Original

Expression of Cathepsin E and Its Suppression with Intestinalization in Epithelial Cells and Tumor Cells in Rat Glandular Stomach Treated with N-methyl-N'-nitro-N-nitrosoguanidine

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Abstract: Expression of cathepsin E in epithelial cells of the normal glandular stomach and small intestine, intestinal metaplasia, stomach and small intestinal tumors, was investigated in rats treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Immunohistochemically, parietal cells were found to be moderately positive while surface mucous and pyloric gland cells demonstrated marked staining. Intestinal metaplastic glands in the stomach and normal small intestinal epithelial cells did not have any cathepsin E reactivity although some regenerative small intestinal epithelium proved positive. Histochemical staining for mucin demonstrated all stomach tumors (adenomatous hyperplasias and well-differentiated adenocarcinomas) to be mainly comprised of gastric epithelial type cells (pyloric gland and surface mucous cells), with intestinal epithelial type cells (goblet and intestinal absorptive cells) being only occasional findings. Almost all of the gastric epithelial type cells showed cathepsin E reactivity in their cytoplasm while the intestinal epithelial cell type cells were mainly consisted of cathepsin E negative one like those in small intestinal cancers. Cathepsin E may thus be a useful marker for cell differentiation of stomach tumors and their intestinalization. Cancers arising in the small intestine consisted of intestinal epithelial cell type cells, but no stomach tumors consisted predominantly of this type, so that there was no suggestion of any derivation from intestinal metaplasias in rats.

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Key words: cathepsin E, intestinal metaplasia, stomach cancer, rats, cellular differentiation

Introduction

A close link between the presence of intestinal metaplasia and development of well-differentiated adenocarcinomas has been observed in the stomach of humans^{1–4} as well as in rats treated with chemical gastric carcinogens^{5, 6}. However, the significance of intestinal metaplasia with regard to the risk of stomach cancer and its origin is by no means clear. Since the phenotypic expression of tumor cells is widely thought to resemble that of the tissue of origin, that of gastric cancers derived from areas of such metaplasia would be expected to demonstrate characteristics of intestinal epithelial cells. Histochemical staining of

mucin [paradoxical concanavalin A (Con A), the galactose oxidase-Schiff (GOS), and the sialidase-GOS] and immunohistochemical staining of pepsinogen, allows human and rat stomach cancer cells to be readily classified into a gastric epithelial cell type, subdivided into pyloric gland and surface mucous cells, as well as an intestinal epithelial cell type, with goblet and intestinal absorptive cells^{2, 7–9}. However, the phenotypic expression of early gastric cancer cells has been consistently found to be of gastric epithelial cell type in man⁷ and the rat², suggesting independent induction of intestinal metaplasia and neoplasia. For confirmation, investigation of other cellular differentiation markers is necessary.

Cathepsin E is known as a major intracellular aspartic proteinase in mammals, but with a limited distribution^{10–13}. In man, as far as has been ascertained, the cathepsin E concentration is highest in cells of the gastric mucosa, followed by the urinary bladder, thymus, spleen, bone marrow, red blood cells, and neutrophils^{10, 11, 14}. In the

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stomach, it has been suggested that cathepsin E may play important roles in protein degradation or in host defense mechanisms as an enzyme functioning in the mucosal barrier¹⁵. Immunohistochemically it appears limited to surface gastric mucous cells¹⁶ and therefore might be expected to be a good marker for the surface mucous cell type phenotype in gastric cancers. In this study we therefore immunohistochemically investigated the localization of cathepsin E in normal gastric epithelial cells, intestinal metaplastic cells, and glandular stomach cancer cells in rats, to address the question of whether intestinal metaplasia is a preneoplastic change relevant to stomach cancers.

