J. Biochem. **137**, 579–586 (2005)
DOI: 10.1093/jb/mvi070

Thrombomodulin Enhances the Invasive Activity of Mouse Mammary Tumor Cells

Shingo Niimi[1,*], Mizuho Harashima[2], Kazuko Takayama[2], Mayumi Hara[2], Masashi Hyuga[1], Taiichiro Seki[2], Toyohiko Ariga[2], Toru Kawanishi[1] and Takao Hayakawa[3]

[1]Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501; [2]Department of Nutrition and Physiology, Nihon University College of Bioresource Sciences, Kameino, Fujisawa 252-8510; and [3]Deputy Director General, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501

Received January 31, 2005; accepted February 18, 2005

Thrombomodulin (TM) is a thrombin receptor on the surface of endothelial cells that converts thrombin from a procoagulant to an anticoagulant. Thrombin promotes invasion by various tumor cells, and positive or negative correlations are found between the expression of TM and tumorigenesis in some patients. In this study, we used an invasion assay to investigate the effect of TM on the invasive activity of a mouse mammary tumor cell line, MMT cells, and the effects of TM were compared with those of thrombin as a positive control. In the presence of 1% fetal calf serum (FCS), TM significantly stimulated MMT cell invasion in a dose-dependent manner, resulting in an approximately 3-fold increase at 1–10 pg/ml over the untreated control. Thrombin also caused a similar degree of stimulation at 50 ng/ml. Since thrombin activity was detected in the components of the assay system, an invasion assay was also performed in a thrombin-activity-depleted assay system constructed to eliminate the effect of thrombin activity; TM (10 pg/ml) plus thrombin (1 pg/ml) stimulated invasion by approximately 3.5-fold in this assay system. Hirudin, a specific thrombin inhibitor, inhibited stimulation by TM as well as by thrombin in both the presence and absence of 1% FCS. Investigations of the effects of TM on proliferation, adhesion and chemotaxis to clarify the mechanism of stimulation by TM revealed that TM does not affect proliferation or adhesion in the presence of 1% FCS, but stimulates chemotaxis by approximately 2.3-fold. Similar results were obtained in experiments using thrombin. TM (10 pg/ml) plus thrombin (1 pg/ml), on the other hand, stimulated chemotaxis by approximately 2.3-fold in the thrombin-activity-depleted assay system. Binding studies using [^{125}I]-thrombin revealed that the cells have specific saturable binding sites for thrombin. These results show that TM stimulates the invasive activity of MMT cells, probably by acting as a cofactor for the thrombin-stimulated invasion of the cells *via* its receptor and lowering the effective concentration of thrombin. The findings also indicate that the stimulation of invasive activity in the presence of 1% FCS and in the thrombin-activity-depleted assay system may mainly be mediated by the stimulation of chemotaxis.

Key words: invasion, thrombin, thrombomodulin.

Abbreviations: TM, thrombomodulin; MEM, modified Eagle's medium; CS, calf serum; FCS, fetal calf serum; MMP, matrix metalloprotease; ECM, extracellular matrix; Boc-Asp(Obzl)-pro-Arg-MCA, Boc-β-benzyl-Asp-Pro-Arg-4-methyl-coumaryl-7-amide; PBS, phosphate-buffered saline.

Thrombomodulin (TM) is a thrombin receptor on the surface of endothelial cells (*1*) that was first discovered as a cofactor for the thrombin-catalyzed activation of the anticoagulant protein C (*2*). Biologically active soluble forms of TM, which probably represent the products of limited proteolytic cleavage of cell-surface TM, were later detected in human plasma (*3*), suggesting a possible role of the soluble forms *in vivo*. TM also positively or negatively regulates various functions of thrombin as described below. TM stimulates the inactivation of pro-urokinase-type plasminogen activator (*4*), the activation of TAF I (*5*), and the activation of progelatinase A (*6*). TM inhibits the activation of platelets (*7*), the activation of factor X (*8*) and human endothelial cells (*9*), the stimulation of fibrin formation (*8*), and the proliferation of arterial smooth muscle cells (*10*) and human umbilical vein endothelial cells (*11*).

On the other hand, there are several direct and indirect lines of evidence indicating that thrombin stimulates invasion and/or metastasis by tumor cells (*12–18*), and it has recently been reported that the expression of TM is increased or decreased in some carcinomas. The expression of TM increases in squamous carcinomas of the lung (*19*), colorectal carcinomas (*20*), and some transitional

*To whom correspondence should be addressed. Tel: +81-3-3700-9347, Fax: +81-3-3700-9084, E-mail: niimi@nihs.go.jp

carcinomas (*21–22*), and its expression level is negatively correlated with the malignancy of carcinoma of the esophagus (*23*), hepatocellular carcinoma (*24*), and ovarian tumors (*25*). There is also evidence of increased serum levels of TM in some tumors, including pancreatic cancer (*26*), digestive tract carcinoma (*27*), and glioblastoma (*28*). Based on this evidence, it is likely that TM plays some role in the regulation of tumor metastasis.

In this study, we investigated the effects of TM on the invasive activity of a mouse mammary tumor cell line, MMT, by an *in vitro* invasion assay, because tumor cell invasion through the basement membrane is a critical step in the process of metastasis (*29–30*). We also compared the effects of TM with those of thrombin as a positive control.

MATERIALS AND METHODS

Materials—TM was a kind gift of Asahi Kasei Pharma, Japan. The TM was prepared as described by Gomi *et al.* (*31*). Plasmids containing the cDNA encoding TM (residues 1–498) were transfected into COS-1 cells, and the recombinant TM was purified from serum-free COS-1-cell-conditioned medium. The purified TM yielded a single band at 90 kDa in SDS-PAGE under reducing conditions. The recombinant TM was confirmed to be thrombin-free by a protein C activating assay developed in our laboratory (*32*). Thrombin (1,140 units/mg protein) was a kind gift of Mochida Pharmaceutical Co., Ltd., Japan. Hirudin was purchased from Wako (Osaka, Japan). A fluorogenic substrate, Boc-β-benzyl-Asp-Pro-Arg-4-methyl-coumaryl-7-amide (Boc-Asp(Obzl)-pro-Arg-MCA), was purchased from Peptide Institute, Inc. (Osaka, Japan).

