Tissue Inhibitor of Metalloproteinase (TIMP)-2 Acts Synergistically with Synthetic Matrix Metalloproteinase (MMP) Inhibitors but Not with TIMP-4 to Enhance the (Membrane Type 1)-MMP-dependent Activation of Pro-MMP-2

The membrane-type 1 matrix metalloproteinase (MT1-MMP) has been shown to be a key enzyme in tumor angiogenesis and metastasis. MT1-MMP hydrolyzes a variety of extracellular matrix components and is a physiological activator of pro-MMP-2, another MMP involved in malignancy. Pro-MMP-2 activation by MT1-MMP involves the formation of an MT1-MMP-tissue inhibitors of metalloproteinases 2 (TIMP-2)-pro-MMP-2 complex on the cell surface that promotes the hydrolysis of pro-MMP-2 by a neighboring TIMP-2-free MT1-MMP. The MT1-MMP-TIMP-2 complex also serves to reduce the intermolecular autocatalytic turnover of MT1-MMP, resulting in accumulation of active MT1-MMP (57 kDa) on the cell surface. Evidence shown here in Timp2-null cells demonstrates that pro-MMP-2 activation by MT1-MMP requires TIMP-2. In contrast, a C-terminally deleted TIMP-2 (Δ-TIMP-2), unable to form ternary complex, had no effect. However, Δ-TIMP-2 and certain synthetic MMP inhibitors, which inhibit MT1-MMP autocatalysis, can act synergistically with TIMP-2 in the promotion of pro-MMP-2 activation by MT1-MMP. In contrast, TIMP-4, an efficient MT1-MMP inhibitor, had no synergistic effect. These studies suggest that under certain conditions the pericellular activity of MT1-MMP in the presence of TIMP-2 can be modulated by synthetic and natural (TIMP-4) MMP inhibitors.

Proteolytic degradation of extracellular matrix (ECM) is a fundamental aspect of cancer development and a key event in tumor-induced angiogenesis and tumor metastasis. A major group of enzymes responsible for ECM degradation in cancer tissue is the matrix metalloproteinase (MMP) family (1–4). The MMPs are zinc-dependent multidomain endopeptidases that, with few exceptions, share a basic structural organization comprising propeptide, catalytic, hinge, and C-terminal (hemopexin-like) domains (1, 5). All MMPs are produced in a latent form (pro-MMP) requiring activation for catalytic activity, a process that is usually accomplished by proteolytic removal of the propeptide domain. Once activated, all MMPs are specifically inhibited by a group of endogenous tissue inhibitors of metalloproteinases (TIMPs) that bind to the active site, inhibiting catalysis (1). Over the last 5 years, the MMP family has been expanded to include a new subfamily of membrane-tethered MMPs known as membrane-type MMPs (MT-MMPs), which to date includes six members (6–12). The MT-MMPs, with the exception of MT4-MMP, are unique because they are anchored to the plasma membrane by means of a hydrophobic stretch of approximately 20 amino acids, leaving the catalytic domain exposed to the extracellular space. This organization makes the MT-MMPs perfectly suited for regulation of pericellular proteolysis. MT1-MMP (MMP-14) was the first member of the MT-MMP family to be discovered and has been shown to be the major physiological activator of pro-MMP-2 (gelatinase A) on the cell surface (6, 12). The role of MT1-MMP in pericellular proteolysis is not restricted to pro-MMP-2 activation, since MT1-MMP is a multifunctional enzyme that can also degrade a variety of ECM components (13–16) and hence can play a direct role in ECM turnover. MT1-MMP has been recently shown to be the first member of the MMP family indispensable for normal growth and development, since mice deficient in MT1-MMP exhibit a variety of connective tissue pathologies and a short life span (17, 18). Furthermore, both MMP-2 (19) and MT1-MMP (20–26) have been associated with metastatic potential in many human cancers, angiogenesis (27), and enhanced tumor cell invasion in experimental systems (28–31). This has raised considerable interest in understanding the regulation of these MMPs because they represent an important target for development of novel drugs aimed at inhibiting tumor metastasis and angiogenesis (3, 32, 33).

