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Role of Tissue Inhibitor of Metalloproteinases-2 (TIMP-2) in Regulation of Pro-Gelatinase A Activation Catalyzed by Membrane-Type Matrix Metalloproteinase-1 (MT1-MMP) in Human Cancer Cells[1]

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To clarify the regulatory mechanism of pro-gelatinase A (proGelA) activation at a cellular level, expression of gelatinase A (GelA), three MT-MMPs, and TIMP-2 was examined with 11 human cancer cell lines cultured in the presence and absence of stimulants. MT1-MMP mRNA was expressed in 8 cell lines, while MT2-MMP and MT3-MMP mRNAs were expressed in fewer cell lines. The cells with high proGelA activation strongly expressed MT1-MMP mRNA but not MT2-MMP and MT3-MMP mRNAs, suggesting that MT1-MMP was responsible for the proGelA activation in the cancer cells. Treatments with concanavalin A (Con A) and a phorbor ester (TPA) enhanced the MT1-MMP expression, but only Con A stimulated the proGelA activation in many cell lines. In HT1080 fibrosarcoma cells, however, TPA also stimulated the activation. The level of TIMP-2 secreted into culture medium inversely correlated with proGelA activation. For example, 2 squamous cell carcinoma lines (HSC-3 and HSC-4) and 3 HT1080 clones, which efficiently activated proGelA, secreted little TIMP-2 into medium, whereas other cell lines and other HT1080 clones, which hardly activated proGelA, secreted TIMP-2 at high levels. When HSC-3 cells were incubated with TIMP-2 protein or transfected with TIMP-2 cDNA, the proGelA activation was strongly inhibited. These results indicated that extracellular TIMP-2 was an important negative regulator of proGelA activation. However, the level of extracellular TIMP-2 was not consistent with that of TIMP-2 mRNA in some cell lines. Other experimental results suggested that TIMP-2 might be rapidly metabolized after binding to MT1-MMP, and Con A treatment might stabilize the complex of TIMP-2 and MT1-MMP on cell membranes.

Key words: concanavalin A, gelatinase A (MMP-2), membrane-type matrix metalloproteinase-1 (MT1-MMP), phorbor ester (TPA), tissue inhibitor of metalloproteinase-2 (TIMP-2).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases involved in turnover of various extracellular matrix (ECM) proteins. More than 15 members of the MMP family have been reported, most of which are secreted in latent forms (pro-MMPs). These MMPs are thought to play an essential role in tissue remodeling under various physiological and pathological conditions such as morphogenesis, angiogenesis, tissues repair, arthritis, and tumor invasion (reviewed in Refs. *1-3*). The activities of MMPs are regulated at many levels including gene expression, activation of the latent MMPs, and inhibition by four kinds of tissue inhibitors of metalloproteinases (TIMPs). Recently much attention has been focused on the activation mechanism of gelatinase A (GelA; MMP-2).

GelA, as well as gelatinase B (GelB; MMP-9), has a potent activity against gelatin and type IV collagen of basement membranes (*4, 5*). GelA is produced by normal stromal fibroblasts and a wide range of tumor cells (*3, 4*). The activation of the latent form of GelA (proGelA) to the mature form appears to be associated with the invasiveness of several types of tumors (*6, 7*). Recently, Sato *et al.* identified a novel membrane-bound MMP, MT1-MMP, as

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Con A, concanavalin A; bp, base pair; CM, conditioned medium; FCS, fetal calf serum; GelA, gelatinase A; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; proGelA, pro-gelatinase A; RT-PCR, reverse transcription/polymerase chain reaction; TIMP, tissue inhibitor of metalloproteinases.

the proGelA-activating enzyme (*8*). MT1-MMP is predominantly detected in malignant cancer tissues, and its expression is associated with the invasiveness of various tumors, such as breast, stomach, and brain tumors (*9-11*). Since the finding of MT1-MMP, three other MT-MMP species (MT2-, MT3-, and MT4-MMPs) have been cloned (*12-14*).

All MT-MMPs but MT4-MMP have been reported to activate proGelA (*8, 15, 16*). Since MT4-MMP does not have a signal sequence, it is unlikely to function for proGelA activation (*14*). The initial cleavage of the propeptide sequence of proGelA by MT-MMPs leads to the subsequent intermolecular, autolytic conversion of the intermediate form of GelA to the mature form (*17*). The trans-membrane domains of MT1-MMP and MT3-MMP seem to be important in the effective conversion of the intermediate form of GelA to the mature form (*16, 18*). TIMP-2 is essential for the binding to cell surface and subsequent activation of proGelA: TIMP-2 seems to bind to cell surface MT1-MMP and form a tri-molecular complex with MT1-MMP and proGelA (*19*). However, the detailed mechanism of proGelA activation in living cells remains unclear. Recent studies have shown that the expression of MT1-MMP in cultured cells does not necessarily correlate with the activation of proGelA (*20, 21*). For example, the treatment of human embryonic lung fibroblasts with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) increases the expression of MT1-MMP mRNA without stimulating proGelA activation (*21*). In contrast, the treatment of human fibrosarcoma cell line HT1080 with concanavalin A (Con A) effectively activates proGelA without increasing the basal expression of MT1-MMP mRNA. Thus, it remains unclear how the activation of proGelA by MT-MMP is regulated in living cells. In addition, specific functions of each MT-MMP species have been poorly understood.

To understand the regulatory mechanism of proGelA activation, we examined the proGelA activation and expression of three types of MT-MMPs and TIMP-2 in 11 human cancer cell lines with or without stimulation. Our results demonstrate that TIMP-2 plays an important role in negative regulation of proGelA activation mediated by MT1-MMP in human cancer cells.

