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Characterization of Matrix-degrading Proteinases and Their Inhibitors Secreted by Human Gynecological Carcinoma Cells

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Matrix-degrading proteinases secreted by tumor cells play crucial roles in tumor cell invasion and metastasis. Serum-free conditioned media of 7 human gynecological carcinoma cell lines were examined for proteinases and their inhibitors by using gelatin zymography, reverse zymography and immunoblotting. All of three ovarian adenocarcinoma cell lines secreted urokinase-type plasminogen activator. Among them, a mucinous cystadenocarcinoma cell line also secreted tissue-type plasminogen activator, plasmin-like enzyme and trypsinogen. On the other hand, two ovarian undifferentiated carcinoma cell lines mainly secreted gelatinase A or B. A choriocarcinoma cell line secreted multiple metalloproteinases in the highest amount, whereas an endometrial adenocarcinoma cell line (HEC-1) derived from an early clinical stage hardly secreted any gelatinolytic enzyme. The five high proteinases producers hardly secreted the corresponding inhibitors, such as tissue inhibitor of metalloproteinases (TIMP)-1, -2 or plasminogen activator inhibitor-1. In contrast to these highly malignant cell lines, a poor proteinase producer, HEC-1, secreted a large amount of TIMPs. Therefore, an enhanced proteolytic tendency appears to be associated with gynecological cancer cells established from highly malignant tumors.

Key words: Proteinase — Proteinase inhibitor — Gynecological cancer — Metastasis

Extracellular matrix-degrading proteinases secreted by malignant tumor cells are thought to play an essential role in the processes of invasion and metastasis. Matrix metalloproteinases (MMPs), such as gelatinase A (M_r 72,000 or 64,000 gelatinase/type IV collagenase) and gelatinase B (M_r 92,000 or 90,000 gelatinase/type IV collagenase), are especially important in the process of metastasis.[1-8] Their secretion is highly correlated with the metastatic potential of tumor cells.[3, 5-8] Recently, not only MMPs but also serine proteinases have been shown to contribute to matrix degradation and the invasion of tumor cells.[9-15] The expression of plasminogen activators, especially urokinase-type plasminogen activator (u-PA), is associated with malignant phenotype of tumor cells.[9-10, 13, 14] We have also demonstrated that human gastric carcinoma cell lines secrete multiple matrix serine proteinases such as trypsin-like, kallikrein-like and plasmin-like enzymes, and suggested that these matrix serine proteinases might play a major role in the matrix degradation by some human carcinoma cells.[15]

In addition to these proteinases, their inhibitors are also involved in invasion and metastasis. Two kinds of tissue inhibitor of metalloproteinases, TIMP-1 and TIMP-2, are secreted by many kinds of normal and malignant cells, and form tight complexes with the latent forms of gelatinase B and gelatinase A, respectively.[16-18] These inhibitors suppress tumor invasion.[19-21] As well as TIMPs, two plasminogen activator inhibitors, PAI-1 and PAI-2, have been reported to function as negative regulators of invasion and metastasis of tumor cells.[22-24]

Although many studies on the secretion of matrix-degrading proteinases by malignant tumor cells have been reported, proteinase molecules secreted by malignant gynecological tumor cells have not been examined in great detail. Moreover, there has been little systematic examination of the secretion of metalloproteinases and serine proteinases, as well as their inhibitors, by tumor cells. In this study, we comprehensively examined metalloproteinases, serine proteinases and their inhibitors secreted by 7 human gynecological carcinoma cell lines. We found that 6 gynecological carcinoma cell lines established from highly malignant tumors secreted significant amounts of MMPs or serine proteinases or both. Five carcinoma cell lines established from highly malignant tumors showed an enhanced proteolytic tendency.

MATERIALS AND METHODS

Cell lines and culture conditions Human gynecological carcinoma cell lines tested were as follows: two cell lines of ovarian clear cell adenocarcinoma, OVISE and OVTOKO; a cell line of ovarian mucinous cystadenocarcinoma, MCAS (JCRB 0240); two cell lines of ovarian undifferentiated carcinoma, KURAMOCHI (JCRB

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0098) and TYK-nu (JCRB 0234); a cell line of endometrial adenocarcinoma, HEC-1 (JCRB 0042); and a cell line of choriocarcinoma, BeWo (CCL 98). OVISE[25] and OVTOKO (to be published) were established by Nakazawa *et al.* from intrapelvic and splenic metastatic lesions of two patients, respectively. The other 5 cell lines were provided by the Japanese Cancer Research Resources Bank (JCRB). Except for HEC-1, which was derived from stage IA endometrial adenocarcinoma, all cell lines were established from specimens of highly malignant tumors or metastatic lesions.

These cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Plastic 150-mm dishes (Falcon) were purchased from Becton Dickinson (Lincoln Park, NJ) and other plasticware was from Sumitomo Bakelite (Tokyo).

Preparation and concentration of serum-free conditioned media of human gynecological carcinoma cells The human gynecological carcinoma cells were grown to semi-confluence in 150-mm dishes containing 30 ml of RPMI 1640 supplemented with 10% fetal calf serum. The cultures were washed twice with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution and then replaced with serum-free RPMI 1640. After a 2-day incubation, the media of 6 gynecological carcinoma cell lines (not BeWo) were discarded and replaced with fresh RPMI 1640 for further incubation. The resultant serum-free conditioned media were harvested 2 days later, and then clarified by 2-step centrifugation at 1,000 rpm for 10 min and 15,000 rpm for 10 min. The conditioned medium of BeWo cells was harvested after cultivation in serum-free RPMI 1640 for 24 h because of the intolerance of this line to longer cultivation in serum-free medium. The clarified media were concentrated (finally 100-fold) and stored following the procedure reported previously.[15]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gelatin zymography SDS-PAGE and zymography using gelatin-containing gel for detecting gelatinolytic activities in each conditioned medium were performed following the procedures reported previously.[4, 15] Bio-Rad molecular weight standards (Bio-Rad, Richmond, CA) were used as molecular weight markers. To classify each proteinase band on the gelatin zymogram into metalloproteinase or serine proteinase, gels after renaturation were incubated 1) in 50 m*M* Tris-HCl buffer (pH 7.5) containing 10 m*M* EDTA or 5 m*M* 1,10-phenanthroline for the detection of serine proteinase, or 2) in 50 m*M* Tris-HCl buffer (pH 7.5) containing 10 m*M* diisopropyl fluorophosphate (DFP) and 5 m*M* $CaCl_2$ for the detection of metalloproteinase.

Reverse zymography For detecting metalloproteinase inhibitors, reverse zymography was performed as reported previously using gelatinase A as an indicator enzyme and the Bio-Rad molecular standards as molecular weight markers.[26]

Immunoblotting analysis Sample proteins were separated on 10% or 12.5% SDS-PAGE under non-reducing or reducing conditions. Rainbow markers (Amersham, Arlington Heights, IL) were used as molecular weight markers. After electrophoresis, the separated proteins on the gels were transferred, probed with specific antibodies and stained using the alkaline phosphatase system as described previously.[27]

Reagents Human gelatinase A was purified as the TIMP-2-bound proenzyme form from the conditioned medium of human glioblastoma cell line T98G following the methods reported previously.[4, 17] DFP was purchased from Wako Pure Chemicals (Osaka) and APMA (*p*-aminophenyl mercuric acetate) from Tokyo Kasei (Tokyo). Rabbit polyclonal antibody against human trypsin was purchased from Athens Research and Technology (Athens, GA); rabbit polyclonal antibody against human urokinase from Japan Chemical Research (Kobe); goat polyclonal antibodies against human melanoma tissue-type plasminogen activator (t-PA) and human plasminogen activator inhibitor-1 (PAI-1) from Biopool (Umea, Sweden); and goat polyclonal antibody against human tissue kallikrein from Protgen (Switzerland). Monoclonal antibodies against human plasminogen and against human PAI-2 were purchased from American Diagnostica (Greenwich, CT). Authentic human high-molecular-weight urokinase was purchased from Japan Chemical Research; and authentic human pancreatic trypsin from Athens Research and Technology. Recombinant human t-PA was kindly provided by Pharmaceuticals Research Center, Toyobo (Shiga).

RESULTS

Molecular weight estimation and classification of secreted proteinases Conditioned media of 7 gynecological carcinoma cell lines were assayed for gelatinolytic activities by gelatin zymography. Many proteinases varying in molecular weight and activity were detected in 50 m*M* Tris-HCl buffer containing 5 m*M* $CaCl_2$ (Fig. 1A). Proteinase inhibitors, EDTA or DFP (Fig. 1B), were used to classify each proteinase band in the zymogram into metalloproteinase or serine proteinase. In some cases, 1,10-phenanthroline was also used.