Studies on the mechanism of activation of pro-MMP-2 by MT1-MMP revealed a complex role for TIMP-2 in this process. A model for the activation of pro-MMP-2 has been proposed in which the catalytic domain of MT1-MMP binds to the N-termi-
nal portion of TIMP-2, leaving the negatively charged C-termina-
lar region of TIMP-2 available for the binding of the he-
mapoxin-like domain of pro-MMP-2 (12, 34–38). This ternary
complex has been suggested to cluster pro-MMP-2 at the cell
surface near a residual TIMP-free active MT1-MMP molecule,
which is thought to initiate activation of the bound pro-MMP-2.
Pro-MMP-2 activation would occur only at low TIMP-2 concen-
trations relative to MT1-MMP, which would permit availability
of active MT1-MMP to activate the pro-MMP-2 bound in the
ternary complex (39). Thus, under restricted conditions,
TIMP-2 is thought to promote the activation process by acting
as a molecular link between MT1-MMP and pro-MMP-2. We
have recently shown that TIMP-2, besides its role in ternary
complex formation, has direct and critical effects on MT1-MMP
processing, which influence the profile and spatial localization
of MT1-MMP forms (40). Biochemical and cellular evidence
showed that binding of TIMP-2 to active MT1-MMP (57 kDa)
inhibits autocatalytic degradation, leading to accumulation of
active MT1-MMP on the cell surface. In the absence of TIMP-2,
MT1-MMP is rapidly processed to a 44-kDa membrane-bound
inactive enzyme (40, 41). Thus, under controlled conditions,
TIMP-2 may act as a positive regulator of MT1-MMP activity
by promoting the availability of active MT1-MMP on the cell
surface and consequently may support pericellular proteolysis.
Since some of the effects of TIMP-2 on MT1-MMP activities are
related to its inhibitory activity, we wished to examine the
effects of synthetic and physiological MMP inhibitors (MMPIs)
onto MT1-MMP processing and its ability to promote pro-MMP-2
activation with TIMP-2. Although several types of MMPIs have
been developed (3, 32, 33, 42–47), little is known about their
effects on the processing and activity of membrane-tethered
MMPs, which exhibit unique properties. Here we show for the
first time that synthetic MMPIs and a C-terminally truncated
TIMP-2 but not TIMP-4, which inhibit MT1-MMP activity, act
synergistically with TIMP-2 to promote pro-MMP-2 activation
by MT1-MMP. These studies demonstrate the complexity of
MT1-MMP regulation and provide new insights into the roles
of TIMP-2, TIMP-4, and MMPIs in this process.

EXPERIMENTAL PROCEDURES

Cell Culture—Nonmalignant monkey kidney epithelial BS-C-1 (CCL-26) and human fibrosarcoma HT-1080 (CCL-121) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. HeLa S3 cells were obtained from ATCC (CCL-2.2) and grown in suspension in MEM Spinnier medium (Quality Biologicals, Inc., Gaithersburg, MD) supplemented with 5% horse serum. All other tissue culture reagents were purchased from Life Technologies, Inc.

Isolation of Immortalized Timp2 Mutant Mouse Fibroblasts—Adult skin fibroblast cells were isolated from heterozygous (+/−) Timp2 mutant mice and immortalized by retroviral infection using a Ha-ras and v-myc-producing, replication-defective retrovirus as described previ-
ously (48). A G418 selection protocol was used to select for homozygous Timp2 (−/−) mutant cells from the immortalized (+/−) mutant clone as described (49). Detailed methods for the isolation and selection of the immortalized (+/+) and (−/−) isogenic cells will be reported elsewhere. The homozygous and heterozygous Timp2 mutant cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics.

Recombinant Vaccinia Viruses—The production of the recombinant vaccinia virus (vTF7–3) expressing bacteriophage T7 RNA polymerase has been described by Fuerst et al. (50). Recombinant vaccinia viruses expressing either human pro-MMP-2, TIMP-2, or MT1-MMP under control of the T7 promoter were obtained by homologous recombination as described previously (35, 40, 51).

Recombinant Proteins, Synthetic MMP Inhibitors, and Antibodies—
Human recombinant pro-MMP-2, TIMP-2, and TIMP-1 were expressed in HeLa S3 cells infected with the appropriate recombinant vaccinia viruses and purified to homogeneity, as described previously (52). Hu-
man recombinant TIMP-4 was expressed in baby hamster kidney cells and purified from the conditioned medium by sequential application to

Red Sepharose, phenyl-Sepharose, Q-Sepharose, and Zn2+-charged che-
late Sepharose columns as described. A C-terminally truncated hu-
man TIMP-2 ending at Cys128 (Δ-TIMP-2) was constructed and ex-
pressed in mammalian cells as described previously (53). The concentrations of TIMP-2 and TIMP-2 were determined by active

Enzyme Inhibition Studies—MT1-MMPcat activity was monitored with the fluorescence-quenched substrate MOCapGLLAc(Dnp)-Apro-
NH2 (62). Fluorescence was measured with a Photon Technology Inter-

duced conditions by active TIMP-2. A Cys1÷ TIMP-2 mutant (Ala1÷ TIMP-2) was kindly provided by Dr. W. G. Stetler-Stevenson (NCI,
National Institutes of Health) (54). A recombinant catalytic domain of
human MT1-MMP (MT1-MMPcat) comprising residues Ile114 to Ile318
was expressed in Escherichia coli, purified, and characterized as de-
scribed previously (55). The concentration of the MT1-MMPcat enzyme
was determined by active titration with recombinant TIMP-2 as
described (56). Batimastat (BB-94) and marimastat (BB-2516), two
hydroxamate-based MMP inhibitors (33, 45, 47), and BB-2116, a
borate-containing MMP inhibitor, were obtained from British Biotech
(Annapolis, MD). The mechanism-based MMP inhibitor SB-3CT was
synthesized and characterized as previously reported (42). Stock solu-
tions of marimastat (1 mM), batimastat (1 mM), BB-2116 (20 mM), and
SB-3CT (30 mM) were prepared in Me2SO. The rabbit anti-TIMP-2 polyclonal antibody (pAb) and the anti-TIMP-2 monoclonal antibody
CA-101 were previously described (57). The rabbit pAb 437 to MT1-
MMP (40, 58) has been previously described. The rabbit pAb 160 to
MT1-MMP (40, 59) and the rabbit pAb to human TIMP-4 were
a generous gift from Dr. Amy Sang (Florida State University, Tallahassee, FL).

