Chapter 4

Cathepsin B on invasion and metastasis of gastric carcinoma

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ABSTRACT

**Objective:** To investigate the expression of cathepsin B (CB) in human gastric carcinoma tissue.

**Methods:** The expression of CB in human gastric tissue was studied by using monospecific polyclonal rabbit antibody raised against human liver CB for immunohistochemistry, and full length cDNA of CB for *in situ* hybridization and dot blot.

**Results:** CB overexpression in gastric carcinoma was found when compared with non-neoplastic gastric tissue at both mRNA and protein levels. Diffuse cytoplasmic CB staining of mRNA and protein were identified in malignant cells of 53.3% and 69.1% of gastric adenocarcinoma respectively. The increased staining of CB in malignant cells was associated with the depth of the invasiveness and growth pattern as well as metastasis of lymph nodes, but not with the histological classification. It was also found that there were the expression of CB in stromal cells of the tumor and the expression localized mainly in the endothelial cells of the microvessels which correlated with angiogenesis.

**Conclusion:** These results indicate that the expression of CB in gastric carcinoma is related to tumor progression, and leads to development of the invasive phenotype.
INTRODUCTION

The biological behavior of neoplasm hinges on its growth pattern and malignant phenotype—invagination and metastasis. The mechanism of tumor invasion and metastasis is complex and multifactorial. Tumor cells are supposed to have a serial enzymatic proteolysis which digest the extracellular matrix for metastasis. The functions of the proteinases produced by tumor cells and/or stromal cells in malignancies have been the focus of active investigation. It has been shown that many classes of proteinases correlate with the invasiveness and metastasis of the tumor, including metalloproteinase, cysteine proteinase, aspartic proteinase and serine proteinase. Cathepsin B (CB) that we studied is one of the cysteine proteinases, which is a lysosomal proteinase that functions in the normal turnover of cell protein. It can degrade several extracellular matrix components such as laminin, fibronectin and Type IV collagen. It has been demonstrated that overexpression of CB is correlated with progression of malignancy. However, most of these evidences were derived from the studies on breast, colorectal or prostatic carcinoma. We have recently demonstrated enhanced activity of CB in gastric carcinoma, and the high CB activity was due to insufficient inhibitory activity of endogenous cysteine proteinase inhibitors. In this study, we used immunohistochemistry, nonradioactive in situ hybridization (ISH) and dot blot to analyze the effect of CB on invasion and metastasis of gastric carcinoma.
METHODS AND MATERIALS

Materials

Polyclonal antibody raised against human liver CB and PTF7 CB plasmid (containing full length cDNA of CB) were developed in Dr. Sloane's laboratory. PK-4001 ABC kit was from Vector Co. (USA). 3'-3 diaminobenzidine tetrahydrochloride (DAB), poly-L-lysine and sperm DNA were from Sigma Co. (USA), Dig-DNA labeling and detection kit, and nitrocellulose membrane were from Boehringer Mannheim Co. (Germany). Restriction enzyme BamH I and Nco I, and protease K were from Promega Co. (USA).

Specimens

Eighty-five specimens of gastric adenocarcinoma and matched non-neoplastic tissues (more than 5 cm from the tumor margin) were obtained from Ren Ji Hospital, Shanghai Second Medical University from 1994 to 1996. The study group comprised 56 men and 29 women with a mean age of 61 (range of 31-86) years. Tumors were classified according to their histological types, \(^1\) well differentiated (n = 27) and poorly differentiated (n=58). The pathological staging was according to the criteria of the depth of invasion (i.e., extension into the muscularis propria) with early stage (n = 16), and advanced stage (n = 69). The growth pattern of tumors as described by Ming, \(^2\) included expanding type (n = 38) and infiltrative type (n = 47).