Cell Culture—MMT mouse mammary tumor cells were obtained from the Japanese Health Science Research Resource Bank and cultured in modified Eagle's medium (MEM) supplemented with 10% calf serum (CS) on 60-mm diameter culture dishes, 4×10^5 cells per dish. After 7 d, the subconfluent MMT cells were detached from the culture dishes with 0.25% trypsin/EDTA, treated with MEM containing 10% CS, and collected by centrifugation. The cells were then washed with MEM and used in experiments. In some experiments, the thrombin activity associated with cells was depleted as described below, and the resultant cells were used for various experiments in which MEM containing 0.1% BSA was used as the basal medium. We refer to this assay system as the thrombin-activity-depleted assay system below. To deplete thrombin activity, the cell suspension (1.5×10^5 cells in 10 ml of MEM) was incubated in a non-adherent form on 100-mm diameter non-treated culture dishes pre-coated with BSA (10 mg/ml) for 2 h in a humidified chamber at 37°C under 5% CO_2, and then washed with MEM.

In Vitro Invasion Assay—In vitro invasion by MMT cells was measured in a Matrigel invasion chamber (Collaborative Biomedical Products, Bedford, MA, USA). The chamber (upper compartment) was placed in a 24-well culture plate (lower compartment), and the cell suspension (1.6×10^5 cells in 500 μl) and the basal medium (750 μl) containing various factors were added to the upper and lower compartments, respectively. MEM containing 1% fetal calf serum (FCS) or 0.1% BSA was used as the basal medium. Matrigel invasion chambers were precoated with fibronectin as described below before use in the thrombin-activity-depleted assay system. Human plasma fibronectin solution (IWAKI, Japan) was diluted to a final concentration of 5 μg/ml with phosphate-buffered saline (PBS), and a 300 μl aliquot was added to the chamber and a 750 μl aliquot to the 24-well culture plate. The chamber and 24-well plate were allowed to stand at 37°C for 2 h and were then washed with PBS. After incubating the cells for 18 h, the filters were fixed with methanol and stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping with cotton swabs, and the number of cells that had migrated to the lower surface of the filters was counted under a microscope.

Measurement of Thrombin Activity—Thrombin activity in FCS, CS, and on cells was measured by the method of Kawabata *et al.* (*33*). A 10 μl volume of 10% FCS or CS was mixed with 90 μl of reaction buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM $CaCl_2$, with or without hirudin (0.5 unit/ml). Packed cells (1×10^6 cells) were suspended in 100 μl of reaction buffer with or without hirudin (0.5 unit/ml). After adding 1 μl of 10 mM substrate, Boc-Asp(Obzl)-pro-Arg-MCA solution to the cell suspension, the mixture was incubated for 20 min at 37°C, and the reaction was stopped by adding 600 μl of 0.6 M acetic acid. The fluorescence of the aminomethylcoumarine released was measured with a fluorospectrophotometer at an excitation wavelength of 380 nm and an emission of 460 nm. A blank solution was prepared by adding 1 μl of substrate solution to the reaction buffer mixed with 600 μl of 0.6 M acetic acid. Thrombin activity was calculated using 1, 2.5, 5 and 10 ng/ml thrombin solutions as standards and subtracting the fluorescence obtained in the presence of hirudin from that in the absence of hirudin. A linear dose-response curve was obtained between 0.5–5 ng/ml of thrombin, and its activity was inhibited by more than 98% by hirudin (0.5 unit/ml). The fluorescence of each sample was within the linear range.

Proliferation Assay—The cell suspension (1×10^5 cells in 4 ml) was seeded on 60-mm diameter culture dishes and incubated with each factor for 18 h. MEM containing 1% FCS was used as the basal medium. The cells were then detached from the culture dishes with 0.25% trypsin/EDTA, treated with MEM containing 10% CS, collected by centrifugation, and counted with a hemocytometer.

Adhesion Assay—Adhesion assays were performed by a modification of the method of Deryugina *et al.* (*34*). A 300 μl aliquot of fibronectin (5 μg/ml), prepared as described above, was added to each well of 24-well plates (IWAKI, Japan). The plates were allowed to stand overnight at 4°C, washed with PBS, blocked with 1% BSA in PBS for 1 h at 37°C, and finally washed in PBS. MMT cells (55×10^4 cells) were exposed to each factor in 2 ml of MEM containing 1% FCS for 30 min at 37°C. After washing with 2 ml of MEM, the cell suspensions (1×10^5 cells in 0.38 ml of MEM) were seeded on each well. After incubation for 30 min at 37°C, non-adherent cells were removed by washing with PBS, and the adherent cells were fixed and stained with 0.2% crystal violet in 10% ethanol for 10 min. After three washes with 2 ml of PBS,

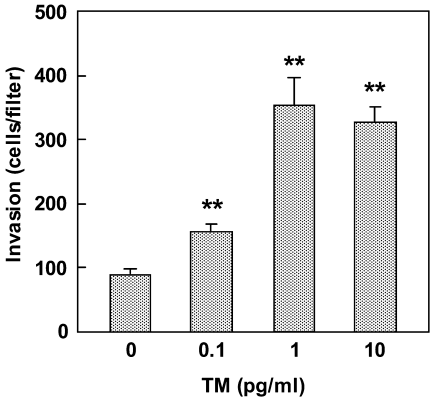

Fig. 1. **Dose dependency of the effect of TM on invasiveness.** MEM containing 1% FCS was used as the basal medium. The concentrations of TM indicated are the concentrations in the lower compartment. The data shown are means ± SD of the data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

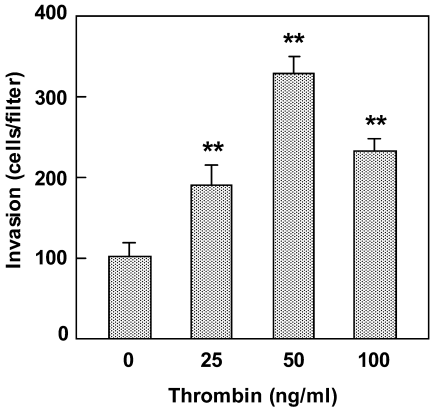

Fig. 2. **Dose dependency of the effect of thrombin on invasiveness.** MEM containing 1% FCS was used as the basal medium. The concentrations of thrombin indicated are the concentrations in the lower compartment. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

the dye was extracted in an end-over-end mixer with 600 μl of 50% ethanol in 50 mM sodium phosphate (pH 4.5) for 10 min, and absorbance was measured at 540 nm. The correlation between absorbance and cell number was confirmed in a preliminary experiment.

