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Overexpression of Tissue Inhibitors of Metalloproteinase-1 and -2 in the Stroma of Gastric Cancer

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The fundamental event of cancer invasion and metastasis is the complicated interaction of cancer cells with host cells, in which event, a number of proteases and their inhibitors are involved. Matrix metalloproteinases are the potent proteases in degrading the basement membrane and extra cellular matrix and are inhibited by specific endogeneous inhibitors, tissue inhibitors of metalloproteinases-1(TIMP-1) and TIMP-2. The expression of mRNA for TIMP-1 and -2 was investigated by Northern blot analysis in specimens taken from 27 patients with primary gastric adenocarcinoma ; 25 samples from the primary site, six from the metastatic lymph nodes and two from the peritoneal fluids. The expression for TIMP-1 and -2 was compared in primary gastric cancer tissues, metastatic lymph nodes and normal gastric mucosae. TIMP-1 mRNA was overexpressed in 24 (96%) out of 25 primary cancer tissues compared with the paired normal mucosae, while TIMP-2 was in 10 (40%). In six specimens of metastatic lymph nodes, TIMP-1 and -2 were overexpressed in 6 (100%) and 4 (67%) specimens, respectively. Of two specimens prepared from the peritoneal fluids, all specimens overexpressed TIMP-1 compared with the those of primary cancer tissues, while one (50%) specimen overexpressed TIMP-2. Immunohistochemical staining was done to investigate the localization of TIMP-1 and -2, demonstrating that the immunoreactivity for TIMP-1 and -2 was clearly detected in the cytoplasm of the stromal cells. These results suggest that both TIMP-1 and -2 are overexpressed by stromal cells in most of primary and some metastatic gastric cancer tissues and that TIMP-1 and TIMP-2, produced by stromal cells, may play an important role in inhibiting the proteolytic activity of matrix metalloproteinases originated from cancer cells, in gastric cancer.

Key Words : *Gastric cancer, Metastasis, TIMP-1, TIMP-2, Northern blot analysis, Immunohistochemistry*

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INTRODUCTION

Most malignant cancers have the natural course of life threatening progression, in which each step of the invasion and metastasis progresses by the complicated interactions between cancer cells and host cells and usually takes an intractable course once the cancer progresses (Liotta, 1986 ; Fidler, 1990). During the cascade of invasion and metastasis, a number of proteases, synthesized and secreted by cancer cells, are identified, such as matrix metalloproteinase(MMP), plasminogen activator, lysosomal hydrolase and cathepsins (Mullins and Rohrlich,1983 ; Hart et al.,1989 ; Buck et al.,1992 ; Stetler-Stevenson et al.,1993). As a response to these proteases, host cells have a barrier, such as basement membrane and extracellular matrix, which contain laminin, various types of collagens, fibronectin and heparan sulfate proteoglycan. In addition, recent studies have clearly demonstrated that some host cells regulate the degradative biochemical activity of cancer cells by producing certain inhibitors for proteases (Welgus et al.,1985a ; Welgus et al.,1985b ; Herron et al.,1986 ; Stetler-Stevenson et al.,1990).

This study was focused on the role of inhibitors of MMP, a kind of collagenase in the invasion and metastasis of gastric cancer. The MMP family consists of eight members and is subdivided into three general classes; interstitial collagenases, stromelysins and gelatinases (previously known as type IV collagenase) (Woessner, 1991 ; Stetler-Stevenson et al., 1993). The proteolytic activity of MMP is inhibited by a specific class of endogenous inhibitors named as tissue inhibitors of metalloproteinases (TIMPs) which are classified into TIMP-1 and -2 (Goldberg et al., 1989; Wilhelm et al., 1989)

TIMP-1 was reported to be a specific inhibitor to latent form of 92-kDa type IV collagenase (progelatinase B), while TIMP-2 was to be specific for latent and activated form of 72-kDa type IV collagenease (progelatinase A and gelatinase A) (Welgus and Stricklin,1983 ; Carmichael et al.,1986 ; Stetler-Stevenson et al.,1990 ; Stetler-Stevenson et al.,1993). The proteolytic activity of gelatinases is now proven to be suppressed by producing its specific inhibitors, TIMP-1 and -2. It is a generally accepted concept that stromal cells adjacent to cancer cells having a highly invasive potential produce a large amount of inhibitors as a defense mechanism against the invasion. Therefore, in tissues containing gastric cancer cells, the level of TIMPs may be increased by the local existence of a high amount of gelatinases, because human gastric cancer cells, in general, have the innately high potential for invasion and metastasis.