Materials and Methods

Animals and chemicals

MNNG was obtained from Aldrich Chemical Co. (Milwaukee, WI). Male Lewis (LEW/Crj) and WKY (WKY/NCrj) rats were purchased from Charles River Japan, Inc., Kanagawa, Japan, 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, Japan) and water. Fifty rats of each strain were used, 40 being given drinking water containing 100 µg/ml of MNNG for 30 weeks and then normal tap water until week 50. Subgroups were sacrificed at weeks 30 and 50. Control

animals (10 rats of each strain) were given normal tap water and sacrificed after 50 weeks. The stomachs were immediately removed, fixed in sublimated formaldehyde, cut into 8 strips, and embedded in paraffin for histopathological assessment. Samples of small intestine were similarly processed.

Mucin histochemistry

Paradoxical Con A staining^{17, 18}, galactose oxidase-Schiff (GOS) staining^{9, 19} and sialidase-GOS (S-GOS) staining^{9, 19} and alcian-blue PAS staining were performed as described previously.

Immunohistochemistry

Anti-rat polyclonal cathepsin E¹³ and intestinal-alkaline phosphatase (I-ALP)²⁰ antibodies were prepared as described previously. The avidin-biotin-peroxidase complex (ABC) method was used to determine the location of cathepsin E and I-ALP in each tissue sample. After deparaffinization, sections were treated with rabbit anti-rat cathepsin E (1:8000) or I-ALP (1:200). Affinity-purified biotin-labeled goat anti-rabbit immunoglobulin IgG and avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit) were obtained from Vector Laboratories (Burlingame, CA, USA). The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were then counterstained with hematoxylin for microscopic examination. As a negative control for the specificity of

Table 1. Numbers and Incidence of Intestinal Metaplasia (IM) in the Pyloric Mucosa of Rats Treated with or without MNNG

Strain	MNNG-treated group		Control group	
	Effective no. of rats	No. of rats with IM (%)	Effective no. of rats	No. of rats with IM (%)
30 weeks				
Lewis	15	2 (13.3)	10	0
WKY	15	3 (20.0)	10	0
50 weeks				
Lewis	24	8 (33.3)	10	6 (60.0)
WKY	23	15 (65.2)	10	4 (40.0)

Table 2. Adenomatous Hyperplasia and Adenocarcinomas in the Glandular Stomach and Small Intestinal Adenocarcinoma 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)}	Incidence	Total no. ^{a)}	No. of IT ^{b)}	Incidence	Total no. ^{a)}	No. of IT ^{b)}
30 weeks										
Lewis	15	10 (66.6)	12	0	2 (13.3)	2	0	10 (66.6)	12	12 (100)
WKY	15	6 (40.0)	9	1 (11.1)	1 (6.7)	1	0	5 (33.3)	7	7 (100)
50 weeks										
Lewis	24	18 (75.0)	22	1 (4.5)	13 (54.2)	15	10 (66.6)	17 (70.8)	21	21 (100)
WKY	23	13 (56.5)	14	0	15 (65.2)	20	11 (55.0)	15 (65.2)	18	18 (100)

^{a)} Total number of tumors detected in histological specimens.

^{b)} Number of tumors containing cells of intestinal epithelial cell type.

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

Results

Histopathological findings at weeks 30 and 50 are summarized in Tables 1 and 2. At week 30, only a few intestinal metaplastic glands consisting of intestinal absorptive cells and goblet cells were found in the normal-looking pyloric mucosa of MNNG treated, but not control animals. They were mainly located adjacent to the small intestinal mucosa. Relatively high incidences of adenomatous hyperplasias and low incidences of adenocarcinomas were observed in both strains given MNNG. In the small intestine, some rats demonstrated epithelial regeneration due to MNNG induced erosion or ulceration, and adenocarcinomas were also found in both strains. At week 50, the incidences of intestinal metaplasia in the normal-looking pyloric mucosa were increased in MNNG treated animals, also appearing in the control group. In the MNNG groups high incidences of adenocarcinomas in the glandular stomach as well as the small intestine had developed in each strain. The incidences of intestinal epithelial cell type cells in adenomatous hyperplasia and adenocarcinoma at 30 weeks were relatively low in both strains. These incidences were increased at 50 weeks in adenocarcinoma but not in adenomatous hyperplasia.

