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Dose-dependent Induction of Both Pepsinogen-altered Pyloric Glands and Adenocarcinomas in the Glandular Stomach of C3H Mice Treated with *N*-Methyl-*N*-nitrosourea

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The dose-response relation for the appearance of pepsinogen isozyme 1 (Pg 1)-altered pyloric glands (PAPG) and the related induction of adenocarcinomas were examined in male C3H mice given *N*-methyl-*N*-nitrosourea (MNU) in their drinking water at the concentration of 120 ppm (group 1), 60 ppm (group 2), 30 ppm (group 3) or 0 ppm (group 4) for 30 weeks and then normal tap water. Animals were killed at weeks 10, 30 and 42. Adenomatous hyperplasias and adenocarcinomas were noted from week 30 and their induction was dose-dependent at week 42. Almost all cells of pyloric gland cell type in those lesions had little or no immunohistochemically demonstratable Pg 1 content, as was also the case for the cells in PAPG, whose numbers per 100 normal-appearing pyloric glands were found to be MNU dose-dependent at all experimental time points. The numbers of PAPG at week 10 significantly correlated with the incidences of adenomatous hyperplasias and adenocarcinomas at week 42. Investigation of proliferation by immunohistochemical detection of bromodeoxyuridine (BrdU) labeling in the PAPG at week 10 demonstrated elevation ($P<0.05$) as compared to normal pyloric glands. Intestinal metaplasia was not a feature in the present experiment and the results suggest that in mice, PAPG might be a preneoplastic lesion involved in gastric chemical carcinogenesis.

Key words: Preneoplasic lesion — *N*-Methyl-*N*-nitrosourea — Pepsinogen 1-altered pyloric gland — Stomach cancer — Mouse

Since the first report of experimental production of adenocarcinomas of the glandular stomach in rats with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG),[1] many other mammals have been shown to be susceptible to this carcinogen, including hamsters,[2] ferrets[3] and dogs.[4] In studies to determine the pathogenesis of gastric cancer, determination of the nature of preneoplastic changes is one of the most important fields for research. We have concentrated attention on pepsinogen isozyme 1 (Pg 1) as a marker of preneoplasia. Pg 1–4 have been isolated from the normal rat glandualr stomach, three (Pg 1,3,4) occurring in the pyloric mucosa, and all four (Pg 1–4) in the fundic glands.[5,6] Pg 1 expression preferentially decreases or disappears in pyloric mucosa during the early stages of rat MNNG-induced gastric carcinogenesis before morphologically distinct preneoplastic changes become evident.[7–9] Immunohistochemically, the alteration of Pg 1 expression can be readily detected as pepsinogen 1-altered pyloric glands (PAPG) in normal-appearing pyloric mucosa, and also consistently in adenomatous hyperplasias and adenocarcinoma.[10,11] Induction of PAPG was found to be dependent on the dose of MNNG administered and the numbers increase with time,[11,12] the PAPG cells having a higher proliferative activity than their normal counterparts. Thus PAPG are now generally accepted as preneoplastic changes in the glandular stomach of rats.

The mouse is a particularly useful species for carcinogenesis studies because of the availability of a number of transgenic, mutant, and chimeric strains. Although some transgenic mouse lines were reported with adenocarcinomas,[13] attempts to establish a chemically induced gastric experimental model were unsuccessful for a long time.[13–16] However, administration of *N*-methyl-*N*-nitrosourea (MNU) to BALB/c or C3H mice was recently shown to result in good yields of adenocarcinomas in the glandular stomach.[17,18]

In the present study, we immunohistochemically examined changes of Pg 1 expression in mouse pyloric mucosa during gastric carcinogenesis induced by MNU, and investigated whether the PAPG might be a preneoplastic change in the mouse stomach as well as in the rat. For this purpose, we evaluated the dose-response relation between induction of PAPG and the incidences of adenomatous hyperplasias and adenocarcinomas, as well as the proliferation kinetics of PAPG at an early stage.

