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Pepsinogens I and II in Gastric Cancer: An Immunohistochemical Study Using Monoclonal Antibodies

Shih Che HUANG,[*1, *6] Kazumasa MIKI,[*1, *7] Junjiro SANO,[*1] Masao ICHINOSE,[*1], Norio KAWAMURA,[*1] Hiroshi OKA,[*1] Kazuyuki HIRANO,[*2] Chie FURIHATA,[*3] Yozo MASUGI[*4] and Kenji TAKAHASHI[*5]

*[1]*First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113,* *[2]*Gifu Pharmaceutical University, 5-6-1 Mitahorahigashi, Gifu 502,* *[3]*Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, 4-6-1 Shiroganedai, Minato-ku, Tokyo 108,* *[4]*First Department of Pathology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113 and* *[5]*Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113*

Monoclonal antibodies were used to examine the immunohistochemical expression of pepsinogens I and II in 31 early and 76 advanced gastric cancers. Of the 107 carcinomas studied, 19 contained pepsinogen II and only 3, found exclusively in pepsinogen II-positive cases, contained pepsinogen I. Gastric cancer produces pepsinogen II more frequently than pepsinogen I, and production of the latter is significantly associated with the former. Histologically, there were 54 intestinal-type and 53 diffuse-type cancers. The former produced pepsinogen II more frequently than the latter. In the diffuse type, the four pepsinogen II-positive cases were found exclusively in females. Although the pepsinogen expression was independent of the macroscopic features in advanced gastric cancer, it was found that the protruded-type early gastric cancer produced pepsinogen II more frequently than the depressed type. Incidences of pepsinogen positivity were not different between early and advanced gastric cancers or between cancers with or without lymph node metastasis, suggesting that production of pepsinogen is independent of tumor growth.

Key words: Pepsinogen — Gastric cancer — Monoclonal antibody

Pepsinogen I (PG I) and pepsinogen II (PG II=progastricsin) have been detected immunohistochemically in gastric cancer, including primary lesions and metastatic lesions in local lymph nodes. In the few reports published,[1–3] pepsinogens have been identified using polyclonal antibodies from rabbit. However, data on early gastric cancer are lacking. We have recently reported the generation of monoclonal antibodies to human PG I and PG II and the development of enzyme-linked immunosorbent assays of serum PG I and PG II using these antibodies.[4, 5] A monoclonal antibody usually binds only one antigenic epitope with uniform affinity and is generally able to give crisp labeling of the antigen with almost no background staining in immunohistochemical work.[6, 7] In this study, we used monoclonal antibodies to investigate pepsinogen production in gastric cancer. The relationship of antigen expression to macro- and microscopic features of tumors was also assessed.

Materials and Methods

Tissues One hundred and seven consecutive cases of primary gastric carcinomas obtained during surgery from 1982 to 1986 were included in this study. Tumor blocks studied were chosen to include adjacent normal mucosa as a positive control. Other tissues included metastatic gastric carcinomas in local lymph nodes from the same primary tumors, as well as normal gastric mucosa, esophagus, duodenum, jejunum and colon of human origin.

Pepsinogens Human pepsinogens I and II were purified as previously reported.[4, 5, 8, 9]

Monoclonal Antibodies Monoclonal antibodies to human pepsinogen I and pepsinogen II were generated and purified as described previously.[4, 5]

*[6] Present address: National Institutes of Health, Digestive Diseases Branch, Building 10, Room 9C-215, MD 20892, USA.

*[7] To whom correspondence should be addressed.

