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Cellular Differentiation and Histogenesis of Rat Glandular Stomach Cancers

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The gastric and intestinal phenotypic expressions of tumor cells in 18 adenomatous hyperplasias, 33 well-differentiated adenocarcinomas, and 16 undifferentiated adenocarcinomas (4 poorly differentiated adenocarcinomas, 10 signet-ring cell carcinomas and 2 mucinous adenocarcinomas) induced by N-methyl-N′-nitro-N-nitrosoguanidine or 4-nitroquinoline-1-oxide in the rat glandular stomach were studied by histochemical stainings for mucin and immunohistochemical staining for pepsinogen isozyme 1 (Pg 1). By histochemical staining for mucin [by the paradoxical concanavalin A method, the modified method with labeled peanut lectin, the galactose oxidase-Schiff (GOS) reaction, and the sialidase-GOS reaction] and immunohistochemical staining of Pg 1, gastric cancer cells of each histological group could be clearly classified into a gastric type, including mucous neck cell pyloric gland cell, and surface mucous cell subtypes, and an intestinal type, including goblet-cell, and intestinal absorptive cell subtypes. All tumors examined in this work consisted mainly of gastric-type cells but intestinal-type tumor cells were occasionally found among the gastric-type tumor cells. The incidences of intestinal-type cells in adenomatous hyperplasias (11.1%) and small well-differentiated adenocarcinomas (28.6%) were significantly less ($P<0.05$) than that in large well-differentiated adenocarcinomas (68.4%). The incidence of intestinal-type cells in small undifferentiated adenocarcinomas (25.0%) was also less than that in large ones (58.3%). The present results suggest the occurrence of change of phenotypic expression of tumor cells from the gastric type to the intestinal type during growth of tumors.

Key words: Mucin histochemistry — Gastric cancer — Histogenesis — Cellular differentiation — Rat

The phenotypic expression of tumor cells is widely thought to resemble that of the tissue of origin of the tumor cells.[1] Thus, examination of phenotypic expression of glandular stomach cancers should reveal their histogenesis. The phenotypic expression of mucin in cells of the alimentary tract is usually determined by paradoxical concanavalin A (Con A) staining.[2,3] Mucins in the alimentary tract can be classified into two types: class III mucins in mucous neck cells, pyloric gland cells and Brunner's gland cells, and class II mucins in surface mucous cells, goblet cells and the surface coat of intestinal absorptive cells. Histochemical investigations by paradoxical Con A staining have demonstrated that mucin-positive gastric cancers (well-differentiated adenocarcinomas and signet-ring cell carcinomas) induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) or 4-nitroquinoline-1-oxide (4-NQO) in rat stomach have either class II or class III mucin.[3] Cancer cells containing class III mucins are considered to show phenotypic expression of pyloric gland cells, and those with class II mucins to show that of surface mucous cells,[3] because chemical carcinogens usually induce gastric cancers in the pyloric region[4–6] with little intestinal metaplasia. However, a detailed histochemical examination of the phenotypic expression of mucins by each epithelial cell type in rodent gastric cancers has not been reported. Recently, new techniques have been developed for histochemical staining of mucins and immunohistochemical staining of pepsinogens to determine the phenotypic expressions of epithelial cells in the alimentary tract. These techniques developed for histochemical staining of mucin include a modified method of staining with labeled peanut lectin (PNA) to differentiate pyloric gland cells from mucous neck cells and Brunner's gland cells,[7,8] the galactose oxidase-Schiff (GOS) reaction to demonstrate mucins in surface mucous cells[9,10] and the sialidase-GOS reaction to detect mucins in goblet cells of the small intestine.[9,10] Rat pepsinogens (Pg) have been classified into 4 isozymes, namely Pg 1 to 4.[11,12] Pg 1 has

Abbreviations: AB, Alcian blue; ABC, avidin-biotin-peroxidase complex; Con A, concanavalin A; GOS, galactose oxidase Schiff; HRP, horseradish peroxidase; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; 4-NQO, 4-nitroquinoline-1-oxide; PA, periodic acid; PAS, periodic acid-Schiff; Pg 1, pepsinogen isozyme 1; PNA, peanut lectin; Re, reduction.

been shown immunohistochemically to be located in mucous neck cells, chief cells, pyloric gland cells and Brunner's gland cells.[13] In this work, we examined the phenotypic expression of gastric cancer cells by using paradoxical Con A staining, the modified method of labeled PNA staining, the GOS reaction, the sialidase-GOS reaction and Pg 1 immunohistochemical staining. The histogenesis of gastric cancers is discussed on the basis of findings on the phenotypic expression of their cells.