Immunohistochemistry (IHC)

All specimens used in this study (n = 55) were routinely fixed in 10% formalin and embedded in paraffin. Sections (5 \(\mu\)m) cut from the tissue blocks were dewaxed in xylene and then rehydrated. The endogenous peroxidase activity was blocked with 0.3% \(\text{H}_2\text{O}_2\) in methanol for 30 minutes at room temperature. Slides were then incubated with normal goat serum for 30
minutes at room temperature, followed by incubation with primary antibody at 4 °C overnight. Control slides were produced by replacing the primary antibody with PBS. After washing in PBS (pH 7.2), the sections were incubated with the second antibody (biotinylated goat anti-rabbit) at a 1: 200 dilution. The slides were washed again in PBS and then incubated for 30 minutes with an avidin and biotinylated peroxidase complex (Vectastain; Vector Laboratories). Peroxidase activity was developed by a solution of 5 mg of 3'-3' diaminobenzidine tetrahydrochloride (DAB) at room temperature under microscopic control. Finally, the sections were washed in double-distilled water, counterstained, and the immunoreactivity was assessed under the light microscope.

Immunohistochemical stains were evaluated by two independent observers. Reactivity was scored using a combined quantitative (percentage of tumor cells with immunoreactivity) and qualitative (intensity of staining as none, weak and strong; staining pattern: granular or diffuse staining).

**In situ hybridization (ISH)**

The probe used was a full length cDNA fragment of CB (1020 bp) obtained from PTF7 CB plasmid with excision of restriction enzyme Nco I and BamH I. CBcDNA probe was labeled with DIG11-dUTP using the random primer labeling method following the manufacturer's procedure (Boehringer Mannheim kit).

All specimens used in this study (n = 30) were snap frozen within 30 minutes after surgical resection and stored at-70 °C until use. Tissue blocks were fixed in 4% paraformaldehyde (PFA) in PBS, pH 7.0 for 2 hours and embedded in paraffin. All sections were cut onto poly-L-lysine-coated slides. H&E stained sections were evaluated to confirm the presence of tumor within the tissue samples and their histological appearance. Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated following standard protocols. The sections were incubated with 0.3 % Triton X-100/PBS for 15 minutes, followed by 0.2 N HC1 for 20 minutes at room temperature. Slides were then
digested with proteinase K (20 μg/ml) and fixed with 4 % paraformaldehyde again. The post-PFA fixed slides were incubated with prehybridization buffer (50% formamide, 1 x denhardt's solution, 5 % dextran sulfate, 200 μg/ml boiled sheared salmon sperm DNA) at 42 °C for 2 hours. After incubation, the prehybridization buffer was removed and the hybridization buffer containing DIG-labeled denatured probe (final concentration 1 μg/ml) was added. The slides were each covered with a coverslip pretreated with Sigmacote (Sigma). The hybridization reaction was performed at 42 °C overnight in a humid box. In addition to using sense probe, negative controls include both elimination of DIG-labeled probe in the hybridization buffer and pretreatment of sections with RNase A (37 °C, 2 hours) before hybridization. After hybridization reaction, the coverslips were gently removed in 2 x standard saline citrate (SSC). Sections were then washed sequentially at 40 °C with 2 x SSC for 20 minutes, 1 x SSC for 15 minutes and 0.1 x SSC for 30 minutes. Sections were incubated with 1 % blocking reagent (Boehringer Mannheim) in buffer I (100 mmol/L Maleic acid, 150 mmol/L NaCl, pH 7.5) for 30 minutes and then incubated with alkaline phosphatase-conjugated antidigoxigenin F (ab) fragments (1 : 1000 dilution, Boehringer Mannheim) at room temperature for 2 hours. The slides were then washed twice in buffer I for 15 minutes and rinsed in buffer II (100 mmol/L NaCl, 100 mmol/L Tris-HCl, 50 mmol/L MgCl<sub>2</sub>, pH 9.5). The sections were covered with chromogen substrate solution (45 μl NBT solution and 35 μlX-phosphate solution in 100% dimethylformamide in 10 ml buffer II) and exposed in the dark for 6-8 hours. After development, the sections were counterstained with fast red.