Immunostaining Titration experiments revealed the optimal dilutions of anti-PG I and anti-PG II to be 1:800 (final concentration 2.7 mg/liter IgG) and 1:200 (final concentration 3.7 mg/liter IgG), respectively. Treatment of sections with protease (as described below) was found to enhance staining with either antibody. Tissues were fixed in 10% unbuffered formaldehyde and embedded in paraffin. Sections (3 μm) were deparaffinized in xylene. The avidin-biotin-peroxidase complex technique (Vectastain ABC Kit, Vector Laboratories, USA) described by Hsu *et al.*[10] was used with a slight modification. All incubations were performed at room temperature. Deparaffinized sections were treated with 0.3% hydrogen peroxide in absolute methanol for 30 min to quench endogenous peroxidase, washed for 20 min with 10 m*M* phosphate-buffered saline (PBS), pH 7.4, and then incubated in 0.01% type XIV protease (Sigma) in PBS for 2 min. Nonspecific binding was blocked by treatment with normal horse serum diluted 1:50 in PBS for 20 min. The normal horse serum was removed and monoclonal antibody solution was applied for 30 min. This was followed by washing in three changes of PBS for 15 min. Sections were then incubated with biotinylated horse antimouse immunoglobulin G for 30 min, washed in PBS, and incubated with avidin DH-biotinylated horseradish peroxidase. After a final wash in PBS, the sections were incubated for 5 min in a solution containing 0.02% diaminobenzidine and 0.005% hydrogen peroxide. Presence of pepsinogen was indicated by a brown coloration. After washing in distilled water, sections were counterstained with Mayer's hematoxylin, washed again in distilled water, dehydrated in alcohol and mounted.

Control incubations included normal mouse immunoglobulin G in place of monoclonal antibodies and monoclonal antibodies absorbed with their homologous antigens by incubation at 37° for 3 hr.

Macro- and Microscopic Features of Gastric Cancer Macroscopically, early gastric cancer, i.e. a cancer whose depth is limited to the mucosa or submucosa, was classified according to the criteria of the Japanese Endoscopic Society[11] into the following types: type I, the protruded type; type II_a, the elevated superficial type; type II_b, the flat superficial type; type II_c, the depressed superficial type without ulcerated lesion and type II_c+III, the depressed superficial type with ulcerated lesion. Advanced gastric cancer was classified according to the criteria of Borrmann *et al.*[12] Microscopically, all cancers were classified according to the criteria of Lauren[13] as intestinal or diffuse types.

Statistics Differences in incidences of pepsinogen positivity were analyzed by the G-test of independence with Williams' correction[14]; $P<0.05$ was considered statistically significant.

Results

Normal Mucosa Examination of sections of normal human gastric and duodenal mucosa showed that PG I staining was present only in chief cells and mucous neck cells of the fundic gland (Fig. 1). The intensity of staining was much greater in the former than in the latter.

In serial sections, PG II staining was present not only in cells positive for PG I, i.e. the chief cells and mucous neck cells (Fig. 1), but also in the pyloric (Fig. 2), cardiac and Brunner's glands cells. The intensity of staining was greatest in the chief cells. Control incubations revealed complete abolition of all staining with PG I and PG II. Neither of the antibodies reacted with normal human esophagus, jejunum or colon.

Gastric Carcinoma Staining, when present, was often of rather lower intensity in carcinoma than in the normal mucosa (Fig. 2), and generally of a focal distribution. Again, control incubations revealed no staining.

Of the 107 carcinomas studied, 19 contained PG II and only 3, all of which also contained PG II, contained PG I (Table I). The overall incidence of pepsinogen II positivity, 17.8%, was significantly higher than that of pepsinogen I, 2.8% ($P<0.001$, Table II). The expression of PG I was highly associated with that of PG II ($P<0.005$, Table III).

Macroscopic Features Of the 107 carcinomas, 31 were early gastric cancer and 76 were advanced gastric cancer. No significant differences in the incidence of pepsinogen positivity were found between the two groups (Table II). All the three PG I-positive cases were advanced gastric cancer (Table I) and of the intestinal type (see below). The macroscopic features of early and advanced gastric cancers with pepsinogen II are summarized in Tables IV and V, respectively. There was no association of the PG II expression with Borrmann types in advanced gastric cancer. However, in early gastric cancer, the incidence of PG II positivity was significantly higher in the protruded type (type I, 66.7%) than in the non-ulcerated depressed type (type II_c, 0%) ($P<0.001$, Table IV).