MATERIALS AND METHODS

Samples Specimens were taken from 18 adenomatous hyperplasias and 33 well-differentiated adenocarcinomas obtained in a previous study.[14] All these tumors had been obtained from rats (SD, Lewis, WKY, Wistar or F344) at 50 weeks of age after treatment with 100 μg/ml of MNNG (Aldrich Chemical Co., Milwaukee, WI) for 30 weeks. The specimens were fixed in sublimed formaldehyde. In addition, specimens from 16 undifferentiated adenocarcinomas (4 poorly differentiated adenocarcinomas, 10 signet-ring cell carcinomas and 2 mucinous adenocarcinomas) induced by MNNG or 4-NQO (Nacalai Tesque, Kyoto) in rats in our previous studies were analyzed.[3, 8, 15–17] These undifferentiated adenocarcinomas had all been found more than 50 weeks after the beginning of carcinogen treatment. These specimens were fixed in 10% phosphate-buffered formalin. Tissues were stained by the following histochemical and immunohistochemical procedures.

Paradoxical Con A staining[2, 3] Most mucins that are stained with Con A-horseradish peroxidase (HRP) lose their stainability when oxidized with periodate. However, some mucins showed paradoxical enhancement of staining after periodate oxidation with or without subsequent reduction (Re) or blockage of oxidized groups. By this paradoxical Con A staining, mucins in epithelial cells of the alimentary tract were classified into 2 main classes (classes II and III). Three procedures of paradoxical Con A staining were used: periodic acid Schiff (PAS)-Con A-HRP staining (class III mucins stained reddish brown and class II mucins stained red to purple); periodic acid (PA)-Re-Con A-HRP staining (class III mucins stained brown and class II mucins were not stained), and PA-Re-Con A-HRP-Alucian blue (AB) staining (class III mucins stained brown and acid class II mucins stained blue).

Pepsinogen immunohistochemical staining Anti-Pg 1 serum was prepared as described previously.[18] The avidin-biotin-peroxidase complex (ABC) method[19] was used to determine the localization of Pg 1 in the gastric mucosa and cancers as described previously.[13] Sections were counter-stained with hematoxylin for microscopic examination. Normal (non-immune) rabbit serum was used instead of anti-Pg 1 antibody as a negative control.

Modified method of labeled PNA staining[7, 8] Some PNA-reactive mucins demonstrated with PNA-HRP lose their affinity for PNA when they are oxidized with periodate. However, some class III mucins are resistant to this treatment. Therefore, oxidation by PA was performed before labeled PNA staining to differentiate cells containing class III mucin. Preparations were oxidized with 1% PA for 4 h before staining with HRP-conjugated PNA (E-Y Laboratories, San Mateo, CA).

GOS staining[9, 10] Galactose oxidase (Worthington Biochemical Corp., Freehold, NJ) oxidizes the C-6 hydroxy bond of galactosyl residues to produce D-galactohexodialdose. This reaction has been shown to be highly specific for galactose and N-acetyl-D-galactosamine residues in complex carbohydrates. Aldehyde groups generated by galactose oxidase are located by their reaction with 2% Schiff's reagent. GOS-reactive mucins were stained red.

Sialidase-GOS staining[9, 10] For detection of sugar residues penultimate to sialic acid, the terminal sialic acid residues were removed by treating sections with sialidase (Nacalai Tesque) for 18 h at 37°C before staining with GOS. Sialidase-GOS-positive mucins were stained red.

