ORIGINAL ARTICLE

The Collagenase Activities, Interstitial Collagenase and Type IV Collagenase, in Human Stomach Cancer: with Special Reference to Local Spreading and Lymph Node Metastasis

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Abstract. In order to investigate the role of collagenase in cancer invasion and metastasis, two collagenase activities of interstitial collagenase and type IV collagen degrading enzyme (type IV collagenase) were determined in 40 cases of human stomach cancer tissue. Elevated cancers which are known to have a propensity to cause blood-borne metastases showed higher activities of both interstitial collagenase and type IV collagenase than flat or ulcerous type of cancer. Using the parameters of lymph node metastasis *vs* tumor size or *vs* depth of cancerous invasion into the stomach wall, classification of the cases was attempted according to the degree of malignancy. In the cases with marked lymph node metastases in spite of small tumor size and/or shallow cancerous invasion into the stomach wall, type IV collagenase activity was higher than that in the cases with lower malignancy ($p < 0.025$, $p < 0.05$, respectively). These results suggest that collagenase in stomach cancer tissue play an important role in the invasion and metastasis of cancer cells. Type IV collagenase activity in stomach cancer tissue could be one of the useful biological markers for the degree of malignancy. (Keio J Med 39 (3): 159–167, September 1990)

Key words: metalloproteinase, invasion, matrix, basement membrane, biological marker

Introduction

Stomach cancer has been the most common cancer in Japan; more than 45,000 patients die from this cancer every year.¹ Regarding the nature of cancer, the most important characteristic is its ability to destroy surrounding connective tissues and to metastasize into distant organs. In 1946, Fischer first described the involvement of proteolytic activity during cancer invasion.² The type of cells at the outer margin of cancer and the source of proteolytic activity may vary considerably. We have focused on detecting interstitial collagenase activity in total tissue homogenate^{3–5} and have shown the increased activity of this enzyme at the advancing front of stomach cancer.⁶

Recent progress in collagen chemistry revealed that the main component of basement membrane is type IV collagen. Basement membrane has a multicomponent structure playing important architectural and functional roles and widely distributes throughout the body. Base-

ment membranes around capillary vessels as well as epithelial layers are penetrated by cancer cells during the invasive and metastatic process. With ultrastructural techniques, Birbeck and Wheatley in 1965 observed the degradation of basement membrane by Ehrlich ascites cells.⁷ Type IV collagen forms a tight three-dimensional network of basement membrane⁸ and is not cleaved by interstitial collagenase.^{9,10} We have established the assay method for detecting type IV collagenase activity and have reported the increased activity in lung cancer tissues as well as in stomach cancer tissues.¹¹

As demonstrated by *in vitro* invasion assay systems^{12–14} and *in vivo* metastatic models,¹⁵ collagenase activities in some animal cell lines have been reported to be positively correlated with the invasive or metastatic potential of the cancer cells, while the relationship with the clinicopathological findings of the cancer is not known.

In the present study, the relationship between two collagenase activities in stomach cancer tissue and the

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clinicopathological findings of the cancer was investigated in order to reveal the role of these enzymes in cancer invasion and metastasis. The possibility of clinical application of these enzyme activities as a biological marker indicating the degree of malignancy of stomach cancer was evaluated as well.

Materials and Methods

Tissue samples

Fresh stomach tissue specimens from 40 patients, 39–81 years of age, were obtained from the operating room of Keio University Hospital, generally within 30 min after therapeutic surgery. Tissue specimens of the advancing front of the cancer were cut out in small pieces ($4 \times 4 \times 4$ mm). Normal tissues, grossly free from cancer in the resected stomach, were used as controls.