Microscopic Features Histologically, 54 of the 107 carcinomas were of the intestinal type and 53 were of the diffuse type. PG II was

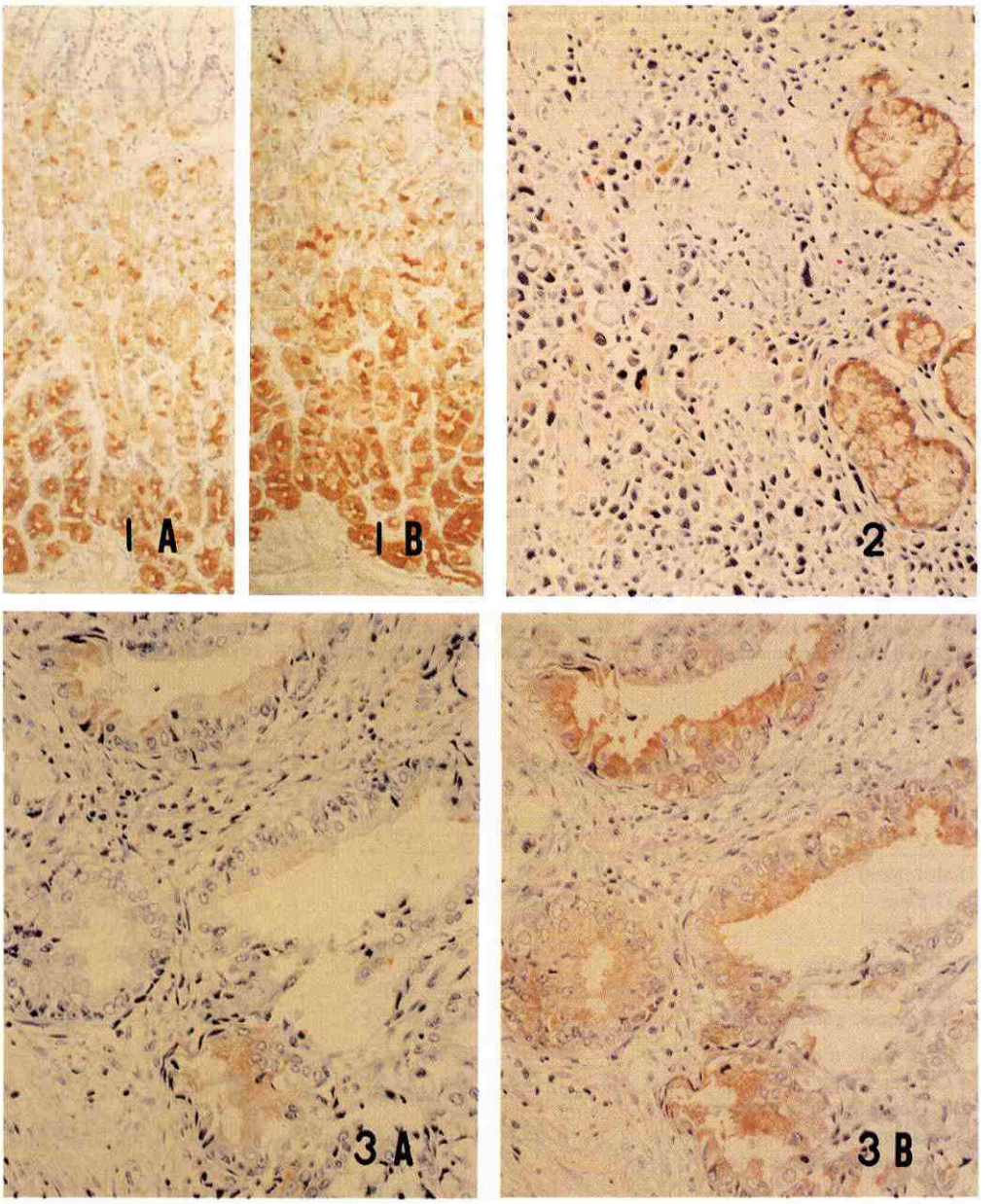

Fig. 1. Serial sections of fundic gland mucosa were stained (avidin-biotin immunoperoxidase complex method, ×100) with pepsinogen I (1A) and pepsinogen II (1B). Chief and mucous neck cells are positive (brown-colored).