RESULTS

Normal gastrointestinal mucosa From the results of paradoxical Con A staining, the mucins in gastric and small intestinal epithelial cells were classified into class III or class II mucins (Figs. 1a, 1b). Class III mucins were found in mucous neck cells, pyloric gland cells and Brunner's gland cells. Class II mucins were found in surface mucous cells, goblet cells and the surface coat on intestinal absorptive cells. On immunohistochemical staining (Figs. 2a, 2b), Pg 1 was detected in cells containing class III mucins (mucous neck cells, pyloric gland cells and Brunner's gland cells) and in chief cells. From the results obtained by the modified method of labeled PNA staining (Figs. 3a, 3b), the class III mucin-positive cells were divided into PNA-positive cells (mucous neck cells and Brunner's gland cells) and PNA-negative cells (pyloric gland cells). After oxidation with periodic acid for 4 h, the class III mucins in pyloric gland cells lost their reactivity with PNA, while the mucins in mucous neck cells and Brunner's gland cells retained PNA-reactivity. By GOS staining (Figs. 4a, 4b), class III mucin-positive cells were not stained or were stained only weakly (Fig. 4a). On the other hand, of the class II mucin-positive cells, surface mucous cells, including gastric pit mucous cells, stained red, whereas the surface coat of intestinal absorptive cells and mucins in goblet cells stained weakly if at all (Fig. 4b). On sialidase-GOS

