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Expression of Interstitial Collagenase (Matrix Metalloproteinase-1) in Gastric Cancers

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The expression of matrix metalloproteinase-1 (MMP-1) gene and the presence of MMP-1 protein in gastric cancer were examined by *in situ* hybridization and immunohistochemistry. Expression of the interstitial collagenase (MMP-1) gene was detected within the stroma of the neoplastic glands, and infiltration of eosinophils was observed to be associated with regions of MMP-1 gene expression. The degree of eosinophilic infiltration correlated with the level of MMP-1 mRNA expression. Immunostaining showed localization of MMP-1 protein in the stromal cells, and additionally in the neoplastic glands. These findings indicate that the stromal cells may play an important role in the expression of MMP-1, and suggest a pathophysiological role for MMP-1 in the invasion and metastasis of gastric cancer.

Key words: Matrix metalloproteinase-1 — Collagenase — Eosinophil — Gastric cancer — Stromal cell

Despite the availability of early detection systems and improvement in operative procedures, the postoperative survival rates of stage III and IV gastric cancer are still low.[1)] Further study to clarify precisely the mechanisms of the invasion and metastasis of gastric cancer is important in the search for new therapeutics for gastric cancer.

During the process of tumor invasion and metastasis, cancer cells cross the extracellular matrix and basement membrane to enter lymphatic and blood vessels. In 1946, Fischer first described the involvement of proteolytic activity during cancer invasion.[2)] The roles of type IV collagenase/gelatinase (MMP-2 and MMP-9) and stromelysin (MMP-3) in the degradation of type IV and V collagens, and laminin and fibronectin, during the course of metastasis have been described.[3,4)] However, little is known about the pathological role of interstitial collagenase (MMP-1), which can degrade type I, II, III, and X collagens. Previously we observed an increased activity of interstitial collagenase and type IV collagenase at the invasive front of gastric cancer.[5)] In this study, we have extended these observations by examining MMP-1 expression using *in situ* hybridization and immunohistochemistry in different gastric cancers.

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The abbreviations used are: MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; SSC, standard saline citrate; HPF, high-power field; HE, hematoxylin and eosin; IL-1, interleukin-1.

MATERIALS AND METHODS

Tissue Twenty cancer tissue specimens were obtained from surgically resected materials within 30 min after operation. Specimens were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 1 h at room temperature and embedded in paraffin. Sections were cut at 5 μm, mounted on microscope slides, dried thoroughly for 3 h on a slide warmer at 42°C, and stored at 4°C.

cDNA probe MMP-1 cDNA (1.7 kb) was isolated from a cDNA library of human synovial fibroblasts by Saus *et al.*[6)] This cDNA probe was labeled with [α-^{35}S]dCTP by an oligolabeling technique.

***In situ* hybridization** The hybridization procedure employed in this study was essentially the same as that described by Otani *et al.*[7)] Predigestion was completed with 0.25 mg/ml of proteinase K (Sigma Co., St. Louis, MO). Samples were washed with PBS and incubated in acetic anhydride in triethanolamine buffer. The slides were washed, dehydrated, and dried in air under sterile conditions. The complete hybridization mixture, which contained the labeled cDNA probe, yeast tRNA, salmon sperm DNA, 50% formamide, 10 m*M* Tris-HCl, pH 7.0, 0.3 *M* NaCl, 1 m*M* EDTA, and 1×Denhardt's mixture (10 mg/ml Ficol, 10 mg/ml polyvinylpyrrolidone, and 10 mg/ml bovine serum albumin, Pentax fraction V) was heated for 3 min at 80°C to denature the probe DNA and chilled quickly in iced water. Twenty microliters of the mixture was spread over the sample on a microscope slide. The sample was covered with a sterile cover glass

and hybridization was performed by placing the incubated slides on an aluminum cake pan floating on a water bath at 45°C for 16 h. After hybridization, the cover glass was removed in 2×SSC, and samples were washed in 2×SSC at room temperature for 20 min, 0.5×SSC at 45°C for 10 min, and three changes of 0.1×SSC at 45°C for 10 min each. The slides were then dipped into Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY), exposed for 3–7 days at 4°C, developed in D19 (Kodak), and stained with hematoxylin.