Preparation of tissue homogenate

Immediately after the stomach specimens were obtained, they were placed into saline and kept at -70°C . Before the assay, they were cut into small pieces and washed five times in cold saline to remove small amounts of serum inhibitors against collagenase like α_2 -macroglobulin, etc.¹⁶ Tissues were collected on filter paper, weighed and homogenized with ice-cold Tris buffer (0.05 M Tris-HCl, pH 7.6 at 5°C with 0.2 M NaCl, 5 mM CaCl_2) using a microglass hand homogenizer. A part of the tissue homogenate was used for protein measurement by the method of Lowry et al.¹⁷

Preparation of type I and type IV collagens

For use as substrate, acid soluble collagen was extracted and purified from rabbit skin by the method of Glimcher et al.¹⁸ Type I collagen was isolated and purified according to the method of Timpl et al.¹⁹ Soluble type IV collagen was isolated from healthy human placenta by the method of Glanville et al.²⁰ Both purified collagens were labeled with ^3H -acetic anhydride according to the method of Gisslow and McBride.²¹ The purity of both type I and type IV collagens was confirmed by polyacrylamide gel electrophoresis.^{6,11}

Direct measurement for two collagenase activities in tissue homogenate

The reaction conditions in these assays were set to exclude the activity of other proteinases.²² The activities of interstitial collagenase and type IV collagenase were measured using purified type I and type IV collagens as substrates either in fibrils or in solution, respectively.

Interstitial collagenase activity was determined by

measuring the release of soluble radioactive products from the reconstituted collagen fibrils, which had been incubated at 37°C overnight to form fibrils. The incubation mixture was adjusted to a final concentration of 0.05 M Tris-HCl, pH 7.6 at 37°C containing 0.2 M NaCl, 10 mM CaCl_2 , and 3 mM para-chloromercuribenzoic acid (p-CMB). The tissue homogenate was used as the enzyme source. Assay blanks contained 3 mM p-CMB, the enzyme solution, and 10 mM ethylenediamine-tetraacetic acid (EDTA) instead of CaCl_2 . Assays were usually performed in triplicate. After incubation at 37°C for 24 h the undigested collagen was spun down at 15,000 G for 5 min at room temperature. The supernatant (100 μl) was transferred into 3 ml of Phase combining system (PCS, Amersham Japan, Tokyo) and the counts were measured through liquid scintillation counting. Complete (100%) lysis by bacterial collagenase was also determined as a positive control and was usually about 4,000 cpm/reaction in this assay system. The buffer blank gave a reading of less than 5% of the complete lysis. In order to evaluate the denaturation of the collagen substrate, assays containing 0.01% trypsin were performed. They released no more than 10% of the complete lysis.

Type IV collagenase activity was measured by our method^{6,11} which was a modification of the method of Liotta et al.²² The incubation buffer and enzyme source were the same as those used in the assay of interstitial collagenase; tissue homogenate was applied as the enzyme source and the reaction condition was adjusted to a final concentration of 0.05 M Tris-HCl, pH 7.6 at 37°C containing 0.2 M NaCl, 10 mM CaCl_2 , and 3 mM p-CMB. After incubation for 24 h, in order to precipitate undigested collagen, trichloroacetic acid (final 2%) and tannic acid (final 0.1%) were added to the reaction, which was then cooled down in ice for 30 min. The precipitate was spun down at 15,000 G for 5 min at room temperature. The radioactivity in 100 μl of the supernatant was measured by liquid scintillation counting.

Collagenase activities against both types of collagen were expressed as unit of μg collagen degraded/hr/mg protein.

Clinicopathological investigation of stomach cancer

The activities of interstitial collagenase and type IV collagenase were analysed by the clinicopathological findings of the cases depicted in Table 1. All cases were clinically and histologically classified based on "The general rules for the stomach cancer study in surgery and pathology."^{23,24}

Statistical analysis

Results were expressed as mean $\pm$ SEM. Significance of difference between them was established by Student's

Table 1 Clinicopathological Investigation.

Patients:

age
sex

Gross Findings:

location of cancer in stomach
tumor size
Borrmann's classification
cancer stage

Histological Findings:

histological feature of epithelial malignancy
amount of interstitial connective tissue
growth pattern
depth of cancer invasion
degree of cancer cell invasion into lymph vessels and veins
degree of lymph node metastasis

t-test or paired t-test. If p value was smaller than 0.05, the difference was considered to be statistically significant.

