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• CLINICAL RESEARCH •

Detection of type IV collagenase activity in malignant ascites

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Abstract

AIM: Type IV collagenase participates in invasion and metastasis of cancer cells. Malignant ascites is a manifestation of advanced malignant disease that is associated with invasion and metastasis of the peritoneal cavity. Thus, it is reasonable to hypothesize that type IV collagenase is linked to malignant ascites. The purpose of our study was to detect type IV collagenase activity in malignant ascites so as to provide the scientific basis for clinic diagnosis and treatment of malignant ascites.

METHODS: Cirrhotic ascites (n=36), tuberculous ascites (n=8) and malignant ascites (n=23) from patients with gastric cancer (n=6), colon cancer (n=5), ovarian cancer (n=8) and other cancers (n=4), including 2 hepatocellular cancers, 1 pancreatic cancer, 1 primary peritoneal carcinoma were collected by paracentesis. The ascites were made cell-free by centrifugation and stored frozen at -70 ℃ before determination. Type IV collagenase activity was determined by gelatin zymography.

RESULTS: The activity of matrix metalloproteinases-2 and -9 could not be detected in ascites of hepatic cirrhosis and tuberculous peritonitis but could be detected in 20 and 18 out of 23 malignant ascites respectively. The positive rate of type IV collagenase (MMP-2, 87.0 % and MMP-9, 78.3 %) was higher than that by routine ascites tests (P<0.01) in malignant ascites. Furthermore, the activity of MMP-2 was higher than that of MMP-9 (P=0.022<0.05).

CONCLUSION: Type IV collagenase is positive in malignant ascites. Detection of type IV collagenase activity is useful in qualitative diagnosis of ascites. Type IV collagenase may play an important role in malignant ascites formation.

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INTRODUCTION

Although tumor invasion and metastasis are considered to be a dynamic, complex and multi-step process, proteolytic degradation of the extracellular matrix (ECM) made of interstitial matrix and basement membrane is the essential step[1-4]. The basement membrane, a specialized ECM containing large amounts of type IV collagen and laminin, serves a barrier function separating epithelial cells from the underlying stroma. For the occurrence of metastasis, a tumor cell must repeatedly cross this basement membrane barrier, a process for which proteolysis of ECM components is required[5,6]. It is reasonable to hypothesize that proteolysis of ECM also plays a key role in intraperitoneal metastasis of tumor cells, such as in disruption of the mesothelial cell layer, during extension of the implanted tumor through the submesothelial basement membrane into the visceral organ stroma, and importantly, in gaining access to the host vascular supply, a necessary step in progression of the implant.

In disruption of the ECM, the contribution of matrix metalloproteinases (MMPs) of proteolytic enzymes is direct and important in that it catalyzes the hydrolysis of numerous ECM molecules[7-11]. While in the matrix metalloproteinase, type IV collagenase, one of the most important members of MPPs family, including a 72 kD enzyme resembling matrix metalloproteinase-2 (MMP-2), also named gelatinase A and a 92 kD enzyme resembling matrix metalloproteinase-9 (MMP-9), also named gelatinase B, has been demonstrated to be closely associated with several tumor systems and linked to invasive potential of tumor cells[12-16]. Type IV collagenase can degrade not only interstitial matrix, but also basement membrane, and malignant ascites is the direct and prominent manifestation of advanced malignant disease that is associated with invasion and metastasis of peritoneal cavity by tumor cells. Thus it is possible and feasible to detect type IV collagenase in malignant ascites. In the present study, we detected type IV collagenase activity in various kinds of ascites by gelatin zymography so as to explore the relationship between type IV collagenase and malignant ascites, and to provide the scientific basis for clinic diagnosis and treatment of malignant ascites.

MATERIALS AND METHODS

Reagents and instruments

The common reagents and principal apparatus used in SDS-PAGE were provided by the Biochemical Laboratory, Medical College, Wuhan University. Gelatin was purchased from Sigma Co. Type IV collagenase was the product of Invitrogen. Matrix metalloproteinase inhibitor, 1,10-phenanthroline, was purchased from Sangon, Shanghai, China.

Clinical specimems

Sixty-seven inpatients with ascites were recruited respectively at Renmin Hospital of Wuhan University, Zhongnan Hospital of Wuhan University and Tumor Hospital of Hubei Province from July 2002 to March 2003. The ascites were obtained by therapeutic or diagnostic paracentesis. In all cases, informed consent of the patient and approval of the hospital were obtained prior to collection of ascites and medical records. All the diagnoses were confirmed by cytologic examination of the ascites, biopsy for pathological examination, B-ultrasound and CT scan. Details of the patient data are shown in Table 1. The ascites were made cell-free by centrifugation at 3 000 rpm for 15 min and stored frozen at -70 ℃ before determination. At the same time, the protein content of the ascites was measured.