Antibodies The primary antibody to MMP-1 was a mouse monoclonal antibody, kindly provided by Professor Yasunori Okada, Kanazawa University. Secondary antibody was rabbit anti-mouse IgG antibody labeled with horseradish peroxidase (DAKO Japan Co., Kyoto).

Immunohistochemistry Deparaffinized sections were subjected to immunohistochemical analysis using a standard immunoperoxidase technique (indirect method). Sections were then rehydrated and blocked with 0.45% H_2O_2 in methanol for 30 min, followed by incubation with normal rabbit serum (DAKO Japan Co.) diluted 1/50 for 20 min. The primary antibody, diluted 1/50, was applied and incubated at 4°C overnight. The sections were washed in PBS three times, then a secondary antibody, diluted 1/100, was applied for 30 min, followed by 0.2 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Co., Tokyo) in 50 m*M* Tris, pH 7.6, containing 0.01% H_2O_2 for 5 min. The sections were counterstained lightly with hematoxylin. The primary antibody was replaced by PBS for control sections.

Microscopic studies Resected specimens stained with HE were histologically diagnosed and classified according to the criteria of the Japanese Research Society for Gastric Cancer in 1995.[8]

On hybridized sections, cells containing silver grains were recorded as positive. The degree of MMP-1 mRNA expression at the invasive front of gastric cancer was determined by counting cells that were recorded as positive in a HPF (×400). In all cases, three randomly selected HPFs were used to evaluate positive cells and the average number of positive cells was determined. To determine the expression of MMP-1 protein, serial sections were examined, and the average number of positive cells was compared in the same HPF as for the hybridized sections.

Expression of MMP-1 mRNA and that of MMP-1 protein were classified as more than 20 MMP-1 positive cells in a single HPF (+++), 10 to 20 (++), less than 10 (+), few (±) or none (−).

Eosinophils infiltrated into the stroma were detected using Luna stain.[9] To determine the number of eosinophils, three HPFs were selected randomly in serial sections and the numbers of eosinophils were averaged.

Statistical analysis Statistical analysis was carried out by simple regression.

Table I. Histopathological Findings and Expression of MMP-1 in Gastric Cancer

Case no.	Macroscopic type	Size (mm)	Stage	Depth	Histology	MMP-1 expression			
						mRNA		protein	
						tumor cells	other cells[a]	tumor cells	other cells
1	3	70	IVb	ss	tub2	(−)	(++)	(++)	(++)
2	4	75	IVb	se	por1	(−)	(++)	(±)	(++)
3	2	62	IVb	se	tub2	(−)	(±)	(+)	(+)
4	3	55	IIIa	mp	por1	(−)	(+++)	(+)	(++)
5	4	115	IVb	se	tub2	(−)	(++)	(+)	(++)
6	0 (IIc)	75	Ia	sm	por1	(−)	(++)	(++)	(+++)
7	3	110	IVb	si	tub2	(−)	(+)	(±)	(+)
8	2	40	II	mp	tub2	(−)	(++)	(+)	(++)
9	3	50	IIIa	se	tub2	(−)	(++)	(±)	(+)
10	3	45	IIIa	ss	tub2	(−)	(+)	(+)	(+)
11	3	40	IIIb	ss	tub2	(−)	(+)	(+)	(+)
12	4	110	IVb	se	por2	(−)	(±)	(−)	(±)
13	1	95	Ib	mp	pap	(−)	(+++)	(++)	(+++)
14	3	40	IIIa	ss	por1	(−)	(+)	(+)	(+)
15	4	120	IVb	se	por2	(−)	(±)	(−)	(±)
16	2	45	IVb	se	tub2	(−)	(++)	(+)	(+++)
17	3	55	IIIa	mp	por1	(−)	(++)	(−)	(++)
18	3	100	IIIb	se	por2	(−)	(±)	(−)	(±)
19	3	85	IVa	ss	por1	(−)	(+++)	(+)	(+++)
20	1	35	IIIa	ss	pap	(−)	(+)	(±)	(+)

a) Stromal cells and inflammatory cells.