Results

Interstitial collagenase activity in stomach cancer

Interstitial collagenase activity in human intact stomach mucosa was 10.37 ± 0.90 (n=23) μg collagen degraded/hr/mg protein. The activity in the advancing front of the cancer was 12.32 ± 1.16 (n=23), which was significantly higher than that in intact mucosae ($p < 0.05$, Fig. 1).

Type IV collagenase activity in stomach cancer

Type IV collagenase activity in intact mucosa was 0.65 ± 0.10 (n=20). The activity in the advancing front of the cancer was 1.39 ± 0.34 (n=20), which was significantly higher than that in intact mucosa ($p < 0.05$, Fig. 1).

Both collagenase activities in stomach cancer tissue and age of patients

Both collagenase activities were slightly higher in patients aged 60 to 69 than in other age groups without a significant difference.

Collagenase activities in stomach cancer tissue and sex of the patients

Interstitial collagenase activity in males was 12.92 ± 1.09 (n=30) and in females was 11.14 ± 1.33 (n=8). Type IV collagenase activity in males was 1.05 ± 0.27 (n=23) and in females was 1.39 ± 0.59 (n=7). There was not a significant difference in either col-

Fig. 1 Comparison of collagenase activities between intact mucosa and stomach cancer in the same individuals. Values are in mean $\pm$ SEM. ■ = interstitial collagenase; ▨ = type IV collagenase

Table 2 Collagenase Activities and Location of Stomach Cancer.

Location	Interstitial Collagenase	Type IV Collagenase
C	$10.03 \pm 2.42^*$ (5) [†]	1.83 ± 1.12 (5)
M	12.04 ± 1.27 (16)	0.92 ± 0.38 (11)
A	13.11 ± 1.41 (17)	1.01 ± 0.22 (14)

Cancers have been classified by the primarily involved portions of stomach, which is separated into three portions, upper (C), middle (M), and lower (A).

* μg collagen degraded/hr/mg protein

[†] number

lagenase activity between males and females.

Collagenase activities and location of the cancer

Between three groups (upper, middle and lower portions) there was no significant difference in either collagenase activities (Table 2).

Collagenase activities and tumor size

Between two groups ($5.0\text{cm} \leq T \leq 8.0\text{cm}$ vs $8.0\text{cm} < T$) significant difference in interstitial collagenase activity was observed (Fig. 2).

Collagenase activities and Borrmann's classification

Applying Borrmann's classification, stomach cancers were classified into 4 types based on gross inspection of the mucosal surface of the fresh specimen. Interstitial collagenase activity in the cancer of Borrmann 1 was

Fig. 2 Collagenase activities and tumor size.
 ■ = interstitial collagenase; ▨ = type IV collagenase

16.25 ± 0.72 ($n=3$), which was significantly higher than that in Borrmann 3 and Borrmann 4 (13.22 ± 1.39 , $n=19$, 10.25 ± 1.86 , $n=6$, respectively) (Fig. 3). Type IV collagenase activity in the cancer of Borrmann 1 was 2.62 ± 1.22 ($n=4$), which was also significantly higher than that in Borrmann 3 (0.90 ± 0.29 , $n=15$) (Fig. 3).

Collagenase activities and cancer stage

Interstitial collagenase activities in the cancer of stage I and IV, (13.44 ± 1.96 , $n=4$, 14.26 ± 2.20 , $n=9$, respec-

tively), were significantly higher than that in stage III, (8.86 ± 1.18 , $n=9$, both $p < 0.05$). Along with the stage of cancer, type IV collagenase activity seemed to increase gradually, but no significant difference was observed.

Collagenase activities and histological feature of epithelial malignancy

No significant difference in the collagenase activities has been observed among well differentiated tubular adenocarcinoma (tub₁), moderately differentiated tubular adenocarcinoma (tub₂) and poorly differentiated adenocarcinoma (por).