Fig. 2. Diffuse-type advanced gastric cancer (avidin-biotin immunoperoxidase complex method, ×200). Pepsinogen II-positive (brown-colored) tumor cells, including some signet ring cells, scattered in clusters. Residual pyloric glands (right) are strongly positive.

Fig. 3. Intestinal-type advanced gastric cancer (avidin-biotin immunoperoxidase complex method, ×200). Serial sections were stained with pepsinogen I (3A) and pepsinogen II (3B), respectively. Pepsinogen II-positive (brown-colored) tumor cells are localized within the acini (3B). Pepsinogen I-positive (brown-colored) tumor cells (3A) are limited to a subpopulation of pepsinogen II-positive tumor cells.

Table I. Characteristics of Pepsinogen-positive Cases of Gastric Cancer

Case no.	Sex	Age	Macroscopic type	Microscopic feature	Location	PG I	PG II
1	M	59	I	Intestinal	Body	−	+
2	M	78	I	Intestinal	Antrum	−	+
3	M	69	II_a	Intestinal	Antrum	−	+
4	M	54	II_c+III	Intestinal	Antrum	−	+
5	M	47	B-2	Intestinal	Cardia	−	+
6	M	78	B-2	Intestinal	Antrum	−	+
7	F	48	B-3	Diffuse	Body	−	+
8	F	48	B-3	Diffuse	Antrum	−	+
9	F	71	B-3	Diffuse	Antrum	−	+
10	F	65	B-3	Diffuse	Antrum	−	+
11	F	62	B-3	Intestinal	Body	−	+
12	M	63	B-3	Intestinal	Body	−	+
13	M	84	B-3	Intestinal	Antrum	−	+
14	M	52	B-3	Intestinal	Antrum	−	+
15	F	76	B-3	Intestinal	Antrum	−	+
16	F	58	B-3	Intestinal	Antrum	−	+
17	M	68	B-3	Intestinal	Antrum	+	+
18	F	80	B-3	Intestinal	Antrum	+	+
19	M	62	B-4	Intestinal	Antrum	+	+

PG, pepsinogen; I, early cancer type I; II_a, early cancer type II_a; II_c+III, early cancer type II_c+III; B-2, advanced cancer Borrmann type 2; B-3, advanced cancer Borrmann type 3; B-4, advanced cancer Borrmann type 4.

Table II. Comparison of Gastric Cancers of Different Types that Stained with Pepsinogens

	PG I		PG II	
	No. positive	(%)	No. positive	(%)
Histological type				
Diffuse	0/53	(0)	4/53	(7.5)
Intestinal	3/54	(5.6)	15/54*	(27.8)
Depth of invasion				
Early gastric cancer	0/31	(0)	4/31	(12.9)
Advanced gastric cancer	3/76	(3.9)	15/76	(19.7)
Lymph node metastasis				
Cancer with lymph node metastasis	1/35	(2.9)	8/35	(22.9)
Cancer without lymph node metastasis	2/72	(2.8)	11/72	(15.3)
Total	3/107	(2.8)	19/107**	(17.8)

PG, pepsinogen. * $P<0.01$ vs. diffuse type. ** $P<0.001$ vs. PG I positive cancer.

Table III. Comparison of Gastric Cancer Stained with Pepsinogens

	PG II-positive	PG II-negative	Total	% PG II-positive
PG I-positive	3	0	3	100*
PG I-negative	16	88	104	15.4
Total	19	88	107	17.8

PG, pepsinogen. * $P<0.005$ vs. the PG I-negative group.