Table 1 Patient data

Characteristics	*n*
Number of patients	67
Male	38
Female	29
Median age (y)	
Male	50.68
Female	48.60
Disease categories	
Cirrhotic ascites	36
Tuberculous ascites	8
Malignant ascites	23
Ovarian cancer	8
Gastric cancer	6
Colon cancer	5
Hepatocellular cancer	2
Pancreatic cancer	1
Primary peritoneal carcinoma	1

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography

Samples were analyzed by SDS-PAGE zymography according to the method of Kleiner and settler-Stevenson[17] to determine the molecular weights and relative abundance of the gelatinases present. Samples containing 70 μg of protein were incubated for 40 min at 37 ℃ and electrophoresed without reduction (no DTT) on 8 % SDS polyacrylamide gels copolymerized with 0.1 % gelatin. Electrophoresis was performed at 4 ℃ at a constant current of 20 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 2.5 % 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 operation was repeated twice both at 4 ℃. Next, 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 μmol/L $ZnCl_2$, 0.5 mol/L NaCl, pH 8.3 , at 37 ℃. At last, following staining with 0.075 % Coomassie blue for 3 h, regions of proteolytic activity were visualized as clear zones against a blue background. Gelatinolytic bands were assessed for semiquantitative analysis using an arbitrarily graded scale. Scale categories were defined as follows: +/-, faint band detected, <1.0 mm in width: 1+, clear band detected, 1.0-1.5 mm in width: 2+, intense band detected, 1.5-3.0 mm in width.

Matrix metalloproteinase inhibition test[18]

In order to verify that the clear zones resembled matrix metalloproteinase, 2.5 mmol/L 1,10- phenanthroline was added into the samples before electrophoresis to inhibit matrix metalloproteinase activities.

Detection of LDH, cytologic examination of ascites and serum complex index

Detection of LDH, cytologic examination of ascites and serum complex index were performed by the Department of Clinical Laboratory and Pathology.

Statistical analysis

Chi-square test and Fisher' s exact probabilities test were used for statistical analysis. Differences were considered significant when *P* value was less than 0.05.

RESULTS

Metalloproteinase inhibition test

Except that when 2.5 mmol/L 1,10-phenanthroline was added to the sample prior to electrophoresis, the result of electrophoresis for the identical samples was negative, indicating what we had detected was matrix metalloproteinase (Figure 1).

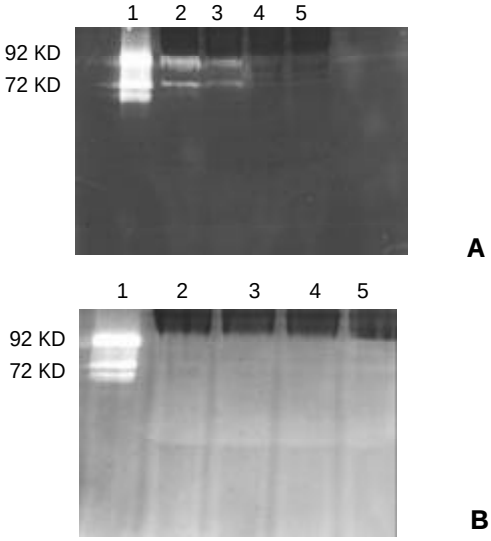

Figure 1 Results of SDS-PAGE zymography. A, Lane 1: type IV collagenase as marker, Lanes 2, 3: cancerous ascites, Lane 4: cirrhotic ascites (no bright bands), Lane 5: tuberculous ascites (no bright bands). B, Lane 1: Marker, Lanes 2-5: identical samples with A, except the addition of 2.5 mmol/L 1,10-phenanthroline. The result was negative.

Qualitative analysis of type IV collagenase activity in various kinds of ascites

Type IV collagenase activity could not be detected in ascites of hepatic cirrhosis and tuberculous peritonitis but could be detected in majority of malignant ascites (Table 2, Figure 1A).

Table 2 Qualitative analysis of type IV collagenase activity in various kinds of ascites

Ascites type	MMP-2 (Positive cases)	MMP-9 (Positive cases)
Cirrhotic ascites	0	0
Tuberculous ascites	0	0
Malignant ascites	20	18
Gastric cancer	5	5
Colon cancer	3	5
Hepatocellular cancer	2	1
Pancreatic cancer	1	0
Ovarian cancer	8	6
Primary peritoneal carcinoma	1	1

Table 3 Semi-quantitative analysis of MMP-2, MMP-9 activity in different kinds of malignant ascites

Type	Case (*n*)	MMP-2 activity (*n*)			MMP-9 activity (*n*)		
		+/-	1+	2+	+/-	1+	2+
Gastric cancer	6	1	4	0	3	2	0
Colon cancer	5	1	2	0	4	1	0
Hepatocellular cancer	2	1	1	0	0	1	0
Pancreatic cancer	1	0	1	0	0	0	0
Ovarian cancer	8	1	5	2	3	1	2
Primary peritoneal carcinoma	1	0	0	1	0	0	1

+/-, faint band detected, <1.0 mm in width; 1+, clear band detected, 1.0-1.5 mm in width; 2+, intense band detected, 1.5-3.0 mm in width.