MATERIALS AND METHODS

Materials—Mouse monoclonal antibody against human TIMP-2 was prepared using a 20-meric synthetic peptide (Lys^{22}-Lys^{41}) as the antigen (*22*). Mouse monoclonal antibody against TIMP-1 was purchased from Fuji Chemical Industries (Toyama). Mouse monoclonal antibody against human MT1-MMP (#114) (*8*), MT1-MMP cDNA (*8*), and proGelA cDNA (*23*) have previously been reported. A cDNA clone for human TIMP-2 and recombinant TIMP-2 protein were kindly provided by Drs. S. Misawa and H. Takaku, Pharmaceuticals and Biotechnology Laboratory, Japan Energy (Saitama). cDNA clones for human MT2-MMP and MT3-MMP were previously obtained from the placenta and fetal brain, respectively, as reported previously (*16*). The plant lectin Con A (type IV, substantially free of carbohydrates) was purchased from Sigma (St. Louis, MO); 12-*O*-tetradecanoylphorbol-13-acetate (TPA) from Wako Pure Chemical Industries (Osaka); [α-^{32}P]dCTP from Amersham (Buckinghamshire, England); and enzymes for DNA digestion and modification from Takara Shuzo (Shiga) or Toyobo (Osaka). Mammalian expression vector pGM, which had been constructed from pCDL-SRα296 (Okayama and Berg 1983), was a kind gift from N. Ohkura (Terumo R&D center, Kanagawa). A synthetic hydroxamic acid inhibitor for MMPs, KB8301, was a generous gift from Dr. K. Yoshino, Kanebo Institute for Cancer Research (Osaka).

Cell Cultures and Preparation of Conditioned Media (CMs) and Cell Lysates—Types of human cancer cell lines used are as follows: HSC-3 and HSC-4, tongue squamous cell carcinomas; A549, lung adenocarcinoma; HT1080, fibrosarcoma; T24, urinary bladder carcinoma; UMK-1, bladder mixed carcinoma; T98G and U-87MG, glioblastoma; G361, malignant melanoma; HeLa S3, cervix epithelioid carcinoma; and K562, chronic myelogenous leukemia. UMK-1 was a kind gift from Dr. M. Koono (Second Department of Pathology, Miyazaki Medical College, Miyazaki), and U-87MG from Dr. A. Nakano (Department of Neurosurgery, Hyogo College of Medicine, Hyogo). The other cell lines were obtained from Japanese Cancer Resources Bank (JCRB). Six morphologically different clones of HT1080 cells were isolated from the parent cell line by the limiting dilution method. Each cell line was grown to semi-confluency in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Gibco; Grand Island, NY), DME/F12, supplemented with 10% fetal calf serum (FCS). The cells were rinsed three times with serum-free DME/F12, and the culture was further continued for 2 days. The resultant CM was collected, clarified by centrifugation, and concentrated by ammonium sulfate precipitation, as reported previously (*24*). The protein precipitate was dissolved in a small volume of 10 mM Tris-HCl buffer (pH 7.5) containing 0.05% of Brij35 and dialyzed against the same buffer. By these procedures, the initial CMs were concentrated 30-fold. To prepare cell lysates, the cells were dissolved in a small volume of the SDS-sample buffer without a reducing reagent.

Northern Blotting Analysis—Total RNAs were prepared from 11 cell lines cultured under different conditions by the acid-guanidinium phenol chloroform method (*25*). Thirty microgram RNA was electrophoresed on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane by capillary elution. The nylon membrane was hybridized with ^{32}P-labeled cDNAs and washed by the standard method. The hybridized signals were visualized by autoradiography. The membranes were then dehybridized and reused.

Transfection of TIMP-2 cDNA into HSC-3 Cells—The cDNA for human TIMP-2 was inserted into a mammalian expression vector pGM for transient expression (*26*). Transfection of the cDNA into HSC-3 cells was performed by the lipofection method described before (*16*). The serum-free CM of the transfected cells was prepared as described above.

Gelatin Zymography—Zymography was carried out on 10% polyacrylamide gels containing 1 mg/ml of gelatin, as described before (*16*). Concentrated CM from each cell line was diluted with the SDS-sample buffer and applied to the assay.

Immunoblotting Analysis—Samples were separated on 10% (for detecting MT1-MMP) or 14% (for detecting

TIMPs) polyacrylamide gels by reducing SDS-PAGE. After the electrophoresis, proteins were electrophoretically transferred onto nitrocellulose membranes. The blotted membranes were reacted with primary antibody. Immunoreactive signals were visualized by the alkaline phosphatase method as described previously (*24*).

RESULTS

Gelatinase Activities Secreted by 11 Human Cancer Cell Lines—To investigate the regulatory mechanism of proGelA activation, we first analyzed gelatinase activities secreted by 11 human cancer cell lines incubated with or without Con A or TPA, which are known to stimulate proGelA activation in some cell types. When analyzed by gelatin zymography, serum-free conditioned media (CMs) of many cell lines showed well-known patterns of the latent and mature forms of GelA and gelatinase B (GelB): apparent molecular mass obtained by the nonreducing SDS-PAGE was 64 kDa for proGelA, 60 kDa for mature GelA, and 90 kDa for pro-gelatinase B (proGelB) (Fig. 1). The 8 cell lines other than UMK-1, HeLaS3, and K562 secreted significant levels of GelA in the absence of Con A and TPA. HeLaS3 cells secreted little gelatinase of any type. In the CMs of two squamous cell carcinoma lines, HSC-3 and HSC-4, about half activity of proGelA had converted to the mature form, whereas in those of the others (A549, HT1080, T24, T98G, U-87MG, and G361) the active form of GelA was little detected.