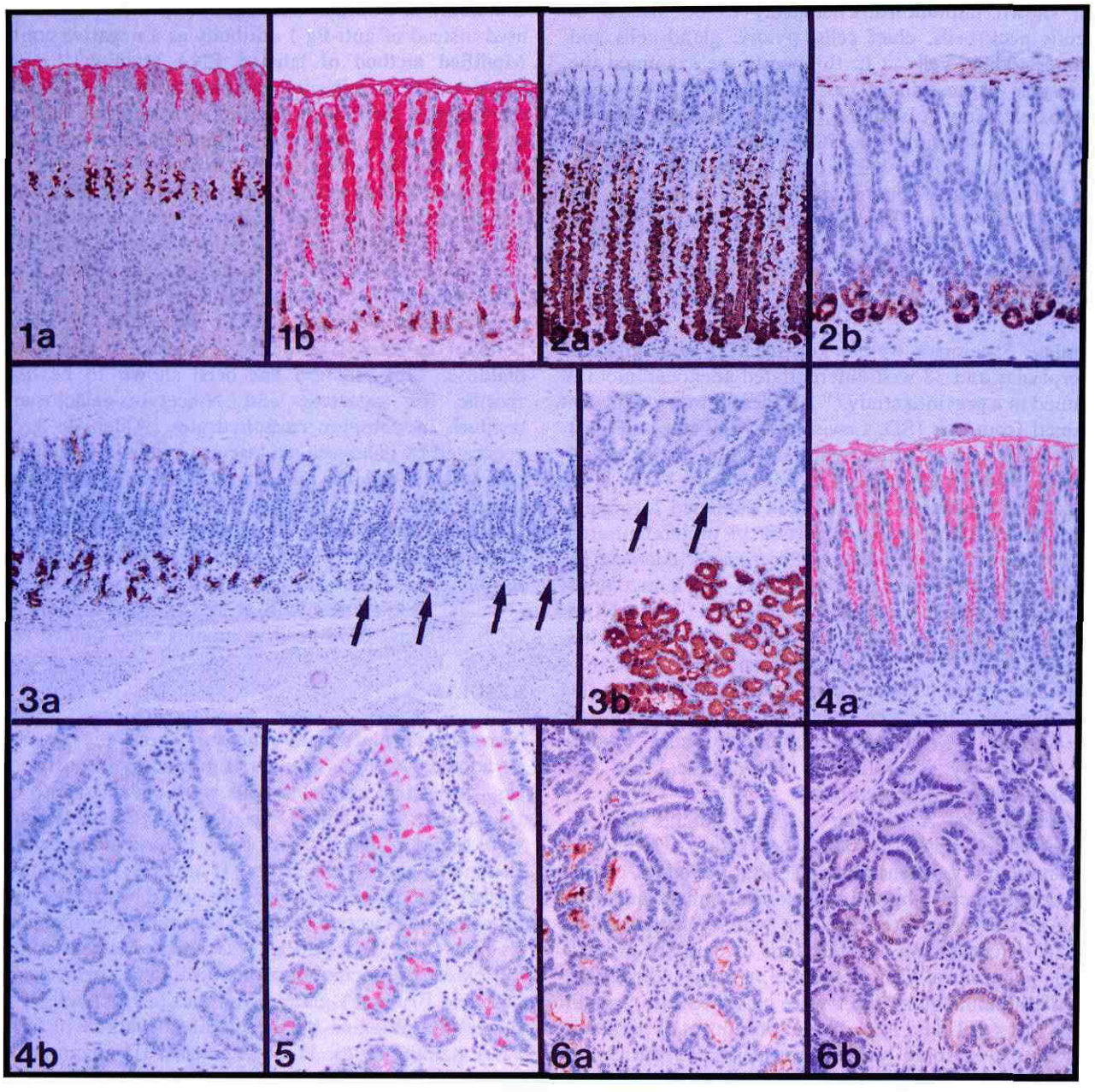

Fig. 1. Appearance on PAS-Con A-HRP staining. Mucous neck cells (a) and pyloric gland cells (b) containing class III mucins were stained reddish brown, and surface mucous cells (a, b) containing class II mucins were stained purple. (a), ×100; (b), ×120.
Fig. 2. Appearance on Pg 1 staining. Pg 1 is strongly stained in mucous neck cells (a), chief cells (a) and pyloric gland cells (b). (a), ×100; (b), ×120.
Fig. 3. Appearance on staining by the modified method with labeled PNA. Mucins in the pyloric gland cells (a, b; arrows) show selective loss of reactivity to PNA but mucous neck cells (a) and Brunner's gland cells (b) retain reactivity with PNA. (a), ×100; (b), ×200.
Fig. 4. Appearance on GOS staining. Mucins in surface mucous cells (a) stained red, whereas those in goblet cells showed weak or no GOS reactivity. (a), ×100; (b), ×200.
Fig. 5. Appearance on sialidase-GOS staining. Goblet cells showed strong GOS reactivity after sialidase digestion. ×200.
Fig. 6. Appearance of a well-differentiated adenocarcinoma consisting of gastric-type cells. Pyloric gland-type cells contain class III mucins (a) and Pg 1 (b). (a), PA-Re-Con A-HRP ×100 and (b) Pg 1 staining. ×100.