OVISE and OVTOKO, derived from ovarian clear cell adenocarcinomas, exhibited a strong and broad gelatinolytic band of approximate M_r 50,000. Two minor bands of M_r 110,000 and 82,000 were detected only in OVTOKO. All these proteinases secreted by OVISE and OVTOKO were inhibited by DFP (Fig. 1B), and therefore were identified as serine proteinases. MCAS, established from

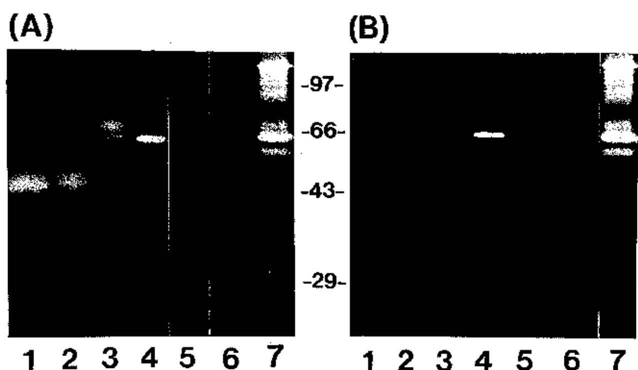

Fig. 1. Zymographic analysis of gelatinolytic enzymes secreted by 7 kinds of gynecological carcinoma cell lines. The serum-free conditioned media of these cell lines were concentrated 100-fold, and aliquots (10 μl) were subjected to zymography on gelatin-containing gels. After electrophoresis, proteins on the gels were renatured and then incubated at 37°C for 20 h in the reaction mixture containing 5 m*M* $CaCl_2$ (A), or 5 m*M* $CaCl_2$ plus 10 m*M* DFP (B). Ordinate, molecular weight in thousands. Other experimental conditions are described in "Materials and Methods." Lane 1, OVISE; lane 2, OVTOKO; lane 3, MCAS; lane 4, KURAMOCHI; lane 5, TYK-nu; lane 6, HEC-1; lane 7, BeWo.

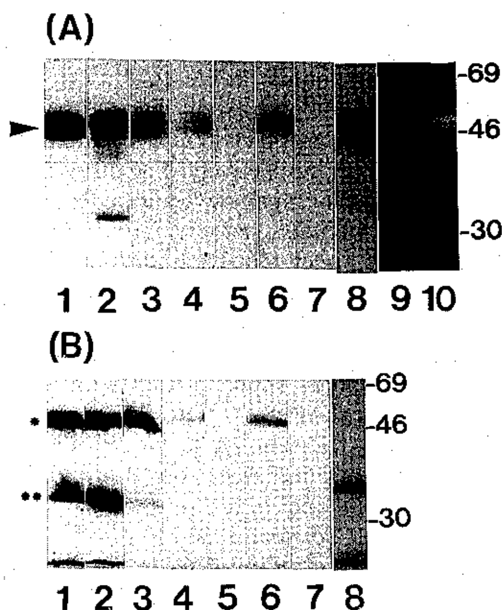

Fig. 2. Immunoblotting analysis of media conditioned by gynecological carcinoma cells with anti-u-PA antibody. The 100-fold-concentrated conditioned media (10 μl) were subjected to immunoblotting analysis with anti-u-PA polyclonal antibody under non-reducing conditions (A) and reducing conditions (B). Rainbow markers (Amersham) were used as molecular weight markers. Other experimental conditions are described in "Materials and Methods." Lane 1, OVISE; lane 2, OVTOKO; lane 3, MCAS; lane 4, KURAMOCHI; lane 5, TYK-nu; lane 6, HEC-1; lane 7, BeWo; lane 8, authentic human u-PA (50 ng) used as a positive control. Lanes 9 and 10 are 50 ng and 100 ng of authentic human u-PA, respectively, detected on the gelatin zymogram in the presence of 10 m*M* EDTA. Ordinate, molecular weight in thousands; arrowhead, an immunostained band with a molecular weight of approximately 50,000; *, single-chain latent u-PA (pro u-PA); **, B-chain of active u-PA.

