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Immunohistochemical Demonstration of Intestinal-type Alkaline Phosphatase in Stomach Tumors Induced by N-Methyl-N′-nitro-N-nitrosoguanidine in Rats

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A polyclonal antibody against rat intestinal-type alkaline phosphatase (I-ALP) was generated and proven to be applicable immunohistochemically to paraffin-embedded sections. Expression of I-ALP in normal tissues, intestinal metaplasia and stomach tumors induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was then investigated in five different strains of rats. Male SD (Crj:CD), Lewis (LEW/Crj), WKY (WKY/NCrj), Wistar (Crj:Wistar) and F344 (F344/DuCrj) animals were given drinking water containing 100 μg/ml of MNNG for 30 weeks and were killed at week 50. Among the 5 strains, stomach adenocarcinomas were found most frequently in the SD case. The susceptibility of rats to induction of stomach carcinoma did not correlate with the development of intestinal metaplasias in each strain. Histochemical staining for mucin demonstrated all stomach tumors (adenomatous hyperplasias and well-differentiated adenocarcinomas) to consist mainly of gastric type cells (pyloric gland cell and surface mucous cell types), with intestinal-type tumor cells (goblet cell and intestinal absorptive cell types) being only occasional findings. Immunohistochemically, I-ALP was strongly positive on the striated cell borders of small intestinal absorptive cells of the villus and on brush borders of epithelial cells of kidney proximal tubules. I-ALP was also detected in the normal stomach, limited to the striated cell borders of absorptive cells of the upper one-fourth of intestinal metaplastic glands. I-ALP may thus be a useful marker for stomach tumor cells of intestinal absorptive cell type, indicative of maturation and differentiation. No stomach tumors consisting mainly of intestinal-type cells were found, and therefore there was no suggestion of any derivation from intestinal metaplasias.

Key words: Intestinal metaplasia — Intestinal-type alkaline phosphatase — Stomach cancer — Rat — Cellular differentiation

The phenotypic expression of tumor cells tends to be the same as that of the tissue of origin of the cells, although, in the stomach cancer case, cellular differentiation is complicated[1-4] because the glandular stomach of the rat consists of fundic and pyloric mucosa containing several kinds of epithelial cells. In addition, intestinal metaplasias are occasionally found in glandular stomach epithelium.[4] Therefore an appreciation of the cellular differentiation of cancer cells of the glandular stomach is necessary for studying the histogenesis of stomach cancers. By histochemical staining of mucin [paradoxical concanavalin A (Con A), galactose oxidase-Schiff (GOS), and sialidase-GOS] and immunohistochemical staining of pepsinogen isozyme 1 (Pg 1), stomach cancer cells were classified into a gastric category, including pyloric gland cell and surface mucous cell types, as well as an intestinal category, including goblet cell and intestinal absorptive cell types.[5] For differentiation of the intestinal absorptive cell type the only aid is a morphologically incomplete striated cell border, and up to the present there has been no good functional marker to indicate selectively cancer cells of intestinal absorptive cell type.

Alkaline phosphatases (ALPs) constitute a group of four related isozymes, i.e. tissue unspecific (liver/bone/kidney), intestinal, placental and the germ cell or placental-like ALPs, whose gene structures have recently been clarified.[6-9] The expression of placental and placental-like ALPs as carcinoplacental or oncofetal antigens has aroused particular interest in terms of tumor biology, because of the common reexpression of typical normal embryonic proteins during malignant transformation.[10, 11] In the case of seminomas, elevation of the placental-like isozyme in tumor tissue and serum is specific.[12] Intestinal ALP (I-ALP) might be expected to be a good marker for the intestinal absorptive cell type in gastric cancers. In this study we therefore prepared polyclonal anti rat I-ALP antibody and immunohistochemically investigated

the localization of I-ALP in normal tissue, intestinal metaplasia and glandular stomach cancers of rats.

