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Induction of Glandular Stomach Cancers in C3H Mice Treated with N-Methyl-N-nitrosourea in the Drinking Water

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Establishment of an animal model of stomach carcinogenesis in mice was attempted using N-methyl-N-nitrosourea (MNU) in the drinking water. One hundred and forty-eight male 6-week-old C3H mice were given MNU in their drinking water at a concentration of 120 ppm (group 1), 60 ppm (group 2), 30 ppm (group 3) or 0 ppm (group 4) for 30 weeks. At the end of this time, dose-related induction of adenomatous hyperplasias was found. From weeks 31 to 54 adenocarcinomas developed in a dose-dependent manner in groups 1, 2 and 3. In total, 6 well differentiated and 5 poorly differentiated adenocarcinomas as well as 6 signet ring cell carcinomas arose in 15 stomach cancer-bearing animals in group 1, 4 well differentiated and 2 poorly differentiated adenocarcinomas with one signet ring cell carcinoma in 5 mice of group 2 and one well differentiated adenocarcinoma in group 3. In the forestomach, only one squamous cell carcinoma was found at week 54 in group 1 along with a single well differentiated adenocarcinoma in the duodenum. Thus, MNU in the drinking water selectively induced neoplastic lesions in the glandular stomach epithelium of mice.

Key words: N-Methyl-N-nitrosourea — Glandular stomach cancer — Mouse — Dose response

For analysis of glandular stomach carcinogenesis, establishment of an optimal experimental model is very important. The availability of a number of mutant mice, transgenic mice and chimeric mice makes this species particularly useful for this purpose and therefore several attempts have been made to establish an experimental system in the mouse glandular stomach. By intragastric instillation of 4-nitroquinoline 1-oxide[1] or 4-hydroxyaminoquinoline 1-oxide,[2] adenocarcinomas can be induced in the glandular stomach, but the mortality rate is high and the cancer incidence is low. Administration of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) in the drinking water to BRSUNT/NJms mice over the life span resulted only in adenomatous hyperplasia of gastric epithelium.[3] Catechol, which induces adenocarcinomas in the rat glandular stomach[4] also failed to cause adenocarcinomas in the mouse organ.[4] Although localized X-irradiation of hypocatalasemic mice (a C3H mouse mutant) was recently reported to induce well differentiated adenocarcinomas and signet ring cell carcinomas in glandular stomach at high incidences,[5] the mouse glandular stomach has generally been considered resistant to chemical carcinogens. However, we found that administration of N-methyl-N-nitrosourea (MNU) by gastric intubation resulted in a good yield of well-differentiated adenocarcinomas, poorly differentiated adenocarcinomas and signet ring cell carcinomas.[6] This glandular stomach cancer model in the mouse has the advantage over that in the rat of exhibiting particular similarities to human gastric cancers in terms of the histopathological changes.[6] The problem with this experimental model was that squamous cell carcinomas developed in the forestomach and caused early death. In rats, administration of MNNG by gastric intubation similarly induces multiple tumors in the forestomach and very few in the glandular stomach,[7,8] but when administered in the drinking water the same carcinogen was found primarily to cause carcinomas in the glandular stomach, especially in the pyloric mucosa, and not in the forestomach.[9,10] The question of whether an equivalent shift in tumor development from the forestomach to the glandular stomach would occur with application of MNU in the drinking water was the subject of the present study, with the aim of establishing a new experimental model for selective induction of glandular stomach cancer in mice.

MATERIALS AND METHODS

Animals and chemicals Male C3H mice (Charles River Japan Inc., Kanagawa) 6 weeks old, were housed in plastic cages with 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 and freshly prepared three times per week. It was given as the drinking water *ad libitum* from light-shielded bottles.

One hundred and forty-eight C3H mice were divided into 4 groups. Mice were given MNU in their drinking water at concentrations of 120 ppm (group 1), 60 ppm (group 2), 30 ppm (group 3) or 0 ppm (group 4 non-treated control). Animals received the MNU solution as their drinking water for 30 weeks and were then given tap water.

