Detection of gelatinase B activity in serum of gastric cancer patients

Vesna V Dragutinović, Nebojša S Radovanović, Lidija T Izrael-Živković, Miroslav M Vrvić

Abstract

AIM: To determine the proteolytic activity and expression of gelatinase B in serum of gastric cancer patients and their correlation with the stage of the tumor.

METHODS: Sera from 23 patients who underwent surgery for primary gastric cancer as the experimental group and from 11 as the control group were used to determine the proteolytic activity and its inhibition by EDTA and 1,10-phenanthroline. Gelatinase B activity was detected by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and SDS-PAGE zymography.

RESULTS: Proteolytic enzyme activity was increased in gastric cancer patients when compared to the control group (P<0.05). The proteinases were determined to be metalloproteinasises upon inhibition test with specific metalloproteinanase inhibitors 1,10-phenanthroline (P<0.05) and EDTA (P<0.01). SDS-PAGE and SDS-PAGE zymography revealed gelatinase B (proMMP-9) activity and its molecular mass of 92 ku.

CONCLUSION: Proteinase activity is overexpressed in serum of gastric cancer patients. Gelatinase B in serum plays an important role in the progression of gastric cancer. ProMMP-9 can be used as a marker for invasiveness of gastric cancer.

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Key words: Matrix metalloproteainase-9; Gastric cancer; Proteolytic activity; Inhibition

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INTRODUCTION

Proteolysis occurs in normal tissue but is limited in duration. A general aspect of malignant neoplasms may be an unbalance of proteolysis, which favors invasion[1].

Tumor progression is a step-wise process. Multiple alterations in normal cells can lead to a localized tumor that can finally invade the surrounding tissues and metastasize. Tumor cell invasion involves attachment of tumor cells to the underlying basement membrane, local proteolysis and migration of tumor cells through the proteolytically modified region[2]. Local proteolysis is facilitated by proteinases outside the tumor cells, perhaps bound to the cell surface and/or secreted from the tumor cells. Recent data suggest that proteinases inside the tumor cells also participate in local proteolysis by digesting phagocytic extracellular matrix. In order to metastasize, cells must be able to move into the vasculature (intravasation), survive in the circulation, move out of the vasculature (extravasation), invade the surrounding tissues and grow. All these steps involve interactions among tumor cells, stromal cells, invading lymphocytic cells, endothelial cells, and extracellular matrix. Proteinases expressed in these cells are believed to participate in many of these steps[8-10].

Matrix metalloproteinases (MMPs) are extracellular enzymes capable of degrading many extracellular matrix proteins. They are classified into five groups according to their structure and substrate specificity: collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs. There is considerable evidence that MMPs play a major role in diverse physiologic processes and pathologic processes, including aspects of embryonic development, tissue morphogenesis, wound repair, inflammatory diseases and cancer. Overexpression and activation of MMPs have been linked with a variety of diseases[8-10]. In the matrix metalloproteinase of MMP family, including a 72 ku enzyme resembling matrix metalloproteinase-2 (MMP-2) known as gelatinase A and
a 92 ku enzyme resembling matrix metalloproteinase-9 (MMP-9) known as gelatinase B, have been demonstrated to be closely associated with several tumor systems and to invasive potential of tumor cells[11-13]. Type IV collagenase can degrade not only interstitial matrix but also the basement membrane. Malignant ascite[14] is the direct and prominent manifestation of advanced malignant diseases associated with invasion and metastasis of peritoneal cavity by tumor cells. In the present study, we detected the gelatinase B activity in the sera from patients with gastric cancer by gelatin zymography in order to provide the scientific basis for clinical diagnosis of gastric cancer.

MATERIALS AND METHODS

Reagents

N\(^7\)-benzoyl-arginine p-nitroanilide hydrochloride (BAPNA), EDTA and 1,10-phenanthroline were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The chemicals used for electrophoresis were from Merck (Darmstadt, Germany). Gelatin was purchased from Difco (Detroit, MN, USA). The mini gel electrophoresis equipment SE260 was from Hoefer Scientific Instruments (San Francisco, CA, USA).