ovarian mucinous cystadenocarcinoma, mainly secreted serine proteinases (M_r 130,000, 70,000, 66,000, 50,000 and 25,000) and also afforded a small amount of a metalloproteinase of approximate M_r 50,000 as a broad band. KURAMOCHI and TYK-nu, derived from ovarian undifferentiated carcinoma, secreted only metalloproteinases. KURAMOCHI secreted three metalloproteinases of M_r 130,000, 80,000 and 64,000. The major M_r 64,000 enzyme in the zymogram corresponded to gelatinase A (M_r 72,000 gelatinase/type IV collagenase) reported previously.[4, 28] TYK-nu was revealed to secrete two metalloproteinases of M_r 90,000 and approximate M_r 50,000. The electrophoretic mobility of the former corresponded to that of authentic gelatinase B (M_r 92,000 gelatinase/type IV collagenase), and that of the latter to that of interstitial collagenase.[4, 6, 8] These results showed that all the ovarian carcinoma cell lines secreted significant amounts of proteinases.

HEC-1, established from endometrial adenocarcinoma at an early clinical stage, showed no gelatinolytic activity in the conditioned medium. In contrast, BeWo, established from brain metastatic lesion of choriocarcinoma, showed the highest gelatinolytic activities of metalloproteinases among the cell lines examined. Three obvious metalloproteinase bands of M_r 130,000, 64,000, and 57,000 were detected in the conditioned medium of BeWo. The gelatinolytic bands of M_r 64,000 and 57,000 corresponded to the precursor and activated forms of gelatinase A, respectively.[4, 28] It is unknown whether the M_r 130,000 enzyme is a dimeric form of gelatinase A or a novel metalloproteinase.

Immunological identification of several serine proteinases To identify the serine proteinases detected by gelatin zymography and to detect other serine proteinases lacking gelatinolytic activity on the gelatin zymogram, immunoblotting analysis of the seven conditioned media was performed.

The immunoblot with the antibody against u-PA under non-reducing conditions exhibited double bands at around M_r 50,000 in 4 cell lines (OVISE, OVTOKO, MCAS and KURAMOCHI) (Fig. 2A). Under reducing conditions, these cell lines showed multiple bands of M_r 50,000, 33,000 and 17,000 (Fig. 2B). These bands seem to correspond to a single-chain latent u-PA (pro-u-PA,

M_r 50,000), and B chain (M_r 33,000) and A chain (M_r 17,000) of active two-chain u-PA. This result indicates that the doublet at M_r 50,000 under non-reducing conditions is composed of single- and two-chain forms of u-PA. On the other hand, HEC-1 cells showed a single band of M_r 50,000 under both non-reducing (Fig. 2A, lane 6) and reducing conditions (Fig. 2B, lane 6), indicating that they secrete only the single-chain latent u-PA. The relative intensity of the M_r 50,000 doublet was high in OVISE and OVTOKO, moderate in MCAS, and low in KURAMOCHI. Its molecular weight was identical to that of the serine proteinase detected in gelatin zymogram (Fig. 1). Authentic u-PA showed a major band at M_r 50,000 under non-reducing conditions (Fig. 2A), and two bands at M_r 33,000 and at M_r 17,000 under reducing conditions (Fig. 2B). Therefore, the authentic u-PA was judged to be a two-chain form. More than 50 ng of the authentic enzyme showed gelatinolytic activity in the zymogram (Fig. 2A, lanes 9 and 10). This confirmed that the gelatinolytic activity at M_r 50,000 in Fig. 1A is due to u-PA.

When t-PA was analyzed by immunoblotting, only the conditioned medium of MCAS showed a clear immunostained band at the same position (M_r 69,000) as authentic t-PA (Fig. 3, lanes 3 and 8). As t-PA showed no gelatinolytic activity up to 5 μg in the gelatin zymogram (data not shown), the gelatinolytic activity of M_r 70,000 detected in the zymogram of MCAS is unlikely to be due to t-PA.