MATERIALS AND METHODS

Animals and chemicals N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Male SD (Crj:CD), Lewis (LEW/Crj), WKY (WKY/NCrj), Wistar (Crj:Wistar) and F344 (F344/DuCrj) rats were purchased from Charles River Japan, Inc., Kanagawa, and used at 7 weeks of age for this study. Animals were housed (5 per plastic cage) on hardwood chips in an air-conditioned room with a 12 h light/dark cycle. They were given continuous access to commercial rat chow (Oriental MF, Oriental Yeast Co., Tokyo) and water. Twenty-five rats of each strain were used. Fifteen of each strain were given drinking water containing 100 μg/ml of MNNG for 30 weeks and then normal tap water until week 50, when the survivors were killed. Control groups (10 rats of each strain) were given normal tap water and killed after 50 weeks. The stomach was removed from each animal, fixed in sublimed formaldehyde, cut into 8 strips, and embedded in paraffin for histopathology. Samples of small intestine were similarly processed. For histochemical investigation of ALP, placentas from 18-day gestation F344 rats and small intestine from control F344 rats were frozen in liquid nitrogen-precooled isopentane at -140°C.

For immunohistochemistry of I-ALP in normal tissues, the brain, lymph nodes, spleen, thymus, thyroid gland, skin, salivary glands, esophagus, stomach, intestine, liver, pancreas, lungs, kidneys, urinary bladder and testis from control F344 rats and placentas from 18-day gestation F344 rats were fixed in sublimed formaldehyde. In addition, human and mouse (C3H) normal tissues (stomach, duodenum, liver and kidneys) fixed in 10% buffered formalin were also subjected to immunohistochemical investigation.

Mucin and enzyme histochemistry Paradoxical Con A staining,[13,14] GOS staining[5,15] and S-GOS staining[5,15] were performed as described previously. ALP activity was demonstrated by using a nitroblue tetrazolium method.[16]

Purification of I-ALP and preparation of antiserum Rat intestinal mucosa was homogenized in 1 volume of butanol and 3 volumes of Tris-HCl buffer, pH 7.5, containing 10 μM $MgCl_2$ and $ZnCl_2$ and 0.02% NaN_3,[17] and cold acetone was added to achieve a final concentration of 60%, the whole mixture being allowed to stand at 4°C for 3 h. The precipitate was dissolved in a minimum volume of buffer and dialyzed against the same buffer. The supernatant fluid was then treated with $(NH_4)_2SO_4$ and the fraction (35–65% saturation of $(NH_4)_2SO_4$) was collected and again dialyzed against buffer. The dialyzed enzyme was applied to a column of DEAE-cellulose (3.0×25 cm) and eluted with a linear gradient of NaCl. Active fractions were concentrated with a membrane filter (YM-10) and applied to a column of Sephadex G-200 (2.5×70 cm) equilibrated with the above buffer. Active fractions were again collected and applied to a reversed-phase HPLC column C4 equilibrated with 0.1% trifluoroacetic acid and eluted with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The main peak on HPLC was collected and lyophilized. A rabbit foot pad was injected with 100 μg of purified ALP in 1 ml of complete Freund's adjuvant. Booster immunization was done by subcutaneous injection twice every two weeks. Antiserum was further purified by DEAE-cellulose and Protein A Sepharose column chromatography after heating at 56°C for 30 min.[18]

Immunohistochemistry The ABC method[19] was used to determine the localization of I-ALP, using polyclonal anti I-ALP diluted 1:200. Affinity-purified biotin-labeled goat anti-rabbit immunogloblin IgG and avidin-biotin-peroxidase complex (Vectastain Elite ABC kit) were obtained from Vector Laboratories (Burlingame, CA, USA). The sites of peroxidase binding were visualized by the diaminobenzidine method. Sections were counterstained with hematoxylin for microscopic examination. As a negative control for the specificity of anti-Pg 1 and

Table I. Incidences and Numbers of Intestinal Metaplasias (IM) in the Pyloric Mucosa of Rats Treated with or without MNNG

Strains	MNNG-treated group (%)			Control group (%)		
	Effective No. of rats	Incidence	No. of IM (No./cm)	Effective No. of rats	Incidence	No. of IM (No./cm)
SD	15	10 (67)	0.2±0.2[a]	10	4 (40)	0.2±0.3
Lewis	14	3 (21)	0.1±0.2	9	6 (67)	0.2±0.1
WKY	13	9 (69)	0.5±0.5	10	4 (40)	0.1±0.2
Wistar	14	7 (50)	0.5±0.6	9	8 (89)	0.5±0.4
F344	14	9 (64)	0.2±0.3	10	5 (50)	0.2±0.2

a) Mean±SD.