Table 4 Comparison of detection of type IV collagenase and other indexes in malignant ascites

	Type IV collagenase		LDH in ascites / serum LDH (>1: positive)	Cytologic examination	Serum complex indexes (AFP, CEA, CA)
	MMP-2	MMP-9			
Positive numbers	20	18	10	11	13
Positive rates	87.0 %	78.3 %	43.4 %	47.8 %	56.5 %

Semi-quantitative analysis of type IV collagenase in different kind of cancerous ascites

Gelatinolytic bands were assessed for semiquantitative analysis using an arbitrary grade scale as described in Materials and Methods. The 72 kD MMP-2 was detected in 87.0 % of malignant ascites (n=23), with 80.0 % being scaled >1+; and the 92 kD MMP-9 was found in 78.3 %, with 44.4 % scaled >1+. The activity of MMP-2 was higher than that of MMP-9 ($0.01<P=0.022<0.05$). Additionally, three cases had the highest type IV collagenase activity (2+) for MMP-2 and MMP-9 respectively. Both included one primary peritoneal carcinoma, and two ovarian cancers (Table 3).

Comparison of detection of type IV collagenase and other indexes in malignant ascites

In 23 cases of carcinoma ascites, MMP-2 was found in 20 (87.0 %), significantly higher than that by routine ascites tests (including LDH, cytologic examination of ascites and complex indexes in serum) ($P<0.01$, respectively). MMP-9 was detected in 18 (78.3 %), higher than that by LDH and cytologic examination. However, compared with the two detection methods of type IV collagenase and serum complex indexes, there was no significant difference ($P>0.05$) (Table 4).

DISCUSSION

Type IV collagenase is closely linked with malignant ascites

Overexpression of type IV collagenase has been demonstrated in a variety of cancers, including colorectal cancer, gastric cancer, and breast cancer. Substantial evidences indicate that type IV collagenase activity or concentration was increased in plasma of patients with advanced carcinoma[19-24]. Recently, some researches found that type IV collagenase activity was increased in urine of patients with metastatic breast cancers[25,26] and in cerebrospinal fluid of patients with brain tumors[27]. However, few reports on type IV collagenase activity in ascites could be seen[18,21]. In the present study, we found that type IV collagenase activity could not be detected in ascites of hepatic cirrhosis and tuberculous peritonitis but could be detected in malignant ascites. At the same time, MMP-2 activity was higher than MMP-9 activity in malignant ascites, which might be attributed to the difference of expression of MMP-2 and MMP-9 in ascites of different kinds of tumor. Furthermore, the detection rate of type IV collagenase in ascites of malignant tumor was higher than that by routine ascites tests. Additionally, 2 out of 4 unidentified cases of ascites were identified by peritoneal biopsy. One was metastatic ovarian cancer, the other was primary peritoneal carcinoma. In these two cases, the activity of MMP-2 and MMP-9 was positive, and scale categories all exceeded 1+. Another 2 cases, gastric cancer and colon cancer, were identified by gastroscopy and colonoscopy respectively. The activity of MMP-2 and MMP-9 in these 2 cases was positive also. It was suggested that the peritoneal implants of tumors might secrete type IV collagenase (MMP-2 and/or MMP-9) into peritoneal cavity. Type IV collagenase activity in ascites reflects tumor biological behavior to some extent and is related with malignant ascites. So detection of type IV collagenase activity may probably provide a new approach with high sensitivity and specificity for differential diagnosis of benign and malignant ascites.

Type IV collagenase is correlated with malignant ascites formation

Some reports demonstrated that MMPs played a key role in intraperitoneal metastasis[21]. Others found metalloproteinase inhibitor could reduce ascites in patients with advanced malignant disease[28-30]. Our experiments indicated no expression of type IV collagenase in nonmalignant ascites. On the contrary, majority of malignant ascites showed higher activity of type IV collagenase. Thus, it is reasonable to infer that type IV collagenase is associated with malignant ascites formation to some extent. The probable mechanism of inducing malignant ascites formation by type IV collagenase may be explained as follows[31-37]: breakdown of apparent physical barriers to metastasis of peritoneal cavity, role of MMPs in angiogenesis, formation of new blood vessels which increases overall capillary membrane surface available for fluid filtration, inducing fluid accumulation. These new observations indicate that use of metalloproteinase inhibitors may offer a novel treatment of malignant ascites.

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