Chemotaxis Assay—Chemotaxis assays were performed with control inserts (Collaborative Biomedical Products, Bedford, MA, USA) in a similar manner to the invasion assay described above. The control inserts were not coated with Matrigel. MEM containing 1% FCS was used as the basal medium. The control inserts were pre-coated with fibronectin (5 μg/ml) as described for the pre-coating of the Matrigel invasion chamber in the thrombin-activity-depleted assay system.

Iodination of Thrombin and Determination of Binding—Thrombin was iodinated to a specific activity of 19.1 × 10^7 cpm/μg by the chloramine T method as described previously (*35–36*). After precoating 24-well plates with fibronectin (5 μg/ml) as described above, the cell suspen-

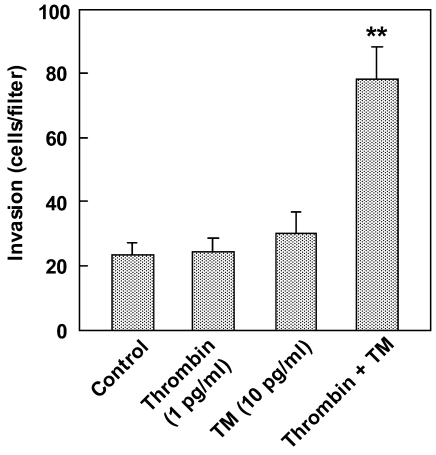

Fig. 3. **Effect of TM and thrombin on invasiveness in the thrombin-activity-depleted assay system.** Cells on which thrombin activity was depleted were used in the experiment. MEM containing 0.1% BSA was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

sions (1.22 × 10^5 cells in 0.45 ml of MEM) were seeded into each well and incubated in a humidified chamber at 37°C under 5% CO_2 for 2 h. The cells were then washed with 0.4 ml of MEM containing 15 mM Hepes (pH 7.2) and 0.1% BSA and incubated for 1.5 h at 37°C in the same buffer with various concentrations of [^{125}I]-thrombin in the presence or absence of a 100-fold excess amount of unlabeled thrombin. After washing the cells four times with the same ice-cold buffer, the cells were solubilized with 0.4 ml of 1 N NaOH for 1 h at 37°C. Specific binding was calculated as the difference between total binding and nonspecific binding.

RESULTS

Effect of TM on Invasiveness—Figure 1 shows the effects of TM on the invasive activity of MMT cells in the presence of 1% FCS. TM significantly stimulated invasive activity in a dose-dependent manner, resulting in an approximately 3-fold stimulation at 1–10 pg/ml. Figure 2 shows the effects of thrombin used as a positive control. Thrombin also stimulated invasive activity in a dose-dependent manner.

On the basis of these findings, we investigated the possibility that the stimulation of invasion by TM might be dependent on thrombin that may have been introduced into the assay system as described below. First, thrombin activity in the assay system was measured. The thrombin concentrations in freshly prepared 10% FCS and CS measured by the thrombin activity assay were 200 pg/ml and 2.8 ng/ml, respectively. The amount of thrombin on the cells measured in a similar manner was 35 pg/10^6 cells. Based on these values, the thrombin concentrations in the assay system with or without 1% FCS were estimated to be 24.48 and 4.48 pg/ml, respectively. Second, the action of TM was examined in the thrombin-activity-depleted assay system described in "MATERIALS AND METHODS," and depletion of thrombin activity in the

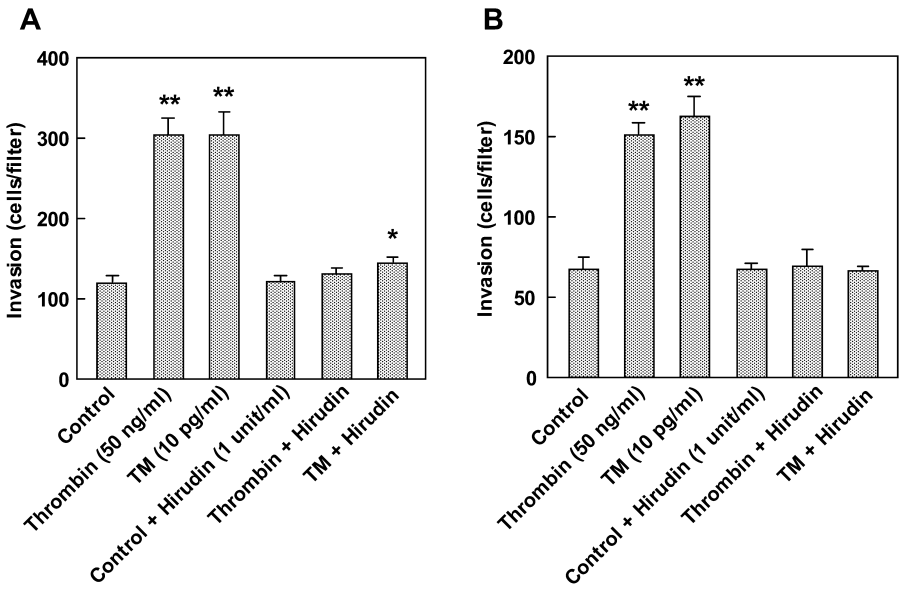

Fig. 4. **Effect of hirudin on the stimulation of invasion by TM.** (A) MEM containing 1% FCS was used as the basal medium. The indicated concentration of each factor is that in the lower compartment. The data shown are means ± SD of data obtained in duplicate wells in three experiments (*$p < 0.05$ *vs.* control; **$p < 0.01$ *vs.* control) (B) MEM containing 0.1% BSA was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

assay system was confirmed by the absence of any detectable thrombin activity on the cells. Figure 3 shows the effects of thrombin (1 pg/ml) and TM (10 pg/ml) on the invasive activity of cells in the thrombin-activity-depleted assay system. While neither thrombin or TM had any effect on invasion, TM plus thrombin stimulated invasion by approximately 3-fold.