MATERIALS AND METHODS

Animals and chemicals A total of 100 male C3H/HeN

mice (Charles River Japan Inc., Kanagawa), 6 weeks old, 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*. The animals were divided into 4 groups. They 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) for 30 weeks and then tap water. MNU (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water (freshly prepared three times per week) and this solution was supplied as the drinking water *ad libitum* from light-shielded bottles.

Sub-groups of animals in all groups were killed at weeks 10, 30, and 42. The selected mice in all groups at week 10 received bromodeoxyuridine (BrdU) (Sigma Chemical Co.) 75 mg/kg body weight i.p., 1 h before they were killed. Necropsies were performed on all animals which died or were killed upon becoming moribund.

As Pg 1-positive controls, stomachs from 3 male F334 rats aged 8 weeks old were used.

Histopathological analyses The excised stomachs were fixed in sublimed formaldehyde,[19] ice-cold acetone or neutral buffered formalin, cut into about 6 strips, and routinely processed for embedding in paraffin. Other tissues were carefully checked with the naked eye, and tumors and related lesions were fixed in neutral buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin for histopathological assessment of lesion development. Before staining, to remove mercuric chloride, the sections fixed with sublimed formaldehyde were treated with Lugol's iodine and then all traces of iodine were removed with 2.5% sodium thiosulfate. Lesions of the pyloric mucosa were classified as adenocarcinomas, adenomatous hyperplasias, and mucosal hyperplasia, according to the criteria previously described.[18]

Ouchterlony assay Antiserum against rat Pg 1 was raised in a New-Zealand-white rabbit as described previously.[6] Glandular stomach mucosa from male F344 rats, and BALB/c and C3H mice at 8 weeks of age were homogenized with 3 volumes of phosphate-buffered saline and centrifuged at 100,000*g* for 1 h at 4°C and the supernatant was used as crude Pg extract for determination of the expression level. Immunodiffusion was performed using the method of Ouchterlony.[20]

Immunohistochemical and histochemical procedures For Pg 1 staining with the anti-rat Pg 1 serum, the ABC (Vectastain ABC kit, PK4001 Vector Laboratories Inc., Burlingame, CA) method was used. Double staining of Pg 1 and BrdU was performed for tissue sections fixed with sublimed formaldehyde using the PAP method (DAKO PAP kit, DAKO Japan Co., Ltd., Kyoto) for BrdU, followed by the ABC method for Pg 1. After Pg 1 staining, the sections were treated with 4 *N* HCl for 20 min at 37°C, neutralized with 0.2 *M* boric acid-borate buffer at pH 7.6, and washed with PBS. Then sections were treated with mouse monoclonal antibody against BrdU (DAKO Japan Co., Ltd.). Visualization of PAP was achieved with antibody-labeled peroxidase 3,3′-diaminobenzidine (brown color) and for the ABC, with alkaline phosphatase-conjugated streptavidin-new fuchsin (red color). For class III mucin histochemistry, paradoxical concanavalin A (ConA) staining was used.[21]

Immunohistochemical analysis Pyloric gland cells in the basal zone of the pyloric mucosa stained strongly for Pg 1 in the control mice. Glands in apparently normal pyloric mucosa whose cells close to the lamina muscularis mucosa stained weakly or negative for Pg 1 were defined immunohistochemically as PAPG. The number of PAPG per 100 pyloric glands was calculated for each animal. Using specimens subjected to double immunohistochemistry for Pg 1 and BrdU, numbers of flash-labeled cells per pyloric gland in normal pyloric mucosa and MNU-treated pyloric mucosa containing PAPG were counted for a total of 20–30 pyloric columns in each individual animal. Class III mucin revealed by paradoxical ConA staining was used as a marker of phenotypic expression for tumor cells of pyloric gland cell type.

Statistical analysis For labeling indices and numbers of PAPG, the two-tailed *t* test was applied to establish the significance of differences. The incidences of stomach lesions and tumors were analyzed using the one-tailed Fisher's exact probability test.