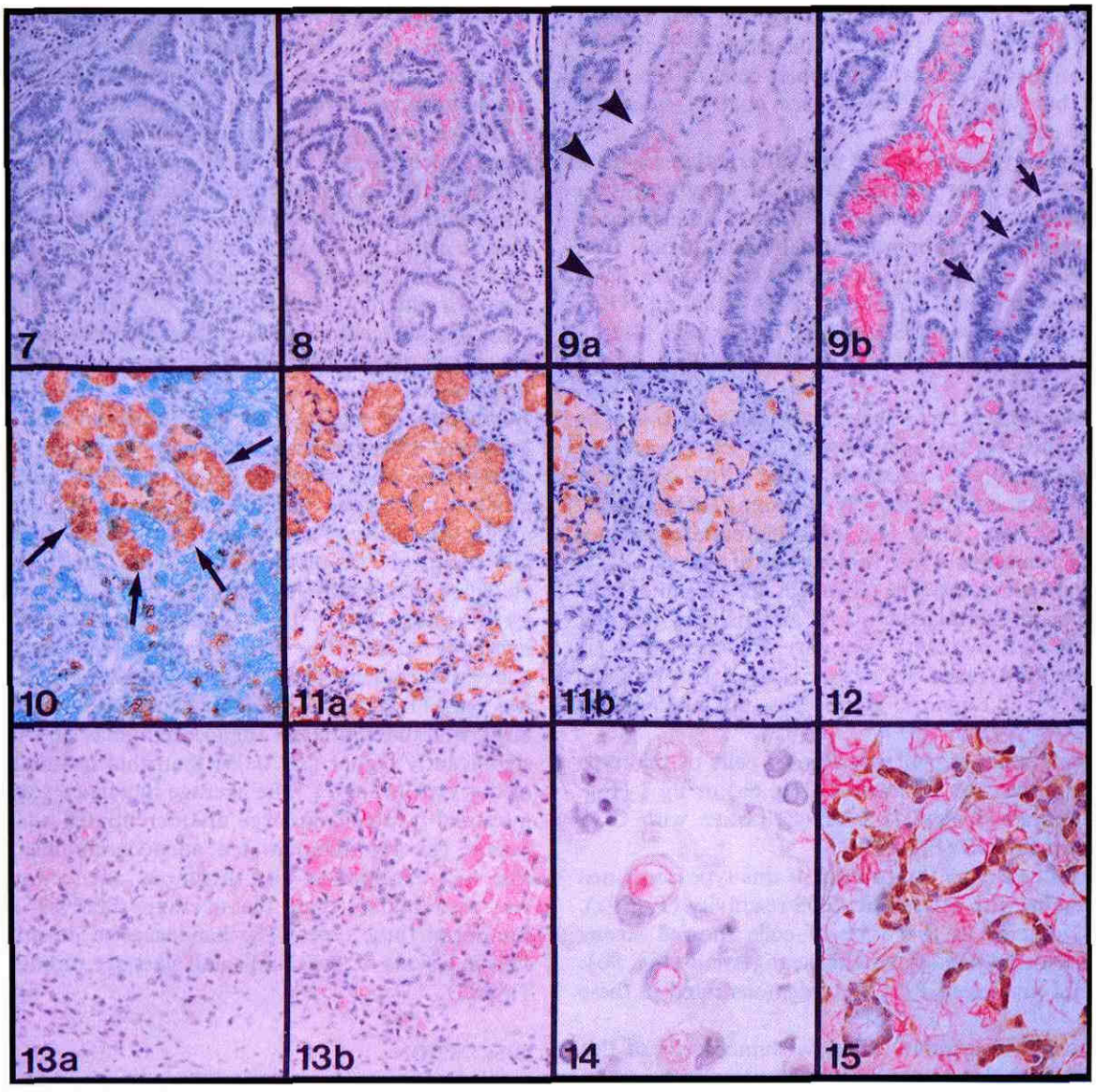

Fig. 7. Serial section of the same specimen as in Fig. 6 stained by the modified method with labeled PNA. Pyloric gland cell-type cells lost PNA reactivity after PA oxidation. ×100.
Fig. 8. Serial section of the same specimen as in Fig. 6 stained with GOS. Surface mucous cell-type cells contain mucins with GOS reactivity.
Fig. 9. Well-differentiated adenocarcinoma consisting of surface mucous cell type cells (a, arrowheads) with GOS-reactive mucins and goblet cell-type tumor cells (b, arrows) with GOS-reactivity after sialidase digestion. (a), GOS, ×200; (b), sialidase-GOS, ×200.
Fig. 10. Signet ring cell carcinoma invading the duodenal mucosa, consisting of class III mucin-positive (brown) cells and class II mucin-positive (blue) cells. Brunner's gland cells (arrows) also contain class III mucins. PA-Re-Con A-HRP-AB. ×200.
Fig. 11. Serial sections of Fig. 10. Pyloric gland cell-type signet-ring cells (a) lost PNA reactivity after PA oxidation (b). (a), PA-Re-Con A-HRP, ×200; (b), modified method with labeled PNA. ×200.
Fig. 12. Surface mucous cell-type signet-ring cells with GOS-reactive mucins. GOS, ×100.
Fig. 13. Goblet cell-type signet-ring cells showing little or no GOS reactivity (a) and strong GOS reactivity after sialidase digestion (b). (a) GOS, ×200 and (b) sialidase-GOS, ×200.
Fig. 14. Signet-ring cells of the intestinal absorptive cell type containing microcysts with class II mucins on the cyst surface and in the lumen. PAS-Con A-HRP. ×1000.
Fig. 15. GOS-positive mucins (red) in a mucin lake of mucinous adenocarcinoma and pyloric gland cell-type tumor cells have Pg 1 (brown) activity. Double staining for GOS and Pg 1. ×200.

staining (Fig. 5), class III mucin-positive cells stained weakly, if at all, whereas class II mucins stained strongly in surface mucous cells and in goblet cells of the small intestine, but weakly in the surface coat of intestinal absorptive cells.