Table II. Adenomatous Hyperplasias and Adenocarcinomas in the Glandular Stomach, and Small Intestinal Adenocarcinomas in Rats Treated with MNNG

Strains	Effective No. of rats	Glandular stomach (%)								Small intestine (%)			
		Adenomatous hyperplasia				Adenocarcinoma				Adenocarcinoma			
		Incidence	Total No.[a]	No. of IT[b]	No. of I-ALP[c]	Incidence	Total No.[a]	No. of IT[b]	No. of I-ALP[c]	Incidence	Total No.[a]	No. of IT[b]	No. of I-ALP[c]
SD	15	9 (60)	12	2 (17)	1 (50)	11 (73)	14	8 (57)	4 (50)	3 (20)	3	3 (100)	3 (100)
Lewis	14	11 (79)	14	1 (8)	0	8 (57)	9	6 (67)	3 (50)	10 (71)	12	12 (100)	12 (100)
WKY	13	7 (54)	8	0	0	9 (69)	12	7 (58)	3 (43)	9 (69)	10	10 (100)	10 (100)
Wistar	14	4 (29)	4	0	0	5 (36)	5	1 (20)	0	6 (43)	6	6 (100)	6 (100)
F344	14	3 (21)	4	0	0	3 (21)	3	0	0	4 (29)	4	4 (100)	4 (100)

a) Total numbers of tumors detected in histological specimens.
b) Numbers of tumors containing cells of intestinal type (IT) per total no. of tumors.
c) Numbers of tumors containing I-ALP-positive tumor cells per total IT-positive tumors.

anti-I-ALP antibody binding, normal (non-immune) rabbit serum was used.

RESULTS

A few intestinal metaplastic glands consisting of intestinal absorptive cells and goblet cells were found in the pyloric mucosa, mainly adjacent to the duodenal mucosa, in MNNG-treated and control group animals of each strain. Only one intestinal metaplastic gland found in a Lewis (control) rat had Paneth cells. No significant incidence relationships were evident between MNNG-treated and control groups for intestinal mataplasia or for stomach adenocarcinomas in any strain of rat (data summarized in Table I). In the MNNG-treated groups, adenomatous hyperplasias were found, especially in Lewis rats, and adenocarcinomas were observed in all strains with particularly high incidences in the SD and WKY cases. Small intestinal adenocarcinomas were also found, almost all of them located in the duodenum and the remainder in the jejunum, the highest incidence being observed for Lewis rats. Data for lesions in the glandular stomach and small intestines are summarized in Table II.

Mucin histochemistry of stomach and small intestinal mucosa and tumors The mucin histochemistry (paradoxical Con A, GOS, and S-GOS) of gastric, small intestinal, and intestinal metaplastic mucosa has been described previously.[5, 14] Briefly, mucous neck cells, pyloric gland cells and Brunner's gland cells contain class III mucins stained by paradoxical Con A. Surface mucous cells show no class III mucins and contain GOS- and S-GOS-reactive mucins. Goblet cells in the small intestine and intestinal metaplasia contain mucins with little or no GOS, and strong S-GOS reactivity. Intestinal absorptive cells in small intestine and intestinal metaplasia have striated cell borders in their apical surface covered by a surface coat with little or no GOS or S-GOS reactivity.

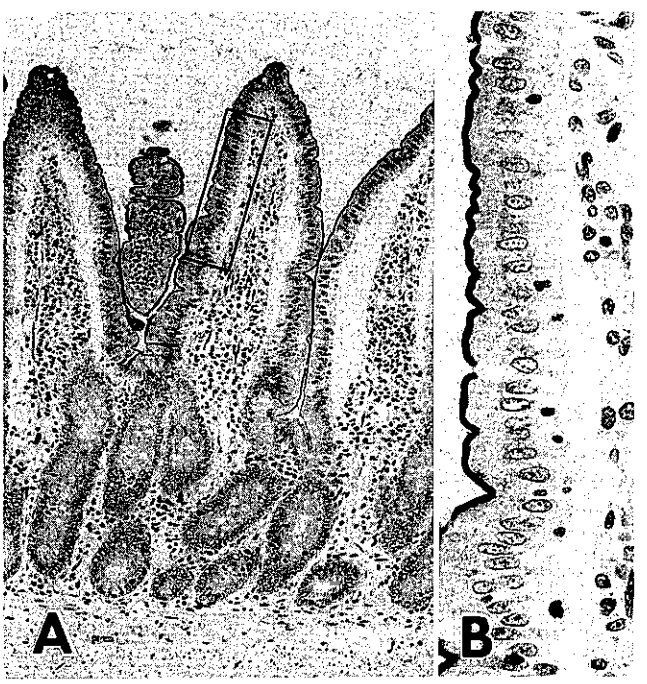

Fig. 1. Strong I-ALP antibody binding demonstrated on the striated cell borders of small intestinal absorptive cells of the villus of an F344 rat (A) and a higher magnification view of I-ALP-positive small intestinal absorptive cells (B). I-ALP immunohistochemistry (A) ×100, (B) ×400.