**Dot hybridization**

Total RNA was extracted from 10 fresh specimens of gastric cancer tissue and the matched neighboring non-neoplastic tissues according to the manufacturer's protocol with TRIZol [Gibco BRL Co.]. RNA concentration was determined by spectrophotometric reading at A<sub>260</sub>. RNA samples (10 μg) were dissolved in formaldehyde plus 10 x SSC and denatured at 68 °C.
for 15 minutes before loading onto the nitrocellulose membrane. After RNA loading, the nitrocellulose membrane was baked at 80 °C for 2 hours. The dried filter was incubated at 42 °C for 3 hours in prehybridization buffer (50 % formamide, 7 x SDS, 5 x SSC, 0.1 % sodium pyrophosphate [ inorganic ], 1 % blocking reagent [ Boehringer Mannheim ] , 1 μg/ml of salmon sperm DNA). Hybridization was performed overnight at 42 °C in a fresh mixture of the prehybridization buffer with DIG-labeled CB cDNA probe as described in *in situ* hybridization. After hybridization reaction, filter was washed sequentially and color reaction was developed with NBT plus BCIP as described in *in situ* hybridization.

**Statistical analysis**

Chi-square test was used for comparison between frequencies. Results were considered significant at P < 0.05 .
RESULTS

Immunostaining distribution of CB in gastric carcinoma

Immunostaining was performed using a polyclonal antibody to detect mature form of CB. The sections of non-neoplastic mucosa showed either a negative or a weak granular CB staining located in perinuclear area. When compared with non-neoplastic mucosa, the intensity of CB staining within gastric cancer tissue was greatly increased. The number of cells staining positive for cathepsin B was much higher within tumor tissue (69.1%, 38/55) than those in non neoplastic mucosa (15%, 3/20) (Fig. 1). In most tumor tissues, neoplastic cells showed a diffuse staining throughout the cytoplasm (Fig. 2a). Increased staining of CB in stromal cells of the tumor also displayed. Occasionally, cathepsin B immunostaining was detected in the endothelial cells within the tumor tissue (Fig. 2b), suggesting that CB expression in these tumors and endothelial cells should play some role in angiogenesis of gastric carcinoma.3

CB mRNA expression in gastric carcinoma

To detect whether or not CB mRNA level was also increased in gastric carcinoma, dot hybridization with digoxin-labeled CB probe was performed using 10 matched samples of gastric carcinoma tissue with neighboring non-neoplastic tissues. As shown in Fig. 3a, CB mRNA levels were increased significantly in all tumor tissues as compared with their matched non-neoplastic mucosa. However, the limitation of dot hybridization was that it could not differentiate the origin of CB signal from the tumor cell itself or from other cells. Therefore, we further studied with in situ hybridization using 30 fresh samples of gastric carcinoma. As shown in Fig. 3b, control sections using anti-sense probe and pretreating with RNase showed negative result. The positive CB staining was much higher in gastric carcinoma tissue (16/30 cases, 53.3%) than that in non-neoplastic tissues (2/20 cases, 10%). The statistical difference is significant (Fig. 1). In addition, CB signal was much stronger in neoplastic cells than in non-neoplastic cells, especially in the tumor nest (Fig. 3c). Interestingly, in some tumor tissue, the positive CB staining was also localized in the endothelial cells of the microvessels near tumors. Whereas in normal tissue, none of the endothelial cells of the microvessels were stained.

CB expression and tumor growth patterns and clinical stage (Figs. 4 and 5)
As described in previous section, we found that CB expression was elevated in gastric carcinoma tissue both at mRNA and protein levels. We also found that the data from both immunohistochemical study and from *in situ* hybridization are comparable and in good agreement (data not shown). Overexpression of CB at both mRNA and protein level was correlated with both growth patterns and clinical stage of gastric carcinoma (Fig. 4) in this study. In addition, a correlation was observed between CB expression and positive lymph node status but not with the histological type of the tumors (Fig. 5).
DISCUSSION

Gastric carcinoma remains as a major cause of death worldwide and is the most common fatal malignancy in China. Majority of these are attributed to local invasion or distant metastases, and the management of these patients is far from satisfaction. Therefore, recognizing the overexpression of CB as a marker of invasion and metastasis, one would be able to choose the right option therapeutically.