To identify the other serine proteinases possessing gelatinolytic activity in gelatin zymography, we attempted to detect trypsin, kallikrein and plasmin, because these enzymes were revealed to be secreted by gastric cancer cells[15] and authentic samples of these serine proteinases showed activity in the gelatin zymogram (data not shown). Only the antibody against human pancreatic trypsin exhibited cross-reactivity with the proteinase secreted by MCAS (Fig. 4). The immunoblots of the conditioned medium of MCAS showed a single band of M_r 31,000 under reducing conditions and two bands of M_r 24,000 and 26,000 under non-reducing conditions (Fig. 4, lanes 2 and 4). Authentic human pancreatic trypsin showed a similar staining pattern, though the bands were significantly lower in molecular weight than those of the enzyme in MCAS (Fig. 4, lanes 1 and 3). These results indicate that the M_r 24,000 and 26,000 proteins of MCAS are very similar or identical to the proform of trypsin, trypsinogen. This is consistent with reports that trypsin-like enzymes are secreted by some kinds of malignant cells in the form of trypsinogen.[15, 29–31] A gelatinolytic band at M_r 25,000 in the zymogram of MCAS conditioned medium was identical to that of a small dose of active-form trypsin (6 ng), which was below the sensitivity limit of immunoblot analysis with

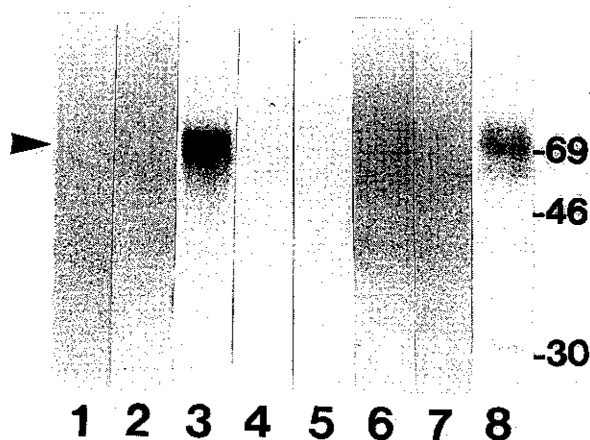

Fig. 3. Immunoblotting analysis of media conditioned by gynecological carcinoma cells with anti-t-PA antibody. The 100-fold concentrated conditioned media (10 μl) were subjected to immunoblotting analysis with anti-t-PA polyclonal antibody under non-reducing conditions. Other experimental conditions are described in "Materials and Methods." Lane 1, OVISE; lane 2, OVTOKO; lane 3, MCAS; lane 4, KURAMOCHI; lane 5, TYK-nu; lane 6, HEC-1; lane 7, BeWo; lane 8, authentic human t-PA (50 ng) used as a positive control. Ordinate, molecular weight in thousands; arrowhead, an immunostained band with a molecular weight of approximately 70,000.

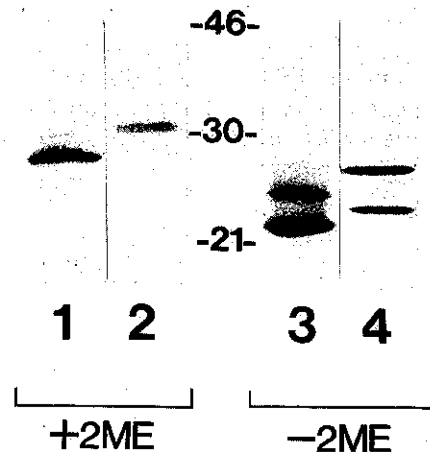

Fig. 4. Immunological detection of trypsin-like protein secreted by MCAS cells. The 100-fold-concentrated conditioned medium (10 μl) of MCAS cells (lanes 2 and 4) and authentic human pancreas trypsin (lanes 1 and 3) were subjected to immunoblotting analysis with anti-trypsin polyclonal antibody under reducing conditions (lanes 1 and 2) or non-reducing conditions (lanes 3 and 4). The trypsin-like protein secreted by MCAS cells exhibited a higher molecular weight than the mature form of authentic pancreatic trypsin. 2ME, 2-mercaptoethanol. Ordinate, molecular weight in thousands.

Table I. Summary of Proteinases Secreted by Human Gynecological Carcinoma Cells

Proteinase molecular weight ($\times 10^3$)	Identified enzyme (type)[b]	Cell line						
		OVISE	OVTOKO	MCAS	KURA.[a]	TYK-nu	HEC-1	BeWo
130	(M)				+			+++
130	(S)			+				
110	(S)		+					
90	Gelatinase B (M)					+		
82	(S)		+					
80	(M)				+			
70	(S)			++				
69	t-PA (S)			+[c]				
66	(S)			+				
64	Gelatinase A (M)				++			+++
57	Gelatinase A[d] (M)							++
50	u-PA (S)	+++	+++	+	+		+[c]	
50	(M)			+				
26	Trypsinogen (S)			+[c]				
24	Trypsinogen (S)			+				