The treatment with Con A promoted the conversion of proGelA to its mature form in all of the proGelA-secreting cell lines but A549 (Fig. 1). In HSC-3, HSC-4, and HT1080, proGelA was almost completely converted to the mature form by the Con A treatment. TPA treatment of the cell lines caused effective conversion of proGelA to the mature form in HT1080 cells, but not in the other cell lines. These results indicate that proGelA activation in the presence and absence of stimulants strikingly varies from one cell type to another.

The zymographic analysis shown in Fig. 1 also showed that proGelB was secreted at high levels from 4 (HSC-3, HSC-4, HT1080, and UMK-1) out of 11 cell lines, and its secretion was strongly induced by TPA in 7 cell lines (HSC-3, HSC-4, A549, HT1080, T24, T98G, and K562). Con A slightly inhibited or did not affect the secretion of proGelB.

Expression of Three MT-MMPs and GelA mRNAs in Various Cancer Cell Lines—Northern blotting analysis was performed to examine expression of three MT-MMP mRNAs in the 11 cell lines cultured in the presence and absence of the stimulants (Fig. 2). Expression of MT1-MMP mRNA was detectable in the 8 cell lines other than A549, HeLaS3, and K562. Con A treatment increased MT1-MMP expression in 6 of the 8 cell lines, while TPA treatment increased it in 7 cell lines. Exceptionally, Con A significantly suppressed the MT1-MMP expression in HT1080 cells.

The Northern blotting analysis also showed that MT2-MMP mRNA was expressed at relatively low levels in five cell lines (HSC-4, HT1080, UMK-1, U-87MG, and HeLaS3), and that MT3-MMP mRNA was expressed at a high level in U-87MG and at a low level in HT1080 and T24. The expression of MT2-MMP mRNA was greatly enhanced by Con A and TPA treatments in HeLaS3 cells, but it was slightly suppressed or not affected in the others. The expression of MT3-MMP mRNA in U-87MG cells was not affected by Con A or TPA.

The data shown in Fig. 1 and Fig. 2 demonstrate three important points. First, the expression of three MT-MMP mRNAs is independently regulated in a cell-type-specific fashion. Second, the cells with high proGelA activation express high levels of MT1-MMP mRNA, but the converse is not always true as shown in the cases of non-treated HT1080 and G361 cell lines. Third, the proGelA activation induced by Con A appears to be associated with the increased level of MT1-MMP mRNA in all cell lines except for HT1080, but the TPA-induced expression of MT1-MMP mRNA does not lead to proGelA activation. These results suggested that MT1-MMP was responsible for proGelA activation in these cancer cell lines, and other

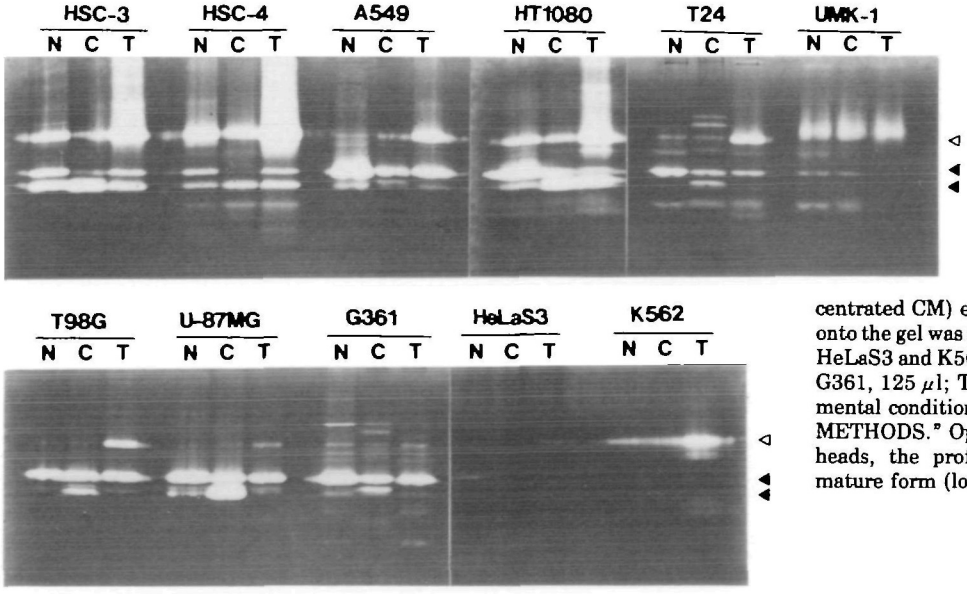

Fig. 1. **Gelatinolytic activities secreted by 11 human cancer cell lines cultured with or without Con A or TPA.** The semi-confluent cultures of the indicated cell lines were incubated in serum-free medium for 2 days in the absence (N) or presence of 100 μg/ml Con A (C) or 100 ng/ml TPA (T). The resultant CMs were concentrated 30-fold and applied to gelatin zymography. The volume of the original CM (unconcentrated CM) equivalent to the sample volume applied onto the gel was as follows; HSC-3, HSC-4, T24, UMK-1, HeLaS3 and K562, 250 μl; A549, 83 μl; HT1080, 50 μl; G361, 125 μl; T98G and U-87MG, 25 μl. Other experimental conditions are described in "MATERIALS AND METHODS." Open arrowheads, proGelB; closed arrowheads, the proform (proGelA; upper band) and the mature form (lower band) of GelA.