Effect of Hirudin on the Stimulation of Invasion by TM—The action of TM was also examined in the presence of the specific thrombin inhibitor hirudin to investigate the possibility described above. Fig. 4, A and B, shows the effects of hirudin on the invasion-stimulating activity of TM in the presence and absence of 1% FCS. We used a 1 unit/ml concentration of hirudin in this experiment, because 50 ng/ml thrombin corresponds to 0.057 unit/ml, and so 1 unit/ml hirudin seemed adequate to inhibit this concentration of thrombin. As expected, hirudin (1 unit/ml) not only inhibited the stimulation by thrombin to control levels, but the stimulation by TM as well.

Effect of TM on Proliferation—Since tumor cell invasion consists of a series of events, including adhesion to the extracellular matrix (ECM) and chemotaxis, we investigated the effects of TM on these two events to clarify the molecular mechanism of the stimulation of invasive activity by TM. Before investigating the effect of TM on these processes, we investigated its effects on cell proliferation to confirm that the stimulation of invasive activity by TM is not an artifact of the enhancement of cell proliferation.

Figure 5 shows the effects of TM on MMT cell proliferation in the presence of 1% FCS. The numbers of cells in the presence of TM or thrombin did not differ from the numbers in the control cultures.

Effect of TM on Adhesion to Fibronectin—Figure 6 shows the effects of TM on adhesion to fibronectin, a basal lam-

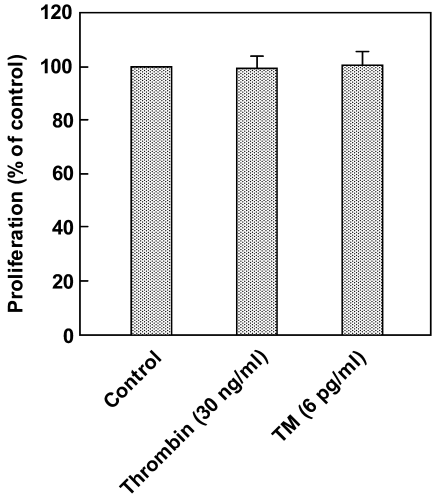

Fig. 5. **Effect of TM on proliferation.** MEM containing 1% FCS was used as the basal medium. The data shown are means ± SD of data obtained in duplicate dishes in three experiments. The deviation in each experiment was less than 10%.

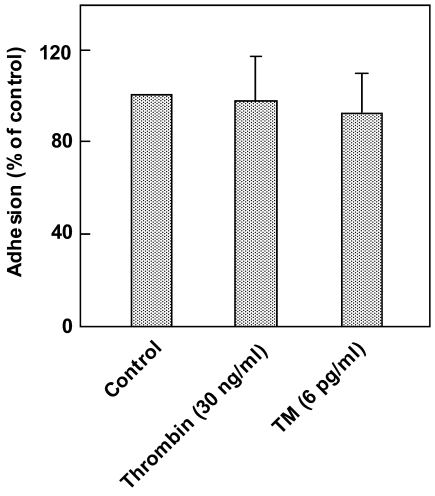

Fig. 6. **Effect of TM on adhesion to fibronectin.** MEM containing 1% FCS was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments. The deviation in each experiment was less than 10%.

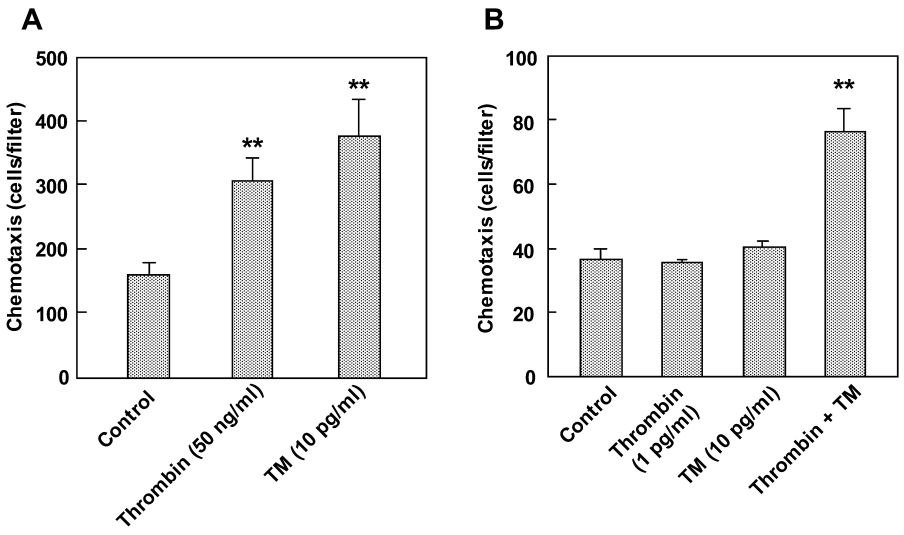

Fig. 7. **Effect of TM on chemotaxis.** (A) MEM containing 1% FCS was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). (B) Cells on which thrombin activity was depleted were used in the experiment. MEM containing 0.1% BSA was used as the basal medium. The data shown are means ± SD of data obtained in duplicate wells in three experiments (**$p < 0.01$ *vs.* control). The deviation in each experiment was less than 10%.

ina component. Neither TM nor thrombin affected adhesion to fibronectin.

Effect of TM on Chemotaxis—Figure 7 (A and B), shows the effects of TM on chemotaxis by MMT cells in the presence of 1% FCS and in the thrombin-activity-depleted assay system, respectively. Both TM (10 pg/ml) and thrombin (50 ng/ml) significantly stimulated chemotaxis by MMT cells by approximately 1.9–2.3-fold in the former system, but neither TM (10 pg/ml) nor thrombin (1 pg/ml) affected chemotaxis in the latter system; TM plus thrombin, on the other hand, stimulated chemotaxis by approximately 2-fold.