Each gelatinolytic activity in gelatin zymography was scored as + (low), ++ (medium), or +++ (high).
a) KURA., KURAMOCHI.
b) (S), serine proteinase; (M), metalloproteinase.
c) Gelatinolytic activity was not detected in gelatin zymography.
d) Active form of gelatinase A.

this anti-trypsin antibody. The M_r 70,000 and 66,000 serine proteinases secreted by MCAS were not immunochemically identified in this study. From their molecular weights, however, they might be plasmin, as we suggested previously.[15]

The results for the proteinases examined are summarized in Table I. By gelatin zymography and immunodetection, many of the proteinases secreted from gynecological carcinomas were identified and a relative increase in secretion by the highly malignant gynecological carcinomas was apparent.

Identification of secreted proteinase inhibitors To evaluate the secretion of metalloproteinase inhibitors, reverse zymography using gelatinase A as the indicator enzyme was performed. Three major inhibitor bands of M_r 100,000, 28,000 and 20,000 were detected in most of the conditioned media, but their intensity varied among samples (Fig. 5). From our previous studies, these inhibitors were identified as a secretory form of amyloid precursor protein (APP; M_r 100,000),[32] TIMP-1 (M_r 28,000) and TIMP-2 (M_r 20,000).[17,26] KURAMOCHI and BeWo scarcely secreted TIMPs, although they secreted metalloproteinases such as gelatinase A. APP was detected in the conditioned media of OVISE, OVTOKO, KURAMOCHI, TYK-nu and HEC-1.

We also examined the secretion of two serine proteinase inhibitors, PAI-1 and PAI-2, by immunoblotting analysis. The antibody against PAI-1 detected the band of M_r 47,000 in four conditioned media (OVISE,

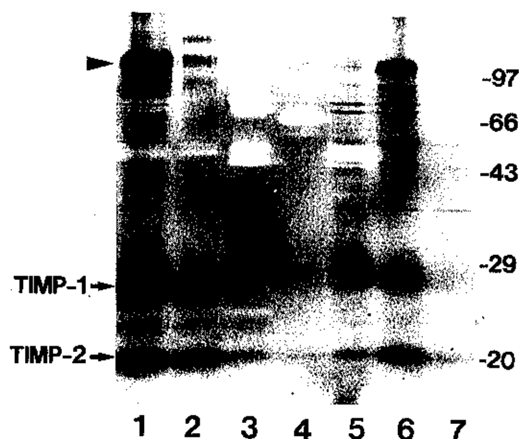

Fig. 5. Analysis of metalloproteinase inhibitors by reverse zymography. The 100-fold-concentrated conditioned media (10 μl) were subjected to reverse zymography. After electrophoresis on a gelatin-containing gel, proteins on the gel were renatured and then incubated at 37°C for 18 h in a reaction mixture of 50 m*M* Tris-HCl (pH 7.5), 10 m*M* $CaCl_2$, 1 m*M* *p*-aminophenyl mercuric acetate and 1 μg/ml purified human TIMP-2-bound gelatinase A. Other experimental conditions are described in "Materials and Methods." Lane 1, OVISE; lane 2, OVTOKO; lane 3, MCAS; lane 4, KURAMOCHI; lane 5, TYK-nu; lane 6, HEC-1; lane 7, BeWo. The metalloproteinase inhibitor activities of M_r 28,000 and M_r 20,000 correspond to TIMP-1 and TIMP-2, respectively. Ordinate, molecular weight in thousands; arrowhead, metalloproteinase-inhibitor activity of approximately M_r 100,000, which was recently identified (see text) as APP (amyloid precursor protein).

OVTOKO, TYK-nu and BeWo) under reducing conditions (Fig. 6). MCAS, a producer of multiple serine proteinases, and KURAMOCHI and HEC-1 did not secrete PAI-1. The secretion of PAI-1 was the highest in TYK-nu. OVISE and OVTOKO, high producers of u-PA, secreted low or moderate amounts of PAI-1. An additional minor band of approximate M_r 62,000 was detected in conditioned media of several cell lines. This might be a reduction-resistant complex of PAI-1 or proteins possessing similar epitopes recognized by the polyclonal antibody against PAI-1. PAI-2 was not detected immunologically in any sample in this study (data not shown). These results are summarized in Table II. A tendency for enhanced proteolytic activity of metalloproteinases was observed in KURAMOCHI and BeWo. A similar tendency of serine proteinases was observed in the serine proteinase-secreting cell lines (OVISE, OVTOKO and MCAS).