The cellular differentiation of tumor cells in adenomatous hyperplasias and well-differentiated adenocarcinomas was classified into 4 types (pyloric gland cell type, surface mucous cell type, goblet cell type and intestinal absorptive cell type) as described previously.[5, 14] Each tumor cell type showed the same mucin histochemical reactivities as the normal counterpart gastric and intesti-

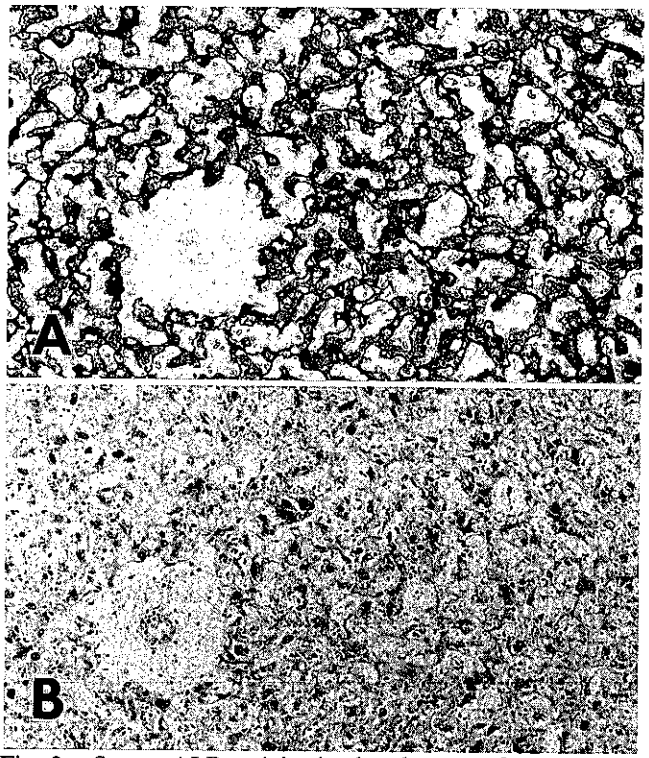

Fig. 2. Strong ALP activity in the placenta of an F344 rat (A) but no I-ALP reaction in a serial section (B). (A) ALP histochemistry ×200, (B) I-ALP immunohistochemistry ×200.

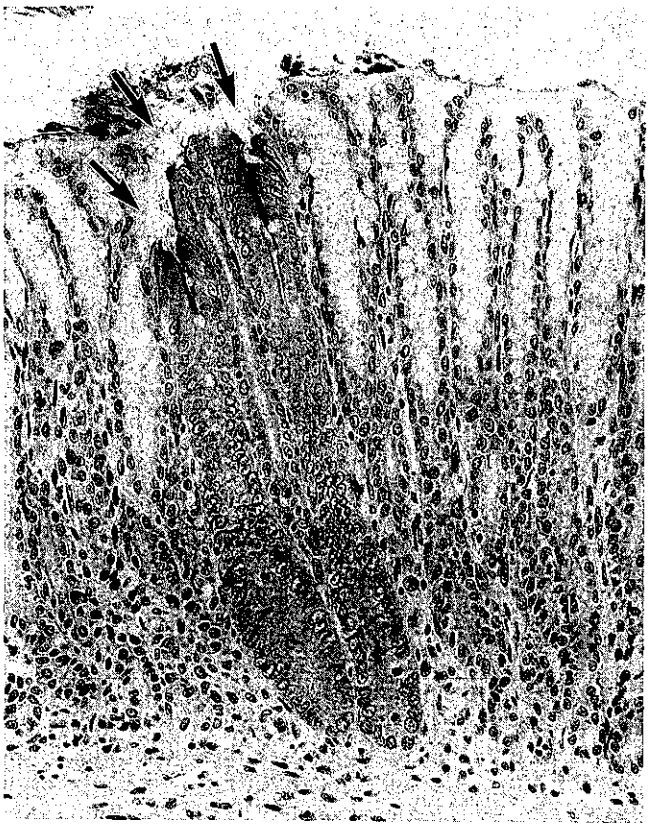

Fig. 3. Intestinal metaplasia with I-ALP positivity (arrows) in the upper one-fourth of the gland of an SD rat. I-ALP immunohistochemistry ×200.

nal epithelial cells. All stomach tumors mainly consisted of gastric-type cells (pyloric gland cell and surface mucous cell types) with rather fewer intestinal-type cells (intestinal absorptive cell and goblet cell types). However, almost all tumor cells in small intestinal lesions proved to be of intestinal type.