Mucin histochemistry and I-ALP immunohistochemistry of stomach mucosa and tumors

The protocols used for mucin histochemistry (paradoxical Con A, GOS, and S-GOS) and I-ALP immunohistochemistry have been described previously^{2,18,20}. 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 but contain GOS and S-GOS reactive mucins. Goblet cells in the small intestine and in areas of intestinal metaplasia contain mucins with no or slight GOS, and strong S-GOS reactivity. Intestinal absorptive cells in the small intestine and intestinal metaplasia have striated cell borders on their apical surfaces covered by a surface coat with no or slight GOS and S-GOS

Fig. 1. Strong binding of anti-cathepsin E antibody demonstrated on surface and in foveolar mucous cells of pyloric glands, and no reactivity in duodenal epithelium. I-ALP (A) and cathepsin E (B) immunohistochemistry $\times 100$.

Table 3. Results of Histochemical and Immunohistochemical Stainings of Normal Stomach and Small Intestine, and Intestinal Metaplasia in Stomach

	Type III mucin	GOS	S-GOS	I-ALP	Cathepsin E
Normal glandular stomach					
Surface mucous cell	—	+	+	—	+
Mucus neck cell	+	—	—	—	+
Pyloric gland cell	+	—	—	—	+
Normal small intestine					
Goblet cell	—	±	+	—	—
Brunner's gland cell	+	—	—	—	—
Absorptive cell	—	—	—	+	—
Intestinal metaplasia*	—	— (+)	— (+)	+	—

*: Results depended on the status of the cell types consisting metaplastic glands.

Fig. 2. No cathepsin E expression in an intestinal metaplastic gland in pyloric epithelium consisting of cathepsin E positive cells. Alcian blue-PAS staining (A) and cathepsin E immunohistochemistry (B) $\times 100$.

Fig. 3. Cathepsin E positive regenerated small intestinal epithelial cells with decreased I-ALP reaction on incomplete striated cell borders (arrows) in serial sections. I-ALP (A) and cathepsin E immunohistochemistry (B) $\times 100$.

Fig. 4. Stomach cancer cells of surface mucous cell type of a WKY rat. GOS and cathepsin E positive cells demonstrated in gastric epithelial cell type (surface mucous cell type and pyloric gland cell type) in serial section. (A) GOS staining $\times 200$ and (B) Cathepsin E immunohistochemistry $\times 200$.

reactivity but strong I-ALP antibody binding. Reactivity of the normal stomach and small intestine and intestinal metaplasia are summarized in Table 3.

The cellular differentiation of tumor cells in adenomatous hyperplasias and well differentiated adenocarcinomas was classified into gastric and intestinal epithelial cell types as described previously^{2,7-9}. Each tumor cell type showed the same mucin histochemistry and I-ALP immunohistochemistry reactivities as its normal counterpart. All adenomatous hyperplasias and adenocarcinomas found at weeks 30 and 50 either consisted solely of gastric epithelial type cells (pyloric gland and surface mucous cells) or mainly of such cells with rather fewer intestinal epithelial type cells (intestinal absorptive and goblet cells). The incidences of the latter in the presently induced adenomatous hyperplasias and adenocarcinomas of the glandular stomach are summarized in Table 2. Almost all tumor cells in the small intestine proved to be of intestinal epithelial cell type.

Localization of cathepsin E in the normal glandular stomach and small intestine

In normal gastric mucosa, cathepsin E was found to be

Fig. 5. Adenocarcinoma consisted of tumor cells of gastric and intestinal epithelial cell types. Alcian blue-PAS positive mucin were found in apical portion of cells of surface mucous cell type and in cytoplasm of cells of goblet cell type compressing adjacent cells. No cathepsin E reactivity was found in intestinal epithelial cell type (goblet cell and intestinal absorptive cell types) tumor cells in serial section. (A) cathepsin E immunohistochemistry $\times 200$ and (B) Alcian blue-PAS staining $\times 200$.

expressed moderately in parietal cells of fundic mucosa, strongly in surface and foveolar mucous cells of the fundus and pylorus and strongly in pyloric gland cells of the pyloric mucosa. Normal small intestinal epithelial cells did not have any cathepsin E reactivity (Fig. 1).