Expression of MT1-MMP by Vaccinia Infection—To express MT1-
MMP, confluent cultures of BS-C-1 or Timp2 mutant cells in 6-
or 12-well plates were co-infected with 5–10 pfu/cell each of vTF7–3 and
vTF7-20 viruses for 45 min in infection medium (DMEM plus 2.5% fetal bovine serum and antibiotics) at 37 °C. As control, the cells were
infected only with the vTF7–3 virus as described (40).

Natural and Synthetic Inhibitor Treatment and Pro-MMP-2 Activa-
tion—After infection, the media were aspirated, and the cells were
rinsed with serum-free DMEM and replaced with fresh serum-free
DMEM supplemented with or without various doses of purified human
recombinant TIMP-2. After various times at 37 °C, the media were
aspirated; the cells were rinsed with DMEM and then incubated (15–30
min, 37 °C) in fresh media supplemented with 10 nm pro-MMP-2. The
media were then collected, and the cells were rinsed twice with cold
phosphate-buffered saline and solubilized in cold lysis buffer (25 mM
Tris-HCl (pH 7.5), 1% IGEPAI CA-630, 100 mM NaCl, 10 μg/ml apro-
tinin, 1 μg/ml leupeptin, 2 mM benzamidine, and 1 mM phenylmeth-
sulfonyl fluoride). The lysate fractions were analyzed for pro-MMP-2
activation by gelatin zymography and/or immunoblot analysis for as-
semination of MT1-MMP forms. To examine the effects of synthetic
TIMP inhibitors, Δ-TIMP-2, Ala1÷ TIMP-2, and TIMP-4 on TIMP-2-depen-
dent activation of pro-MMP-2, the MT1-MMP-infected cells were treated
(16 h, 37 °C) with the appropriate MMP inhibitors (various doses)
diluted in serum-free DMEM. Then the media were aspirated, and the
cells were rinsed with DMEM. TIMP-2 (10 nm) was then added to the
cells in serum-free DMEM for a 5–30-min incubation at 37 °C. The
media were aspirated followed by a wash with DMEM to remove un-
bound TIMP-2. The cells were then incubated (15 min, 37 °C) with
serum-free DMEM supplemented with 10 nm pro-MMP-2. Analysis of
pro-MMP-2 activation and of the profile of MT1-MMP forms in the cell
lysates were carried out as described below.

Gelatin Zymography and Immunoblot Analysis—Gelatin zymo-
graphy was performed using 10% Tris-glycine SDS-polyacrylamide gels
containing 0.1% gelatin. Briefly, samples of lysates or media were
mixed with Laemmli sample buffer (60) without reducing agents and
without heating and then subjected to SDS-polyacrylamide gel electro-
phoresis (SDS-PAGE) as described previously (61). For immunoblot
analysis, the cell lysates were subjected to reducing SDS-PAGE follow-
ing by transfer to a nitrocellulose membrane essentially as described
(61). Detection of the immune complexes was performed using the
enhanced chemiluminescence system (Pierce) according to the manu-
facturer’s instructions.

2 Bigg, H. F., Morrison, C. J., Butler, G. S., Bogoyevitch, M.A., Wang, Z., Soloway, P. D., and Overall, C. M., submitted for publication.
Substrate hydrolysis was monitored at emission and excitation wavelengths of 328 and 393 nm, respectively. Fluorescence measurements were taken every 4 s. Less than 10% hydrolysis of the fluorogenic substrate was monitored, as described by Knight (62). For slow binding inhibition, progress curves were obtained by adding enzyme (0.5 nM) to a mixture of fluorogenic substrate (7 μM) and varying concentrations of inhibitor in buffer R (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, 0.01% Brij-35, and 1–5% Me₂SO; final volume 2 ml) in acrylic cuvettes with stirring and monitoring the increase in fluorescence with time for 15–30 min. The progress curves were nonlinear least squares fitted to Equation 1 (63),

\[ F = v_i + [v_o - v_i] \left(1 - \exp(-kt)\right)/k + F_0 \]  
(Eq. 1)

where \(v_o\) represents the initial rate, \(v_i\) is the steady state rate, \(k\) is the apparent first order rate constant characterizing the formation of the steady-state enzyme-inhibitor complex, and \(F_0\) is the initial fluorescence, using the program SCIENTIST (MicroMath Scientific Software, Salt Lake City, UT). The obtained \(k\) values, \(v_o\), and \(v_i\) were further analyzed according to Equations 2 and 3 for a one-step association mechanism.

\[ k = k_{cat} + k_{off}[I] \left(1 + [S]/K_a\right) \]  
(Eq. 2)

\[ (v_o - v_i)v_i = [I]/K_a \left(1 + [S]/K_a\right) \]  
(Eq. 3)