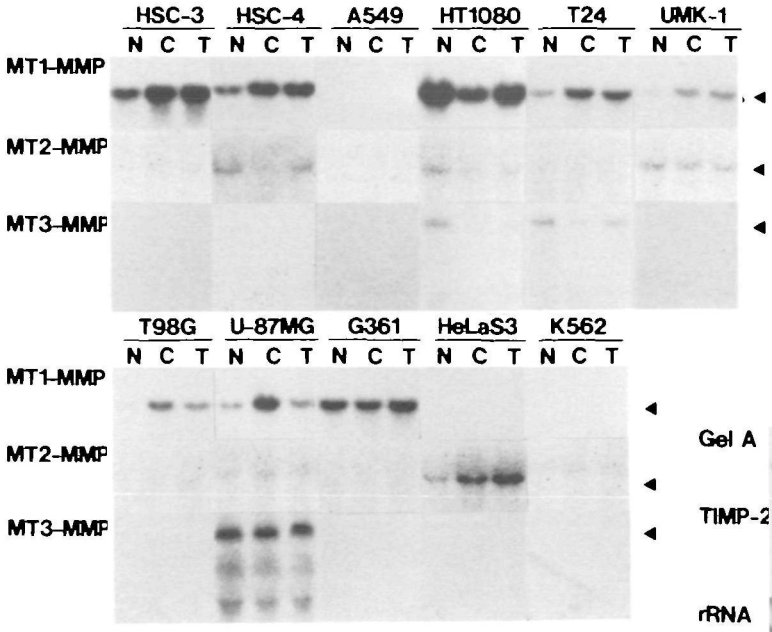

Fig. 2. **Expression of MT1-MMP, MT2-MMP, and MT3-MMP mRNAs in 11 human cancer cell lines cultured with or without Con A or TPA.** mRNAs were prepared from the indicated cells which had been cultured in serum-free medium without (N) or with 100 μg/ml Con A (C) or 100 ng/ml TPA (T) for 24 h. Thirty micrograms of purified RNA was electrophoresed, blotted to a nylon membrane and sequentially hybridized to MT1-MMP, MT2-MMP, and MT3-MMP cDNA probes, as described in "MATERIALS AND METHODS." Arrowheads indicate the bands of MT1-MMP, MT2-MMP, and MT3-MMP mRNAs.

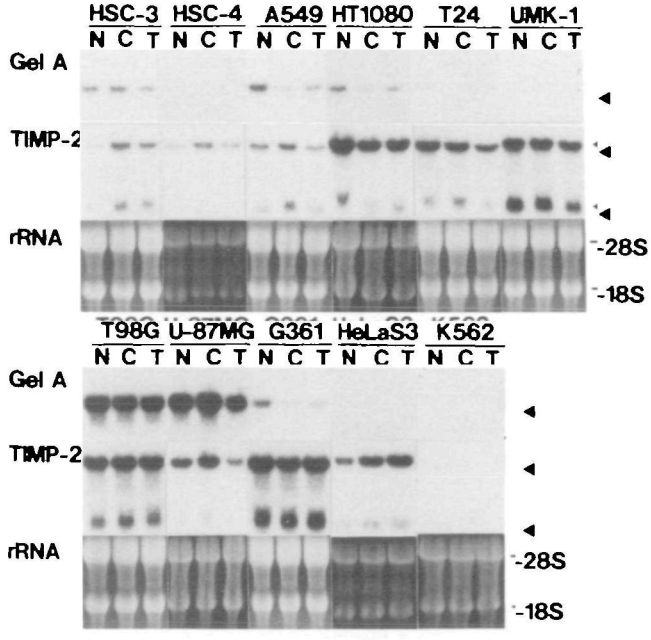

Fig. 3. **Expression of GelA and TIMP-2 mRNAs in 11 human cancer cell lines cultured with or without Con A or TPA.** The same mRNA blots used for Fig. 2 were hybridized with GelA and TIMP-2 cDNA probes, as described in Fig. 2 and in "MATERIALS AND METHODS." To verify the quantity and quality of RNA samples, ribosomal RNA (rRNA) in each sample was stained with ethidium bromide and photographed. N, non-treated; C, Con A-treated (100 μg/ml); T, TPA-treated (100 ng/ml).

factors might be involved in the regulation of proGelA activation.

We also examined expression of GelA mRNA in the cancer cell lines treated with or without Con A or TPA (Fig. 3). The levels of GelA mRNA were nearly proportional to those of proGelA/GelA activity secreted by the cells. Con A and TPA treatments decreased the expression of GelA mRNA in some cell lines such as A549, HT1080, and G361.

Expression of TIMP-2 in Various Cancer Cell Lines—Studies have shown that TIMP-2 affects the proGelA activation on cell membranes (*19, 27*). Therefore, we next analyzed the expression of TIMP-2 in the 11 cancer cell lines at the levels of mRNA and its protein product by Northern blotting and immunoblotting analyses, respectively. In the absence of Con A and TPA, the expression of TIMP-2 mRNA was very low or negligible in HSC-3 and HSC-4, low or moderate in A549, U-87MG, and HeLaS3 and high in HT1080, T24, UMK-1, T98G, and G361 (Fig. 3). Con A and TPA treatments significantly increased TIMP-2 expression in HSC-3, HSC-4 and HeLaS3. In contrast, the TIMP-2 expression in HT1080 was significantly decreased by these treatments.

When TIMP-2 protein secreted into the culture medium was analyzed by immunoblotting, the expression patterns were considerably different from those of the mRNA expression (Fig. 4). The level of TIMP-2 was very low or undetectable in HSC-3, HSC-4, U-87MG, and K562, low in T24, UMK-1, and T98G, and high in A549, HT1080, G361, and HeLaS3. In HT1080, T24, and UMK-1, the TIMP-2 level was significantly decreased by Con A and TPA treatments. It should be noted that A549 and HeLaS3, which expressed TIMP-2 mRNA at relatively low levels, had high amounts of TIMP-2 protein in the medium, while T24, UMK-1, T98G, and U-87MG, which expressed TIMP-2 mRNA at higher levels, had low or negligible levels of TIMP-2 protein in the medium.

