerous conditions.[12, 13] Findings of hypo-acidity in these patients might merely be one of the phenomena accompanying these mucosal lesions. In our animal models, however, differentiated-type tumors were induced by operatively creating hypo-acidic conditions after the completion of carcinogenic initiation. These results suggest that the hypo-acidic state itself may play an important role in the occurrence of differentiated-type gastric cancer.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan. We would like to thank Mr. N. Sunaga, Mrs. M. Kuwahara, Mr. R. Miyazawa and Mr. T. Sato for their technical assistance. We are also very grateful to Prof. E. Tahara and Dr. W. Yasui for their advice and assistance in immunohistochemical staining.

(Received December 7, 1992/Accepted February 18, 1993)

REFERENCES

1) Tatsuta, M., Yamamura, H., Iishi, H., Ichii, M., Noguchi, S., Baba, M. and Taniguchi, H. Promotion by vagotomy of gastric carcinogenesis induced by N-methyl-N′-nitro-N-nitrosoguanidine in Wistar rats. *Cancer Res.*, **45**, 194–197 (1985).
2) Deveney, C. W., Freeman, H. and Way, L. W. Experimental gastric carcinogenesis in the rat: effects of hypergastrinemia and acid secretion. *Am. J. Surg.*, **139**, 49–54 (1980).
3) Kurihara, M., Shirakabe, H., Murakami, T., Yasui, A., Izumi, T., Sumida, M. and Igarashi, A. A new method for producing adenocarcinomas in the stomach of dogs with N-ethyl-N′-nitro-N-nitrosoguanidine. *Gann*, **65**, 163–177 (1974).
4) Sunagawa, M., Takeshita, K., Nakajima, A., Ochi, K., Habu, H. and Endo, M. Duration of ENNG administration and its effect on histological differentiation of experimental gastric cancer. *Br. J. Cancer*, **52**, 771–779 (1985).
5) Kobori, O., Vuillot, M. T. and Martin, F. Growth responses of rat stomach cancer cells to gastro-enteropancreatic hormones. *Int. J. Cancer*, **30**, 65–67 (1982).
6) Watson, S., Durrant, L. and Morris, D. Gastrin: growth enhancing effects on human gastric and colonic tumour cells. *Br. J. Cancer*, **59**, 554–558 (1989).
7) Salmon, R. J., Laurent, M. and Thierry, J. P. Effect of taurocholic acid feeding on methyl-nitro-N-nitrosoguanidine induced gastric tumors. *Cancer Lett.*, **22**, 315–320 (1984).
8) Kobori, O., Shimizu, T., Maeda, M., Atomi, Y., Watanabe, J., Shoji, M. and Morioka, Y. Enhancing effect of bile and bile acid on stomach tumorigenesis induced by N-methyl-N′-nitro-N-nitrosoguanidine in Wistar rats. *J. Natl. Cancer Inst.*, **73**, 853–861 (1984).
9) Kurihara, M., Shirakabe, H., Yamaya, F., Miyazaki, K., Maruyama, T., Izumi, T., Yasui, A. and Kamano, T. Gastric carcinoma in dogs produced by the combined use of N-ethyl-N′-nitro-N-nitrosoguanidine (ENNG) and gastrin. *Acta Pathol. Jpn.*, **29**, 171–176 (1979).
10) Tatsuta, M., Itoh, T., Okuda, S., Wada, A., Taniguchi, H., Tamura, H. and Yamamura, H. Effects of gastrin and histamine on gastric carcinogenesis induced in rats by N-methyl-N′-nitro-N-nitrosoguanidine. *Eur. J. Cancer*, **16**, 631–638 (1980).
11) Tani, N., Harasawa, S., Suzuki, S., Miwa, M., Nomiyama, T., Senoue, I., Kikuchi, K., Makino, T., Shibata, H. and Miwa, T. Gastric acid secretion and plasma gastrin response to test meal in patients with gastric cancer. *Tokai J. Exp. Clin. Med.*, **7**, 169–172 (1982).
12) Morson, B. C., Sobin, L. H., Grundmann, E., Johansen, A., Nagayo, T. and Serck-Hanssen, A. Precancerous conditions and epithelial dysplasia in the stomach. *J. Clin. Pathol.*, **33**, 711–721 (1980).
13) Correa, P. Precursors of gastric and esophageal cancer. *Cancer*, **50**, 2554–2565 (1982).