The \(K_a\) and \(k_{cat}\) values for the reaction of MT1-MMP \(_{\text{cat}}\) with the fluorogenic substrate were determined to be 6.9 ± 0.5 μM and 0.67 ± 0.03 s⁻¹, respectively. Intercept and slope values, obtained by linear regression of the \(k\) versus inhibitor concentration plot (Equation 2), yielded the association and dissociation rate constants \(k_{cat}\) and \(k_{off}\) respectively, and the inhibition constant \(K_i\) (\(k_{off}/k_{cat}\)). Alternatively, \(K_i\) was determined from the slope of the \((v_o - v_i)v_i\) versus [I] plot according to Equation 3. The dissociation rate constant was determined independently from the enzyme activity recovered after dilution of a pre-formed enzyme-inhibitor complex. To this end, typically 50 μl of enzyme was incubated with 80 nM of inhibitor for a sufficient time to reach equilibrium (~45 min) at 25.0 °C. The complex was diluted 400-fold into 2 ml of buffer R containing fluorogenic substrate (10 μM final concentration). Recovery of enzyme activity was monitored for ~60 min. The fluorescence versus time trace was fitted, using the program SCIENTIST, to Equation 4.

\[ F = v_i + [v_o - v_i] \left(1 - \exp(-kd)t\right)/k_{cat} + F_0 \]  
(Eq. 4)

where \(v_o\) represents the initial rate (very small), \(v_i\) is the rate observed when the EI complex is completely dissociated, and \(k_{cat}\) is the first order rate constant of EI dissociation. In light of the slow dissociation of the MT1-MMP \(_{\text{cat}}\)-TIMP-2 complex, the direct analysis of the \(k_{off}\) parameter for the wild type TIMP-2 was not possible and was estimated based on a 10-fold difference observed between the slopes of the linear portions of the dissociation curves for the complexes of MT1-MMP \(_{\text{cat}}\) with Δ-TIMP-2 (steady state rate) and wild type TIMP-2. For competitive inhibition, initial rates were obtained by adding enzyme to a mixture of fluorogenic substrate (7 μM) and varying concentrations of inhibitor in buffer R (final volume 1 ml) in quartz semimicro cuvettes and monitoring the increase in fluorescence with time for 5–10 min. The initial velocities were determined by linear regression analysis of the fluorescence versus time traces using FeIIX™. The initial rates were fitted to Equation 5 (64).

\[ v/v_o = (K_a + [S])/K_a(1 + [I]/K_a) + [S] \]  
(Eq. 5)

where \(v_i\) and \(v_o\) represent the initial velocity in the presence and absence of inhibitor, respectively, using the program SCIENTIST.

RESULTS

Pro-MMP-2 Activation and MT1-MMP Processing in Timp2 Mutant Cells—Using a vaccinia expression system, we have recently shown that immortalized monkey kidney epithelial BS-C-1 cells infected to express MT1-MMP could activate pro-MMP-2 (40). Under these conditions, this process appeared to be independent of TIMP-2, since in infected BS-C-1 cells expression of the endogenous inhibitor was significantly suppressed (35, 40, 51). However, these studies were inconclusive in regard to the requirement of TIMP-2 for pro-MMP-2 activation, since a residual amount of endogenous inhibitor could not be ruled out. To establish the importance of TIMP-2 in the activation of pro-MMP-2 by MT1-MMP, we used homozygous (−/−) and heterozygous (+/−) Timp2 mutant mouse fibroblasts (65) that were immortalized by retroviral infection. The infected cells were used as the control-infected cells (Fig. 1A, TIMP-2-only mutant cells), and the T7 RNA polymerase virus (vTF7–3) mutant cells (65) to express MT1-MMP (Fig. 1B, but not in the control-infected cells (Fig. 1B, lane 1) in both cell types (only the homozygous cells are shown in Fig. 1B).

The ability of the expressed MT1-MMP to promote pro-MMP-2 activation with or without TIMP-2 in this cellular system was examined by gelatin zymography of the lysate fraction. To this end, after infection, the cells were incubated with or without exogenous TIMP-2, washed to remove unbound inhibitor, and incubated with exogenous pro-MMP-2. The latter was added, since both the (+/−) and (−/−) Timp2 mutant cells failed to activate pro-MMP-2 regardless of exogenous TIMP-2 presence (Fig. 1C, lane 6, without TIMP-2; lane 7, with TIMP-2). Control-infected homozygous and heterozygous Timp2 mutant cells consistently failed to activate pro-MMP-2 regardless of exogenous TIMP-2.
puriﬁed recombinant TIMP-4.