Collagenase activities and the amount of connective tissue

There was no correlation between interstitial collagenase activity and the amount of connective tissue observed by microscopic examination, that is, no significant difference in the enzyme activity among three types (medullary, intermediate and scirrhous) of cancer was observed. On the other hand, type IV collagenase activity in medullary type of cancer was 1.52 ± 0.28 ($n=10$), which was significantly higher than those in the intermediate and the scirrhous types of cancer (1.02 ± 0.22 , $n=12$, 0.73 ± 0.18 , $n=6$, respectively).

Collagenase activities and growth pattern

The growth pattern of the cancer was classified histologically according to the rule²⁴ into three groups as follows: INF α (expansive growth), INF β (intermediate), and INF γ (infiltrative growth). No significant difference of collagenase activities was observed among these three groups.

Collagenase activities and lymph node metastasis

Lymph node metastasis was evaluated histologically. Interstitial collagenase activity in the cases with lymph node metastasis was 12.59 ± 1.15 ($n=24$), which was slightly higher than that in the cases without lymph node metastasis (9.94 ± 1.55 , $n=7$) (Fig. 4); type IV collagenase activity in the cases with lymph node metastasis was 1.22 ± 0.30 ($n=24$), which was higher than that in the cases without lymph node metastasis (0.74 ± 0.25 , $n=6$), but without statistical significance (Fig. 4).

Collagenase activities and cancer cell invasion into lymph vessels and/or veins of stomach wall

Interstitial collagenase activity in the cases with marked invasion of cancer cells into lymph vessels and/or veins of the stomach wall was 11.81 ± 1.27 ($n=18$),

Fig. 3 Collagenase activities and Borrmann's classification of stomach cancer.
 ■ = interstitial collagenase; ▨ = type IV collagenase

Fig. 4 Collagenase activities and lymph node metastasis.
 ■ = interstitial collagenase; ▨ = type IV collagenase

Fig. 5 Collagenase activities and cancer cell invasion into lymph vessels and/or veins of stomach wall.

vascular invasion (-), (+): ly_{0-1} and/or v_{0-1} ,
 vascular invasion (++) , (+++): ly_{2-3} and/or v_{2-3}

■ = interstitial collagenase; ▨ = type IV collagenase

Fig. 6 Collagenase activities and degree of malignancy of the cancer: lymph node metastasis and tumor size.

The regional lymph nodes of the stomach are to be histologically designated into five groups:

no lymph node metastasis ($n(-)$)

metastasis to lymph nodes group 1 ($n_1(+)$)

metastasis to lymph nodes group 2 ($n_2(+)$)

metastasis to lymph nodes group 3 ($n_3(+)$)

metastasis to lymph nodes located beyond group 3 ($n_4(+)$)

■ = interstitial collagenase; ▨ = type IV collagenase

which was higher than that in the cases with less invasion (11.60 ± 1.54 , $n=13$) (Fig. 5). Type IV collagenase activity in the cases with marked vascular invasion was 1.36 ± 0.30 ($n=17$), which was higher than that in the cases with less invasion (0.83 ± 0.21 , $n=13$, $p<0.1$) (Fig. 5).

Collagenase activities and degree of malignancy of the cancer

Lymph node metastasis and tumor size (Fig. 6): For the purpose of detecting highly malignant cases, the 30 cases of stomach cancer were re-evaluated through two parameters, lymph node metastasis and tumor size. In regard to 8 cases of group I, shown in Fig. 6, their tumor sizes were larger than the median of the each group, but they had less lymph node metastases than the cases of group II, which showed marked lymph node metastases in spite of relatively small tumor size. Interstitial collagenase activity in the cases of group I was 11.80 ± 1.35 ($n=8$), which was lower than that in group II, without a significant difference (14.01 ± 4.49 , $n=3$). Type IV collagenase activity in the cases of group II, presumed to be highly malignant, was 1.74 ± 0.39 ($n=3$), which was higher than that of group I, presumed to be less

malignant (0.72 ± 0.16 , $n=8$, $p<0.025$).