Binding of Thrombin—Figure 8 shows the binding curves for specific [^{125}I]-thrombin binding sites on cells in the presence and absence of TM (10 pg/ml). These binding curves show the specific [^{125}I]-thrombin binding to be saturable at approximately 40 ng/ml, and that TM has no effect on the specific binding of [^{125}I]-thrombin. A similar binding experiment was performed in the [^{125}I]-thrombin concentration range of 1–10 pg/ml, but no specific binding was detected independent of the presence or absence of TM, probably because the absolute amount of radioactivity used was too low to be detected as specific binding.

DISCUSSION

In the present study, we show that, at its maximum effective dose, TM stimulates the invasive activity of MMT cells *in vitro* by approximately 3-fold. As far as we know, this is the first time that TM has been shown to stimulate the invasive activity of tumor cells *in vitro*. Similarly, exogenous thrombin causes maximal stimulation of invasion at 50 ng/ml, which is a concentration more than 1,000-fold higher than the maximum effective dose of TM.

Since TM acts as a cofactor for the thrombin-catalyzed activation of protein C and increases the rate of the reaction by >1,000-fold (*8*), the stimulation by TM may have been due to TM interacting with thrombin, which had been introduced into the assay system, and acting as a cofactor for thrombin-stimulated invasion of MMT cells, thus lowering the effective concentration of thrombin. This possibility seems to be supported by the detection of thrombin activity in the assay systems, the requirement for thrombin for stimulation by TM in the thrombin-activity-depleted assay system, and the inhibition of stimulation by hirudin. It is noteworthy that the thrombin concentration required for stimulation by TM in the thrombin-activity-depleted assay system is more than 20,000-fold less than the concentration required for thrombin to stimulate invasion. On the other hand, the control level was not inhibited by hirudin in the presence of 1% FCS, probably due to the lower thrombin concentration in the assay system compared with the effective concentration of exogenous thrombin.

There have been two studies examining the effect of TM on the invasive activity of tumor cells. Matsushita *et al*. showed that a subcloned human esophageal squamous cell carcinoma line with low TM expression is more invasive than a high TM-expressing clone (*37*). In their study, the action of TM does not seem to be due to an acceleration of its thrombin cofactor activity, because the difference between the cell lines with low and high TM expression with respect to their cofactor activity for protein C activation by thrombin was less than 13% and significantly lower than their TM levels and invasive activities. Hosaka *et al*. showed that TM (10–100 ng/ml)

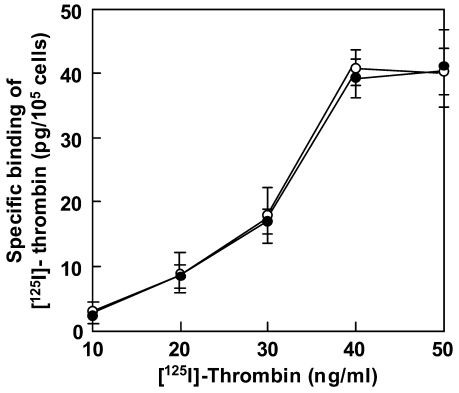

Fig. 8. **Binding of [^{125}I]-thrombin.** Specific binding of [^{125}I]-thrombin to cells in the absence (solid circles) and presence of TM (10 pg/ml) (open circles) was measured. The data shown are representative specific binding curves for [^{125}I]-thrombin binding sites and are means ± SD of data obtained in triplicate wells.

inhibits the invasive activity of mouse melanoma cells *in vitro* (*38*), and TM has also been found to decrease the proliferation of tumor cell lines subcloned from patients with malignant melanomas (*39*). None of these inhibitory effects were inhibited by hirudin.

In contrast to these studies, the present study shows that TM enhances the invasive activity of MMT cells and indicates that the action of TM is entirely dependent on thrombin as described above. Therefore, the mode and mechanism of action of TM in MMT cells seems to be different from its mode and mechanism of action in the squamous cell carcinoma line and melanoma cells.

It is useful to speculate on the role of TM in tumorigenesis based on the findings of our study, because tumor cell invasion through the basement membrane is a critical step in the process of metastasis (*29–30*). Several studies have shown that TM levels in serum increase in patients with certain tumors (*26–28*), as described in the Introduction. Thus, the results of this study suggest that the soluble form of TM may play a positive role in the malignancy of some kinds of tumors, probably by enhancing the metastatic potential of thrombin. On the other hand, TM on the cell surface may act as a negative regulator to thrombin, because thrombin is degraded as the thrombin-TM complex by its internalization after binding to TM on the cell surface (*40*). This possibility may be supported by the findings that the expression level of TM is negatively correlated with the malignancy of some carcinomas (*23–25*), as described in the Introduction.

Tumor cell invasion is a complex process that involves adhesion to ECM, degradation of ECM, and chemotaxis (*41*). Chemotaxis is the essential step in invasion as reviewed by Wells (*42*). The results of the present study show that both TM and thrombin stimulate chemotaxis in the presence of 1% FCS, and that TM plus thrombin stimulate chemotaxis in the thrombin-activity-depleted assay system. Both of these findings are consistent with previous reports that thrombin stimulates chemotaxis (*17*, *18*), and the presence of specific binding sites for thrombin on cells indicates that these actions are mediated by thrombin receptors.

However, other actions of thrombin may also be involved in the stimulation of tumor cell invasion, because the enhancement of chemotaxis alone is insufficient to account for the increase in tumor cell invasion. One such other possible action of thrombin is the stimulation of the matrix metalloprotease (MMP)–mediated degradation of a variety of ECM proteins, including collagens type IV, V, VII, and X, fibronectin, laminin (*43–47*), elastin (*48–49*), proteoglycans (*49–51*), and entactin (*52*). Several reports have indicated that thrombin increases the active forms of MMP-2 and MMP-9 (*53–59*), and plays important roles in the enhancement of tumor cell invasion and metastasis (*34*, *60–68*). There are also reports that thrombin stimulates the release of MMP-2 (*69*), the expression of MMP-1 and MMP-3 (*70*), and the expression of MMP-9 mRNA (*71*). Another possible action is the stimulation of MMP-independent degradation of ECM. This possibility appears to be supported by the finding that thrombin stimulates the expression of urokinase-type plasminogen activator, a factor involved in the degradation of ECM protein (*72*), and stimulates the heparinase-mediated release of heparan sulfate from ECM (*73*).