This study was conducted to examine the mRNA expression for TIMP-1 and -2 by Northern blot analysis to investigate the expression of TIMPs in gastric cancer tissues. We thought that it might be of great importance to clarify the localization of these inhibitors to understand the role of TIMPs. For this purpose, we have also compared the distribution of TIMP-1 and -2 by immunohistochemical staining in gastric cancer tissues.

MATERIALS AND METHODS

Specimens :

Twenty-five specimens of primary gastric cancer tissues and paired normal gastric mucosae were taken perioperatively from 25 patients with gastric adenocarcinoma treated at the Korea Cancer Center Hospital. During operation, metastatic abdominal lymph nodes were found in six patients. The presence of metastasis in lymph nodes was confirmed histologically before the specimens were examined. In two patients with malignant peritonitis, cancer cells were prepared from the peritoneal fluids without paired specimens. The specimens were immediately frozen in liquid nitrogen and stored at -70℃ until experimentation.

Isolation of RNA and Northern blot analysis :

Frozen tissues were pulverized and total RNA was extracted by guanidine thiocyanate-phenol-chloroform extraction method. Twenty g of RNA in each sample were size-fractionated on 1% agarose gel containing 2.0M formaldehyde and transferred to a nylon membrane (Schleicher & Schuell, Germany) by capillary transfer in 10×standard saline citrate (SSC) overnight. Before blotting, each gel was stained by ethidium bromide in order to visualize ribosomal RNA by ultraviolet (UV)-lighting to monitor the quality and quantity of mRNA of each specimen. The membranes were washed in 2×SSC and UV-cross-linked using UV Stratealinker 2400 (Stratagene, USA). The membranes were prehybridized overnight at 42℃ in 50% formamide, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 100 g/ml of salmon sperm DNA and 5×SSC and then hybridized overnight at 45℃. The proves for TIMP-1 and -2, kindly provided by Dr. Stetler-Stevenson WG, National Cancer Institute, USA, were radio labeled with [^{32}P] dCTP by the random primer method. Hybridized

filters were washed in 0.2×SSC and 1% SDS at 65℃ for 30 min. before autoradiography at -70℃ with intensifying screens. Overexpression of TIMP-1 and -2 was considered to be the increased intensity of the band in test specimen compared with that in corresponding control specimen. In two specimens prepared from the peritoneal fluids, the expression was determined by comparison with the expression of other specimens of the representative cases, because paired control tissues were not available for these specimens.

Immunohistochemistry :

Frozen cancer tissue blocks, prepared from the specimens taken during the operation for gastric cancers, were processed for the determination of TIMP-1 and -2 by immunohistochemical method using primary anti-TIMP-1 and -2 antibody (American diagnostica inc. Greenwich, USA). The details have been described previously (Hong et al.,1994). In brief, sections were cut from the frozen cancer blocks and mounted on silane-coated glass slides. To block the endogenous peroxidase activity, the slides were incubated in methanol with 0.3% hydrogen peroxide for 30 min. at room temperature. The slides were treated with 3% normal goat serum for 15 min. to block the nonspecific binding and then incubated with primary anti-TIMP-1 and -2 antibody diluted 1:100 in a moisture chamber for 2 hr at room temperature. After washing in phosphate buffered saline, the slides were incubated with a avidin-biotinyl peroxidase complex using a Vectastain ABC kit (Vector Laboratories, CA) for 30 min. The slides were washed again with phosphate buffered saline and then incubated in a solution containing 0.02% diaminobenzidine substrate and 0.03% hydrogen peroxide. As a negative control, phosphate buffered saline was used instead of the antibody. Nuclear counterstaining was performed with Mayer's hematoxylin solution.

RESULTS

The expression of mRNA for TIMP-1 and -2 was investigated by Northern blot analysis in 33 specimens taken from 27 patients with gastric adenocarcinoma ; 25 samples from the primary site, six from the metastatic lymph nodes and two from the peritoneal fluids. The expression for TIMP-1 and -2 was compared between primary gastric cancer tissues, metastatic lymph nodes and normal gastric mucosae (Fig. 1). TIMP-1 mRNA was overexpressed in 24 (96%) out of 25 primary gastric cancer tissues compared to the paired normal gastric mucosae (Table 1). Overexpressed TIMP-2 was observed in 10 (40%) of 25 primary gastric cancer tissues.