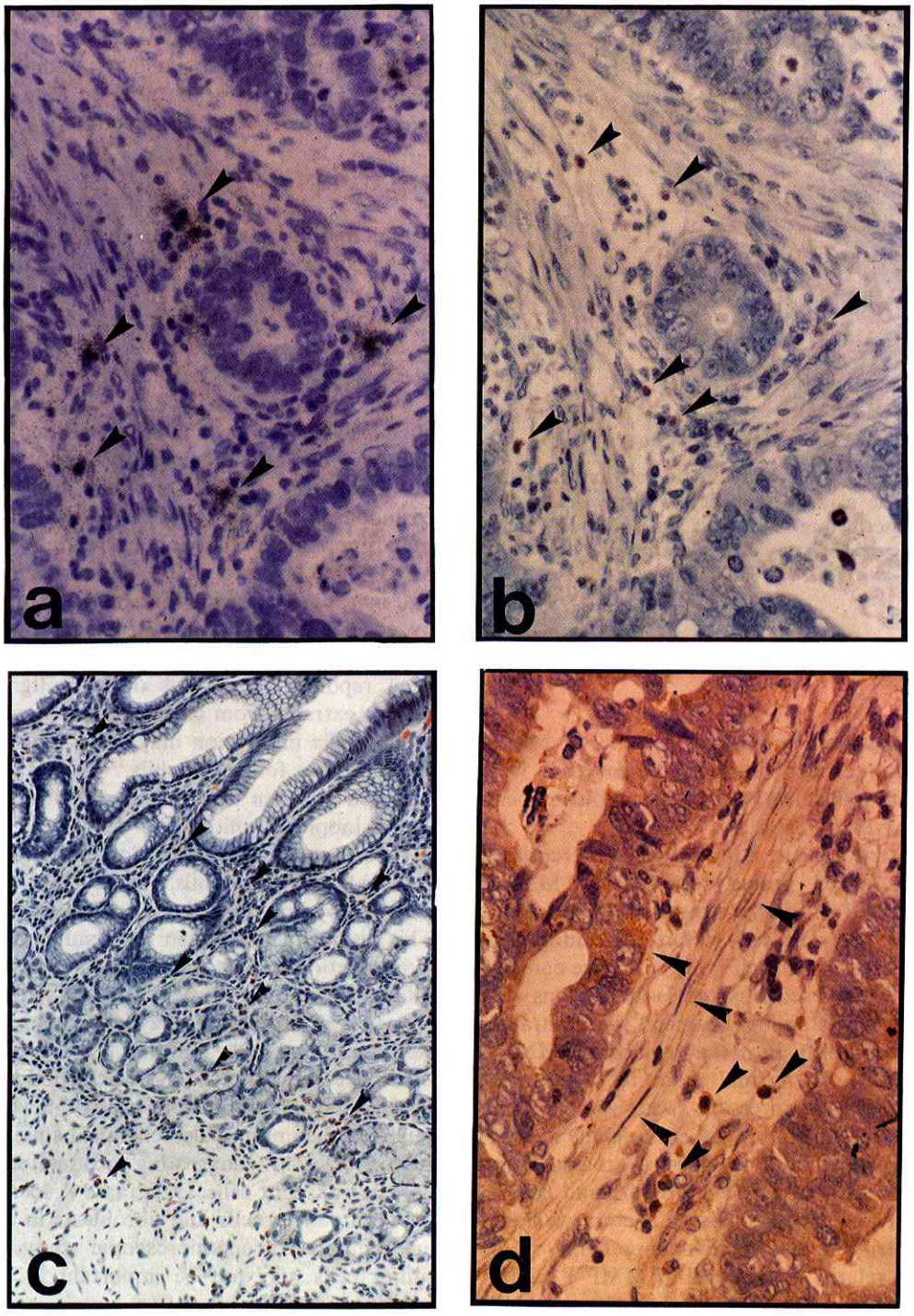

Fig. 1. Results in case 1, type 3 gastric cancer, moderately differentiated adenocarcinoma. a, *In situ* hybridization. Silver grains representing mRNA for MMP-1 are detected within the stroma of the neoplastic glands (arrowheads), while no definite signals are detected within endothelial, smooth muscle, or cancer cells. ×200, Original magnification. b, Luna staining. Eosinophils infiltrate the same regions where signals for MMP-1 are detected (arrowheads). ×200, Original magnification. c, Luna staining (non-cancerous region). Some infiltration of eosinophils is observed in the stroma (arrowheads). ×100, Original magnification. d, Immunohistochemistry for MMP-1 protein. Immunostaining for MMP-1 shows a localization not only to the stromal cells (granulocytes, fibroblasts), but also to the neoplastic glands (arrowheads). ×200, Original magnification.

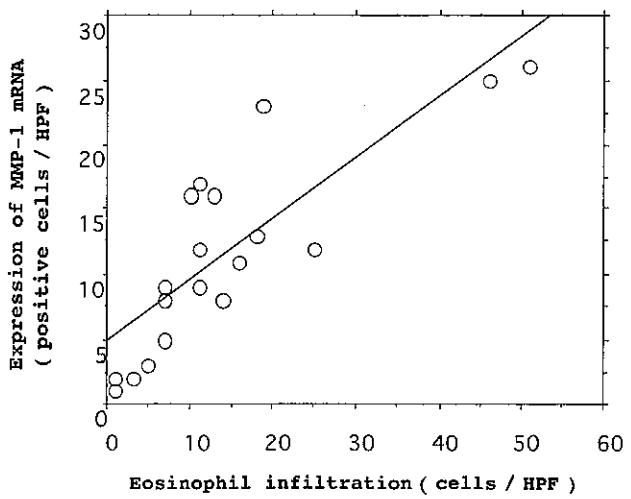

Fig. 2. Expression of MMP-1 mRNA in relation to eosinophil infiltration. The average number of positive cells (○) was obtained as described in "Materials and Methods." Statistical analysis was carried out by simple regression. $y=0.464x+4.993$, $r=0.817$, $P=0.0001$.

RESULTS

The histopathological findings of a total of twenty samples of gastric cancer tissue specimens that were subjected to *in situ* hybridization and immunohistochemistry are summarized in Table I.