Adenomatous hyperplasias and well-differentiated adenocarcinomas Cellular differentiation of tumor cells in 18 adenomatous hyperplasias and 33 well-differentiated adenocarcinomas were investigated by paradoxical Con A staining, Pg 1 immunohistochemical staining for Pg 1, the modified method of labeled PNA staining, GOS staining and sialidase-GOS staining. Tumor cells in each histological category could be clearly classified into 5 types:

a) Mucous neck cell type: tumor cells of this type contained class III mucins, showed low Pg 1 reactivity and retained PNA reactivity after PA oxidation (modified method of labeled PNA staining), like normal mucous neck cells. Only one well-differentiated adenocarcinoma contained a few tumor cells of this type.

b) Pyloric gland cell type (Figs. 6a, 6b, 7): tumor cells of this type also contained class III mucins (Fig. 6a) and showed low Pg 1 reactivity (Fig. 6b). However, class III mucins lost PNA reactivity when oxidized with periodic acid (Fig. 7), like pyloric gland cells, but not mucous neck cells.

c) Surface mucous cell type: tumor cells of this type did not stain for class III mucins (Fig. 6a) or Pg 1 (Fig. 6b) and contained class II mucins reactive with GOS (Fig. 8) and S-GOS (Fig. 9b).

d) Goblet cell type: tumor cells of this type contained class II mucins with little or no GOS reactivity (Fig. 9a). However, mucins in this type of cells showed strong reactivity with S-GOS after sialidase digestion (Fig. 9b). No class III mucins or Pg 1 were demonstrated in these cells.

e) Intestinal absorptive cell type: tumor cells of this type formed tubular or papillary structures with incomplete striated cell borders at their apical surface and were covered by a class II reactive surface coat with little or no GOS- or S-GOS-reactivity (Figs. 9a, 9b).

Undifferentiated adenocarcinomas The tumor cells in 4 poorly differentiated adenocarcinomas, 10 signet-ring cell carcinomas and 2 mucinous adenocarcinoma were investigated. Mucin-containing tumor cells were first classified into class III mucin-positive cells and class II mucin-positive cells by paradoxical Con A staining (Fig. 10). Then they were classified into a) a mucous neck cell type, b) pyloric gland cell type (Figs. 11a, 11b), c) surface mucous cell type (Fig. 12), d) goblet cell type (Figs. 13a, 13b) and intestinal absorptive cell type (Fig. 14) by the various staining methods used in this work. Tumor cells of the mucous neck cell type were found in only one signet-ring cell carcinoma. Signet-ring cells of the intestinal absorptive cell type (Fig. 14) had a microcyst in their cytoplasm. The internal surface of this cyst had an incomplete striated cell border covered with a class II mucin reactive surface coat with little or no GOS- or S-GOS-reactivity. Accumulation of the surface coat was often observed as a core in the center of the microcyst. Tumor cells of mucinous adenocarcinomas were found in lakes of mucins of the surface mucous cell type (Fig. 15), pyloric gland cell type and/or goblet cell type. Therefore, cells in undifferentiated adenocarcinomas were functionally well differentiated, although the cellular differentiations of some cells in poorly differentiated adenocarcinomas could not be determined because they produced too little mucin.

Incidences of gastric-type and intestinal-type cells in lesions of different sizes All tumors consisted mainly of gastric-type cells (pyloric gland cell type and surface mucous cell type) but intestinal-type cells (intestinal absorptive cell type and goblet cell type) were occasionally found in cells of the gastric type. The tumors were classified into small (less than 5.0 mm in diameter) and large (more than 5.0 mm) tumors. All adenomatous hyperplasias were less than 5 mm in diameter. The incidence of intestinal-type cells in adenomatous hyperplasias was only 11.1%. The incidence of intestinal-type cells in large well-differentiated adenocarcinomas (68.4%) was significantly higher ($P<0.05$) than that in small tumors of this type (28.6%). The average incidence (58.3%) of intestinal-type cells in large undifferentiated adenocarcinomas (poorly differentiated adenocarcinomas, signet-ring cell carcinomas and mucinous adenocarcinomas) was also higher than the average (25.0%) in small tumors of these types. The incidences of intestinal type cells in lesions of large and small sizes are summarized in Table I.

DISCUSSION

In this work, differentiation of gastric cancer cells of well-differentiated adenocarcinomas and undifferentiated adenocarcinomas (poorly differentiated adenocarcinomas, signet-ring cell carcinomas and mucinous adenocarcinomas) in the glandular stomach of rats were classified into 5 types (mucous neck cell type, pyloric gland cell type, surface mucous cell type, goblet cell type, and intestinal absorptive cell type) by paradoxical Con A staining, a modified method of labeled PNA staining, GOS staining, and sialidase-GOS staining. Human gastric cancer cells have also been classified into these 5 types.[20, 21]