The relative levels of proGelA activation, MT1-MMP mRNA, and TIMP-2 protein in the eight proGelA-secreting cell lines are summarized in Table I. The proGelA activation was associated with a high level of MT1-MMP mRNA expression and a low level of TIMP-2 protein in the culture medium. For example, HSC-3 and HSC-4, which showed a high level of MT1-MMP mRNA but a negligible level of TIMP-2 protein, could effectively activate proGelA in the absence of stimulants, whereas HT1080 and G361, which were high in both MT1-MMP mRNA and TIMP-2 protein, hardly activated proGelA. Con A treatment stimulated MT1-MMP mRNA expression in most cell lines, leading to proGelA activation. In HT1080, however, Con A significantly decreased the levels of both MT1-MMP mRNA and TIMP-2. Although TPA, like Con A, stimulated MT1-MMP mRNA expression, it did not promote proGelA activation in any cell lines but HT1080. This suggests that another factor is involved in the Con A-induced proGelA activation.

When TIMP-1 in CMs was analyzed by immunoblotting, all 11 cell lines showed an intense band of TIMP-1 and its level was specially high in A549, HT1080, T24, and

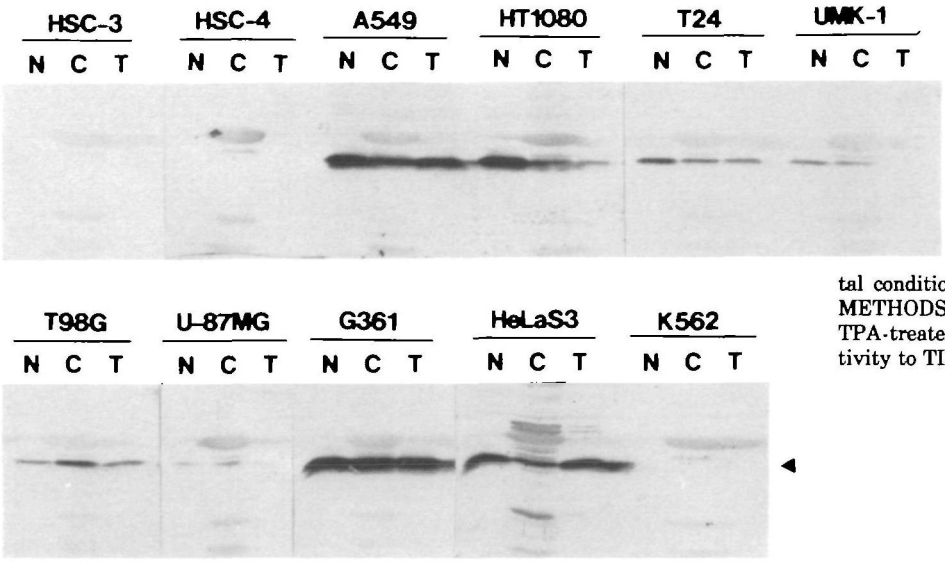

Fig. 4. **Immunoblotting analysis of TIMP-2 secreted into culture medium by 11 human cancer cell lines incubated with or without Con A or TPA.** The same CMs as used in Fig. 1 were subjected to immunoblotting with an anti-TIMP-2 antibody. The sample volume was equivalent to 500 μl of the original CM (unconcentrated CM) in each cell line. Other experimental conditions are described in "MATERIALS AND METHODS." N, non-treated; C, Con A-treated; T, TPA-treated. Arrowheads denote the immunoreactivity to TIMP-2 (approximately 20 kDa).

TABLE I. **Comparison of levels of proGelA activation, MT1-MMP mRNA, TIMP-2 mRNA, and extracellular TIMP-2 protein in eight proGelA-secreting cell lines.**

Treatment	HSC-3			HSC-4			A549			HT1080		
	N	C	T	N	C	T	N	C	T	N	C	T
GelA activation	+	++	+	+	++	+	−	−	−	−	++	++
MT1-MMP mRNA	++	+++	+++	++	+++	+++	−	−	−	+++	++	+++
TIMP-2 protein	−	−	−	−	−	−	+++	++	+++	+++	+	+
TIMP-2 mRNA	±	+	+	±	+	+	+	++	+	+++	++	++
Treatment	T24			T98G			U-87MG			G361		
	N	C	T	N	C	T	N	C	T	N	C	T
GelA activation	−	+	−	−	+	−	−	+	−	−	+	−
MT1-MMP mRNA	+	++	++	+	++	+	+	++	+	++	++	++
TIMP-2 protein	++	+	+	+	++	+	+	+	−	+++	+++	+++
TIMP-2 mRNA	++	++	+	+++	+++	+++	++	+++	+	+++	+++	+++

N, non-treated; C, Con A-treated; T, TPA-treated; +++, strongly detectable; ++, moderately detectable; +, weakly detectable; −, undetectable or negligible.

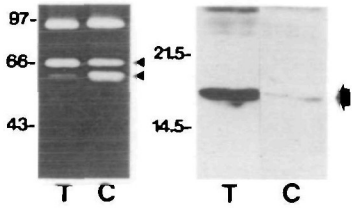

Fig. 5. **Effect of transfection of TIMP-2 cDNA into HSC-3 cells on proGelA activation.** TIMP-2 cDNA was transfected into HSC-3 cells by the lipofection method and transiently expressed. Serum-free CMs were prepared from the cells transfected with the control vector (C) and with the TIMP-2 cDNA vector (T) after 72-h incubation, concentrated and applied to gelatin zymography and immunoblotting analysis with the anti-TIMP-2 antibody. A: (left panel), gelatin zymography. Arrowheads denote proGelA (upper band) and the active GelA (lower band). B: (right panel), immunoblotting analysis. The arrow indicates the immunostained TIMP-2 band. The ordinates of the two panels denote molecular weight values in thousands.