Lymph node metastasis and depth of cancer invasion (Fig. 7): Also for the detection of highly malignant cases, the 30 cases were again evaluated by another two parameters: lymph node metastasis and depth of cancerous invasion into the stomach wall. In regards to 6 cases of group I shown in Fig. 7, they showed deep cancerous invasion into the stomach wall, but had no or slight metastases only in perigastric lymph nodes. On the other hand, 7 cases of group II, whose cancerous invasion remained relatively shallow in the stomach wall, had already remarkable lymph node metastases. Interstitial collagenase activity in the cases of group II was 11.29 ± 1.52 ($n=7$), which was lower than that of group I (11.87 ± 3.22 , $n=6$), without a significant difference. Type IV collagenase activity in the cases of group II, presumed to be highly malignant, was 1.18 ± 0.28 ($n=7$), which was significantly higher than that of group I, presumed to be less malignant (0.44 ± 0.13 , $n=6$, $p<0.05$).

Discussion

The present study was undertaken as an attempt to

Fig. 7 Collagenase activities and degree of malignancy of the cancer: lymph node metastasis and depth of cancer cell invasion into stomach wall.

The deepest layer of the cancerous invasion in the stomach wall is expressed by the following abbreviations.

m: Tunica mucosa (including muscularis mucosae)

sm: Tela submucosa

pm: Tela muscularis propria

ss: Tela subserosa

s: Tunica serosa

■ = interstitial collagenase; ▨ = type IV collagenase

clarify the relationship between collagenase activities in human stomach cancer tissue and clinicopathological findings of the cancer.

During invasion of cancer, normal interstitial connective tissue is known to be destroyed by the cancer.²⁵ Fischer in 1946 first described the involvement of the proteolytic activity in the dissolution of extracellular matrix by invasive tumor cells.² During the steps of invasion into submucosal layer from the state of carcinoma *in situ*, the cancer cells must destroy and penetrate basement membrane as a first event of metastatic process.²⁶ After penetrating the basement membrane underneath the mucosal layer, the cancer cells migrate into the submucosal matrix which is mainly composed of type I and III collagens. Intact capillary vessels are surrounded by basement membrane.²⁷ Actually it is believed theoretically that cancer cells traverse capillary walls twice during the metastatic process, entering the vessels near the primary lesion and exiting from the vessels after reaching distant organs where metastatic nests will be formed.²⁸ When cancer cells penetrate capillary walls, they need again to degrade proteolytically the basement membrane components. Using ultrastructural techniques, the evidence for the involvement of the degradation of basement membrane by malignant cells has been shown by Birbeck and Wheatley⁷ and Tarin.²⁵ Type IV collagen, a major structural protein in basement membrane, is resistant to interstitial collagenase.^{9,10} Liotta et al. in 1979 demonstrated the existence of a proteolytic enzyme specific for type IV collagen isolated from metastatic murine fibrosarcoma cells.²⁹ Fessler et al. in 1984 reported that a specific enzyme isolated from metastatic mouse PMT sarcoma cells cleaved murine type IV procollagen into two segments with an approximate mass ratio of 3:1.³⁰

A few investigators have reported on collagenase activity in stomach cancers. Honya revealed higher interstitial collagenase activity in stomach cancer tissue than in intact mucosa.³¹ Particularly at the advancing front of cancer, where active tissue degradation was presumed to occur, high collagenase activity was detected.^{6,11,32} In this study of 40 cases of stomach cancer, two activities of interstitial collagenase and type IV collagenase were determined, and they were compared between cancer tissue and intact mucosa in the same individuals. The higher activities of both enzymes were detected in cancer tissue, so that the reliability of our previous observation has been confirmed.

The cancers which were 5 to 8 cm in size showed significantly higher activity of interstitial collagenase than the larger cancers. Since larger cancers showed lower collagenase activity, the medium-size cancers could be more actively destroying the surrounding matrix or the turnover of the matrix between the cancer and intact mucosa could be accelerated in these medium-size

cancers.