MMP forms as a function of TIMP-2 concentration. As shown in the cell system, the homozygous Timp2 mutant cells were analyzed for pro-MMP-2 activation and MT1-MMP processing in the enzyme form (40). To examine the relationship between pro-MMP-2 activation and MT1-MMP processing, we used a C-terminally truncated form of TIMP-2, Δ-TIMP-2, incapable of binding pro-MMP-2 (data not shown) and therefore unable to form the ternary complex (36, 53). In addition, we tested recombinant TIMP-4, known to bind to pro-MMP-2 (68). Due to their lower afﬁnity for MT1-MMP, Δ-TIMP-2 (shown in Table I) and TIMP-1 (36, 69) were added to the homozgyous cells at concentrations 10-fold higher than that of wild type TIMP-2. As shown in the zymogram of Fig. 2B, 2.5 nM TIMP-2 (Fig. 2B, lane 1) efﬁciently promoted pro-MMP-2 activation. In contrast, Δ-TIMP-2 (Fig. 2B, lane 2; 25 nM) and TIMP-1 (Fig. 2B, lane 4; 25 nM) failed to induce pro-MMP-2 activation. Interestingly, exogenous TIMP-4 also had no effect on activation (Fig. 2B, lane 3; 1–100 nM, only 10 nM shown), consistent with the results of Bigg et al.2 In addition, co-expression of MT1-MMP with TIMP-4 in the (−/−) Timp2 mutant cells using the vaccinia expression system had no effect on pro-MMP-2 activation.4 Immunoblot analysis demonstrated the cell association of the exogenous TIMP-4 with (−/−) Timp2 mutant cells expressing MT1-MMP (Fig. 2C, lane 2) suggesting the binding of TIMP-4 to MT1-MMP. Taken together, these results demonstrate that only full-length TIMP-2 can promote the MT1-MMP-dependent activation of pro-MMP-2 in a process that is dependent on ternary complex formation.

TIMP-2 and MMP Inhibitors Act Synergistically to Enhance Pro-MMP-2 Activation—Previous studies suggested that, in addition to ternary complex formation, the enhancing effect of TIMP-2 on pro-MMP-2 activation was the result of a speciﬁc inhibition of MT1-MMP autocatalytic turnover on the cell surface (40). Indeed, TIMP-2 induces the accumulation of the 57-kDa form of MT1-MMP (shown in Fig. 2A). It was hypothesized that at low inhibitor concentrations relative to MT1-MMP and continuous enzyme synthesis by the cells, this process would slow down enzyme turnover, generating a fraction of inhibitor-free active MT1-MMP and hence increase pericellular proteolysis (40). Since this effect is due to inhibition of MT1-MMP activity, we hypothesized that synthetic MMPiPs may mimic TIMP-2 in its ability to reduce MT1-MMP turnover. We asked whether reduction of MT1-MMP autocatalytic turnover by MMPiPs together with ternary complex formation by TIMP-2 would enhance pro-MMP-2 activation when compared with activation promoted by TIMP-2 alone.

To test this hypothesis, the Timp2 (−/−) null cells were incubated overnight with a variety of synthetic and natural MMPiPs to inhibit MT1-MMP autocatalysis and induce accumulation of the active 57-kDa species. The cells were then washed to remove excess unbound inhibitors and then exposed to TIMP-2 to generate the ternary complex. After a rinse to remove unbound TIMP-2, the cells received pro-MMP-2. We tested the effects of marimastat and batimastat, two hydroxamate-based inhibitors (33, 45, 47); SB-3CT, a mechanism-based inhibitor (42); and Δ-TIMP-2 (53). As shown in the zymogram of Fig. 3, administration of TIMP-2 alone for 5 min was sufﬁcient to promote pro-MMP-2 activation, as expected (Fig. 3, lane 2). However, in the cells pretreated with a 1 μM concentration of either marimastat (Fig. 3, lane 3) or batimastat-

3 M. Toth, unpublished results.

4 S. Hernandez-Barrantes, Y. Shimura, and R. Fridman, unpublished results.
Aspirated and replaced with serum-free DMEM (1 ml/well) supplemented with 1 μM marimastat (lane 3), 1 μM batimastat (lane 4), 1 μM SB-3CT (lane 5), or 100 nM Δ-TIMP-2 (lane 6) followed by a 16-h incubation at 37 °C. Some wells received DMEM without inhibitors (lanes 1 and 2). The cells were washed once to remove excess inhibitors and then incubated with DMEM (1 ml/well) supplemented without (lane 1) or with (lanes 2–6) 10 nM TIMP-2 for 5 min at 37 °C. The media were then aspirated and replaced with fresh DMEM containing 10 nM pro-MMP-2. After 15 min at 37 °C, the cells were rinsed with phosphate-buffered saline and solubilized in lysis buffer. The lysates were analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. The asterisk shows the pro-MMP-2 added to the media. The ~50-kDa band is nonspecific. This experiment was repeated at least three times with similar results.

**Fig. 3. Synthetic MMPIs enhance pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2.** The TIMP2 (−/−) mutant cells in 12-well plates were co-infected to express MT1-MMP as described in the legend to Fig. 1. After the infection, the media were aspirated and replaced with serum-free DMEM (1 ml/well) supplemented with 1 μM marimastat (lane 3), 1 μM batimastat (lane 4), 1 μM SB-3CT (lane 5), or 100 nM Δ-TIMP-2 (lane 6) followed by a 16-h incubation at 37 °C. Some wells received DMEM without inhibitors (lanes 1 and 2). The cells were washed once to remove excess inhibitors and then incubated with DMEM (1 ml/well) supplemented without (lane 1) or with (lanes 2–6) 10 nM TIMP-2 for 5 min at 37 °C. The media were then aspirated and replaced with fresh DMEM containing 10 nM pro-MMP-2. After 15 min at 37 °C, the cells were rinsed with phosphate-buffered saline and solubilized in lysis buffer. The lysates were analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. The asterisk shows the pro-MMP-2 added to the media. The ~50-kDa band is nonspecific. This experiment was repeated at least three times with similar results.