RESULTS

General and histopathological findings During the first 10 weeks of the experiment, total intakes of MNU per mouse in groups 1, 2 and 3 were 22.1 mg, 16.8 mg and 11.9 mg, respectively. The body weights in groups 1, 2, 3, and 4 at the end of experimental week 42, were depressed in a dose-dependent manner, being 28.3±2.6 g, 30.6±1.6 g, 33.4±1.6 g, and 34.8±2.1 g, respectively.

Histological findings for the glandular stomach are summarized in Table I. At week 10, the pyloric mucosa of treated animals show slight irregularities in arrangement, with some hyperplastic elongated or shortened atrophic crypts. At week 30 adenomatous hyperplasias were found in groups 1, 2, and 3, and their numbers were increased with dose. One poorly differentiated adenocarcinoma was observed in group 1 and one well differentiated adenocarcinoma in group 2. At week 42, 4 well differentiated, and 2 poorly differentiated adenocarcinomas, as well as one signet ring cell carcinoma were found in 6 stomach cancer-bearing mice in group 1, and one well differentiated adenocarcinoma was observed in group 2. The incidences of adenomatous hyperplasias

Table I. Sequential Changes in Numbers of PAPG and Incidences of Neoplastic Lesions in Mice Treated with MNU

Week Groups	Histology			Pg 1 immunohistochemistry	
	No. of[a] mice	Adenomatous hyperplasia (%)	Adenocarcinoma (%)	No. of[b] mice	No. of[c] PAPG
10 weeks					
Group 1 (120 ppm)	11	0	0	11	26.7±8.0[g,h,i]
Group 2 (60 ppm)	10	0	0	10	8.6±5.8[f]
Group 3 (30 ppm)	10	0	0	10	5.4±2.0[f]
Group 4 (0 ppm)	5	0	0	5	1.0±0.7
30 weeks					
Group 1 (120 ppm)	8	5 (62.5)	1 (12.5)	4	13.0±5.3[d,h,i]
Group 2 (60 ppm)	8	2 (25.0)	1 (12.5)	4	3.7±1.4[f]
Group 3 (30 ppm)	8	2 (25.0)	0	4	1.8±2.0
Group 4 (0 ppm)	4	0	0	4	1.1±0.7
42 weeks					
Group 1 (120 ppm)	10	10 (100.0)[e,i]	6 (60.0)[d,f,h]	8	18.2±8.7[e,i]
Group 2 (60 ppm)	10	7 (70.0)[f]	1 (10.0)	8	13.8±5.2[i]
Group 3 (30 ppm)	10	6 (60.0)[f]	0	8	10.1±6.0[f]
Group 4 (0 ppm)	6	0	0	5	3.2±0.6

a) Total number of mice available for this study. Fixation was in sublimed formaldehyde, acetone and/or neutral buffered formalin.
b) Number of mice with stomachs fixed in sublimed formaldehyde.
c) Number: mean±SD.
d) Significantly different from group 2 at $P<0.05$.
e) Significantly different from group 3 at $P<0.05$.
f) Significantly different from group 4 at $P<0.05$.
g) Significantly different from group 2 at $P<0.01$.
h) Significantly different from group 3 at $P<0.01$.
i) Significantly different from group 4 at $P<0.01$.
Pg, pepsinogen isozyme; PAPG, Pg 1-altered pyloric gland; MNU, *N*-methyl-*N*-nitrosourea.

and adenocarcinomas were dose-dependent. Both lesion types developed mainly in the pyloric mucosa but some were found at the fundopyloric border.

Intestinal metaplasias were not observed in any of the groups.

Ouchterlony assay As shown in Fig. 1 mouse Pg reacted with antiserum to rat Pg 1, and the precipitin line fused completely with that of rat Pg 1.