In conclusion, the results of this study show that TM stimulates the invasive activity of MMT cells, probably by acting as a cofactor for the thrombin-stimulated invasion of cells mediated by thrombin receptors, and by lowering the effective concentration of thrombin. Further, the results indicate that the stimulation is mainly caused by an enhancement of chemotaxis.

This study was supported, in part, by a Grant-in-Aid for research on health sciences focusing on drug innovation from the Japan Health Sciences Foundation.

REFERENCES

1. Esmon, C.T. and Owen, W.G. (1981) Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc. Natl Acad. Sci. USA* **78**, 2249–2252
2. Esmon, C.T. (1989) The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J. Biol. Chem.* **264**, 4743–4746
3. Ishii, H. and Majerus, P.W. (1985) Thrombomodulin is present in human plasma and urine. *J. Clin. Invest.* **76**, 2178–2181
4. Molinari, A., Giorgetti, C., Lansen, J., Vaghi, F., Orsini, G., Faioni, E.M., and Mannucci, P.M. (1992) Thrombomodulin is a cofactor for thrombin degradation of recombinant single-chain urokinase plasminogen activator "in vitro" and in a perfused rabbit heart model. *Thromb. Haemost.* **67**, 226–232
5. Bajzar, L., Morser, J., and Nesheim, M. (1996) TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *J. Biol. Chem.* **271**, 16603–16608
6. Pekovich, S.R., Bock, P.E., and Hoover, R.L. (2001) Thrombin-thrombomodulin activation of protein C facilitates the activation of progelatinase A. *FEBS Lett.* **494**, 129–132
7. Esmon, N.L., Carroll, R.C., and Esmon, C.T. (1986) Thrombomodulin blocks the ability of thrombin to activate platelets. *J. Biol. Chem.* **258**, 12238–12242
8. Esmon, C.T., Esmon, N.L., and Harris, K.W. (1983) Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. *J. Biol. Chem.* **257**, 7944–7947
9. Parkinson, J.F., Bang, N.U., and Garcia, J.G. (1993) Recombinant human thrombomodulin attenuates human endothelial cell activation by human thrombin. *Arterioscler. Thromb.* **13**, 1119–1123
10. Li, J., Garnette, C.S., Cahn, M., Claytor, R.B., Rohrer, M.J., Dobson, J.G. Jr., Gerlitz. B., and Cutler, B.S. (2000) Recombinant thrombomodulin inhibits arterial smooth muscle cell proliferation induced by thrombin. *J. Vasc. Surg.* **32**, 804–813
11. Lafay, M., Laguna, R., Le Bonniec, B.F., Lasne, D., Aiach, M., and Rendu, F. (1998) Thrombomodulin modulates the mitogenic response to thrombin of human umbilical vein endothelial cells. *Thromb. Haemost.* **79**, 848–852
12. Nierodzik, M.L., Plotkin, A., Kajumo, F., and Karpatkin, S. (1991) Thrombin stimulates tumor-platelet adhesion *in vitro* and metastasis *in vivo*. *J. Clin. Invest.* **87**, 229–236
13. Nierodzik, M.L., Kajumo, F., and Karpatkin, S. (1992) Effect of thrombin treatment of tumor cells on adhesion of tumor cells to platelets *in vitro* and tumor metastasis *in vivo*. *Cancer Res.* **52**, 3267–3272
14. Esumi, N., Fan, D., and Fidler, J.I. (1991) Inhibition of murine melanoma experimental metastasis by recombinant desulfatohirudin, a highly specific thrombin inhibitor. *Cancer Res.* **51**, 4549–4556
15. Wojtukiewicz, M.Z., Tang, D.G., Ben-Josef, E., Renaud, C., Walz, D.A., and Honn, K.V. (1995) Solid tumor cells express functional 'tethered ligand' thrombin receptor. *Cancer Res.* **55**, 698–704
16. Even-Ram, S., Uziely. B., Cohen, P., Grisaru-Granovsky, S., Maoz, M., Ginzburg, Y., Reich, R., Vlodavsky, I., and Bar-