The incidences of tumor cells of intestinal type are summarized in Table II.

Localization of I-ALP in normal tissues Histochemically, ALP activity was found to be strong on the striated cell borders of small intestinal (duodenum, jejunum and ileum) absorptive cells of the villus. Immunohistochemically, I-ALP antibody binding was also strongly detected in the same region (Fig. 1). Virtually no staining was detected in any epithelial cells within the lower crypt region but I-ALP was found on the striated cell borders of absorptive cells located in the upper one-fourth of large intestinal crypts, although their reactivities were extremely weak. In the kidney, strong immunohistochemical staining for I-ALP were found on brush borders of epithelial cells of proximal tubules. Immunoreactivity for I-ALP was not evident in the placenta, despite strong histochemical ALP activity (Fig. 2). No significant positive immunohistochemical binding was found for any other of the investigated normal organs. Species cross-reaction was evident from the positive reactions observed for small intestinal absorptive cells of both man and mice.

Localization of I-ALP in intestinal metaplasia and tumor cells I-ALP was detected immunohistochemically on the striated cell borders of intestinal absorptive cells located in the upper one-fourth of intestinal metaplastic glands, but not in the lower parts (Fig. 3). In adenomatous hyperplasia and well-differentiated adenocarcinoma of the stomach, a few intestinal absorptive-type cells showed I-ALP reactivity on their incomplete striated cell borders (Fig. 4). The other tumor cell types did not have any I-ALP reactivity. In all adenocarcinomas of the small intestine, some intestinal absorptive cell-type cancer cells showed an I-ALP reaction but many were negative, like those in normal crypts (Fig. 5). In neither stomach nor small intestinal tumors were cancer cells found with I-ALP activities higher than those of normal small intestinal epithelial cells of the villus.

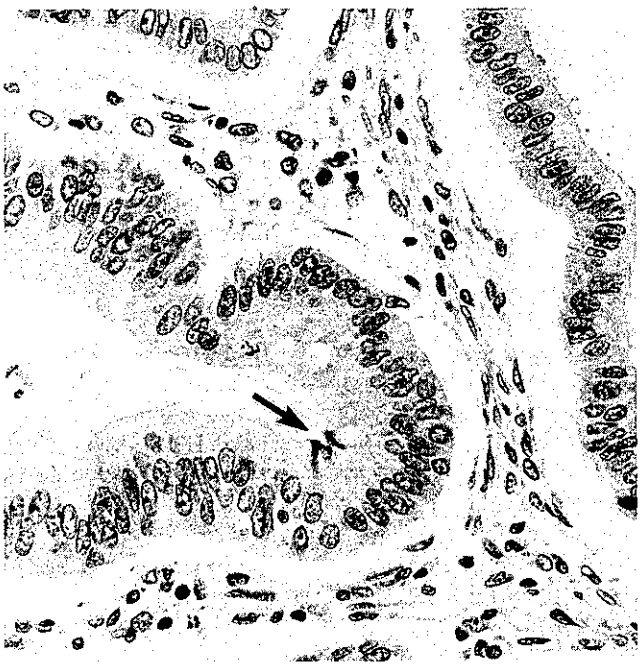

Fig. 4. Positive I-ALP reaction on incomplete striated cell borders (arrow) of stomach cancer cells of intestinal absorptive cell type of a WKY rat. I-ALP immunohistochemistry ×200.