Immunohistochemistry of Pg 1 and histochemistry of class III mucin In control mice, Pg 1 (Fig. 2a) and class III mucins were present in pyloric gland cells in the pyloric mucosa and in mucous neck cells in fundic mucosa, with only Pg 1 being positive in chief cells, completely corresponding to the control rat glandular stomach case. In normal-looking mucosa of mice treated with MNU, class III mucin-positive pyloric glands with a low or no Pg 1 content were found as PAPG (Fig. 2b). The numbers of such PAPG in the pyloric mucosa for each group are summarized in Table I. They were found at all time points in a dose-dependent manner. The numbers at week 30 were about half those at week 10, but at week 42, an increase was again registered. In altered

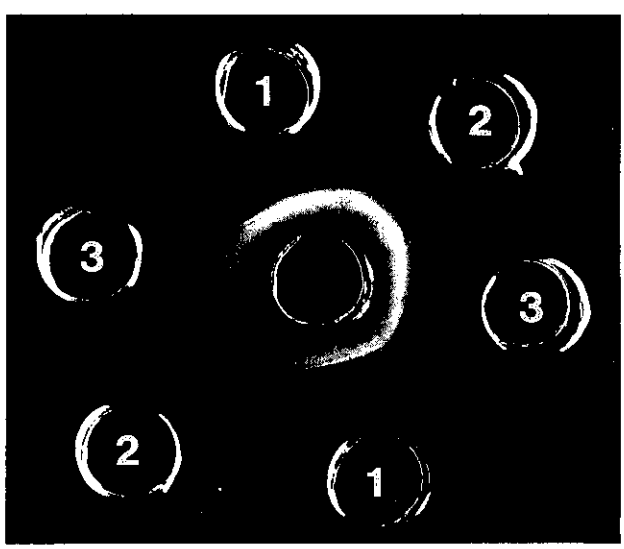

Fig. 1. Ouchterlony assay. Antiserum to rat pepsinogen isozyme (Pg) 1 was raised in a New-Zealand-white rabbit. (1) Pg 1 extract from a F344 rat; (2) Pg extract from a BALB/c mouse; (3) Pg extract from a C3H mouse.