**Table I**

| Inhibitor          | kₐ  | kᵦ  | Kᵦ  |
|--------------------|-----|-----|-----|
| Wild type TIMP-2   | (2.74 ± 0.14) × 10⁶ | 2 × 10⁻⁴ | na  |
| Δ-TIMP-2           | (2.68 ± 0.12) × 10⁶ | (1.95 ± 0.03) × 10⁻³ | 0.07 |
| MARIMASTAT         | 8 ± 1 | 8 ± 1 | 110 ± 11 |
| BATIMASTAT         | 8 ± 1 | 8 ± 1 | 110 ± 11 |
| BB-2116            | 8 ± 1 | 8 ± 1 | 110 ± 11 |
| SB-3CT             | 8 ± 1 | 8 ± 1 | 110 ± 11 |

*Estimated value based on a 10-fold difference between the slopes of the linear portions of the dissociation curves for the complexes of MT1-MMPcat with Δ-TIMP-2 (steady state rate) and wild type TIMP-2.

The results above indicated a differential inhibition of MT1-MMP turnover by various synthetic MMPIs and Δ-TIMP-2, which inhibit MT1-MMP activity and consequently autocatalytic degradation, can enhance MT1-MMP-dependent pro-MMP-2 activation in the presence of TIMP-2.

**The Enhancing Effect of MMPIs on Pro-MMP-2 Activation by MT1-MMP Requires TIMP-2 for Ternary Complex Formation**—To examine the relationship between the effects of the MMPIs on pro-MMP-2 activation (inhibition of MT1-MMP autocatalysis) and ternary complex formation, the TIMP2 (−/−) mutant cells were pretreated with marimastat to accumulate the ~57-kDa form of MT1-MMP. Pretreatment of the cells with TIMP-4 (0–100 nM) followed by TIMP-2 addition, had no enhancing effect on pro-MMP-2 activation (Fig. 4B). Interestingly, TIMP-4 induced the accumulation of ~57-kDa form of MT1-MMP consistent with its inhibitory activity. These results suggest that, with the exception of TIMP-4 and SB-3CT, certain synthetic MMPIs and Δ-TIMP-2, which inhibit MT1-MMP activity and consequently autocatalytic degradation, can enhance MT1-MMP-dependent pro-MMP-2 activation in the presence of TIMP-2.

**Effect of Δ-TIMP-2 and Synthetic MMPIs on MT1-MMP Activity**—The results above indicated a differential inhibition of MT1-MMP autocatalytic turnover by various synthetic MMPIs and Δ-TIMP-2. In order to elucidate the inhibitor effects on MT1-MMP activity observed in the cells, the interactions of the catalytic domain of MT1-MMP (MT1-MMPcat) with natural and synthetic inhibitors were characterized in a purified system. As depicted in Table I, both TIMP-2 and Δ-TIMP-2 exhibit slow binding kinetics with similar association rate constants of

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MT1-MMPcat (0.5 nM) was added to a solution of MOCAcPLGLA pró(Dnp)-AR-NH₂ (7 μM) and varying concentrations of inhibitor in buffer R at 25.0 °C. Substrate hydrolysis was monitored at excitation and emission wavelengths of 328 and 393 nm, respectively, for up to 30 min. To determine the dissociation rate constants, kₐ, a mixture of enzyme (50 nM) and inhibitor (80 nM) in buffer R was incubated for ~1 h at 25 °C and diluted 400-fold in a solution of fluorogenic substrate (10 μM) in the same buffer containing 1% Me₂SO. Substrate hydrolysis was monitored for up to 1 h. The kinetic parameters were evaluated as described under “Experimental Procedures.” Analogous results were obtained from at least two independent experiments.

**Fig. 4. Synthetic MMPIs enhance pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2.** The TIMP2 (−/−) mutant cells in 12-well plates were co-infected to express MT1-MMP as described in the legend to Fig. 1. After the infection, the media were aspirated and replaced with serum-free DMEM (1 ml/well) supplemented with 1 μM marimastat (lane 3), 1 μM batimastat (lane 4), 1 μM SB-3CT (lane 5), or 100 nM Δ-TIMP-2 (lane 6) followed by a 16-h incubation at 37 °C. Some wells received DMEM without inhibitors (lanes 1 and 2). The cells were washed once to remove excess inhibitors and then incubated with DMEM (1 ml/well) supplemented without (lane 1) or with (lanes 2–6) 10 nM TIMP-2 for 5 min at 37 °C. The media were then aspirated and replaced with fresh DMEM containing 10 nM pro-MMP-2. After 15 min at 37 °C, the cells were rinsed with phosphate-buffered saline and solubilized in lysis buffer. The lysates were analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. The asterisk shows the pro-MMP-2 added to the media. The ~50-kDa band is nonspecific. This experiment was repeated at least three times with similar results.
MMP Inhibitors and MT1-MMP Activity