Localization of cathepsin E in histopathological lesions in the glandular stomach and small intestine

Glandular stomach: Cathepsin E was strongly expressed in regenerating pyloric mucosa but could not be detected immunohistochemically in epithelial cells of intestinal metaplastic glands (Figs. 2, 3). In adenomatous hyperplasias and well-differentiated adenocarcinomas of the stomach, almost all gastric epithelial cell type tumor cells showed cathepsin E reactivity in their cytoplasm, sometimes with a greater intensity than their normal counterparts (Figs. 4, 5). Tumor cells of intestinal epithelial cell type occasionally exhibited cathepsin E reactivity, in contrast to normal intestinal epithelium.

Small intestine: In regenerated small intestinal epithelium, some cells with a decreased I-ALP reaction in their striated cell border showed cathepsin E reactivity. In cancers, very small proportions of cells were also positive

(Fig. 6).

Discussion

In the present study, immunohistochemical localization of cells with strong expression of cathepsin E, a major intracellular proteinase with a limited organ distribution¹⁰⁻¹⁵, revealed surface and foveolar mucous cells and pyloric gland cells to be positive. Cells of the intestinal type were, on the other hand, typically negative. In previous studies with neutrophils²¹, the subcellular localization suggested function as a cytosol enzyme.

In contrast to the squamous epithelium of the esophagus and forestomach which is protected by keratinizing layers, the glandular stomach mucosa is relatively lacking in defense. Since it is the first barrier against ingested food, chemical substances, antigens, and bacteria, some repair and/or self-destruction upon becoming damaged might be expected. Indeed, a relationship between the existence of cathepsin E and such mechanisms has been speculated¹⁵. In this study, regenerating small intestinal epithelium also atypically showed cathepsin E expression. This might have been related to destruction of the defensive barrier

Fig. 6. Small intestinal adenocarcinoma consisted of cathepsin E positive and negative tumor cells. Cathepsin E immunohistochemistry $\times 200$.

constituted by the striated cell borders since these were incomplete with decreased activities of I-ALP in the affected areas. On the other hand, the lack of reactivity in intestinal metaplastic cells of the pyloric mucosa appeared to reflect suppressive regulation of cathepsin E like that in the normal intestinal epithelium.

In our previous work^{2,7-9} we established that stomach cancer cells can be classified into a predominant gastric epithelial cell type, comprising the pyloric gland and surface mucous cells, and minor intestinal epithelial cell type, represented by goblet and intestinal absorptive cells. Almost all the gastric epithelial cell type tumor cells in adenomatous hyperplasia and adenocarcinomas in the present study had retained the normal cathepsin E expression of the glandular stomach epithelium. On the other hand, the majority of intestinal epithelial type cells in areas of metaplasia and tumors, also derived from gastric cells, did not show cathepsin E. Considering the limited distribution of cathepsin E, its expression must be highly regulated²¹, and moreover, closely linked to cellular differentiation as revealed here. Our classification^{2,7-9} of phenotypic expression of stomach cancer cells therefore is validated.

While the presence of cells of intestinal type in cancers has been interpreted as giving support to a precancerous nature for intestinal metaplasia in human^{1,3,4,22} and rats^{5,6}. The fact that almost all adenomatous hyperplasias consist

solely of gastric epithelial cell type tumor cells^{2,9,18} would origin to the contrary. Furthermore, all early gastric cancers found at week 30 in the present study were similarly of gastric epithelial cell type so that the goblet cells and absorptive cells found within advanced gastric adenocarcinomas at week 50, were presumably due to secondary metaplasia. This is in clear contrast to the intestinal phenotypic expression of small intestinal tumors at both early (week 30) and late (week 50) stages showing a continuous histogenetic link with the normal epithelium. Thus the data suggest that a phenotypic shift from gastric to intestinal epithelial cell type occur with progression of gastric cancer in line with earlier findings^{2,23}. Rather than intestinal metaplasia, pepsinogen altered pyloric glands in rats, which can be detected immunohistochemically, have recently been suggested to be preneoplastic lesions in rats²⁴⁻²⁷.

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