Jpn. J. Cancer Res. **89**, 385–391, April 1998

N-Methyl-*N*-nitrosourea Concentration-dependent, Rather than Total Intake-dependent, Induction of Adenocarcinomas in the Glandular Stomach of BALB/c Mice

Takasuke Yamachika,[1,2] Hayao Nakanishi,[1] Ken-ichi Inada,[1] Tetsuya Tsukamoto,[1] Nobuyuki Shimizu,[1] Kiyoshi Kobayashi,[1] Shoji Fukushima[2] and Masae Tatematsu[1]

[1]Laboratory of Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464 and [2]First Department of Pathology, Osaka City University Medical School, 1-4-54 Asahi-machi, Abeno-ku, Osaka 54

The influence of the level of carcinogen exposure on histopathological types and cellular differentiation of the induced tumors was examined in 100 male BALB/c mice given *N*-methyl-*N*-nitrosourea (MNU) in their drinking water at 240 ppm on alternate weeks (total exposure: five weeks) (group 1), at 120 ppm similarly (total exposure: ten weeks) (group 2), at 60 ppm for 20 weeks continuously (group 3), or at 30 ppm for 40 weeks continuously (group 4). Forty-three differentiated and 17 undifferentiated type adenocarcinomas were induced. Glandular stomach carcinomas and undifferentiated type lesions were more common in mice treated with a high concentration of MNU for a short period than with a low concentration of MNU for a long period, even though total measured intake of MNU was smaller ($P<0.01$). All the induced glandular stomach carcinomas, independent of the treatment schedule, consisted entirely of gastric phenotype cells. In conclusion, the induction of glandular stomach cancers and the proportion of undifferentiated type lesions depend not on the total quantity, but rather on the concentration of the carcinogen, while the phenotypic expression of tumor cells is not affected by the differences in the administration protocol.

Key words: *N*-methyl-*N*-nitrosourea — Stomach cancer — Concentration-dependence — Cell differentiation — Mouse

Histologically, human gastric carcinomas have been classified into two main groups, namely, the intestinal and diffuse types of Lauren,[1)] and the differentiated and undifferentiated types of Sugano *et al*.[2)] Their histogenesis and progression have been said to differ,[3–5)] although the underlying mechanisms remain to be clarified. One of the reasons for this is the lack of an animal experimental gastric cancer model in which the various histological type carcinomas can be specifically induced. Carcinomas induced by MNNG in rats and hamsters are almost all of differentiated type[6–9)] and although canine signet ring carcinomas can be induced,[10)] statistical analysis is very difficult because it is impractical to use large numbers of dogs. However in a recently established mouse experimental gastric cancer model using MNU the types of tumors proved more similar to human gastric cancers in terms of their histopathological features than those typically found in rats.[11, 12)]

Abbreviations: MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; HE, hematoxylin and eosin; PCS, paradoxical concanavalin A staining; GOS, galactose oxidase Schiff; S-GOS, sialidase GOS; Pg 1, pepsinogen isozyme 1; IgG, immunoglobulin G.

The present study was performed to assess the effects of carcinogen concentration on the histopathological type and incidence of gastric carcinomas induced in our mouse model. For this purpose MNU was given at different concentrations for different periods so that all groups would receive the same total quantity of carcinogen, in theory. The phenotypic expression of the induced tumors was examined using mucin histochemistry and immunohistochemistry, to provide a basis for discussion of differentiation and histogenetic features.

MATERIALS AND METHODS

Experimental design A total of 100 male 6-week-old BALB/c mice (Clea Japan, Inc., Tokyo) were housed in plastic cages on hard wood chips in an air-conditioned room with a 12 h light-12 h dark cycle. They were given food (Oriental NMF, Oriental Yeast Co., Tokyo) and water *ad libitum*. MNU (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water, freshly prepared three times per week, and provided as the drinking water *ad libitum* from light-shielded bottles.

The animals were divided into 4 groups. They were given MNU in their drinking water at 240 ppm on alter-

nate weeks (total exposure: five weeks) (group 1), at 120 ppm similarly (total exposure: ten weeks) (group 2), at 60 ppm for 20 weeks continuously (group 3), or at 30 ppm for 40 weeks continuously (group 4) so that each group of mice received the same quantity of MNU in theory (Fig. 1). All survivors were killed at 50 weeks and autopsied. Necropsies were performed on all animals which died or were killed upon becoming moribund. Animals that survived more than 30 weeks, when the first tumor appeared, were included in the effective numbers with regard to the incidence of tumors.