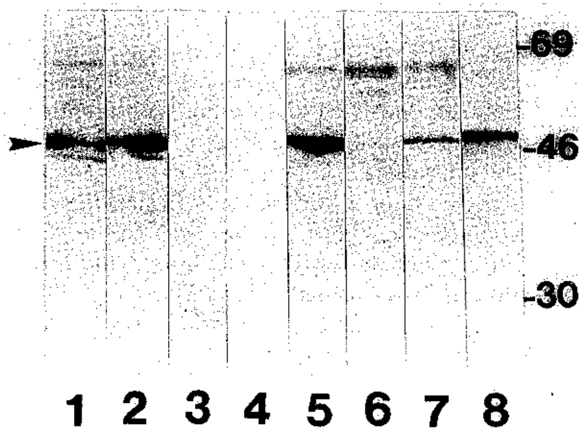

Fig. 6. Immunoblotting analysis of media conditioned by gynecological carcinoma cells with anti-PAI-1 antibody. The 100-fold-concentrated conditioned media (10 μl) were subjected to immunoblotting analysis with anti-PAI-1 polyclonal antibody under reducing conditions. Other experimental conditions are described in "Materials and Methods." Lane 1, OVISE; lane 2, OVTOKO; lane 3, MCAS; lane 4, KURAMOCHI; lane 5, TYK-nu; lane 6, HEC-1; lane 7, BeWo; lane 8, 30-fold-concentrated conditioned medium (10 μl) of human diploid fibroblasts (WI-38) as a positive control. Ordinate, molecular weight in thousands; arrowhead, an immunostained band of PAI-1 (approximately M_r 47,000).

DISCUSSION

Advanced ovarian carcinomas are characterized by high frequency of metastasis to lymph nodes, invasive growth into multiple organs after peritoneal dissemination and poor prognosis. So far, there have been few reports on the secretion of matrix-degrading proteinases in ovarian carcinomas, except u-PA and t-PA. u-PA has been reported to be produced more frequently than t-PA by some kinds of carcinoma cells, including ovarian carcinomas,[9, 14] and to contribute to their invasive phenotype *in vitro* and *in vivo*.[10, 11, 13, 14] In the present study, u-PA was immunologically detected in culture media conditioned by 4 of 5 ovarian carcinoma cell lines. The enzymes secreted from malignant gynecological carcinomas were detected as a mixture of single-chain and two-chain forms. Moreover, three ovarian adenocarcinoma cell lines contained sufficient amounts of u-PA to show the activity on a gelatin zymogram. This raises the possibility that u-PA may directly degrade some extracellular matrix proteins. These results suggest that in ovarian adenocarcinomas u-PA may play a key role in breakdown of the extracellular matrix by direct degradation of its components or through activation of plasminogen or other proteinases derived from plasma and sur-

Table II. Proteinase Inhibitors Secreted by Human Gynecological Carcinoma Cells

Inhibitor	Cell line						
	OVISE	OVTOKO	MCAS	KURA.[a]	TYK-nu	HEC-1	BeWo
APP	+++	++	−	+	+	+++	−
TIMP-1	+++	+++	++	+	+++	+++	+
TIMP-2	+++	+++	++	+	++	+++	+
PAI-1	+	+	−	−	++	−	+
PAI-2[b]	−	−	−	−	−	−	−

APP (amyloid precursor protein) and TIMPs (tissue inhibitors of metalloproteinases) were detected by reverse zymography, and each inhibitor activity against gelatinase A was scored as + (low), ++ (medium), or +++ (high). PAIs (plasminogen activator inhibitors) were detected by immunoblotting analysis, and the intensity of each immunostained band was scored as − (undetectable), + (low), ++ (medium), or +++ (high).

a) KURA., KURAMOCHI

b) Data not shown in the text.

rounding normal tissues. This interpretation is supported by the previous report,[10] which showed that the expression of u-PA was sufficient to confer an experimental invasive phenotype on non-invasive mouse L cells.