Clinical specimens

In this study, we used 23 patients with gastric cancer as the experimental group and 11 patients as the control group. All patients with gastric cancer underwent surgery in the Institute for Digestive Diseases, Clinical Center of Serbia, from June 2002 to January 2004 and received neither chemotherapy nor radiation therapy before surgery. Of these patients, 15 (65%) were men and 8 (35%) were women with a mean age of 58 years (range: 38-75 years).

We had preoperative pathological diagnoses for all patients. Eleven patients underwent abdominal exploration or feeding jejunostomy because of liver metastases, peritoneal dissemination or malignant ascites. Twelve patients underwent radical surgery. In these patients, pathological examinations including depth of the tumor invasion, vascular invasion, lymphatic permeation, and lymph node metastasis were made according to the general rules of gastric cancer outlined by the Japanese Research Society for Gastric Cancer. In the control group, all the 11 patients were diagnosed to have groin hernia.

According to the TNM Classification System of the UICC, there were 2 stage 1, 3 stage 2, 5 stage 3, and 2 stage 4 tumors. According to their histological differentiation, there were 3 well, 4 moderately, and 5 poorly differentiated tumors. The clinicopathological features were found by reviewing all HE stained tissue sections.

Proteolytic activity

Proteolytic activity was determined using the method described by Ebeling et al[15].

Metalloproteinase inhibition test

The effect of EDTA and 1,10-phenanthroline (in concentration of 5 mmol/L) on proteolytic activity of the serum was examined. The serum was incubated at 37 °C for 30 min and the remaining proteolytic activity was determined under standard conditions.

SDS-PAGE

SDS-PAGE was performed with 75 g/L polyacrylamide gel[16] under no reduction conditions using a solution mixture of protein markers containing ovalbumin (45 ku), bovine serum albumin (BSA, 67 ku), β-galactosidase (116 ku) and myosin (200 ku). Serum was diluted in 200 g/L sucrose to prepare the samples. The samples were analyzed by SDS-PAGE to determine the molecular mass.

SDS-PAGE zymography

Samples were analyzed by SDS-PAGE zymography according to the method of Kleiner and Stetler-Stevenson[17] to determine the molecular mass and relative abundance of the gelatinases present. Samples were incubated for 40 min at 37 °C and electrophoreses were performed without reduction of 75 g/L polyacrylamide gels copolymerized with 0.01 g/L gelatin at 4 °C at a constant current of 15 mA. When the tracking dye at the front reached the bottom of the gel, the gel was removed and shaken gently for 45 min in 0.25 g/L Triton x-100 to remove SDS. Then the gel slabs were transferred to a bath (without Triton x-100) and washed for 20 min to remove Triton x-100. The above procedure was repeated twice at 4 °C. Then the gels were incubated and shaken for 60 h in 0.1 mol/L glycine, 50 mmol/L Tris-HCl, 5 mmol/L CaCl\(_2\), 1 mmol/L ZnCl\(_2\), 0.5 mol/L NaCl, pH 8.3, at 37 °C. Regions of proteolytic activity were visualized as clear zones against a blue background after 3-h staining with Coomassie brilliant blue.

MMP inhibition test on zymography

In order to verify that the clear zones resembling matrix metalloproteinase, 5 mmol/L EDTA was added into the samples before incubation to inhibit MMP activities on gelatin zymography.

Statistical analysis

Mann-Whitney test and Wilcoxon signed rank test were used for statistical analysis. P<0.05 was considered statistically significant.

RESULTS

Proteolytic activity

Proteolytic activity was increased (P<0.05) in gastric cancer patients compared to the control group (Tables 1 and 2). On the other hand, there was no significant correlation in proteolytic activity among the patients after radical or palliative surgery.