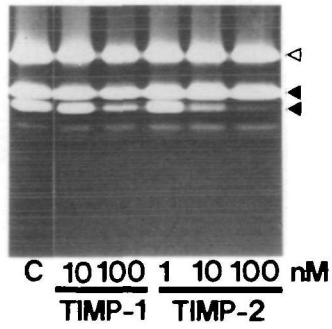

Fig. 6. **Effect of purified TIMPs on proGelA activation in HSC-3 cells.** Semi-confluent culture of HSC-3 cells was incubated for 2 days in serum-free medium without (C) or with the indicated concentrations of purified TIMP-1 or TIMP-2 protein. The resultant CMs were concentrated and applied to gelatin zymography. Open arrowhead, proGelB; closed arrowheads, the proform (upper band) and the mature form (lower band) of GelA.

U-87MG (data not shown). The TIMP-1 levels in CMs appeared not to correlate with proGelA activation.

Relationship between TIMP-2 and ProGelA Activation— The experimental results described above suggested an important role of TIMP-2 in the regulation of proGelA activation. To verify the role of TIMP-2, three different experiments were carried out. First, TIMP-2 cDNA was transfected to and transiently expressed in HSC-3 cells, which showed high proGelA activation and a low TIMP-2

protein content in the culture medium. As shown in Fig. 5, the transfection of TIMP-2 cDNA greatly increased the level of TIMP-2 protein in the medium (right panel) and markedly inhibited the activation of proGelA (left panel). Second, purified TIMP-1 and TIMP-2 proteins were added into the culture of HSC-3 cells, and their effects on proGelA activation were examined (Fig. 6). TIMP-2 clearly inhibited proGelA activation at a concentration of 10 nM, and at 100 nM it almost completely inhibited the activation. TIMP-1 weakly inhibited the proGelA activation at 100 nM.

Third, we isolated six morphologically different clones of HT1080 cells and compared proGelA activation and TIMP-2 content in culture medium among them (Fig. 7). Three clones (N1, N2, and N3) hardly activated proGelA, while the other three clones (A1, A2, and A3) effectively converted proGelA to the intermediate and mature forms (Fig. 7A). The three N clones, which were unable to activate proGelA, had much higher amounts of TIMP-2 in the culture media than the three A clones. When the N clones were treated with TPA, the TIMP-2 level was markedly decreased, leading to the efficient conversion of proGelA to the intermediate and mature forms. When the proGelA-activating and non-activating groups were analyzed for MT1-MMP expression, no significant difference was found (Fig. 7C). In addition, TPA treatment slightly decreased the MT1-MMP expression in all of the clones. The results from the three experiments strongly suggest that TIMP-2 in the culture medium inhibits the activation of proGelA, possibly by binding to both MT1-MMP and proGelA.

GelA, MT1-MMP, and TIMP-2 in Cell Lysate—To examine the interaction of proGelA, MT1-MMP, and TIMP-2, their relative levels in the CM and lysate of HSC-3 cells which had been treated with or without Con A or TPA in the presence or absence of a synthetic MMP inhibitor (KB8301) were analyzed by zymography or immunoblotting (Fig. 8). The addition of KB8301 to the culture effectively inhibited proGelA activation in all cases of the control, Con A-treated, and TPA-treated HSC-3 cells, as shown in gelatin zymography of the CMs (Fig. 8, top panel). To detect low levels of TIMP-2 in CMs, the dose of CMs applied to immunoblotting was increased in this experiment. The treatment with the MMP inhibitor KB8301 strongly increased the TIMP-2 level of CM in the control, Con A-treated, and TPA-treated HSC-3 cells (Fig. 8, second panel). The TIMP-2 level of the Con A-treated cells was far lower than those of the control and TPA-treated cells, regardless of the presence or absence of KB8301. In contrast, TIMP-2 of cell lysate was detected only in the Con A-treated cells, and its level was lowered by the presence of KB8301 (Fig. 8, fourth panel). Gelatin zymography of cell lysates detected the mature form of GelA only in the Con A-treated cells without KB8301 (Fig. 8, third panel). On the other hand, immunoblotting analysis with an anti-MT1-MMP antibody showed a clear band of MT1-MMP only in the cells treated with both Con A and

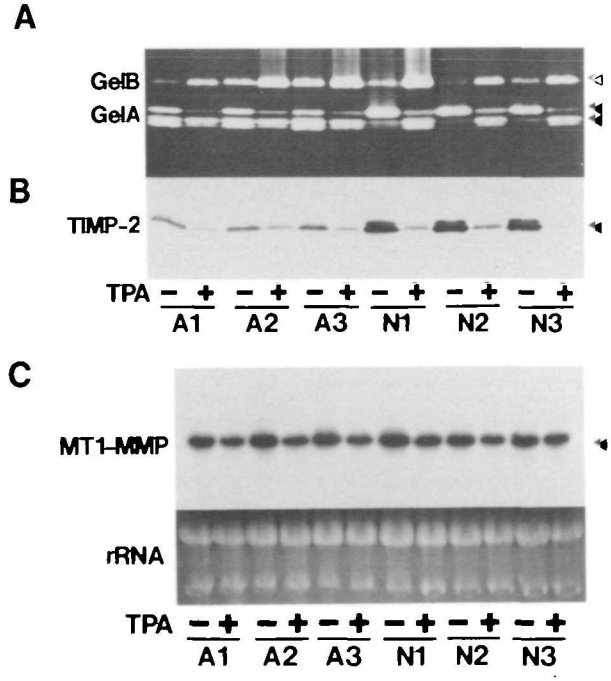

Fig. 7. **Gelatinolytic activities and TIMP-2 secreted by six HT1080 cell clones.** Six clones of HT1080 cells (A1, A2, A3, N1, N2, and N3) were incubated in serum-free medium with (+) or without (−) 100 ng/ml TPA for 2 days. Gelatinase activities and TIMP-2 secreted into the culture media were analyzed by gelatin zymography and immunoblotting, respectively, as described in Figs. 1 and 4. A: gelatin zymography. Open arrowhead, proGelB; closed arrowheads, the proform (upper band) and the mature form (lower band) of Gel. B: immunoblotting analysis of TIMP-2. Arrowhead, TIMP-2. C: Northern blotting analysis of MT1-MMP. Arrowhead, MT1-MMP mRNA. To verify the quality and quantity of RNA samples, ribosomal RNA (rRNA) in each sample was stained with ethidium bromide and photographed.