In a type 3 gastric cancer, a moderately differentiated adenocarcinoma, silver grains representing MMP-1 mRNA were detected within the stroma of the neoplastic glands, but were not detected within the endothelial, smooth muscle, or tumor cells (Fig. 1a). Precise comparison between hybridized sections and serial sections with special staining (Luna staining) revealed the infiltration of eosinophils specifically into the regions of MMP-1 expression (Fig. 1b). In normal tissue from the same case, MMP-1 mRNA expression was not detected, though a degree of infiltration of eosinophils was observed (Fig. 1c). These results suggested an association between the expression of MMP-1 mRNA and eosinophilic infiltration, and prompted us to examine whether eosinophilic infiltration in the advancing front of the primary lesion is related to the level of MMP-1 mRNA expression. As shown in Fig. 2, the degree of eosinophilic infiltration was correlated to the level of MMP-1 mRNA expression. Immunostaining for MMP-1 showed localization of protein in the stromal cells (granulocytes, fibroblasts), and additionally in the neoplastic glands (Fig. 1d).

In contrast, type 4 gastric cancers (poorly differentiated adenocarcinoma, scirrhous type, por2), exhibited little or no expression of MMP-1 mRNA in the stromal or tumor cells (Table I). MMP-1 mRNA was found in the normal tissue associated with some gastric cancers (9/20). However, in six of these nine cases expression was at the lower limit of detection.