In six specimens of metastatic lymph nodes, TIMP-1 and -2 were overexpressed in 6 (100%) and 4 (67%) specimens, respectively, compared to the paired normal gastric mucosae. However, when compared with those of primary gastric cancer tissues, TIMP-1 and -2 were

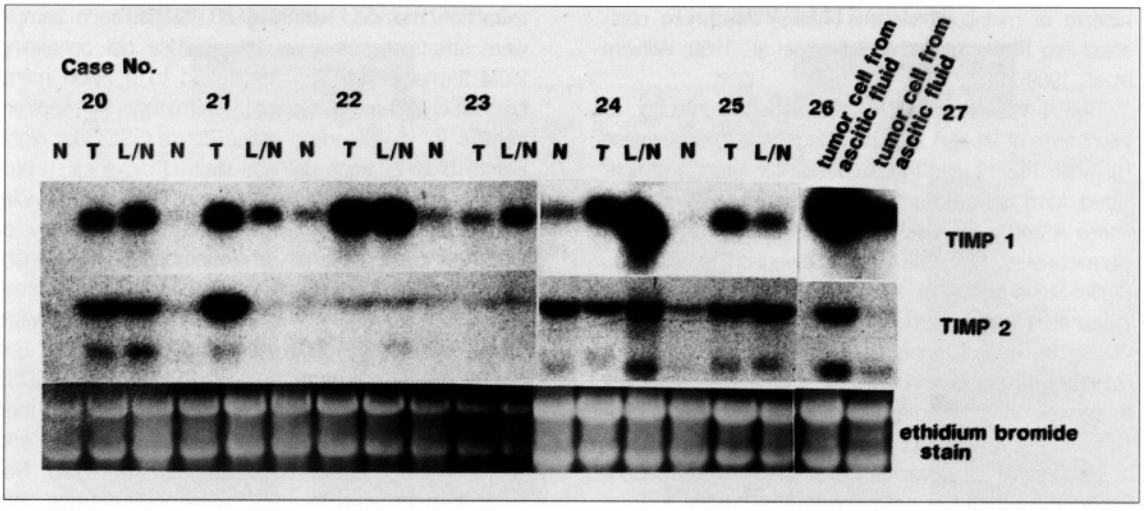

Fig. 1. Representative Northern blot analysis of normal gastric mucosa, primary gastric cancer tissue and metastatic lymph node. Blots were hybridized with probes for TIMP-1 and -2. N, normal gastric mucosa ; T, primary gastric cancer tissue ; L/N, metastatic lymph node.

Table 1. Overexpression of TIMP-1 and TIMP-2, compared with normal gastric mucosa, in 25 primary gastric cancer tissues, six metastatic lymph nodes and two specimens prepared from the malignant peritoneal fluids.

	Primary gastric cancer	Metastatic lymph nodes	Peritoneal fluids
No. of specimens	25	6	2
TIMP-1	24 (96%)*	6 (100%)	2 (100%)
TIMP-2	10 (40%)	4 (67%)	1 (50%)

* number of specimen overexpressed (%)
Overexpression of TIMP-1 and TIMP-2 was determined by comparing the mRNA expression of mRNA of tested tissue with that of control tissue.

overexpressed in 2 (33%) and 1 (17%) specimens, respectively. TIMP-1 and -2 were also tested in two specimens prepared from the peritoneal fluids. Of these two specimens, all specimens overexpressed TIMP-1 compared to the normal gastric mucosae, while one (50%) specimen overexpressed TIMP-2.

In order to investigate the localization of TIMP-1 and -2, we conducted immunohistochemical staining in primary gastric cancer tissues. As shown in Fig. 2, the positive reaction to immunohistochemical staining for TIMP-1 and -2 was clearly detected in the cytoplasm of stromal cells, suggesting that stromal cells are the main site of the production of these inhibitors.

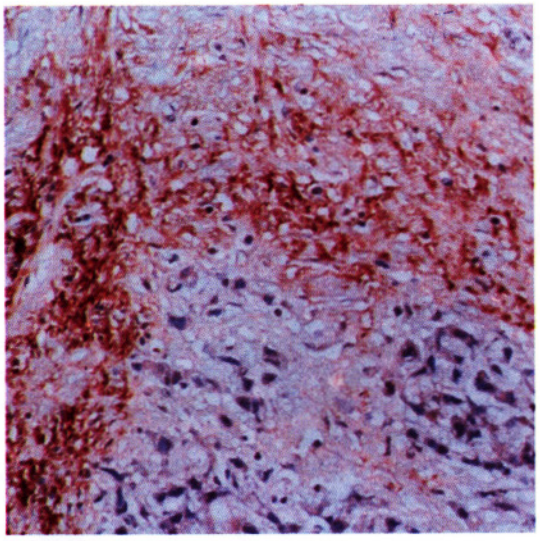

Fig. 2. Representative case of immunohistochemistry for TIMP-1 in gastric adenocarcinoma tissue. Positive stain was evident in the stromal cells.