The experimental animals were weighed every week until week 20, then every two to three weeks. Sub-groups of animals in groups 1, 2, 3 and 4 were killed at weeks 18 (group 1 only), 30, 42 and 54. Necropsies were performed on all animals which died or were killed when they became moribund. Animals that survived more than 30 weeks, when the first tumors appeared, were included in the effective numbers as regards incidence of tumors. Animals necropsied between weeks 31 and 41 were added to the numbers of effective animals killed at week 42 and those after week 42 added to the numbers of animals killed at week 54. At the end of the 54th experimental week, all surviving animals were killed and autopsied. Survival curves of mice were calculated without including mice killed on schedule at weeks 18, 30 and 42.

Histopathological and histochemical analyses The excised stomachs were fixed in sublimed formaldehyde or ice-cold acetone and cut into about 6 strips, which were embedded in paraffin. Other tissues were carefully checked with the naked eye and tumors and related lesions were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (HE) and by the paradoxical concanavalin A (Con A) method.[11] Neoplastic lesions of the glandular stomach were classified as adenomatous hyperplasia and adenocarcinomas.[6] Adenomatous hyperplasias consisted of excessive glandular proliferation with scanty cellular atypia. Adenocarcinomas of the glandular stomach were classified into well differentiated adenocarcinomas characterized by tubular structures, poorly differentiated adenocarcinomas characterized by little tendency to form glandular structures with severe cellular atypia and signet ring cell carcinomas characterized by isolated tumor cells containing abundant mucin. Well differentiated adenocarcinomas were further classified into intramucosal and invasive carcinomas. As intramucosal carcinomas showed severe cellular atypia, they could be clearly distinguished from adenomatous hyperplasias with little cellular atypia.[6]

RESULTS

Reductions in survival rate and of body weights were observed in mice treated with MNU in a dose-dependent manner (Figs. 1 and 2). No animals died until week 30, when 8 mice in group 1, 2 and 3, and 4 mice in group 4 were killed. From 31 to 41 weeks, 17 mice in group 1, 5 mice in group 2 and one mouse in group 3 died or became moribund. At 42 weeks, 10 mice in groups 1, 2 and 3 and 6 mice in group 4 were randomly selected and killed. After 42 weeks 3 mice in group 1, 11 mice in group 2 and 4 mice in group 3 died or became moribund. The remaining mice, 2 (group 1), 5 (group 2), 16 (group 3) and 10 (group 4), were killed at week 54. After week 30, hemorrhage from hemangioendothelial sarcomas in the spleen was one of the main causes of death of mice. Total intakes of MNU per mouse during the experiment were 64.9 mg in group 1, 38.9 mg in group 2 and 25.5 mg in group 3.

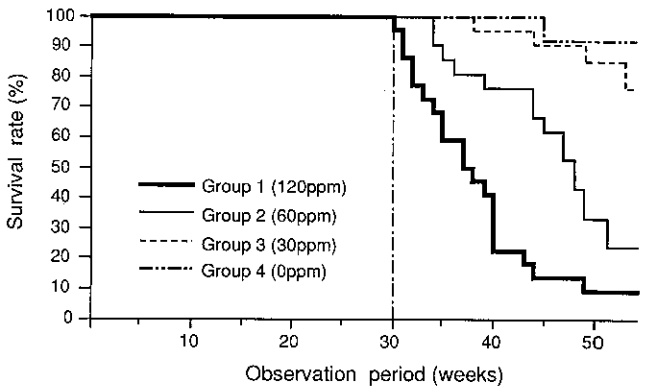

Fig. 1. Survival curves of mice treated with MNU.