Histopathological analysis The excised stomachs were fixed in buffered formalin, cut into about 5 strips, and routinely processed for embedding in paraffin. Tissue sections were stained with HE for histopathological assessment of lesion development and those through the longest tumor diameters were selected for mucin histochemical and immunohistochemical investigation of phenotypic expression in the tumor cells. Neoplastic lesions were classified as adenocarcinomas, adenomas and sarcomas. Adenomas consisted of excessive glandular proliferation with mild cellular atypia. Adenocarcinomas were classified as well differentiated, poorly differentiated and signet ring cell types. Well differentiated adenocarcinomas were characterized by tubular structures and their poorly differentiated counterparts by a more solid pattern with severe cellular atypia. Signet ring cell carcinomas were characterized by isolated tumor cells containing abundant mucin.[12] Differentiated type carcinomas were assigned to well differentiated adenocarcinomas, and undifferentiated type carcinomas were assigned to poorly differentiated adenocarcinoma and signet ring cell carcinoma categories. In cases in which 2 histological types were found in the same stomach, each type of carcinoma was counted. Furthermore, adenocarcinomas were classified into early and advanced carcinomas on the basis of invasion below the muscularis propria.

Mucin histochemistry and immunohistochemistry For mucin histochemistry PCS,[13] GOS[14, 15] and S-GOS[14, 15] staining techniques were applied. Mucins identified by PCS were classified into four types. In this study, PCS identifying class III mucins was used to detect mucous neck and pyloric gland cells. GOS and S-GOS staining procedures were performed as described previously.[14, 15] Surface mucous cells were GOS-positive and S-GOS stained surface mucous and goblet cells. For immunohistochemistry a polyclonal anti-Pg 1 antibody kindly donated by Dr. Chie Furihata (Department of Molecular Oncology, University of Tokyo) was applied.[16] Pg 1 was thus recognized in mucous neck, pyloric gland and chief cells. After deparaffinization and dehydration, sections were incubated with fresh 3% hydrogen peroxide in methanol, and treated sequentially with normal goat serum, rabbit anti-rat Pg 1 (1:2000), biotin-labeled goat anti-rabbit IgG, and the avidin-biotin-peroxidase complex.[17] The sites of peroxidase binding were visualized using diaminobenzidine. Sections were lightly counterstained with hematoxylin for microscopic examination.

Phenotypic expression of tumor cells Tumor cells were classified into gastric epithelial cell (surface mucous and pyloric gland cells) and intestinal epithelial cell (goblet and intestinal absorptive cells) types with regard to their histochemical and HE staining features.[18, 19]

Stastistical analysis The χ^2 test was applied for comparison of tumor incidences in each group and the χ^2 test for trend was used for tendencies. Student's *t* test was performed for comparison of the numbers of induced carcinomas per mouse.

RESULTS

Survival and total intake of MNU Survival curves and body weights of each group of mice are shown in Figs. 2 and 3, respectively. Survival rates of each group of mice at

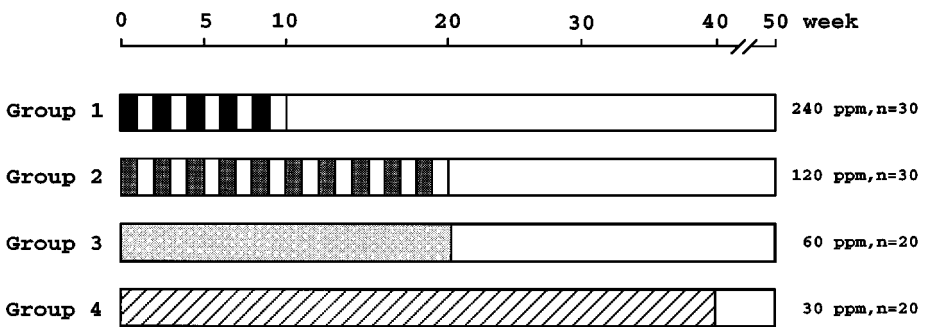

Fig. 1. Experimental design. Group 1: MNU at 240 ppm for alternate weeks to give a total exposure of five weeks, Group 2: MNU at 120 ppm for alternate weeks to give a total exposure of ten weeks, Group 3: MNU at 60 ppm for 20 weeks continuously, Group 4: MNU at 30 ppm for 40 weeks continuously.