Elevated type of advanced gastric cancers, Borrmann 1 type, showed significantly higher levels of activity of both collagenases than flat or ulcerous type of cancer. Generally, elevated type of cancers in stomach and colon are known to have a tendency to cause blood-borne metastasis to liver or bone marrow.³³ These results suggest that both collagenases could play an important role in causing metastasis.

Type IV collagenase activity appeared to increase with the advance in stage of the cancer. Not only local conditions but also systemic factors specific for cancer patients could affect the activity. Circulating agents such as plasmin,³⁴ plasma kallikrein,³⁴ acute phase reactant such as α_2 -macroglobulin,¹⁶ TIMP (tissue inhibitor of metallo-proteinases),³⁵ and cytokines³⁶ are candidates for these factors. Although there are a few reports about how these factors affect proteinase activities in cancer patients, it would be interesting to investigate the relationship between systemic conditions and local proteolytic activities.

The amounts of connective tissue showed a negative correlation with type IV collagenase. The source of proteinases may not necessarily be derived solely from cancer cells.³⁷ While there are many studies of the proteolytic activity from cancer cells, some evidence supports the production of proteinases by stromal cells.³⁸ Ellis et al. showed that LX-1 lung carcinoma cells produced a 58 kD protein, TCSF (tumor cell collagenase-stimulatory factor), which induced collagenase production in cultured fibroblasts.³⁹ In the present study, the enzyme activity could be from both cancer cells and stromal cells since the enzyme has been derived from tissue homogenate. Simply, the negative correlation between type IV collagenase activity and the amounts of interstitial connective tissue could be a result of the imbalance between degradation and synthesis of matrix in the local region. In order to clarify the whole scene of the connective tissue metabolism in cancer tissue, the characterization and localization of the proteolytic enzymes using monoclonal antibodies as well as cDNA probes⁴⁰ specific for these enzymes cannot be avoidable as a next step of the investigation.

As for the lymph node metastasis, both collagenase activities were higher in the cases with lymph node metastasis than in the cases without it. Moreover, the cases with marked vascular invasion showed higher activity of type IV collagenase than the cases with less invasion ($p < 0.1$). It might be possible to regard type IV collagenase as playing an important role in the cancer cell invasion into lymph vessels and veins of stomach wall. This speculation is also in line with the previous observation about the involvement of basement membrane degradation during the step of the vascular invasion.^{6,11}

In regards to the potential for the malignancy of cancer, the speed of local spreading and the degree of metastasis to distant organs can be two major parameters by which we can judge the character of the malignancy. From this stand point, lymph node metastasis, tumor size, and depth of invasion into the stomach wall have been re-evaluated in this study, so that it was possible to isolate the cases belonging to the highly malignant group. The cases which were suspected of being highly malignant showed higher activity of type IV collagenase than the less malignant cases, while interstitial collagenase activity showed no significant difference. Recently, Ostrowski et al. also have demonstrated a positive correlation between the degree of malignancy and transin gene expression, which is believed to be the rat homologue of stromelysin,⁴¹ using chemically induced rat squamous cell carcinoma.⁴²

The present study suggests that type IV collagenase activity in cancer tissues could be an useful biological marker for the degree of malignancy of stomach cancer. The postoperative 20-year-survival rate for early stomach cancer in National Cancer Center Hospital in Japan is over 94%.⁴³ A few percent of early stomach cancer recurred after radical surgery and most of them had metastases in distant organs such as liver, bone, and brain which were certainly caused by blood-borne route.^{44,45} Retrospective studies have revealed that the histological finding of positive vascular invasion was one of the important risk factors for the recurrence after curative operation.^{44,46} Based on the results of this study, type IV collagenase activity correlates with the severity of vascular invasion and the degree of malignancy of stomach cancer. Recently we have demonstrated that the postoperative adjuvant chemotherapy for early stomach cancer was not always effective.⁴⁷ The rate of recurrence may be reduced by intensive lymph node dissection as well as strong anticancer chemotherapy for the cases which are regarded as possessing highly malignant potential based on the preoperative evaluation using the biological marker in the biopsied specimen.

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