Fig. 4. Synergistic effect of TIMP-2 with synthetic MMPIs and TIMP-4 on pro-MMP-2 activation. A, Temp2 (-/-) mutant cells in 12-well plates were infected to express MT1-MMP as described in Fig. 1. After the infection, the media were aspirated and replaced with serum-free DMEM (1 ml/well) supplemented without (lanes 1 and 2) or with BB-2116 (lane 3, 20 nM; lane 4, 500 nM), SB-3CT (lane 5, 20 nM; lane 6, 500 nM) or marromastat (lane 7, 20 nM; lane 8, 500 nM). After a 16-h incubation at 37°C, the media were aspirated and the cells were washed with DMEM to remove excess inhibitors and then incubated with DMEM (1 ml/well) supplemented without (lane 1) or with (lanes 2–8) 10 nM TIMP-2 for 5 min at 37°C. The media were then aspirated and replaced with DMEM containing 10 nM pro-MMP-2. After 15 min at 37°C, the cells were solubilized in lysis buffer and analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. B, Temp2 (-/-) mutant cells in 12-well plates expressing MT1-MMP were incubated (16 h, 37°C) without (lane 1) or with TIMP-4 (lane 2, 1 nM; lane 3, 10 nM; lane 4, 100 nM). After removal of the unbound TIMP-4, the cells received 10 nM TIMP-2 for a 5-min incubation at 37°C followed by the addition of 10 nM pro-MMP-2 as described above. Analysis of pro-MMP-2 activation and MT1-MMP forms were monitored by gelatin zymography and immunoblot analysis, respectively.

Fig. 5. TIMP-2 is required for the enhancing effect of marimastat on pro-MMP-2 activation by MT1-MMP. Temp2 (-/-) mutant cells infected to express MT1-MMP in 12-well plates were treated (lanes 2–5) or not (lane 1) with 1 µM marromastat overnight at 37°C. The medium was then aspirated and replaced with medium supplemented with either 10 nM TIMP-2 (lanes 1 and 2), Δ-TIMP-2 (lanes 3), or TIMP-1 (lane 4) or medium without TIMPs (lane 5). After a 30-min incubation, the medium was aspirated, and the cells were rinsed with DMEM and incubated (30 min, 37°C) with 10 nM pro-MMP-2. The cell lysates were analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. The asterisk shows the pro-MMP-2 added to the medium.

(2.74 ± 0.14) × 10^8 and (2.68 ± 0.12) × 10^8 m^−1 s^−1, respectively. The latter value is in agreement with that reported by Butler et al. (36) for the interaction of the (Δ128–194) TIMP-2 mutant with the catalytic domain of MT1-MMP (2.80 ± 0.45 × 10^8 m^−1 s^−1). TIMP-2 bound with a picomolar Ki (0.07 nM) and showed significant inhibition at a concentration similar to that of the enzyme itself. Δ-TIMP-2 exhibits reduced affinity (K_i = 0.75 ± 0.03 nM) due to a 10-fold higher dissociation rate constant (k_{off} = 1.95 ± 0.03 × 10^−3 s^−1) relative to the value for the full-length TIMP-2. The synthetic MMP inhibitors marromastat, batimatstat, BB-2116, and SB-3CT show competitive inhibition, and, with the exception of SB-3CT, exhibit K_i values in the low nanomolar range. These data are in agreement with the IC_{50} values for marromastat and batimatstat reported by Yamamoto et al. (70) for a mutant MT1-MMP lacking the transmembrane domain. In addition, the K_i value for marromastat with the MT1-MMP cat compares with IC_{50} values reported for the inter-

action of this inhibitor with the gelatinases (IC_{50} = 3–6 nM), fibroblast collagenase (IC_{50} = 5 nM), and matrilysin (IC_{50} = 16 nM) consistent with marromastat being a nonspecific (i.e., broad-spectrum) MMP inhibitor (33, 45, 47, 71). Interestingly, SB-3CT shows an ~10–1600-fold reduced affinity (K_i = 110 nM) for MT1-MMP cat compared with the other MMPIs, in agreement with its inability to induce accumulation of the 57-kDa species of MT1-MMP and pro-MMP-2 activation with TIMP-2.