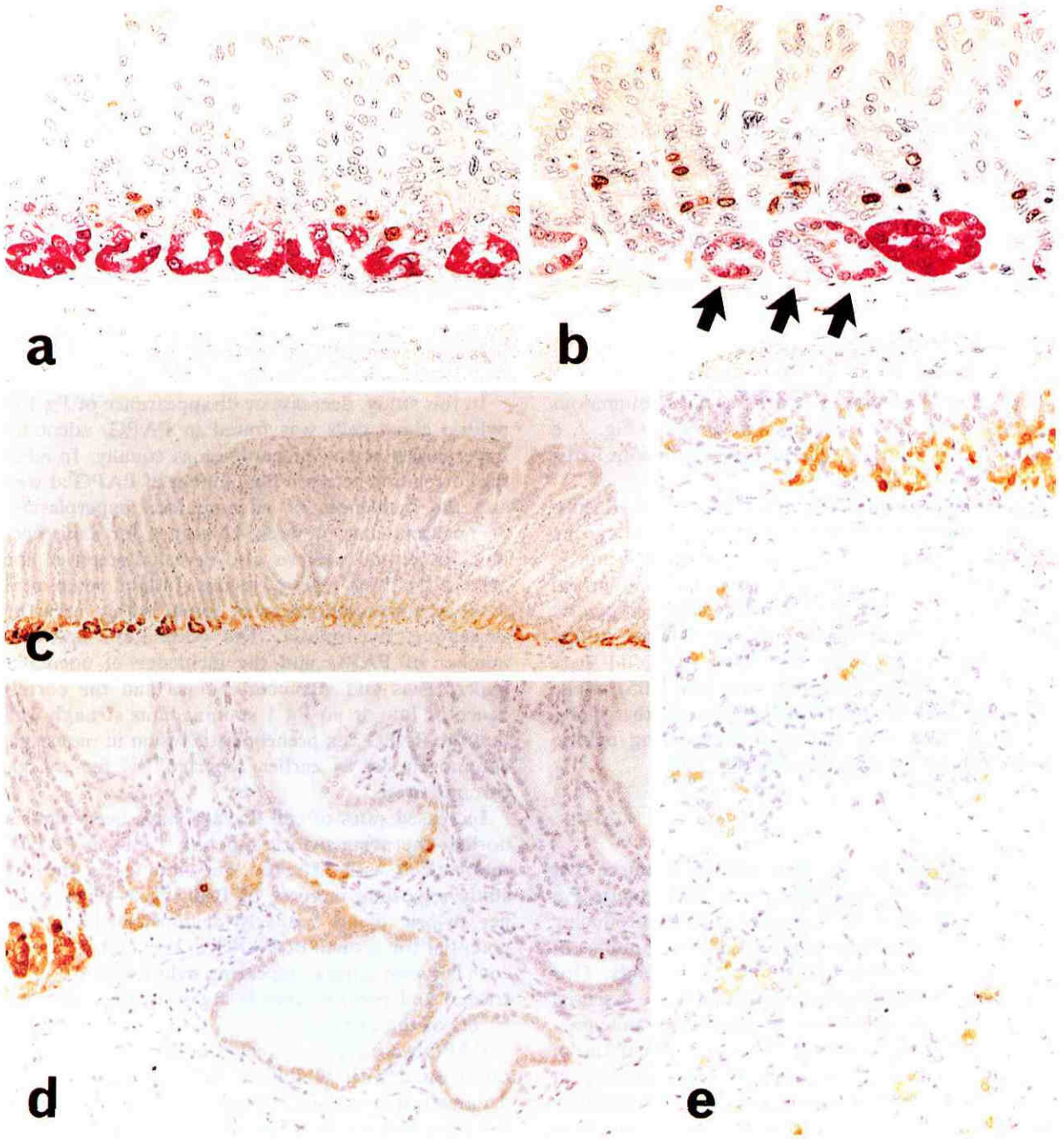

Fig. 2. (a) Pyloric mucosa of a control mouse at week 10. Double immunohistochemical staining of bromodeoxyuridine (BrdU, brown), and pepsinogen isozyme (Pg) 1 (red) 60 min after a single injection of BrdU. Pyloric gland cells have high Pg 1 content. (b) Pyloric mucosa after treatment with 120 ppm *N*-methy-*N*-nitrosourea at week 10. Same staining as in (a). Pg 1-altered pyloric gland consisting of cells with low or no Pg 1 content are apparent (arrows). (c) Adenomatous hyperplasia in a group 2 mouse at week 42. (d) Well differentiated adenocarcinoma in a group 1 mouse at week 42. (e) Poorly differentiated adenocarcinoma in a group 1 mouse at week 42. (c), (d), (e) demonstrating immunohistochemical staining for Pg 1 (brown). Almost all tumor cells of pyloric gland cell type have a very low or no Pg 1 content.

Table II. Bromodeoxyuridine Flash-labeling Indices at Week 10[a)]

	Control group		MNU treated group		
	No. of animals		No. of animals	Normal-[b)] appearing	PAPG[c)]
No. of labeled cells /pyloric gland	5	2.1±0.3	6	3.2±0.9[d)]	4.4±0.6[e,f)]

a) Number: mean±SD.
b) Normal-appearing pyloric gland with high Pg 1 content.
c) Pepsinogen 1-altered pyloric gland.
d) Significantly different from the control at $P<0.05$.
e) Significantly different from the control at $P<0.001$.
f) Significantly different from the normal-appearing group at $P<0.05$.
MNU, *N*-methyl-*N*-nitrosourea; Pg, pepsinogen isozyme.

pyloric mucosa, showing atrophic or hyperplastic changes, some pyloric gland cells had a high Pg 1 content, and others had little or no Pg 1. In adenomatous hyperplasias (Fig. 2c) and adenocarcinomas (Fig. 2, d and e), almost all tumor cells with class III mucins had a low or no Pg 1 content.

Double immunohistochemistry of Pg1 and BrdU Results of double staining of control and MNU-treated (group 1) mice at week 10 are illustrated in Fig. 2, a and b. Flash-labeling indices at this time point are summarized in Table II. The numbers of labeled cells per pyloric gland in the normal-appearing pyloric mucosa in the MNU-treated group (MNU 120 ppm, group 1) were higher than in the control group case ($P<0.05$), while the values for PAPG showed a significant further increment as compared with the normal-appearing pyloric glands featuring Pg 1-positive cells ($P<0.05$).