Table IV. Macroscopic Features of Early Gastric Cancer with Pepsinogen II

Macroscopic feature	PG II-positive	Total	% PG II-positive	
Protruded or elevated type				
Type I	2 (2)[a]	3 (3)	66.7*	(66.7)*
Type II_a	1 (1)	5 (5)	20.0	(20.0)
Subtotal	3 (3)	8 (8)	37.5**	(37.5)
Depressed type				
Type II_c	0 (0)	19 (11)	0	(0)
Type II_c+III	1 (1)	4 (1)	25.0	(100)
Subtotal	1 (1)	23 (12)	4.3	(8.3)
Total	4 (4)	31 (20)	12.9	(20.0)

PG II, pepsinogen II.

a) Numbers in parentheses identify intestinal-type gastric cancers.

* $P<0.001$ vs. type II_c. ** $P<0.05$ vs. subtotal of the depressed types, i.e. type II_c and type II_c+III.

Table V. Gross Features of Advanced Gastric Cancer with Pepsinogen II

Borrmann type	PG II-positive	Total	% PG II-positive
Type 1	0	0	0
Type 2	2	9	22.2
Type 3	12	54	22.2
Type 4	1	13	8.3
Total	15	76	19.7

PG II, pepsinogen II.

found in 15 (27.8%) of the intestinal-type gastric carcinomas and in only 4 (7.5%) of the diffuse type. The incidence in the former was significantly higher than in the latter ($P<0.01$, Table II). In the diffuse-type tumors, PG II-positive cells, including some signet ring cells, were scattered in clusters (Fig. 2). In contrast, there was a much more distinct separation of PG II-positive and -negative cells in the intestinal type. The staining was generally limited in tumor cells within the acini (Fig. 3). In one carcinoma that contained areas which differed markedly in the degree of histologic differentiation, PG II-positive tumor cells were found only in the better differentiated component (data not shown).

PG I was found in only 3 (5.6%) of the intestinal-type cancers and in none of the diffuse type. In all of these 3 cancers, PG II was also detected and the distribution of PG II was much more extensive than that of PG I. In 2 of the 3 cases, all tumor cells positive for PG I were positive for PG II, i.e. PG I positivity was limited to a subpopulation of PG II-positive tumor cells (Fig. 3). The third case exhibited a similar tendency but some PG I-positive, PG II-negative tumor cells did exist (data not shown).

Clinical Features The age and sex of patients and the location of tumors are summarized in Table VI. No association of the presence of staining with tumor location or patients' age was found. No association with sex could be found in the intestinal type, either. However, in the diffuse type, the four PG II-positive carcinomas were found exclusively in female patients. The male/female sex ratio in PG II-positive diffuse type was 0, significantly different from that in PG II-negative, 2.06 (Table VI).

Lymph Node Metastasis No significant difference in the incidence of pepsinogen positivity was found between cancers with or without local lymph node metastasis (Table II). In the pepsinogen-positive carcinomas with lymph node metastasis, the staining of PG I and PG II in the tumor cells of lymph node metastatic lesions was also found focally in 1 of the 1 PG I-positive and 2 of the 8 PG II-positive cases, respectively. The single PG I-positive metastatic tumor also contained PG II and its primary gastric carcinoma contained both antigens. As in the primary lesion, PG I positivity was limited to a subpopulation of PG II-positive neoplastic cells in that metastasis.

Table VI. Patients' Age, Sex Ratio and Tumor Location or Gastric Cancer with Pepsinogen

	Age (median)	Sex M/F (ratio)	Location Body	Location Antrum
Intestinal type				
PG II-positive	63	11/4 (2.75)	4	11
PG II-negative	69	29/10 (2.90)	9	30
Subtotal	68.5	40/14 (2.86)	13	41
Diffuse type				
PG II-positive	56.5	0/4* (0)	1	3
PG II-negative	56	33/16 (2.06)	18	31
Subtotal	56	33/20 (1.65)	19	34
Total	64	73/34 (2.15)	32	75

PG, pepsinogen.
* $P<0.01$ vs. PG II-negative diffuse type.