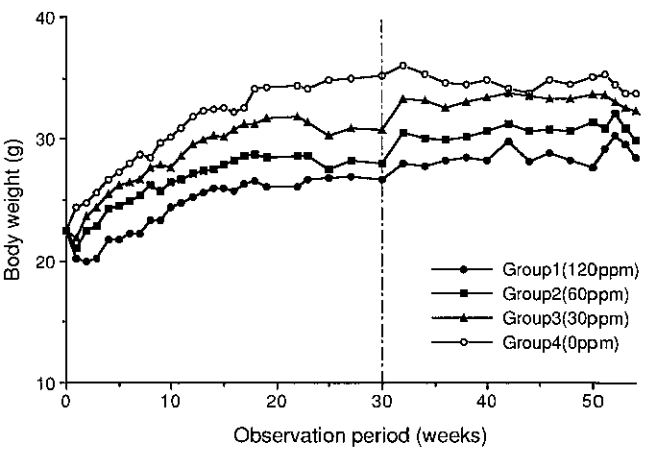

Fig. 2. Body weight curves of mice treated with MNU.

Gross glandular stomach findings At week 18. Shallow ulcers were occasionally found in the pyloric mucosa. Slightly raised tiny plaques to nodules were also apparent in the lesser curvature of the pyloric region. After 30 weeks, nodules with elevated borders developed with time and some of them were ulcerative and umbilicated.

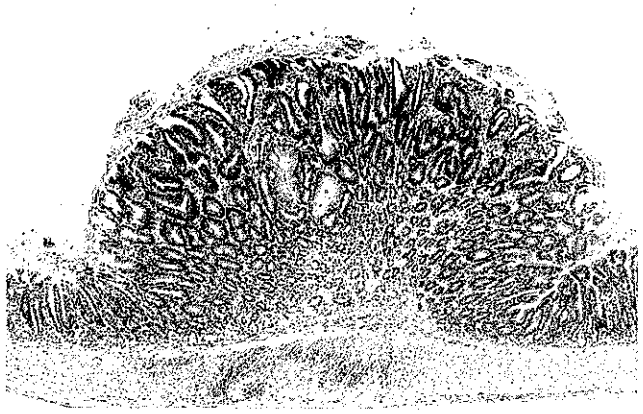

Fig. 3. Adenomatous hyperplasia in a group 1 animal at week 30. HE, ×100.

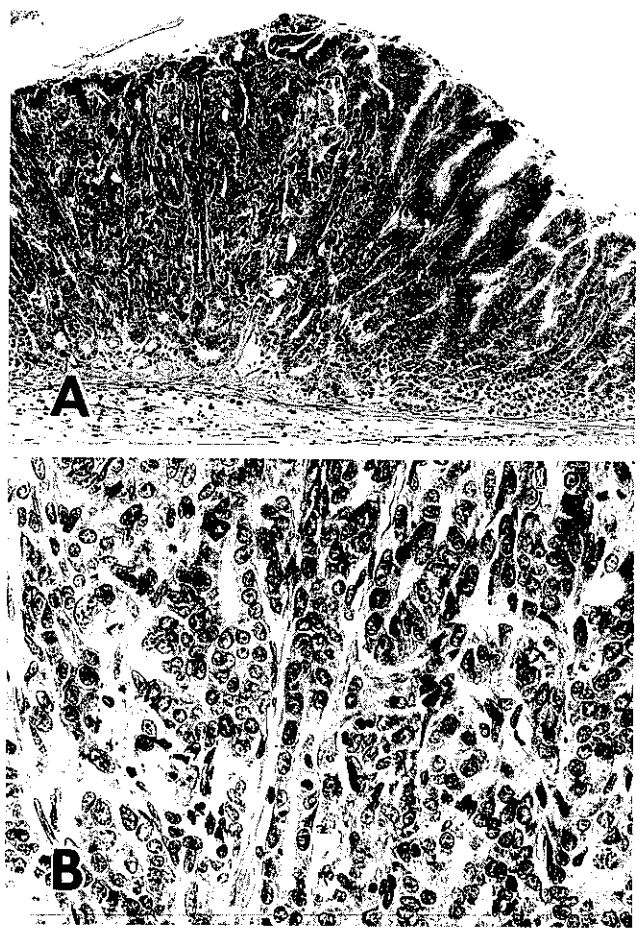

Fig. 4. A: Intramucosal well differentiated adenocarcinoma in a group 1 mouse at week 42. HE, ×40. B: Higher magnification of the same specimen as in Fig. 4A. HE, ×200.