DISCUSSION

During the invasion and metastasis cascade with MMP, the balance between active MMPs and TIMPs, which is endogenous potent inhibitors for MMPs, is believed to be the critical determinant for the degradation of local matrix (Herron et al.,1986 ; Stetler-Stevenson et al.,1990 ; Stetler-Stevenson et al.,1993). TIMP-1 is a glycoprotein which has 184 amino acids and six intramolecular disulfide bonds (Welgus et al., 1985a ; Fidler, 1990). TIMP-1 was known to form a complex of 1:1 stoichiometry with activated interstitial collagenase, stromelysin and gelatinase B (Welgus and Stricklin,1983 ; Stetler-Stevenson et al.,1993). Although in vivo distribution and the role of TIMP-1 are not clear at present, cells producing gelatinase B are also reported to synthesize and secrete TIMP-1 (Herron et al., 1986 ; Goldberg et al., 1989 ; Sato et al., 1992).

TIMP-2, a recently isolated second member of the TIMP family, is the glycoprotein which inhibits the latent and active forms of gelatinase A (Ward et al., 1991 ; Stetler-Stevenson et al., 1993). Recent studies have shown that the levels of TIMP-1 and -2 are inversely correlated with the invasive potential in both murine and human cancer (Kubota et al., 1991 ; DeClerck et al., 1992 ; Testa, 1992). Therefore, the balance of local concentrations of gelatinases and TIMPs is of great importance in the invasion of cancer cells.

In human cancers, the invasive characteristics of TIMP-1 and -2 have not been widely examined so far. Stetler-Stevenson et al. (1990) reported that TIMP-1 level was elevated in all five tissues obtained from colonic adenocarcinoma. In contrast, Sato et al. (1992) reported that TIMP-1 mRNA was undetectable in 50% of cancer cell lines tested, while TIMP-2 mRNA was expressed in the majority of 26 cancer cell lines originated from mesenchymal tumors and epithelial tumors.

This study was conducted to investigate the expression and role of TIMP-1 and TIMP-2 in gastric cancer tissues and demonstrated that TIMP-1 was overexpressed in 96% of primary gastric cancer tissues, while TIMP-2 was in 40%. In six specimens of metastatic lymph nodes, TIMP-1 and -2 were overexpressed in 100% and 67%, respectively, when compared with that of normal mucosa. However, when compared with those of primary gastric cancer tissues, TIMP-1 and -2 were overexpressed in 2 (33%) and 1 (17%) specimens, respectively.

These results indicated that TIMP-1 expression was higher than TIMP-2 in primary gastric cancer tissues. In

metastatic lymph nodes, the intensity of the expression for TIMP-1 and -2 decreased compared with that of primary cancer tissues. The reason for the decreased expression of TIMP-1 and -2 in metastatic lymph nodes is not clear at present. One explanation is that the production and secretion of gelatinases may be decreased by immune cells in lymph node or that lymph node cells invaded by cancer cells can not produce TIMPs appropriately. However, to find out the exact mechanisms, further studies will be necessary.

TIMPs are known to be produced either by cancer cells for the protection of themselves against various degradation enzymes or by adjacent normal cells as a defense against cancer invasion (Stetler-Stevenson et al., 1993). The main site of the production of TIMPs, however, has not been fully established. In order to identified the major site and cells producing TIMPs, immunohistochemical staining was performed on the gastric cancer tissues, demonstrating that TIMPs were detected clearly in the cytoplasm of stromal cells and faintly in cytoplasm of tumor cells, which was consistent with the finding observed by Hoyhtya et al (1994). If the localization indicates the site of synthesis of TIMPs, the majority of TIMPs, present in the cancer tissues, are the product of stromal cells.

From these results, we suggest that TIMPs are produced by stromal cells to decrease the activity of gelatinases which are produced by cancer cells to facilitate invasion using gelatinases at the surface of the cancer cell. However, to establish the precise role of TIMPs in stromal cells and cancer cells in tumor tissues, further studies are required.

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