Table VII. Immunohistochemical Studies of Gastric Cancer with Pepsinogens

Antibody	PG I positivity (%)	Antibody	PG II positivity (%)	Author	Reference
Anti-pepsin	7/125 (5.6)	Anti-gastricsin	33/114 (28.9)	Reid *et al.*, 1985	1
Anti-PG I	24/32 (75.0)	Anti-PG II	7/32 (21.9)	Busby-Earle *et al.*, 1986	2
Anti-PG I	2/64 (3.1)	Anti-PG II	19/64 (29.7)	Stemmermann *et al.*, 1986	3
Anti-PG I[a)]	3/107 (2.8)	Anti-PG II[a)]	19/107 (17.8)	Huang *et al.*	This work

PG, pepsinogen.
a) Monoclonal antibody.

Discussion

This is the first immunohistochemical study of pepsinogens in gastric cancer with monoclonal antibodies. Monoclonal antibodies are potentially superior immunological reagents because of their homogeneity of affinity. However, since each monoclonal antibody is directed against a single determinant, if that determinant is significantly altered, often no antibody binding will occur. It seems such a phenomenon did not occur here. One line of evidence for this is that the cellular distributions of PG I and PG II in normal mucosa found in this study accord well with those seen in previous studies employing polyclonal antibodies.[15–18] Compared with polyclonal antibodies, sections stained with monoclonal antibodies showed crisper labeling of pepsinogens (our unpublished data).[6, 7]

The results of this study indicate that gastric cancer produced PG II more often than PG I: 18% of the tumors contained PG II, while only 3% contained PG I. Previous immunohistochemical studies with polyclonal antibodies are summarized and compared with our data in Table VII.[1–3] As in our study, incidences of PG II positivity reported

were around 20–30%, and those of PG I were around 3–6%. One exception is the PG I incidence of 75% found by Busby-Earle *et al.*[2] The reason for this is unknown.

This is also the first report on the association of macroscopic appearance with pepsinogen expression in gastric cancer. Our data suggest a positive association of pepsinogen II with protruded type of early gastric cancer. However, no association between the gross features and pepsinogen expression in advanced gastric cancer could be found.

In this study we also observed that intestinal-type gastric cancer produced PG II more commonly than the diffuse type. PG II was found in 28% of the intestinal type, but only 8% of the diffuse type. A similar tendency was reported by Stemmermann *et al.*[3]

The four diffuse-type cancers that contained PG II were all from female patients. The sex distribution in the PG II-positive diffuse type was significantly different from that in the PG II-negative diffuse type. With 18 cases of diffuse-type gastric cancers, Stemmermann *et al.* observed 2 PG II-positive cases from 9 females and 1 from 9 males,[3] suggesting a tendency for female predominance in PG II-positive diffuse-type cancer. This accords with our result. The diffuse-type gastric cancers from female patients seem to produce PG II more frequently than those from males. In rats, sex hormones were reported to influence the growth or progression of gastric cancer.[19] Whether similar hormonal factors influence the production of pepsinogen in human gastric cancer requires further elucidation.

In cancer, production of PG I appeared to be highly associated with that of PG II. PG I-producing tumors were found exclusively in PG II-producing ones. Further, in serial sections of both primary tumor and its lymph node metastases, we observed that PG I staining, if present, tended to exist in a subpopulation of PG II-positive tumor cells. In normal tissue, the cellular distribution of PG I is limited to a subpopulation of that of PG II. Productions of PG I and PG II seem to be closely associated in both normal and cancer tissues.

Incidences of pepsinogen positivity were not different between early and advanced gastric cancers or between cancers with or without lymph node metastasis, suggesting that production of pepsinogen by cancer is independent of tumor growth.

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