DISCUSSION

Our findings demonstrate that MMP-1 mRNA is actively expressed by eosinophils associated with the invasive front of gastric cancer tissues and that tumor cells, eosinophils and fibroblasts contain MMP-1 protein. These conclusions are based on *in situ* hybridization observations, which revealed the presence of MMP-1 mRNA specifically in stromal cells, and on immunohistochemical staining, which demonstrated enzyme protein in the stromal cells (eosinophils, fibroblasts), as well as in the neoplastic glands. Our findings suggest that tissue eosinophils actively produce MMP-1, and that the degree of eosinophilic infiltration correlates to the degree of MMP-1 mRNA expression in gastric cancer tissue. Several investigators have reported the expression of MMPs by eosinophils in colonic neoplasia and squamous cell carcinoma[10,11] and the stromal expression of MMP-1 has been reported in various other malignant tissues.[12–14] Tumor extracts from gastric cancer tissues with marked eosinophilic infiltration may show high chemotactic activity *in vitro* for eosinophils.[15] A relationship has been reported between the clinical prognosis of patients and the eosinophilic infiltration.[16–18]

In our study, the expression of MMP-1 mRNA was detected in eosinophils, while MMP-1 protein was detected by immunostaining in stromal and cancer cells. This discrepancy between the results obtained by *in situ* hybridization and immunohistochemistry may be due to differences in the quantity of MMP-1 mRNA and in the capacity for storage of enzyme protein between stromal cells and tumor cells. Another possible explanation is the difference in the thresholds of detection by *in situ* hybridization and immunohistochemistry. Several studies have reported the immunohistochemical staining of MMPs in tumor cells,[19,20] suggesting production or uptake of MMPs by tumor cells. Gelatinase A localization differed between cancer cells and fibroblasts in colon cancers in an immunoelectron microscopic study, suggesting that tumor cells may not be important in the secretion of gelatinase A.[21]

In scirrhous-type gastric cancer tissues, extremely low or no expression of MMP-1 mRNA and protein was observed in the stroma or tumor cells. We observed a large quantity of collagen fibers in the stroma of scirrhous type gastric cancers, whereas only a small number of inflammatory cells (neutrophils, eosinophils, lymphocytes) was detected (data not shown). These findings

may account for the very low expression of MMP-1 mRNA and protein observed in the stroma of scirrhous-type gastric cancers. In the early stages of scirrhous-type gastric cancer, when tumor cells are migrating into the stroma with inflammatory cells, high levels of MMP-1 expression would be detected. Alternatively these results may be due to the presence of adhesion molecules, such as E-cadherin,[22] laminin,[23] fibronectin[24] and tumor motility factor.[25]

Interstitial collagenase (MMP-1), which degrades the triple-helical domains of the fibrillar collagens (types I, II, III and X[4]), is augmented in many human tumors. Since Bassett *et al.* first described collagenolytic activity in eosinophils from rat peritoneal exudate,[26] eosinophil-mediated collagenolytic activity has been reported in inflammatory tissues.[27, 28] We also detected strong expression of MMP-1 mRNA and protein in gastric ulcer tissue, and a large number of eosinophils was detected in the same region (data not shown). Therefore eosinophil-derived MMP-1 may play a role not only in cancer invasion, but also in the remodeling of cancer tissues. In this manner, MMP-1 may have an influence on the histological and macroscopic types of gastric cancer.

Studies on the regulatory mechanism for expression of MMPs suggest that several cytokines and oncogenes may control transcription. The activity of MMPs in tumor-related stroma may be associated with IL-1,[29, 30] tumor necrosis factor α,[31] interferon-γ,[32] and transforming growth factor-β.[33] In our study, immunostaining for IL-1 was observed in granulocytes infiltrating into the stroma of neoplastic glands (data not shown). On the other hand, tissue inhibitor of metalloproteinases share the biochemical properties of inhibitors of MMPs.[34, 35] Details of the regulatory mechanisms that determine the expression of MMPs in tumors and in other diseases remain obscure, and further study is required.

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