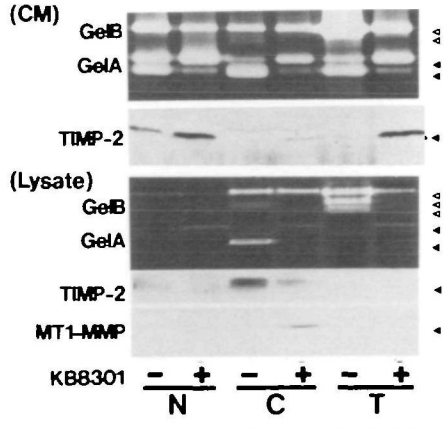

Fig. 8. **Effects of Con A, TPA, and synthetic MMP inhibitor on levels of gelatinases, TIMP-2, and MT1-MMP in CM and cell lysate of HSC-3 cells.** HSC-3 cells were incubated for 2 days in serum-free medium without (N) or with 100 μg/ml of Con A (C) or 100 ng/ml TPA (T) in the presence (+) and absence (−) of the synthetic MMP inhibitor KB8301 (10 μM). CMs and cell lysates were prepared from the incubated cells and subjected to gelatin zymography and immunoblotting as described in "MATERIALS AND METHODS." Top panel, gelatin zymography of CMs; 2nd panel, immunoblotting of TIMP-2 in CMs; 3rd panel, gelatin zymography of cell lysates; 4th panel, immunoblotting of TIMP-2 in cell lysates; 5th (bottom) panel, immunoblotting of MT1-MMP in cell lysates. In the gelatin zymograms, open arrowheads indicate the proform (upper band) and the mature form (lower band) of GelB, and closed arrowheads indicate the proform (upper band) and mature form (lower band) of GelA. In the immunoblots, closed arrowheads indicate the immunostained band of TIMP-2 or MT1-MMP.

KB8301 (Fig. 8, fifth panel). The apparent lack of MT1-MMP in the Con A-treated cells without KB8301 seems due to the low sensitivity of the immunoblotting system. The results described above suggest that the synthetic inhibitor KB8301 binds to the active site of MT1-MMP, leading to inhibition of the binding of TIMP-2 to MT1-MMP on the cell surface and to the elevated release of TIMP-2 into the culture medium. Furthermore, these results suggest that Con A treatment might lead to accumulation of MT1-MMP and TIMP-2 on the cell surface, presumably by suppressing their internalization.

DISCUSSION

We previously reported that MT1-MMP is more widely and more strongly expressed than MT2-MMP and MT3-MMP in various tissues of normal rats (*16*). The present study with 11 human cancer cell lines demonstrated that among the three MT-MMPs, MT1-MMP was most frequently expressed in cancer cells. Importantly, MT1-MMP, but not MT2-MMP and MT3-MMP, was highly expressed in the cells capable of activating the self-producing proGelA in the presence or absence of stimulants. The expression of MT2-MMP and MT3-MMP did not correlate with proGelA activation. Ueno *et al.* recently examined the expression of three MT-MMPs in 20 human breast carcinoma tissues by Northern blotting and detected MT1-MMP in all cases, MT2-MMP in five cases and MT3-MMP in no case (*28*). They also showed that proGelA activation in the tumor tissues correlated with the expression of MT1-MMP but not MT2-MMP. Together with their results, our present results indicate that MT1-MMP plays a major role in proGelA activation in human cancer cells and tissues. In the present study, MT3-MMP was strongly expressed only in the U-87MG glioma cells, without response to stimuli. This result, as well as our previous finding that MT3-MMP is highly expressed in the brain and lung (*16*), suggests that it plays specific roles in more restricted tissues.

More importantly, the present study indicates that TIMP-2 plays a key role in the regulation of proGelA activation. Strongin *et al.* reported that TIMP-2 is required for proGelA activation on the plasma membrane, but an excess amount of TIMP-2 inhibited the activation (*19*). They also demonstrated that TIMP-2 mediates the binding of proGelA to cell membranes by forming a tri-molecular complex of MT1-MMP, TIMP-2, and proGelA. Recent studies have shown that TIMP-2 and TIMP-3, but not TIMP-1, potently inhibit proGelA activation catalyzed by recombinant soluble MT1-MMP and MT2-MMP (*18, 29, 30*). However, the role of TIMP-2 in the proGelA activation by living cells has poorly been understood. In this study, the level of extracellular TIMP-2 protein correlated well with the cellular proGelA activation. For example, HSC-3 and HSC-4, which effectively activated proGelA without any stimulants, secreted a negligible level of TIMP-2 into culture medium, while HT1080 and G361, which secreted a high level of TIMP-2, hardly activated proGelA in the absence of stimulants in spite of their high expression of MT1-MMP mRNA. This relationship was more evident in the comparison between the proGelA-activating and non-activating clones of HT1080 cells. The causal relationship between the extracellular TIMP-2 level and the proGelA activation was directly shown by adding exogenous TIMP-2 into the culture of HSC-3 cells or by transfecting TIMP-2 cDNA into the same cells. The tri-molecular complex model proposed by Strongin *et al.* (*19*) suggests the possible mechanism in which TIMP-2 binds to both the reactive site of MT1-MMP and the hemopexin-like domain of proGelA on cell membranes, and another active MT1-MMP molecule cleaves the propeptide sequence of the proGelA molecule. An excess amount of TIMP-2 will bind to most of both MT1-MMP and proGelA molecules, resulting in the inhibition of proGelA activation. Our results with living cells are consistent with this model. Down-regulation of TIMP-2 might increase the proportion of active MT1-MMP free of TIMP-2, stimulating the proGelA-activating system.