Table I. Incidences of Glandular Stomach Tumors in Mice Treated with MNU

Groups	Effect. no. of mice	Intake of MNU (mg)	Tumor-bearing (%)					Number of carcinomas (mean±SD per mouse)			
			Ade-noma	Adenocarcinoma			Sar-coma	Diff. type	Undiff. type		Advan. type
				Diff. type	Undiff. type	Total		Well.	Por.	Sig.	
Group 1	22	11.2	14 (63.6)	17 (77.3)[a,b,c]	9 (40.9)[a,b,c]	17 (77.3)[a,b,c]	1 (4.5)[e]	19 (0.86±0.56)[b,c,d]	11 (0.5±0.67)[a,c]	3 (0.14±0.35)[d]	8 (0.36±0.49)[a,e]
Group 2	28	11.9	15 (53.6)	10 (35.7)	2 (7.1)	10 (35.7)	1 (3.6)[c]	13 (0.46±0.74)	2 (0.07±0.26)	0	0
Group 3	12	19.8	7 (58.3)	4 (33.3)	1 (8.3)	4 (33.3)	1 (8.3)	5 (0.42±0.67)	1 (0.08±0.29)	0	1 (0.08±0.29)
Group 4	17	23.8	9 (52.9)	5 (29.4)	0	5 (29.4)	6 (35.3)	6 (0.35±0.61)	0	0	1 (0.06±0.24)

Effect. no. of mice, effective number of mice; Diff. type, differentiated type; Undiff. type, undifferentiated type; Well., well differentiated adenocarcinoma; Por., poorly differentiated adenocarcinoma; Sig., signet ring cell carcinoma; Advan. type, Advanced carcinoma.
a) Significantly different from group 2 at $P<0.01$.
b) Significantly different from group 3 at $P<0.05$.
c) Significantly different from group 4 at $P<0.01$.
d) Significantly different from group 2 at $P<0.05$.
e) Significantly different from group 4 at $P<0.05$.

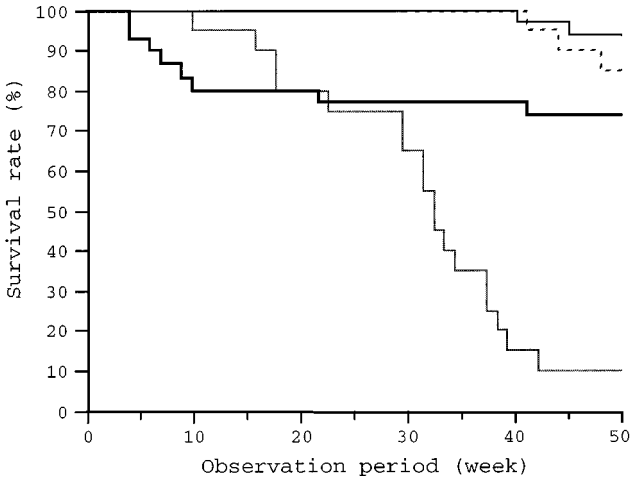

Fig. 2. Survival curves of mice treated with MNU. —Group 1, — Group 2, — Group 3, - - - Group 4.

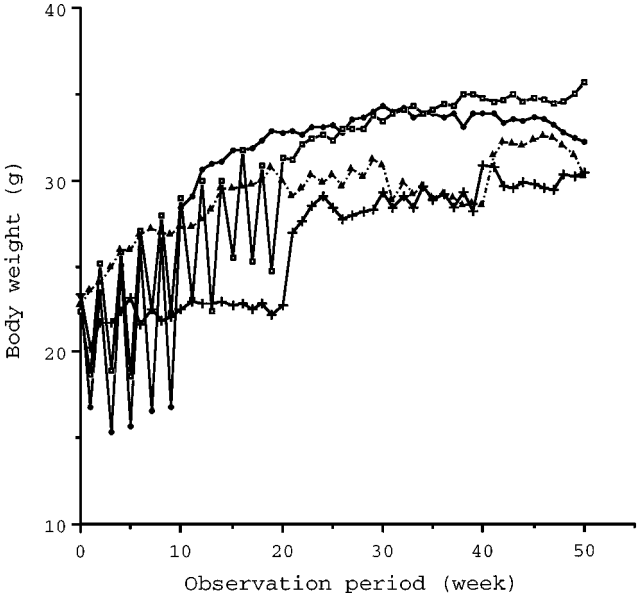

Fig. 3. Body weight curves of mice treated with MNU. ● Group 1, □ Group 2, + Group 3, ▲ Group 4.