Shavit, R. (1998) Thrombin receptor overexpression in malignant and physiological invasion processes. *Nat. Med.* **4**, 909–914
17. Hernandez-Rodriguez, N.A., Correa, E., Contreras-Paredes, A., and Green, L. (1999) Evidence that thrombin present in lungs of patients with pulmonary metastasis may contribute to the development of the disease. *Lung Cancer* **26**, 157–167
18. Henrikson, K.P., Salazar, S.L., Fenton, J.W. II., and Pentecost, B.T. (1999) Role of thrombin receptor in breast cancer invasiveness. *Br. J. Cancer* **79**, 401–406
19. Ogawa, H., Yonezawa, S., Maruyama, I., Matsushita, Y., Tezuka, Y., Toyoyama, H., Yanagi, M., Matsumoto, H., Nishijima, H., Shimotakahara, T., Aikou, T., and Sato, E. (2000) Expression of thrombomodulin in squamous cell carcinoma of the lungs: its relationship to lymph node metastasis and prognosis of the patients. *Cancer Lett.* **149**, 95–103
20. Takebayashi, Y., Yamada, K., Maruyama, I., Fujii, R., Akiyama, S., and Aikou, T. (1995) The expression of thymidine phosphorylase and thrombomodulin in human colorectal carcinomas. *Cancer Lett.* **92**, 1–7
21. Ordonez, N.G. (1997) Value of thrombomodulin immunostaining in the diagnosis of transitional cell carcinoma: a comparative study with carcinoembryonic antigen. *Histopathology* **31**, 517–524
22. Ordonez, N.G. (1998) Thrombomodulin expression in transitional cell carcinoma. *Am. J. Clin. Pathol.* **110**, 385–390
23. Matsumoto, M., Natsugoe, S., Nakashima, S., Shimada, M., Nakano, S., Kusano, C., Baba, M., Takao, S., Matsushita, Y., and Aikou, T. (2000) Biological evaluation of undifferentiated carcinoma of the esophagus. *Ann. Surg. Oncol.* **7**, 204–209
24. Suehiro, T., Shimada, M., Matsumata, T., Taketomi, A., Yamamoto, K., and Sugimachi, K. (1995) Thrombomodulin inhibits intrahepatic spread in human hepatocellular carcinoma. *Hepatology* **21**, 1285–1290
25. Wilhelm, S., Schmitt, M., Parkinson, J., Kuhn, W., Graeff, H., and Wilhelm O.G. (1998) Thrombomodulin, a receptor for the serine protease thrombin, is decreased in primary tumors and metastases but increased in ascitic fluids of patients with advanced ovarian cancer FIGO IIIc. *Int. J. Oncol.* **13**, 645–651
26. Lindahl, A.K., Boffa, M.C., and Abildgaard, U. (1993) Increased plasma thrombomodulin in cancer patients. *Thromb. Haemost.* **69**, 112–114
27. Boffa, M.C., Lapeyrere, C., Berard, M., Lindahl, A.K., Flageul, B., Chemaly, P., Abildgaard, U., and Dubertret, L. (1994) Plasma thrombomodulin level in malignancy varies according to the tumor type. *Nouv. Rev. Fr. Hematol.* **36**, S87–88
28. Salmaggi, A., Eoli, M., Frigerio, S., Ciusani, E., Silvani, A., and Boiardi, A. (1999) Circulating intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and plasma thrombomodulin levels in glioblastoma patients. *Cancer Lett.* **146**, 169–172
29. Liotta, L.A. (1986) Tumor invasion and metastases-role of the extracellular matrix: Rhoads Memorial Award lecture. *Cancer Res.* **46**, 1–7
30. Terranova, V.P., Hujanen, E.S., and Martin, G.R. (1986) Basement membrane and the invasive activity of metastatic tumor cells. *J. Natl Cancer Inst.* **77**, 311–316
31. Gomi, K., Zushi, M., Honda, G., Kawahara, S., Matsuzaki, O., Jr., Kanabayashi, T., Yamamoto, S., Maruyama, I., and Suzuki, K. (1990) Antithrombotic effect of recombinant human thrombomodulin on thrombin-induced thromboembolism in mice. *Blood* **75**, 1396–1399
32. Niimi, S., Oshizawa, T., Naotsuka, M., Ohba, S., Yokozawa, A., Murata, T., and Hayakawa, T. (2002) Establishment of a standard assay method for human thrombomodulin and determination of the activity of the Japanese reference standard. *Biologicals* **30**, 69–76
33. Kawabata, S., Miura, T., Morita, T., Kato, H., Fujikawa, K., Iwanaga, S., Takada, K., Kimura, T., and Sakakibara, S. (1988) Highly sensitive peptide-4-methylcoumaryl-7-amide substrates for blood-clotting proteases and trypsin. *Eur. J. Biochem.* **172**, 17–25
34. Deryugina, E.I., Luo, G.X., Reisfeld, R.A., Bourdon, M.A., and Strongin, A. (1997) Tumor cell invasion through matrigel is regulated by activated matrix metalloproteinase-2. *Anticancer Res.* **17**, 3201–3210
35. Greenwood, F.C., Hunter, W.M., Glover, J.S. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**, 114–123
36. Haeuptle, M.T., Aubert, M.L., Djiane J., Kraehenbuhl, J.P. (1983) Binding sites for lactogenic and somatogenic hormones from rabbit mammary gland and liver. *J. Biol. Chem.* **258**, 305–314
37. Matsushita, Y., Yoshiie, K., Imamura, Y., Ogawa, H., Imamura, H., Takao, S., Yonezawa, S., Aikou, T., Maruyama, I., and Sato, E. (1998) A subcloned human esophageal squamous cell carcinoma cell line with low thrombomodulin expression showed increased invasiveness compared with a high thrombomodulin-expressing clone—thrombomodulin as a possible candidate for an adhesion molecule of squamous cell carcinoma. *Cancer Lett.* **127**, 195–201
38. Hosaka, Y., Higuchi, T., Tsumagari, M., and Ishii, H. (2000) Inhibition of invasion and experimental metastasis of murine melanoma cells by human soluble thrombomodulin. *Cancer Lett.* **161**, 231–240
39. Zhang, Y., Weiler-Guettler, H., Chen, J., Wilhelm, O., Deng, Y., Qiu, F., Nakagawa, K., Klevesath, M., Wilhelm, S., Bohrer, H., Nakagawa, M., Graeff, H., Martin, E., Stern, D.M., Rosenberg, R.D., Ziegler, R., and Nawroth, P.P. (1998) Thrombomodulin modulates growth of tumor cells independent of its anticoagulant activity. *J. Clin. Invest.* **101**, 1301–1309
40. Maruyama, I. and Majerus, P.W. (1985) The turnover of thrombin-thrombomodulin complex in cultured human umbilical vein endothelial cells and A549 lung cancer cells. Endocytosis and degradation of thrombin. *J. Biol. Chem.* **260**, 15432–15438
41. Hart, I.R., Goode, N.T., and Wilson, R.E. (1989) Molecular aspects of the metastatic cascade. *Biochim. Biophys. Acta* **989**, 65–84
42. Wells, A. (2000) Tumor invasion: role of growth factor-induced cell motility. *Adv. Cancer Res.* **78**, 31–101
43. Matrisian, L.M. (1990) Metalloproteinases and their inhibitors in matrix remodeling. *Ternds Genet.* **6**, 121–125
44. Collier, I.E., Wilhelm, S.M., Eisen, A.Z., Marmer, B.L., Grant. G.A., Seltzer. J.L., Kronberger, A., He. C.S., Bauer, E.A., and Goldberg, G.I. (1988) H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. *J. Biol. Chem.* **263**, 6579–6587
45. Wilhelm, S.M., Collier, I.E., Marmer, B.L., Eisen, A.Z., Grant, G.A., and Goldberg, G.I. (1989) SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J. Biol. Chem.* **264**, 17213–17221
46. Fessler, L.I., Duncan, K.G., Fessler, J.H., Salo, T., and Tryggvason, K. (1984) Identification of the procollagen IV cleavage products produced by a specific tumor collagenase. *J. Biol. Chem.* **259**, 9783–9789
47. Woessner, J.F. Jr. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* **5**, 2145–2154
48. Senior, R.M., Griffin, G.L., Fliszar, C.J., Shapiro, S.D., Goldberg, G.I., and Welgus, H.G. (1991) Human 92- and 72-kilodalton type IV collagenases are elastases. *J. Biol. Chem.* **266**, 7870–7875
49. Murphy, G., Cockett, M.I., Ward, R.V., and Docherty, A.J. (1991) Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitive comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinae (PUMP). *Biochem. J.* **277**, 277–279
50. Nguyen, Q., Murphy, G., Hughes, C.E., Mort, J.S., and Roughley, P.J. (1993) Matrix metalloproteinases cleave at two distinct sites on human cartilage link protein. *Biochem. J.* **295**, 595–598