The present study revealed a considerable difference between the relative levels of intracellular TIMP-2 mRNA and TIMP-2 protein in culture medium. For example, although HSC-3, HSC-4, and A549 expressed TIMP-2 mRNA at similar levels, the TIMP-2 content in the medium was negligible in the former two lines but very high in A549, which expressed no MT-MMPs (Figs. 3 and 4). Furthermore, UMK-1, T98G, and U-87MG secreted very low amounts of TIMP-2 into the culture medium in spite of their relatively high expression of TIMP-2 mRNA. These apparent inconsistencies suggest two possible mechanisms, postranscriptional regulation and metabolic regulation of the TIMP-2 level. In the experiment with HSC-3, the TIMP-2 content in the culture medium inversely correlated with that in the cell lysate (Fig. 8). Con A treatment of HSC-3 cells decreased the extracellular TIMP-2 level, whereas it increased the levels of TIMP-2 and active GelA in the cell lysate. On the other hand, the MMP inhibitor KB8301 markedly increased the TIMP-2 content in the culture medium but decreased that in the cell lysate. It also increased the level of MT1-MMP in the lysate of Con A-treated cells. However, the inhibitor did not increase the extracellular level of TIMP-2 in A549 cells. These results suggest the following hypothetical mechanism. TIMP-2 efficiently binds to the cell surface receptor MT1-MMP, and the complex is rapidly internalized and degraded. Therefore, TIMP-2 and MT1-MMP are hardly detected in cell lysate. KB8301 competitively blocks the binding of TIMP-2 to MT1-MMP, increasing the extracellular TIMP-2 level, and it also inhibits the metabolism of MT1-MMP. Con A treatment appears to stabilize the MT1-MMP/TIMP-2 complex on the cell surface, promoting the formation of the MT1-MMP/TIMP-2/proGelA complex and resultant proGelA activation. Although extracellular TIMP-2 strongly inhibits proGelA, the binding of a proper level of TIMP-2 to a cell surface receptor is essential for efficient activation of proGelA (*19*). A recent kinetic study with MT1-MMP-containing cell membrane has shown that a wide range of TIMP-2 levels relative to a constant amount of MT1-MMP allow proGelA activation (*31*).

We have previously reported that KB8301 inhibits proGelA activation catalyzed by MT3-MMP and causes accumulation of MT3-MMP protein on cell membranes, possibly by inhibiting its degradation (*16*). As a clearance mechanism of proteinase/inhibitor complex, it has been reported that neutrophil elastase/α1-antitrypsin is complexed with the cell surface serpin-enzyme complex receptor (SEC-R), internalized, and degraded (*32, 33*). MT-

MMPs, like stromelysin-3 (MMP-11) (*34*), are thought to be intracellularly activated by furin-like proteinases (*29*), although many other MMPs are secreted from the cells in latent proforms. Therefore, the rapid metabolism of MT-MMPs seems to be an important mechanism to regulate their excess action.

Con A, a plant lectin derived from jack bean, binds to cell surface glycoproteins with a high specificity for gluco- and manno-pyranosides and acts as a potent mitogen for lymphocytes. Many studies have shown that Con A promotes proGelA activation in cultured cells (*20, 35, 36*). In the present study, Con A promoted proGelA activation in most of the cell lines producing proGelA and MT1-MMP (Figs. 1 and 2). This activity seems in part due to the stimulation of MT1-MMP expression. However, although Con A and TPA stimulated the MT1-MMP expression to similar levels in several cell lines, such as HSC-3 and HSC-4, the proGelA activation was much higher in Con A than TPA. In addition, Con A rather inhibited the MT1-MMP expression in HT1080 cells. When treated with Con A, cells became markedly round, indicating that Con A had a specific morphological effect on cells. As described above, Con A treatment appears to suppress the rapid turnover of MT1-MMP and TIMP-2. It has been reported that Con A stimulates the phosphorylation of the β-subunit of the insulin receptor, mainly by promoting the aggregation of the receptor (*37*). In addition, Con A is known to inhibit the receptor-mediated internalization of interferon-α, EGF, and TNF-α (*38*). These facts also support the above mechanism that Con A might stabilize the MT1-MMP/TIMP-2 complex by preventing its internalization and clearance. However, natural factors or conditions that mimic the effect of Con A are not clear. It is known that proGelA is effectively activated when fibroblasts are cultured within collagen lattices, where the actin stress fibers can not be formed. Using a culture within collagen lattices, Tomasek *et al.* showed that proGelA activation is negatively regulated by the organization of the actin cytoskeleton (*39*). On the other hand, Lee *et al.* found that proGelA is intracellularly activated on Golgi membranes in the collagen lattice culture (*40*). The Con A treatment and the collagen lattice culture may share a common mechanism for proGelA activation.

Under pathological conditions, some tissues, such as invasive cancers (*6, 7, 10*) and injured blood vessels (*41*), produce the active form of GelA. The present study suggests that the proGelA activation in cancer tissues is caused by both up-regulation of MT1-MMP and down-regulation of TIMP-2. In addition, an unknown mechanism to stabilize the MT1-MMP/TIMP-2 complex on cell membranes may exist for the aberrant expression of GelA activity.

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