The mucin-positive tumor cells were first classified into those containing class II mucin and those containing class III mucin by paradoxical Con A staining. Class III mucin is a marker of phenotypic expression of mucous

Table I. Incidence of Intestinal-type Cells in Glandular Stomach Lesions of Different Sizes in Rats Treated with Chemical Carcinogens

Lesion	Diameter of lesion			
	< 5 mm		5 mm≦	
	No. of lesions	Incidence of IT (%)	No. of lesions	Incidence of IT (%)
Adenomatous hyperplasia	18	2 (11.1)	0	—
Well-differentiated adenocarcinoma	14	4 (28.6)	19	13 (68.4)[a]
Poorly differentiated adenocarcinoma	2	1 (50.0)	2	1 (50.0)
Signet-ring cell carcinoma	2	0 (0.0)	8	5 (62.5)
Mucinous adenocarcinoma	0	0 (0.0)	2	1 (50.0)

IT: Intestinal-type cells.
a) Significantly different from the value for small lesions at $P<0.05$ (χ^2-test).

neck cells and pyloric gland cells.[2, 22] Class III mucin reacts with PNA, but that in pyloric gland cells lost reactivity with PNA when oxidized with periodate. Thus, differentiation of cells containing class III mucin in the gastric mucosa could be further classified into a mucous neck cell type and pyloric gland cell type by the modified method of labeled PNA staining.[7, 8] Pg 1 is a marker of the phenotypic expressions of chief cells, mucous neck cells and pyloric gland cells.[13, 23] No cancer cells of the chief cell type with Pg 1 activity with class III mucin reactivity were found. Only a few gastric cancer cells showed phenotypic expression of the mucous neck cell type with class III mucin, Pg 1, and PNA reactivity after periodate oxidation.[8] Therefore, almost all cancer cells with class III mucin and Pg 1 showed phenotypic expression of the pyloric gland cell type without PNA reactivity after periodate oxidation.[8] The phenotypic expression of gastric cancer cells was not that of fundic glands but was that of pyloric gland, even in tumors developed in the fundic mucosa.[8] Similar gastric phenotypic expressions were found in human stomach cancers.[21] Further studies on gene expressions in gastric mucosa are necessary for analysis of the unstable phenotypic expression of the phenotype of fundic glands.

Class II mucins were found in surface mucous cells (foveolar cells), intestinal goblet cells and the surface coat of intestinal absorptive cells.[2, 7] Tumor cells containing class II mucins have also been classified into a GOS-reactive surface mucous cell type and a GOS-negative type.[20, 24] The GOS-negative type was further classified into a sialidase-GOS positive goblet cell subtype and a sialidase-GOS negative one.[20, 24] The sialidase-GOS negative tumor cells in well-differentiated adenocarcinomas have an incomplete striated cell border with a surface coat like that of intestinal absorptive cells.[21] The intestinal absorptive cell type of signet ring cells has a microcyst in the cytoplasm[20] and intracellular microcysts are covered by incomplete intestinal microvilli[20, 25, 26] with a class II mucin-positive surface coat.[27]

In humans, well-differentiated adenocarcinomas have been suggested to arise from intestinal metaplastic mucosa[28–30] and undifferentiated adenocarcinomas to arise from gastric mucosa.[31–33] Phenotypic expression of gastric cancer originating from intestinal metaplasia should be of the intestinal type. Even if the phenotypic expression of intestinal-type cells gastric cancer cells is unstable, the incidence of intestinal-type cells small gastric cancers should be higher than that in larger gastric cancers, although the phenotypic expression of gastric cancer cells in the intestine is stable.[34] However, the incidence of intestinal-type cells increased significantly ($P<0.05$) in gastric tumors with progression from adenomatous hyperplasia through small well-differentiated adenocarcinomas to large well-differentiated adenocarcinomas. Moreover, the incidence of intestinal-type cells in small undifferentiated adenocarcinomas was lower than that in large ones. These data suggest that the phenotypic expressions of gastric-type cancer cells of both well-differentiated and undifferentiated adenocarcinomas occasionally change to the intestinal types with tumor progression. The data in this work are consistent with the conclusion from our previous studies[34, 35] that intestinal metaplasia may not be a preneoplastic change in gastric carcinoma, but rather that cells of the intestinal type may appear independently in gastric cancers and in gastric mucosa.

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