51. Fosang, A.J., Last, K., Knauper, V., Neame, P.J., Murphy, G., Hardingham, T.E., Tschesche, H., and Hamilton, J.A. (1993) Fibroblast and neutrophil collagenases cleave at two sites in the cartilage aggrecan interglobular domain. *Biochem. J.* **295**, 273–276
52. Sires, U.I., Griffin, G.L., Broekelmann, T.J., Mecham, R.P., Murphy, G., Chung, A.E., Welgus, H.G., and Senior, R.M. (1993) Degradation of entactin by matrix metalloproteinases. Susceptibility to matrilysin and identification of cleavage sites. *J. Biol. Chem.* **268**, 2069–2074
53. Zucker, S., Conner, C., DiMassmo, B.I., Ende, H., Drews, M., Seiki, M., and Bahou, W.F. (1995) Thrombin induces the activation of progelatinase A in vascular endothelial cells. Physiologic regulation of angiogenesis. *J. Biol. Chem.* **270**, 23730–23738
54. Galis, Z.S., Kranzhofer, R., Fenton, J.W. II., and Libby, P. (1997) Thrombin promotes activation of matrix metalloproteinase-2 produced by cultured vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **17**, 483–489
55. Nguyen, M., Arkell, J., and Jackson, C.J. (1999) Thrombin rapidly and efficiently activates gelatinase A in human microvascular endothelial cells via a mechanism independent of active MT1 matrix metalloproteinase. *Lab. Invest.* **79**, 467–475
56. Pekovich, S.R., Bock, P.E., and Hoover, R.L. (2001) Thrombin-thrombomodulin activation of protein C facilitates the activation of progelatinase A. *FEBS Lett.* **694**, 129–132
57. Lafleur, M.A., Hollenberg, M.D., Atkinson. S.J., Knauper, V., Murphy, G., and Edwards, D.R. (2001) Activation of pro-(matrix metalloproteinase-2) (pro-MMP-2) by thrombin is membrane-type-MMP-dependent in human umbilical vein endothelial cells and generates a distinct 63 kDa active species. *Biochem. J.* **357**, 107–115
58. Maragoudakis, M.E., Kraniti, N., Giannopoulou, E., Alexopoulos, K., and Matsuokas, J. (2001) Modulation of angiogenesis and progelatinase A by thrombin receptor mimetics and antagonists. *Endothelium* **8**, 195–205
59. Liu, Y., Gilcrease, M.Z., Henderson, Y., Yuan, X.H., Clayman, G.L., and Chen, Z. (2001) Expression of protease-activated receptor 1 in oral squamous cell carcinoma. *Cancer Lett.* **169**, 173–180
60. Stetler-Stevenson, W.G., Aznavoorian, S., and Liotta, L.A. (1993) Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell. Biol.* **9**, 541–573
61. Himelstein, B.P., Canete-Soler, R., Bernhard, E.J., Dilks, D.W., and Muschel, R.J. (1994) Metalloproteinases in tumor progression: the contribution of MMP-9. *Invasion Metastasis.* **14**, 246–258
62. Bernhard, E.J., Gruber, S.B., and Muschel, R.J. (1994) Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. *Proc. Natl Acad. Sci. USA* **91**, 4293–4297
63. MacDougall, J.R., and Matrisian. L.M. (1995) Contribution of tumor and stromal matrix metalloroteinases to tumor progression, invasion and metastasis. *Cancer Metastasis Rev.* **14**, 351–362
64. Hua, J. and Muschel, R.J. (1996) Inhibition of matrix metalloproteinase 9 expression by a ribozyme blocks metastasis in a rat sarcoma model system. *Cancer Res.* **56**, 5279–5284
65. Kim, J., Yu, W., Kovalski, K., and Ossowski, L. (1998) Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell* **94**, 353–362
66. Hahn-Dantona, E., Ramos-DeSimone, N., Sipley, J., Nagase, H., French, D.L., and Quigley, J.P. (1999) Activation of proMMP-9 by a plasmin/MMP-3 cascade in a tumor cell model. Regulation by tissue inhibitors of metalloproteinases. *Ann. N. Y. Acad. Sci.* **878**, 372–387
67. John, A. and Tuszynski, G. (2001) The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. *Pathol. Oncol. Res.* **7**, 14–23
68. Giannelli, G. and Antonaci, S. (2002) Gelatinases and their inhibitors in tumor metastasis: from biological research to medical applications. *Histol. Histopathol.* **17**, 339–345
69. Fernandez-Patron, C., Zhang, Y., Radomski, M.W., Hollenberg, M.D., and Davidge, S.T. (1999) Rapid release of matrix metalloproteinase (MMP)-2 by thrombin in the rat aorta: modulation by protein tyrosine kinase/phosphatase. *Thromb. Haemost.* **82**, 1353–1357
70. Duhamel-Clérin, E., Orvain, C., Lanza, F., Cazenave, J.P., and Klein-Soyer, C. (1997) Thrombin receptor-mediated increase of two matrix metalloproteinases, MMP-1 and MMP-3, in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **17**, 1931–1938
71. Liu, W.H., Chen, X.M., and Fu, B. (2000) Thrombin stimulates MMP-9 mRNA expression through AP-1 pathway in human mesangial cells. *Acta. Pharmacol. Sin.* **21**, 641–645
72. Yoshida, E., Verrusio, E.N., Mihara, H., Oh, D., and Kwaan, H.C. (1994) Enhancement of the expression of urokinase-type plasminogen activator from PC-3 human prostate cancer cells by thrombin. *Cancer Res.* **54**, 3300–3304
73. Benezra, M., Vlodavsky, I., and Bar-Shavit, R. (1992) Thrombin enhances degradation of heparan sulfate in the extracellular matrix by tumor cell heparanase. *Exp. Cell Res.* **201**, 208–215