50 weeks was more than 70% except for group 3 in which mice became moribund after about 30 weeks and were autopsied. Body weights of each group of mice oscillated with the intermittent exposure and increased gradually after administration of MNU was completed. Total intakes of MNU per mouse are given in Table I. The values were lower for groups 1 and 2 receiving high concentrations for a short period.

Features and incidences of induced glandular stomach tumors The lesions induced in this experiment were 56 adenomas, 60 carcinomas and 7 sarcomas. Among the 60 carcinomas were 43 well differentiated adenocarcinomas (Fig. 4), 14 poorly differentiated adenocarcinomas (Fig. 5) and 3 signet ring cell carcinomas (Fig. 6). Most well differentiated carcinomas were recognized as polypoid lesions in the lesser curvature of the pyloric gland region. With regard to undifferentiated type carcinomas, early lesions showed morphologically the appearance of flat or

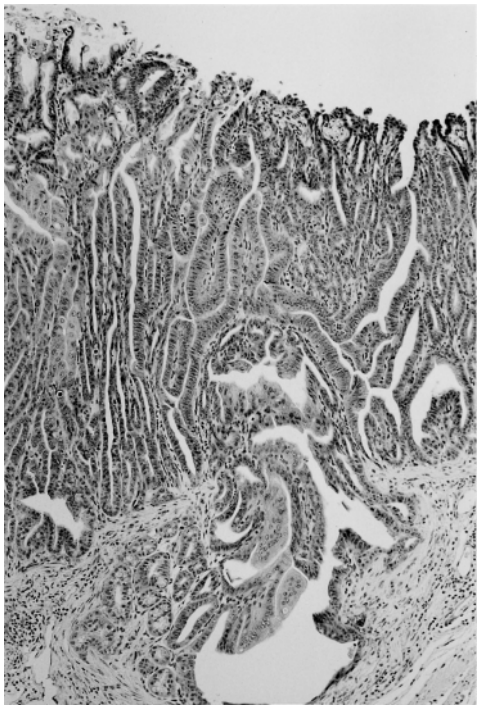

Fig. 4. A well differentiated adenocarcinoma in a group 1 mouse at week 50. HE, ×20.

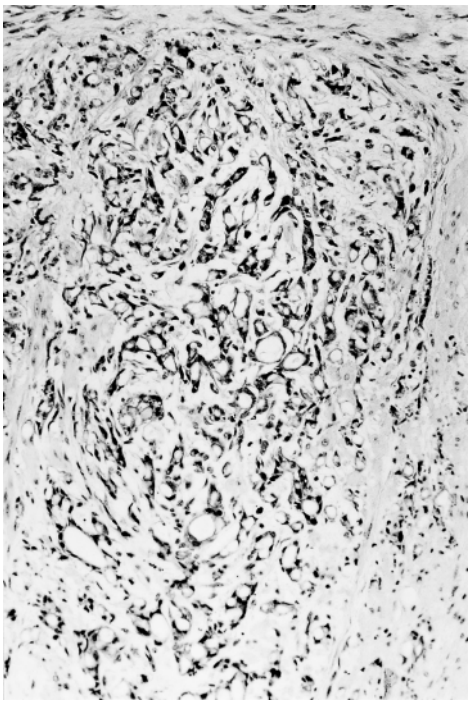

Fig. 5. A poorly differentiated adenocarcinoma in a group 1 mouse at week 50. HE, ×40.