Fig. 5. Well differentiated adenocarcinoma in a group 1 mouse at week 42. HE, ×100.

Marked thickening of the wall of the stomach due to diffuse infiltration of cancer cells was found occasionally. No inter-group differences in the distribution of tumors were found.

Microscopic glandular stomach findings At week 18, hyperplasia of pyloric mucosa was found in 7 out of 10 mice. Adenomatous hyperplasias (Fig. 3) were also found in a few mice, although cancers were absent. At 30 weeks, hyperplasias were found at high incidence in each MNU-treated group. Adenomatous hyperplasias were found in 5 (group 1), 2 (group 2), and 2 (group 3) out of 8 mice in each case. Adenocarcinomas (Figs. 4 to 7) were found from week 30, with one poorly differentiated adenocarcinoma (group 1), one well differentiated adenocarcinoma (group 2) and no cancer (group 3), respectively. From weeks 31 to 42, adenomatous hyperplasias and adenocarcinomas developed in a dose-dependent manner in groups 1, 2 and 3, although carcinomas were not found in group 3. From weeks 43 to 54, adenomatous hyperplasia was found at high incidence (over 60%), and adenocarcinomas developed in a dose-dependent manner in groups 1, 2 and 3. As different types of adenocarcinomas (Figs. 4 to 7) were occasionally found in the same stomach, actual numbers of glandular stomach tumor-bearing animals are indicated in Table I. Among 5 intramucosal well differentiated adenocarcinomas, two were present within areas of adenomatous

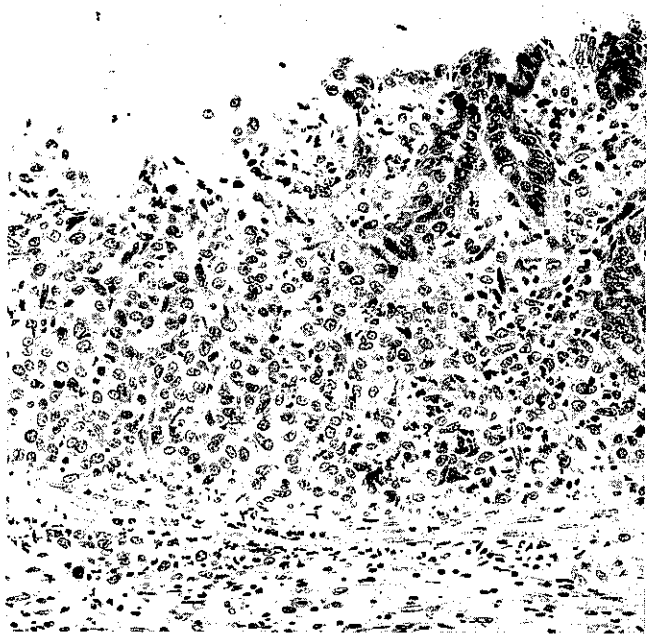

Fig. 6. Poorly differentiated adenocarcinoma in a group 1 mouse at week 42. HE, ×100.