Metalloproteinase inhibition test

EDTA and 1,10-phenanthroline inhibited proteolytic activity on BAPNA superstrate in the sera of patients with gastric cancer. 1,10-phenanthroline (P<0.05) showed less inhibition on proteolytic activity than EDTA (P<0.01) (Table 1).

Detection of gelatinase B in serum

The samples of gastric cancer patients were shown on
Table 1: Proteolytic activity and inhibition of metalloprotease activity in serum of patients with gastric cancer

| Sample | BAPNA (mU) | EDTA (%) | Inhibition in presence of EDTA (%) | 1,10-Phen (mU) | Inhibition in presence of 1,10-phen (%) | TNM stage |
|--------|------------|----------|-----------------------------------|----------------|----------------------------------------|-----------|
| 1      | 8.6        | 1.4      | 83.7                              | 0.9            | 89.5                                   | I         |
| 2      | 3.1        | 1.7      | 45.2                              | 1.6            | 51.6                                   | II        |
| 3      | 9.0        | 2.3      | 74.4                              | 1.7            | 81.1                                   | IV        |
| 4      | 4.0        | 2.8      | 30.0                              | 4.2            | -                                      | III       |
| 5      | 8.8        | 0.0      | 100.0                             | 2.2            | 75.0                                   | IV        |
| 6      | 4.5        | 5.2      | -                                 | 1.4            | 68.9                                   | -         |
| 7      | 0.7        | 2.1      | -                                 | 3.5            | -                                      | -         |
| 8      | 4.9        | 5.3      | -                                 | 8.0            | -                                      | -         |
| 9      | 7.0        | 3.3      | 52.9                              | 5.7            | 18.6                                   | III       |
| 10     | 6.2        | 0.0      | 100.0                             | 3.8            | 38.7                                   | -         |
| 11     | 2.8        | 5.6      | -                                 | 4.2            | -                                      | II        |
| 12     | 1.7        | 1.5      | 11.8                              | 6.1            | -                                      | II        |
| 13     | 6.9        | 0.6      | 91.3                              | 0.7            | 89.9                                   | -         |
| 14     | 8.0        | 0.3      | 96.2                              | 1.9            | 76.2                                   | -         |
| 15     | 5.2        | 0.1      | 98.1                              | 1.5            | 71.2                                   | III       |
| 16     | 1.8        | 0.7      | 61.1                              | 2.5            | -                                      | I         |
| 17     | 0.8        | 0.7      | 12.5                              | 2.3            | -                                      | -         |
| 18     | 8.8        | 1.1      | 87.5                              | 5.0            | 43.2                                   | -         |
| 19     | 4.7        | 1.9      | 99.6                              | 3.6            | 23.4                                   | III       |
| 20     | 4.8        | 1.5      | 68.8                              | 1.9            | 60.4                                   | III       |
| 21     | 5.3        | 0.0      | 100.0                             | 2.4            | 54.7                                   | -         |
| 22     | 4.1        | 0.6      | 88.7                              | 2.4            | 41.7                                   | -         |
| 23     | 1.3        | 0.5      | 61.5                              | 1.1            | 15.4                                   | I         |

1Radical surgery.

SDS-PAGE Coomassie brilliant blue staining bands at the mass position of 92 ku. The protein molecular mass of 92 ku was detected in 82% of patients with gastric cancer. Molecular mass of 92 ku indicated MMP-9 protein (Figure 1). In the control group, MMP-9 protein was not detected.

The gelatinase B activity was detected by SDS-PAGE zymography as the clear bands against the blue background (Figure 1) in the sera of gastric cancer patients. There were no clear bands in the control group. The clear bands detected by gelatin zymography were characterized by the activity of gelatinases A (72 ku) and B (92 ku). The reaction was positive for band migrating at approximately 220 and 92 ku, and for bands at 200 and 116 ku in some samples. The 220-ku band was strongly positive for gelatinase B, suggestive of homodimer. The 200- and 116-ku bands were interpreted as proMMP-9/TIMP-1 complexes.