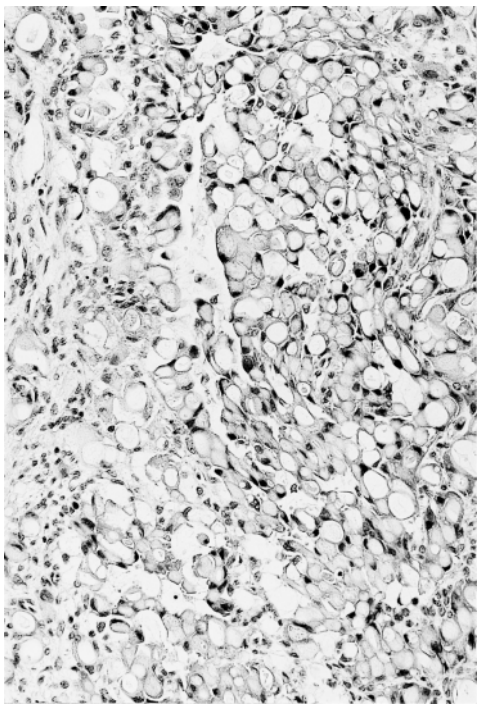

Fig. 6. A signet ring cell carcinoma in a group 1 mouse at week 50. HE, ×55.

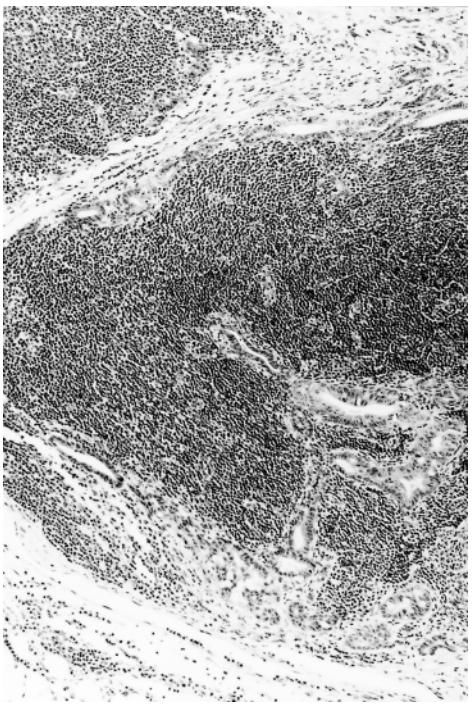

Fig. 7. A metastasis from a well differentiated adenocarcinoma in a regional lymph node of a group 1 mouse at week 50. HE, ×27.

polypoid lesions and were located in both the fundic and pyloric gland regions. Advanced lesions exhibited a polypoid appearance with ulceration in their center, as well as marked thickening of the wall of the stomach due to diffuse infiltration of carcinoma cells. No intestinal metaplasia was found. One well differentiated adenocarcinoma (group 1 lesion) showed metastasis to regional lymph nodes (Fig. 7). The total of 7 sarcomas observed were all hemangiosarcomas.

Incidences of adenomas, glandular stomach carcinomas and sarcomas in each group are summarized in Table I. Values were higher in mice treated with a high concentration in spite of a lower total intake of MNU (χ^2 test for trend, $P<0.01$). With regard to histological types, both differentiated and undifferentiated type lesions showed similar tendencies for increase with the concentration (χ^2 test for trend, $P<0.01$). With regard to the multiplicity of carcinomas per mouse, group 1 demonstrated the greatest numbers for each histological type. Incidences of advanced carcinomas were also significantly higher in group 1 than in groups 2 and 4. No statistically significant differences in incidences of adenomas were found. The rates for sarcomas showed an opposite tendency to carcinomas, being most common in group 4 (χ^2 test for trend, $P<0.01$).

Phenotypic expression of induced tumor cells Examination of the phenotypic expression of tumor cells in the 60 glandular stomach carcinomas and 56 adenomas revealed predominance of the surface mucous cell type in all cases. Pyloric gland cell types were found in 15 carcinomas and 4 adenomas. No goblet or intestinal absorptive cell types were found in either carcinomas or adenomas. Thus, tumor cells of adenomas and carcinomas induced in this study expressed only gastric phenotypes.

Tumor cells of surface mucous cell type contained GOS and S-GOS reactive mucins, but showed no class III mucin or Pg 1 reactivity. In contrast, tumor cells of pyloric gland cell type contained class III mucin and a low Pg 1 reactivity, but showed no GOS or S-GOS reactivity.