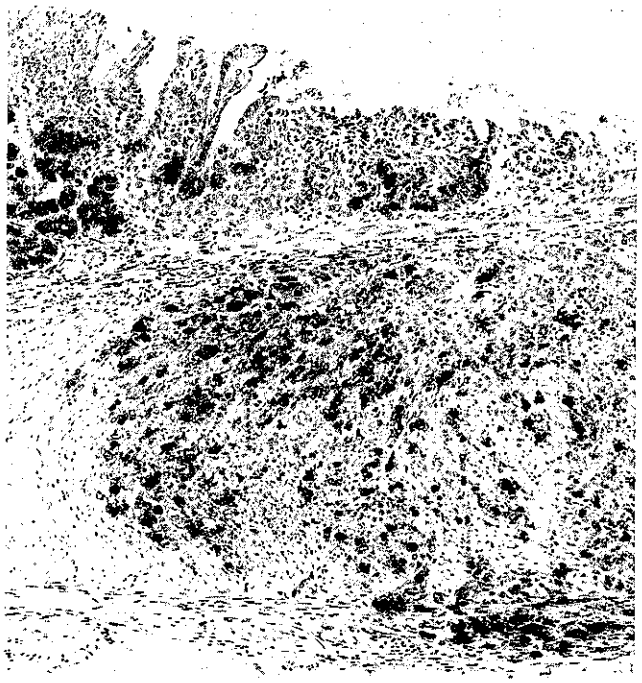

Fig. 7. Signet ring cell carcinoma demonstrating class III mucin-positive cells in a group 1 animal at week 42. Paradoxical Con A staining, ×100.

Table I. Sequential Changes in Incidences of Neoplastic Lesions in the Glandular Stomach of Mice Treated with MNU

Weeks Groups	Effective no. of mice	Adenomatous hyperplasia (%)	Adenocarcinoma				Sarcoma
			Total cancer-bearing	Well Differentiated	Poorly Differentiated	Signet-ring[a]	
18 weeks							
Group 1 (120 ppm)	10	3 (30.0)	0	0	0	0	0
30 weeks							
Group 1 (120 ppm)	8	5 (62.5)	1 (12.5)	0	1	0	0
Group 2 (60 ppm)	8	2 (25.0)	1 (12.5)	1	0	0	0
Group 3 (30 ppm)	8	2 (25.0)	0 —	0	0	0	0
Group 4 (0 ppm)	4	0 —	0 —	0	0	0	0
42 weeks							
Group 1 (120 ppm)	27	22 (81.5)[b]	12 (44.4)[b, c]	6	2	6	2
Group 2 (60 ppm)	15	10 (60.7)[b]	1 (6.7)	1	0	0	0
Group 3 (30 ppm)	11	7 (63.6)[b]	0 —	0	0	0	0
Group 4 (0 ppm)	6	0 —	0 —	0	0	0	0
54 weeks							
Group 1 (120 ppm)	5	4 (80.0)[b]	2 (40.0)[d, e]	0	2	0	1
Group 2 (60 ppm)	16	15 (93.8)[b]	3 (18.8)	2	1	1	2
Group 3 (30 ppm)	20	16 (80.0)[b]	1 (5.0)	1	0	0	1
Group 4 (0 ppm)	10	0 —	0 —	0	0	0	0

a) Signet ring, signet ring cell carcinoma.
b) Significantly different from group 4 at $P<0.01$.
c) Significantly different from group 2 at $P<0.01$.
d) Significantly different from group 3 at $P<0.05$.
e) Significantly different from group 4 at $P<0.05$.

Table II. Sequential Changes in Incidences of Neoplastic Lesions in the Spleen, Liver, Kidneys and Lungs of Mice Treated with MNU[a]