The results depicted in this paper showed overexpression of CB in gastric carcinoma when compared with neighboring non-neoplastic tissue at both mRNA and protein levels ($P < 0.01$). We found marked difference in the staining of CB in non-neoplastic tissue and in malignant cells: non-neoplastic cells showed characteristic punctuate paranuclear staining, which was consistent with the localization of a lysosomal enzyme. In contrast, the neoplastic cells showed diffuse cytoplasmic pattern or localization of the enzyme at the basolateral part of the cells. We speculated that there might be specific plasma membrane-associated CB in the cytoplasm of the tumor cells. Another possibility is due to the lack of a signal peptide during tumor progression. The enzyme cannot enter the lysosome and cause a shift in the distribution of the active enzyme from lysosomal to plasma membrane. A similar pattern had also been observed by Campo, in colorectal carcinoma. The human CB gene was composed of 13 exons and 11 introns, the translation initiation site was in exon 3. But in case of neoplasm, mRNA transcriptions missing exon 2 and exon 3 might generate a truncated procathepsin B protein (CB mRNA 2'3'). Because of lack of a signal peptide, this truncated protein would be expected to be cytoplasmic rather than lysosomal. That is to say CB protein traffics from lysosome to cytoplasm. Such forms are perhaps responsible for the diffuse cytoplasmic staining, no matter whether lysosomal CB and/or cytoplasmic CB are important to invasion and metastases of the tumors. The subcellular changes may facilitate the exposure of the active enzyme to extracellular substrate. Sloane also demonstrated the increased activity of CB in malignant tumors, the correlation between high CB activity and the alteration in CB
synthesis and/or in the endogenous inhibitors of CB as well as in intracellular trafficking and delivery.

The expression of CB in gastric carcinoma is increased at both mRNA and protein levels. The high CB expression in the tumor cells further correlates with the depth of the invasive tumor cells, its growth pattern as well as metastasis to lymph nodes. It means: at both levels the expression of CB in infiltrative type is higher than that in expanding type, higher in advanced stage than that in early stage, higher in lymph nodes metastasis than those without. This discrepancy clearly indicates a fundamental difference in their growth potential and the power of penetration and invasion. It also shows the close relationship between the aggressive behavior of gastric carcinoma and CB expression. It may eventually influence the survival and prognosis. Our results are in good agreement with the finding in both prostatic carcinoma\(^6\) and colorectal carcinoma.\(^4\) The data suggest that CB is a marker of the malignant phenotype--invasion and metastasis.

CB expression at mRNA level correlates well with the histological type. Although there is a trend that the positive percentage of CB in poor-differentiated gastric adenocarcinoma (66.7 %) is higher than that in well-differentiated type (33.3 %), it has not reached statistical significance due in part to the small number of patients within each subset. The expression of CB is not associated with the histological classification at protein level. These apparent contradictory results may reflect regional heterogeneity in regulation at transcription and/or post-transcription level.

Our most interesting finding was the increased staining of CB in stromal cells of the tumor and the expression localized mainly in the endothelial cells of the microvessels. In normal condition, CB is secreted by stromal cells too, such as fibroblast and macrophage. But in case of neoplasm, the CB expression in stromal cells is increased. This suggests that the presence of CB in stroma of gastric carcinoma should be associated with a leakage and overproduction of the enzyme, and a cooperation between the stromal cells and the tumor cells in the tumor...
progression. The similar results have been reported in colorectal carcinoma by Campo\textsuperscript{4}. The development of malignancy is concerned in tumor-induced angiogenesis that not only allows for expansion of the primary tumor, but also permits easy access to the vascular compartment because of defects in the basement membrane of newly formed vessels. This may facilitate hematogenous spread of the tumors. The positive staining of CB in the endothelial cells of the microvessels suggests that CB should play a role in angiogenesis of gastric carcinoma.

REFERENCES


Fig. 1. CB expression in tumor cells and non-neoplastic cells.
Fig. 2a. Immunostaining for CB in papillary gastric carcinoma with diffuse cytoplasmic staining. (x 280)

Fig. 2b. The relative density and distribution of CB immunostained microvessels in the stroma of the gastric carcinoma. (x 280)
Fig. 3a. The comparison of CB mRNA content between gastric carcinoma tissue and matched pairs of normal tissue by dot blot.

Fig. 3b. Negative CB signal in gastric carcinoma without using sense probe. (x 100)

Fig. 3c. Increased expression of CB in the tumor nest by using in situ hybridization. (x 280)
Fig. 4. CB expression in gastric carcinoma with reference to growth pattern and clinical stage

Fig. 5. CB expression in gastric carcinoma with reference to histological classification