DISCUSSION

In experimental cancer models using a single carcinogen, the main factors affecting tumor induction are assumed to be the concentration and the total intake. Although it has already been reported that the induction of gastric carcinomas in rodent experimental models[12, 20] depends on the dose of the carcinogen and dose-dependence of tumor induction is now widely accepted,[21] the present findings suggest an additional level of complication. Yamamoto *et al*. reported that a high concentration of the carcinogen 3′-methyl-4-dimethylaminoazobenzene predominantly induced hepatocellular carcinomas (HCCs) of high grade malignancy in a short latent period, whereas lower concentrations were primarily associated with low grade HCCs in hepatocarcinogenesis.[22] Our present study similarly showed clear evidence of an increase in the induction of tumors of undifferentiated type, as well as differentiated type, by application of a high carcinogen concentration in an animal experimental gastric cancer model, with an inverse relationship to the total intake of the inducing agent.

Carcinomas induced by MNNG or MNU in the rat are mainly of differentiated type and the induction of undifferentiated lesions is relatively difficult without additional surfactant or salt treatment.[23, 24] Therefore, the establishment by Tatematsu *et al*. of a mouse experimental gastric cancer model using MNU to yield both differentiated and undifferentiated type carcinomas was important.[11, 12] In the present study undifferentiated type carcinomas were similarly induced, being positively linked to high concentrations of MNU. It has been suggested that differentiated and undifferentiated types of gastric carcinomas in man may develop through different genetic pathways.[25] Genes controlling tubule formation such as the E-cadherin-catenin complex[26–28] may be more affected upon exposure to high concentrations of a carcinogen. The possibility that this might have contributed to the formation of undifferentiated type carcinomas warrants further attention.

The converse finding that induction of sarcomas depended on not the concentration of MNU, but rather the total intake is also of interest. The details remain unclear, but it can be speculated that the administration period of a carcinogen (exposure time) is a more significant factor than the concentration in this case.

Mucin histochemical staining (PCS, GOS, S-GOS) and immunohistochemical reactivities (Pg 1, intestinal alkaline phosphatase) allow gastric carcinoma cells (in both man and the rat) to be clearly classified into a gastric epithelial cell type (comprising surface mucous and pyloric gland cells) and an intestinal epithelial cell type (goblet and intestinal absorptive cell types).[29–33] By sequential observation of rat glandular stomach carcinomas and comparisons of phenotypic expression of human gastric carcinoma cells between early and advanced cases, a phenotypic shift from gastric to intestinal epithelial cell type expression has been recognized with progression of each histological type of gastric carcinomas.[33–36] The present results regarding phenotypic expression of tumor cells showed that mouse glandular stomach adenomas and carcinomas consist solely of tumor cells of gastric epithelial cell type, without any intestinal metaplasia in the mucosa surrounding carcinomas. Thus, differentiated and undifferentiated type carcinomas arise from gastric proper mucosa independently of intestinal metaplasia in this model. In the mouse, intestinal metaplasia and intestinal phenotype carcinoma cells might also be expected to appear if the ani-

mals were followed for periods longer than one year. A phenotypic shift from gastric to intestinal type with aging is common in both human and experimental animals.

In conclusion, the induction of glandular stomach cancers and the proportion of undifferentiated type lesions in the mouse treated with MNU depend not on the total carcinogen exposure, but rather on the concentration. The phenotypic expression of the tumor cells does not appear to be affected by such differences in the administration protocol.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan, by a Grant for Cancer Research from the Ministry of Health and Welfare of Japan, and by funds for Core Research for Evolutional Science and Technology from the Japan Science and Technology Corporation.

(Received September 12, 1997/Revised November 7, 1997/2nd Revised January 29, 1998/Accepted February 2, 1998)