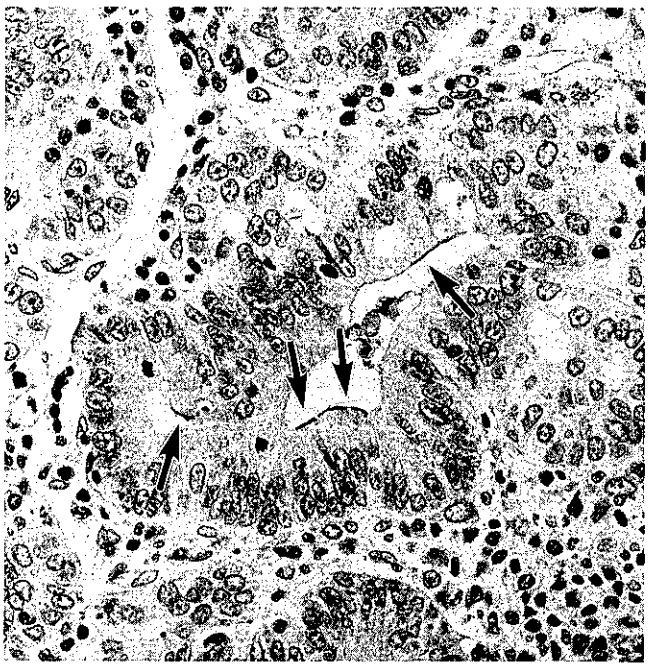

Fig. 5. Positive I-ALP reaction on incomplete striated cell borders (arrows) of small intestinal adenocarcinoma cells of intestinal absorptive cell type of a WKY rat. I-ALP immunohistochemistry ×200.

DISCUSSION

I-ALP was originally considered to be uniquely expressed in the intestine. However, approximately 25% of the total ALP content of human renal tissue at the transition between the cortex and medulla was reported to be of intestinal type with a location on the brush borders of the proximal tubules.[20] In this work, localization of I-ALP was also clearly demonstrated immunohistochemically at the striated cell borders of intestinal absorptive cells in the small intestinal villus of rats as well as the brush borders of renal epithelial cells of proximal tubules. In addition, investigation of human and mouse tissues using anti rat I-ALP antibodies revealed positive immunohistochemical reactions in the same cells as in rats, indicating species cross-reactivity. The lack of any significant I-ALP reaction in other normal organs indicates that the positive immunohistochemical expression observed with our anti rat I-ALP antibodies for intestinal metaplasia in glandular stomach and in stomach cancer cells actually reflects the presence of the isozyme. In our previous work,[4, 5, 14] stomach cancer cells could be classified into a gastric type, including pyloric gland cells and surface mucous cells, and an intestinal type, including goblet cells and intestinal absorptive cells. Expression of I-ALP was found only in cancer cells of intestinal absorptive cell type. Retrospectively, our classification[4, 5, 14] of phenotypic expression of stomach cancer cells therefore appears appropriate.

In the gastrointestinal tract, the epithelial renewal system can usually be readily divided into proliferative and functional compartments.[21] Cells born in the proliferative compartment migrate into the functional compartment, losing their capacity for division and acquiring the characteristics of mature, functionally differentiated cells. In the small intestine, the villus epithelium is the functional compartment and the proliferative compartment is found in the crypt.[21] Immunohistochemically, I-ALP was clearly located in the absorptive cells in the functional compartment (villus epithelium) suggesting that its presence reflects maturation of intestinal absorptive cells. Although villus structures are not clear in intestinal metaplastic glands, the upper one-fourth of such glands might be considered as the functional compartment. On the other hand, many small intestinal cancer cells, despite an unequivocal origin from small intestinal epithelium, and many stomach cancer cells of intestinal absorptive cell type did not show I-ALP. The few small intestinal cancer cells and the few stomach cancer cells of intestinal absorptive cell type in each

tumor showing I-ALP activities in their incomplete striated cell borders might either express villus epithelial function, or their expression of I-ALP might indicate a maturation process.

Although a close relationship between intestinal metaplasia and well-differentiated gastric adenocarcinomas in rats treated with MNNG and related chemicals has been reported,[22] the origin of stomach cancer cells of intestinal absorptive cell type is complicated. The degrees of induction of intestinal metaplasias and adenocarcinomas were not parallel in the present work. If stomach cancer cells of intestinal absorptive cell type originate from intestinal metaplasias, the phenotypic expression of early stage lesions would be expected to be of intestinal type. However, almost all the observed adenomatous hyperplasias consisted only of gastric type tumor cells.[4, 5, 14] Furthermore, the stomach cancers, without exception, also consisted mainly of gastric-type cancer cells with intestinal-type cells, with or without I-ALP activities being found only occasionally. These data suggest that gastric-type tumor cells of adenomatous hyperplasia and well-differentiated adenocarcinomas occasionally change their phenotypic expression to that of intestinal types with tumor progression.[4, 5, 23] As demonstrated earlier, cells of the intestinal type may appear independently in normal gastric mucosa as intestinal metaplasia and in gastric cancer as cancer cells of intestinal type.[4]

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