DISCUSSION

In the present study, we were able to confirm that antiserum against rat Pg 1 also reacts with mouse Pg. Previously, Kataoka *et al.*[22] showed that Pg-producing cells in the mouse fundic mucosa could be immunohistochemically stained using anti-rat Pg 1 antibody. Our immunohistochemical findings further indicate essential similarities between the rat and mouse pyloric mucosa.

Induction of PAPG was MNU dose-dependent and in line with the tumor yield at week 42. The decrease in number observed at week 30 might be due at least partly to the development of adenomatous hyperplasias from PAPG precursors, and therefore replacement. Although the administration of MNU was stopped at week 30, the numbers of PAPG increased thereafter. That this could have been age-related is evidenced by the slight increase in number which was also evident in the control group with time. However, the large increase from weeks 30 to 42 (especially in groups 2 and 3) is more likely to have been due to the elevated cell proliferation in these lesions, so that more became visible.

In this study, decrease or disappearance of Pg 1 in the pyloric gland cells was found in PAPG, adenomatous hyperplasias and adenocarcinomas equally. In addition, the correlation between the number of PAPG at week 10 and the incidences of adenomatous hyperplasias and adenocarcinomas at week 42 argues for a histogenetic link. Sequential analysis also revealed stepwise appearance of the three types of lesions. In fact, some intramucosal adenocarcinomas were found within areas of adenomatous hyperplasia. The correlation between the number of PAPG and the incidence of adenomatous hyperplasias and adenocarcinomas and the correspondence of low or no Pg 1 staining thus strongly suggest that the PAPG is a preneoplastic lesion in mouse gastric carcinogenesis, as earlier reported[10–12] for rat gastric carcinogenesis.

Increased rates of cell division have been reported in normal-appearing pyloric mucosa,[23] and in PAPG[24] in rats treated with MNNG. The findings in the present study regarding increase of BrdU flash-labeling indices per pyloric gland similarly demonstrated an elevated potential for growth of PAPG in the mouse. The difference between normal-appearing pyloric glands in MNU-treated and control animals is consistent with the toxic effects of the carcinogen.

PAPG were earlier detected in five different strains of rats treated with MNNG.[25] In addition to the present demonstration in the C3H mouse, the presence of PAPG has been recognized in the pyloric glands of FVB/N and MT100 (a transgenic mouse expressing human transforming growth factor α) strains given MNU by intragastric intubation.[26] Although adenocarcinomas induced by MNNG,[1,27,28] and MNU[29] in the rat stomach are mainly of well differentiated type, in the present investigation three types of malignant tumors developed in mouse glandular stomach, i. e., well differentiated and

poorly differentiated adenocarcinomas and signet ring cell carcinomas. They all had the same characteristic of weak or no Pg 1. Thus, PAPG can be regarded as a common change in a number of mouse and rat strains, acting as a precursor for a variety of adenocarcinoma types.

In the human stomach, intestinal metaplasia has been suggested to be a preneoplastic change for well differentiated adenocarcinomas.[30] In rat stomach treated with MNNG, intestinal metaplasia is found, but no consistent relationship with well differentiated adenocarcinoma exists.[31] In this study, no intestinal metaplasia was noted, precluding any histogenetic role.

To conclude, our results indicate the following. 1) Weak or no Pg 1 staining is found in PAPG, adenomatous hyperplasias and adenocarcinomas in the mouse stomach. 2) Numbers of PAPG correspond with the incidences of adenomatous hyperplasias and adenocarcinomas in an MNU dose-dependent manner. 3) The mouse PAPG are characterized by altered cell kinetics. Thus, the PAPG detected immunohistochemically may be considered to be a preneoplastic change preceding development of morphologically detectable lesions in mouse stomach carcinogenesis.

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