Weeks and Groups	Effective no. of mice	Spleen (%)	Liver (%)			Kidney (%)	Lung (%)
		HS[b]	NN	HCC	HS	Adenoma	Adenoma
42 weeks							
Group 1	27	18 (66.7)[c,d]	1 (3.7)[e]	0 —	1 (3.7)	1 (3.7)	6 (22.2)
Group 2	15	7 (46.7)[d]	1 (6.7)	1 (6.7)	0 —	1 (6.7)	4 (26.7)
Group 3	11	2 (18.2)	3 (27.3)	1 (9.1)	0 —	0 —	1 (9.1)
Group 4	6	0 —	0 —	0 —	0 —	0 —	0 —
54 weeks							
Group 1	5	5 (100)[c,d]	1 (20.0)	0 —	0 —	0 —	2 (40.0)[f]
Group 2	16	15 (93.8)[c,d]	2 (12.5)[e]	0 —	4 (25.0)	6 (37.5)[f]	5 (31.3)[f]
Group 3	20	8 (40.0)[f]	9 (45.0)	2 (25.0)	1 (5.0)	3 (15.0)	10 (50.0)[f]
Group 4	10	0 —	3 (30.0)	1 (10.0)	0 —	0 —	0 —

a) HS, hemangioendothelial sarcoma; NN, neoplastic nodule; HCC, hepatocellular carcinoma.
b) HS was found in one of 8 mice in group 1 at week 30.
c) Significantly different from group 3 at $P<0.01$.
d) Significantly different from group 4 at $P<0.01$.
e) Significantly different from group 3 at $P<0.05$.
f) Significantly different from group 4 at $P<0.05$.

hyperplasia. All cancers originating from the glandular stomach contained tumor cells with Class III mucins as detected by paradoxical Con A staining (Fig. 7). Intestinal absorptive cell-type cancer cells with incomplete striated cell borders and goblet cell-type cancer cells were not found in morphologically well differentiated adenocarcinomas. Intestinal metaplasias were not found in the stomach of any mouse in this experiment. In control animals (group 4) no tumors were found. Sarcomas also developed in the glandular stomach, with 2 hemangioendothelial sarcomas and one leiomyosarcoma found in group 2, 2 hemangioendothelial sarcomas in group 2 and 2 leiomyosarcomas in group 3. In the forestomach, only one squamous cell carcinoma was found at week 54 in group 1, and in groups 2, 3 and 4, no squamous cell carcinomas were apparent at any time. In the small intestine, including the duodenum, one adenocarcinoma was found in group 1 and hemangioendothelial sarcomas were found in one group 1, one group 2 and 3 group 3 animals. No tumors were found in the large intestine.

Thus, almost all tumors in the gastrointestinal tract developed in the glandular stomach. Histopathological findings for the glandular stomach are summarized in Table I.

In the spleen, hemangioendothelial sarcomas were found at high incidence and with carcinogen dose-dependence in the MNU-treated groups. Some of them were very large and occasionally they showed hemorrhage into the abdominal cavity. In the liver, neoplastic nodules and hepatocellular carcinomas were occasionally found in both MNU-treated and non-treated groups, and hemangioendothelial sarcomas were occasionally found in MNU-treated animals. Tiny adenomas were found in the kidneys and the lungs in MNU-treated groups. In addition, one lymphoma and one rhabdomyosarcoma in group 1, one lymphoma in group 2 and one lymphoma and one thymoma in group 3 were found. Neoplastic changes in the spleen, liver, kidneys and lungs are summarized in Table II.

DISCUSSION

The present investigation clearly confirmed that MNU can induce neoplasia in the C3H mouse glandular stomach. Earlier, ten weekly intragastric intubations of 0.5 mg/mouse were shown to cause adenocarcinomas at high incidence.[6] However, the incidence of squamous cell carcinomas in the forestomach was 77.8% at week 20, rising to 100% at week 40 and many animals died as a result. Forestomach resection was performed for small squamous cell carcinoma development.[6] With the present protocol, in contrast, only one squamous cell carcinoma developed in a mouse receiving 120 ppm MNU and none in those receiving lower doses. This dramatic decrease of tumor development in the forestomach has also been observed with the shift in application of MNNG from gastric intubation[7,8] to the drinking water route[9,10] in rats. Although the experimental model still suffers from the disadvantage of high yields of hemangioendothelial sarcomas in the spleen there is no longer the complication of squamous cell carcinomas in the forestomach invading the glandular stomach and influencing the pathological analysis of gastric cancer development. Thus, for investigation of tumor development in the

glandular stomach in mice, the carcinogen should be given in the drinking water.