REFERENCES

1) Lauren, P. The two histological main types of gastric carcinomas: diffuse and so called intestinal-type carcinoma. An attempt at a histoclinical classification. *Acta Pathol. Microbiol. Scand.*, **64**, 31–49 (1965).
2) Sugano, H., Nakamura, K. and Kato, Y. Pathological studies of human gastric cancer. *Acta Pathol. Jpn.*, **32** (Suppl. 2), 329–347 (1982).
3) Nakamura, K. Special features of intestinal metaplasia and its relation to early gastric carcinoma in man: observation by a method in which leucine aminopeptidase activity is used. *J. Natl. Cancer Inst.*, **61**, 693–702 (1978).
4) Nakamura, K., Sugano, H. and Takagi, K. Carcinoma of the stomach in incipient phase: its histogenesis and histological appearances. *Gann*, **59**, 251–258 (1968).
5) Nagayo, T. Microscopical cancer of the stomach: a study on histogenesis of gastric carcinoma. *Int. J. Cancer*, **16**, 52–60 (1975).
6) Sugimura, T. and Fujimura, S. Tumor production in glandular stomach of rats by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. *Nature*, **216**, 934–944 (1967).
7) Sugimura, T., Fujimura, S. and Baba, T. Tumor production in the glandular stomach and alimentary tract of the rat by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. *Cancer Res.*, **30**, 455–465 (1970).
8) Saito, T., Inokuchi, K., Takayama, S. and Sugimura, T. Sequential morphological changes in *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine carcinogenesis in the glandular stomach of rats. *J. Natl. Cancer Inst.*, **44**, 769–783 (1970).
9) Fujimura, S., Kogure, K., Oboshi, S. and Sugimura, T. Production of tumors in glandular stomach of hamsters by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. *Cancer Res.*, **30**, 1444–1448 (1970).
10) Sugimura, T., Tanaka, N., Kawachi, T., Kogure, K., Fujimura, S. and Shimosato, Y. Production of stomach cancer in dogs by N-methyl-N′-nitro-N-nitrosoguanidine. *Gann*, **62**, 67 (1971).
11) Tatematsu, M., Ogawa, K., Hoshiya, T., Shichino, Y., Kato, T., Imaida, K. and Ito, N. Induction of adenocarcinomas in the glandular stomach of BALB/c mice treated with N-methyl-N-nitrosourea. *Jpn. J. Cancer Res.*, **83**, 915–918 (1992).
12) Tatematsu, M., Yamamoto, M., Iwata, H., Fukami, H., Yuasa, H., Tezuka, N., Masui, T. and Nakanishi, H. Induction of glandular stomach cancers in C3H mice treated with N-methyl-N-nitrosourea in the drinking water. *Jpn. J. Cancer Res.*, **84**, 1258–1264 (1993).
13) Katsuyama, T. and Spicer, S. S. Histochemical differentiation of complex carbohydrates with variants of the concanavalin A horseradish peroxidase method. *J. Histochem. Cytochem.*, **26**, 233–250 (1978).
14) Katsuyama, T., Ono, K. and Nagata, T. Application of galactose oxidase mucosubstance histochemistry: galactose oxidase-Schiff reaction. *J. Histochem. Cytochem.*, **30**, 555 (1982).
15) Schulte, B. A. and Spicer, S. S. Light microscopic histochemical detection of sugar residues in secretory glycoproteins of rodent and human tracheal glands with lectin-horseradish peroxidase conjugates and the galactose oxidase-Schiff sequence. *J. Histochem. Cytochem.*, **31**, 391–403 (1983).
16) Furihata, C., Saito, D., Fujiki, H., Kanai, Y., Matsushima, T. and Sugimura, T. Purification and characterization of pepsinogens and a unique pepsin from rat stomach. *Eur. J. Biochem.*, **105**, 43–50 (1980).
17) Hsu, S. M., Raine, L. and Fanger, H. The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase techniques. *Am. J. Clin. Pathol.*, **75**, 816–821 (1981).
18) Tatematsu, M., Katsuyama, T., Fukushima, S., Takahashi, M., Shirai, T., Ito, N. and Nasu, T. Mucin histochemistry by paradoxical concanavalin A staining in experimental gastric cancers induced in Wistar rats by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine or 4-nitroquinoline 1-oxide. *J. Natl. Cancer Inst.*, **64**, 835–843 (1980).
19) Tatematsu, M., Katsuyama, T., Furihata, C., Fukushima, S., Shirai, T., Kato, T., and Ito, N. Cellular differentiation and histogenesis of rat glandular stomach cancers. *Jpn. J. Cancer Res.*, **81**, 760–767 (1990).
20) Yamamoto, M., Furihata, C., Fujimitsu, Y., Imai, T., Inada, K., Nakanishi, H. and Tatematsu, M. Dose-dependent induction of both pepsinogen-altered pyloric glands and adenocarcinomas in the glandular stomach of C3H mice treated with *N*-methyl-*N*-nitrosourea. *Jpn. J. Cancer Res.*, **88**, 238–244 (1997).