A trypsin-like serine proteinase was secreted only by an ovarian mucinous adenocarcinoma cell line, MCAS. Its secretion has been detected in colon carcinoma, erythroleukemia and fibrosarcoma cell lines in culture.[30] Such heterotropic expression of a trypsin-like enzyme was first found in cyst fluids of patients with mucinous ovarian tumor.[29] The cells producing the enzyme, however, were not identified. To our knowledge, the present report is the first to describe secretion of a trypsin-like enzyme from an ovarian carcinoma cell line. We also observed the secretion of a trypsin-like enzyme by two gastric carcinoma cell lines,[15] and recently, this enzyme was identified as trypsinogen-1.[33] Trypsin secreted by malignant tumor cells is thought to be a key enzyme to promote invasive potential, because of its high activity to degrade extracellular matrix components such as fibronectin, laminin and some kinds of collagens.[15, 30, 31] In addition, this enzyme is thought to be one of the principal initiators of the proteinase cascade for the activation of proenzymes of some metalloproteinases and serine proteinases.[15, 29–31]

MCAS showed secretion of multiple serine proteinases, among which we immunologically identified trypsinogen, u-PA and t-PA. We have previously reported that two gastric carcinoma cell lines secrete multiple serine proteinases. In that study, a serine proteinase with approximate M_r 70,000 was identified as plasmin or a related enzyme.[15] In this study, we failed immunologically to identify the M_r 70,000 enzyme secreted by MCAS, but its electrophoretic mobility and gelatinolytic profile in a gelatin zymogram were very similar to those of plasmin.

There are many reports that gelatinases A and B (type IV collagenases) have important roles in the metastasis of some tumor cells. In the present study, we revealed that two kinds of undifferentiated ovarian carcinoma cells, KURAMOCHI and TYK-nu, secreted gelatinases A and B, respectively. KURAMOCHI also secreted an unidentified metalloproteinase band of M_r 80,000. Very recently, a novel metalloproteinase of the same molecular weight was reported.[34] It seems possible that the band detected in KURAMOCHI may correspond to the novel enzyme. As described above, among five ovarian carcinoma cell lines tested, three adenocarcinoma cell lines mainly secreted serine proteinases, whereas two undifferentiated carcinoma cell lines mainly secreted metalloproteinases, including type IV collagenases. On the other hand, an immunohistochemical study[35] has shown that gelatinase A showed moderate to intense positive staining in clinical samples derived from invasive tumors and metastasis of ovarian serous adenocarcinoma. There may be a difference in proteinase secretion between the serous type and non-serous type of ovarian adenocarcinomas.

Extremely strong gelatinolytic activity was detected in the conditioned medium of choriocarcinoma cells (BeWo) (Fig. 1). High-grade invasive potential and collagenolytic activity of BeWo cells have been reported,[36] but the molecular species involved have not been identified yet. Our data revealed that the proteinase predominantly secreted by BeWo was gelatinase A, and a part of it was processed into its active form of M_r 57,000. In addition, low secretion of TIMPs was noted in its conditioned medium. Such elevated proteolytic activity in BeWo corresponds well to the clinical observation that choriocarcinomas easily metastasize to the lung or brain through blood vessels.

In our preliminary experiments, in which the gynecological carcinoma cell lines used in this study were transplanted into nude or SCID mice by subcutaneous or intraperitoneal injections, all of them retained the original pathological features (data not shown). Furthermore, *in vitro* proteolytic capability, which was assessed by zymography for example, was highly correlated with the pathological invasive potential of each cell line in the transplanted mice. BeWo cells, which showed the highest activity of metalloproteinases and low activity of TIMPs, formed the most invasive and destructive tumors after subcutaneous implantation. In addition, MCAS cells, which secreted multiple serine proteinases including trypsinogen, a plasmin-like enzyme, u-PA and t-PA, and low activity of PAIs, showed the most aggressive and metastatic phenotype among five ovarian carcinoma cell lines after intraperitoneal injection.

There have been a number of studies on proteolytic activity of cancer cells, but most of them examined the production of proteinases and their inhibitors independently. Therefore, there is little information about the balance between proteinases and their inhibitors produced by the same tumor cell lines. In the present study, we identified matrix metalloproteinases, matrix serine proteinases and their inhibitors secreted by gynecological tumor cell lines. We suggest that the balance between the proteinases and their inhibitors might determine the potential of the tumor cells to degrade matrix proteins and, at least in part, their capability of invasion and metastasis.

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