The results gained from the present investigation clearly demonstrated dose-dependent intake of MNU and dose-dependent effects in terms of mouse body weight, survival time and degree of pathological lesion development in the glandular stomach and the spleen. The dose-dependence of survival time was considered to be due to toxic effects of MNU, in view of the dose-response effects on body weight and dose-dependent induction of hemangioendothelial sarcomas in the spleen.

Some intramucosal adenocarcinomas were found within areas of adenomatous hyperplasia, suggesting a histogenetic link. Furthermore, clear dose-related induction of adenomatous hyperplasias was observed at week 30, after which time its incidence increased until the yield in groups 2 and 3 finally reached the high level of group 1. Dose-related induction of adenocarcinomas was found at weeks 42 and 54. Thus adenocarcinomas in adenomatous hyperplasia and the time differential in occurrence of dose-related induction of adenocarcinomas and adenomatous hyperplasias provide evidence that the latter act as progenitors of the malignancies.

Human gastric cancers have been classified into differentiated (papillary and tubular adenocarcinomas) and undifferentiated (poorly differentiated adenocarcinomas and signet ring cell carcinomas) types.[12] Therefore, in addition to high cancer induction, an optimal experimental animal model of human gastric cancers should also involve both differentiated and undifferentiated type lesions. Adenocarcinomas induced by MNNG[9, 13, 14] or MNU[15] in the rat stomach are mainly of differentiated type and induction of undifferentiated type stomach cancers in rats is relatively difficult. For this purpose, surfactant,[16] salt,[17] gastrin or serotonin[18] has been combined with MNNG treatment, but the incidences of the targeted lesions were nevertheless low. Previously, we reported[6] that ten weekly intragastric intubations of 0.5 mg/mouse of MNU induced 4 poorly differentiated adenocarcinomas and 5 signet ring cell carcinomas among 36 glandular stomach cancers in 27 glandular stomach tumor-bearing animals. In this experiment, administration of 120 ppm MNU in drinking water induced 6 well differentiated adenocarcinomas, 5 poorly differentiated adenocarcinomas and 6 signet ring cell carcinomas in 15 stomach cancer-bearing animals. Thus, the similarities in histopathological types of stomach cancers in mice and man are strongly indicative of the advantages of this experimental model.

In rats, cellular atypia in well differentiated adenocarcinomas is slight and occasionally distinction from adenomatous hyperplasia is difficult, with invasion of cancer cells becoming a necessary criterion of cancers.[13, 19] However, cellular atypia of cancer cells comprising well differentiated adenocarcinomas in mice is severe[6] and it is therefore relatively easy to distinguish intramucosal adenocarcinomas from adenomatous hyperplasia. The fact that it is possible to make a diagnosis of intramucosal well-differentiated adenocarcinomas in this mouse experimental stomach cancer model is a further point favoring its use in analysis of early gastric carcinogenesis.

Intestinal metaplasia in humans has been considered to be a preneoplastic change[20–22] for well differentiated adenocarcinomas. However, well-differentiated adenocarcinomas contain gastric-type cancer cells with class III mucins, pepsinogens or mucins specific to gastric surface mucous cells, and no relationship between phenotypic expression of stomach cancer cells and the phenotypic expression of surrounding mucosa has been reported.[23] In the present experiment no intestinal metaplasia was noted in any of the stomachs and well differentiated adenocarcinomas clearly consisted of gastric-type cancer cells. No relationship between intestinal metaplasia and MNU-induced glandular stomach cancers in mice was found. The data in this work are also consistent with the conclusion derived from our previous studies,[24, 25] that intestinal metaplasia may not be a preneoplastic change of any relevance to stomach carcinomas.

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