Metalloprotease inhibition test by zymography

Gelatinase B activity in the serum of gastric cancer patients was inhibited by EDTA.

DISCUSSION

Proteolytic activity in the sera of patients with gastric cancer was higher than that in the control group, indicating that proteolysis can be degraded by ECM. For the occurrence of metastasis, tumor cells must repeatedly cross over the basement membrane barrier, a process for which proteolysis of ECM components is required. Some of the proteins associated with invasion and metastases of tumors are produced by tumor cells. Then, the proteins (whole or fragments) may accumulate in blood or urine of patients.

According to the inhibition test with EDTA and 1,10-phenanthroline, proteinases are found to be metalloproteinases and the inhibition is an additional biochemical parameter for correlation of proteolytic activity and gastric cancer. Increased levels of metalloproteinase have been implicated in the invasive potential of tumors. These results suggest that overexpression of metalloproteinases in the serum plays an important role in the progression of gastric cancer.

Overexpression of type IV collagenase has been demonstrated in a variety of cancers including colorectal cancer, gastric cancer, and breast cancer. There is evidence that type IV collagenase activity or concentration is increased in the plasma of patients with advanced carcinoma. It was reported that type IV collagenase activity is increased in urine and ascites of cancer patients.

In the present study, we initially measured the gelatinase B activities in the serum of gastric cancer patients. The

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**Table 2: Proteolytic activity and inhibition of metalloprotease activity in serum of control group**

| Control | BAPNA (mU) | EDTA (%) | Inhibition in presence of EDTA (%) | 1,10-Phen (mU) | Inhibition in presence of 1,10-phen (%) |
|---------|------------|----------|-----------------------------------|----------------|----------------------------------------|
| 1       | 1.3        | 4.3      | -                                 | 0.4            | 69.2                                   |
| 2       | 0.7        | 3.2      | -                                 | 4.3            | -                                      |
| 3       | 0.4        | 2.3      | -                                 | 1.7            | -                                      |
| 4       | 4.4        | 6.8      | -                                 | 0.9            | 79.5                                   |
| 5       | 0.3        | 1.3      | -                                 | 1.7            | -                                      |
| 6       | 2.2        | 0.9      | 59.1                              | -              | -                                      |
| 7       | 0.9        | 1.6      | -                                 | 2.5            | -                                      |
| 8       | 1.0        | 0.0      | 100.0                             | 3.2            | -                                      |
| 9       | 0.3        | 0.9      | -                                 | 2.6            | -                                      |
| 10      | 0.3        | 1.1      | -                                 | 2.7            | -                                      |
| 11      | 1.7        | 0.0      | 100.0                             | 0.0            | 100.0                                  |

Figure 1: Results of SDS-PAGE and SDS-PAGE zymography. 1: Serum of gastric cancer patients with EDTA inhibitor; 2: serum of control group; 3: molecular mass determination; 4: Protein marker mixture.

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results demonstrated that proteolytically active proMMP-9 was significantly associated with cancer. Proteolytic activity was shown in tumor patients. On the basis of molecular size and inhibition by EDTA, the bands were respectively interpreted as proMMP-9 (92 kD) and its putative dimer 220 kD and proMMP-9/TIMP-1 complexes (200 and 116 kD). The activated form of gelatinase B (83 kD) was not detected in the serum of cancer patients. Gelatinase A activity was not detected in the serum of gastric cancer patients. The gelatinases, particularly gelatinase A, seem to be important in the initial stage of tumor invasion [12] as they degrade the components of the basement membrane, while other MMPs contribute to the later stages of tumor invasion [13]. In some reports [30], gelatinase B in gastric carcinoma is positively correlated with the existence of vessel permeation, lymph node metastasis or the depth of tumor invasion.

In conclusion, gelatinase B protein may serve as a marker for invasiveness and metastasis of gastric cancer [37-39]. ProMMP-9 can be used for the detection of primary or recurrent cancer and for the estimation of tumor extent.

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