21) Gehring, P. J. and Blau, G. E. Mechanisms of carcinogenesis. *J. Environ. Pathol. Toxicol.*, **1**, 163–179 (1978).
22) Yamamoto, N., Nomura, K., Kayano, T. and Kitagawa, T. Carcinogen daily dose-dependence of the biological features and development rate of hepatocellular carcinomas induced by 3′-methyl-4-dimethylaminoazobenzene in the rat. *Cancer Lett.*, **83**, 59–68 (1994).
23) Takahashi, M., Fukushima, S. and Sato, H. Carcinogenic effect of N-methyl-N′-nitro-N-nitrosoguanidine with various kinds of surfactant in the glandular stomach of rats. *Gann*, **64**, 211–218 (1973).
24) Tatematsu, M., Takahashi, M., Fukushima, S. Hananouchi, M. and Shirai, T. Effects in rats of sodium chloride on experimental gastric cancers induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine or 4-nitroquinoline-l-oxide. *J. Natl. Cancer Inst.*, **55**, 101–106 (1975).
25) Tahara, E. Molecular mechanism of stomach carcinogenesis. *J. Cancer Res. Clin. Oncol.*, **119**, 265–272 (1993).
26) Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science*, **251**, 1451–1455 (1991).
27) Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S. and Takeichi, M. Identification of a neural α-catenin as a key regulator of a cadherin function and multicellular organization. *Cell*, **70**, 293–301 (1992).
28) Mayer, B., Johnson, J. P., Letil, F., Jauch, K. W., Heiss, M. N., Schildberg, F. W., Birchmeier, W. and Funke, I. E-Cadherin expression in primary and metastatic gastric cancer: down-regulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Res.*, **53**, 1690–1695 (1993).
29) Furihata, C., Tatematsu, M., Miki, K., Katsuyama, T., Sudo, K., Miyagi, N., Kubota, T., Jin, S., Kodama, K., Ito, N., Konishi, Y., Suzuki, K. and Matsushima, T. Gastric- and intestinal-type properties of human gastric cancers transplanted into nude mice. *Cancer Res.*, **44**, 727–733 (1984).
30) Fujimori, Y., Akamatsu, T., Ota, H. and Katsuyama, T. Proliferative markers in gastric carcinoma and organoid differentiation. *Hum. Pathol.*, **26**, 725–734 (1995).
31) Tatematsu, M., Ichinose, M., Miki, K., Hasegawa, R., Kato, T. and Ito, N. Gastric and intestinal phenotypic expression of human stomach cancers as revealed by pepsinogen immunohistochemistry and mucin histochemistry. *Acta Pathol. Jpn.*, **40**, 494–504 (1990).
32) Yuasa, H., Hirano, K., Kodama, H., Nakanishi, H., Imai, T., Tsuda, H., Imaida, K. and Tatematsu, M. Immunohistochemical demonstration of intestinal-type alkaline phosphatase in stomach tumors induced by N-methyl-N′-nitro-N-nitrosoguanidine in rats. *Jpn. J. Cancer Res.*, **85**, 897–903 (1994).
33) Tatematsu, M., Furihata, C., Katsuyama, T., Hasegawa, R., Nakanowatari, J., Saito, D., Takahashi, M., Matsushima, T. and Ito, N. Independent induction of intestinal metaplasia and gastric cancer in rats treated with N-methyl-N′-nitro-N-nitrosoguanidine. *Cancer Res.*, **43**, 1335–1341 (1983).
34) Tatematsu, M., Katsuyama, T., Furihata, C., Tsuda, H. and Ito, N. Stable intestinal phenotypic expression of gastric and small intestinal tumor cells induced by N-methyl-N′-nitro-N-nitrosoguanidine or methylnitrosourea in rats. *Gann*, **75**, 957–965 (1984).
35) Tatematsu, M., Hasegawa, R., Ogawa, K., Kato, T., Ichinose, M., Miki, K. and Ito, N. Histogenesis of human stomach cancers based on assessment of differentiation. *J. Clin. Gastroenterol.*, **14**(Suppl. 1), 1–7 (1992).
36) Yamachika, T., Inada, K., Fujimitsu, Y., Nakamura, S., Yamamura, Y., Kitou, T., Itzkowitz, S. H., Werther, J. L., Miki, K. and Tatematsu, M. Intestinalization of gastric signet ring cell carcinomas with progression. *Virchows Arch.*, **431**, 103–110 (1997).