Synergistic Effects of MMPI Inhibitors on Pro-MMP-2 Activation in a Background of Endogenous Expression of TIMP-2—To further examine the synergistic effects of MMPIs and TIMP-2 on pro-MMP-2 activation, we used BS-C-1 cells infected to express MT1-MMP. BS-C-1 cells produce low levels of endogenous TIMP-2, which are further suppressed but not completely eliminated upon viral infection (data not shown). Consistently, BS-C-1 cells infected to express MT1-MMP can activate pro-MMP-2 without the addition of exogenous TIMP-2 (40). Thus, we used BS-C-1 cells to examine the effects of MMPIs on pro-MMP-2 activation in a cellular system expressing a background level of endogenous TIMP-2. BS-C-1 cells infected to express MT1-MMP were incubated with increasing concentrations of TIMP-2 (0–20 nM), Δ-TIMP-2 (0–500 nM), SB-3CT (0–1 µM), or marromastat (0–10 µM) followed by the addition of pro-MMP-2. In addition, we tested the effects of Ala + TIMP-2 (0–100 nM), a mutant TIMP-2 devoid of inhibitory activity, as a negative control inhibition of MT1-MMP autocatalytic turnover. As shown in Fig. 6, pro-MMP-2 activation is greatly enhanced after administration of exogenous TIMP-2 (TIMP-2 panel). Both marromastat and Δ-TIMP-2 enhance pro-MMP-2 activation when compared with the basal activation detected in BS-C-1 cells in the absence of inhibitors (due to endogenous TIMP-2). Consistently, activation under these conditions is associated with accumulation of the 57-kDa species of MT1-MMP as shown in the immunoblots of Fig. 6. Both Ala + TIMP-2 and SB-3CT have no significant effects, suggesting that inhibition of the MT1-MMP autocatalytic turnover is required for the synergistic effect of the synthetic and natural MMPIs with the endogenous TIMP-2. Indeed, neither Ala + TIMP-2 nor SB-3CT induces a detectable accumulation of the 57-kDa form (Fig. 6, immunoblot). Taken together, these studies indicate that inhibition of MT1-MMP turnover (accumulation of 57-kDa form) by synthetic MMPIs can enhance the effect of the endogenous TIMP-2 in pro-MMP-2 activation in the BS-C-1 cell system.

DISCUSSION

The studies presented here provide conclusive evidence for the complex regulation of MT1-MMP activity by TIMP-2 and further demonstrate that some synthetic MMPIs might have the potential to promote MT1-MMP-dependent activation of pro-MMP-2. Our results clearly show that pro-MMP-2 activation requires the presence of TIMP-2, since the Temp2-null cells are unable to activate pro-MMP-2 even after expression of MT1-MMP. However, a short (5-min) incubation with exogenous TIMP-2 and a brief incubation (15 min) with pro-MMP-2 result in a significant conversion of pro-MMP-2 to its active form. This rapid activation of pro-MMP-2 is unprecedented in a cellular system and demonstrates the high catalytic efficiency of MT1-MMP for this substrate under optimal conditions. The dependence on TIMP-2 for activation is also evident from the results with the heterozygous Temp2 (+/-) mutant cells and the BS-C-1 cells, both of which contain endogenous TIMP-2 and are able to activate pro-MMP-2 after expression of MT1-MMP without requirement of exogenous TIMP-2. Strongin et al. (12) proposed that the effect of TIMP-2 on activation is mediated by a ternary complex formed between active MT1-MMP, TIMP-2, and pro-MMP-2, where the C-terminal region of TIMP-2 binds...
ternary complex formation is not a limiting step. The result is the result of the inhibition of MT1-MMP autocatalysis and digestion of small molecule inhibitors concurrently with TIMP-2 to active MT1-MMP (57 kDa) to its inactive 44-kDa species, and as a consequence the 57-kDa species accumulates on the cell surface (40). As shown here, a similar effect is induced by synthetic MMPIs (41) as well as by Δ-TIMP-2 and TIMP-4. We show for the first time that some synthetic MMPIs and Δ-TIMP-2 can enhance pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2. This effect is due to the accumulation of the 57-kDa MT1-MMP species as a consequence of inhibition of MT1-MMP turnover. Our kinetic data suggest the possibility that the increase in MT1-MMP-TIMP-2 complexes may be a consequence of a displacement of the bound synthetic MMPI by TIMP-2 (K_i for TIMP-2 is approximately 1–2 orders of magnitude lower than that for the synthetic MMPI, a process that will generate more pro-MMP-2 “receptors.” However, binding of small molecule inhibitors concurrently with TIMP-2 to active MT1-MMP cannot be ruled out. Regardless of the mechanism involved, pro-MMP-2 activation would require a “catalytic” quantity of the inhibitor-free MT1-MMP to hydrolyze the Asn37-Leu38 bond of pro-MMP-2 as previously shown (69). It should be noted that the enhancing effects of the MMPIs on pro-MMP-2 activation in the Timp2 null cells in the presence of TIMP-2 were evident only when the synthetic inhibitors (up to 10 μM) to avoid toxic effects) were administered to the cells and removed from the system prior to the administration of the exogenous TIMP-2. Preincubation of the cells with the MMPIs was necessary to induce accumulation of the 57-kDa form of MT1-MMP, a fraction of which would be expected to be inhibitor-free, since the cells are continuously producing MT1-MMP. We postulate that these conditions (removal of excess synthetic inhibitor prior to the addition of TIMP-2 and pro-MMP-2 and continuous replenishment of MT1-MMP by the cells) generate sufficient catalytically active MT1-MMP to generate ternary complex and to process pro-MMP-2. In contrast, simultaneous administration of TIMP-2 with various doses of marimastat inhibits pro-MMP-2 activation in a dose-dependent manner (data not shown). This inhibitory effect is likely to be due to competition between TIMP-2 and marimastat for MT1-MMP binding, resulting in decreased ternary complex formation (38, 72). However, BS-C-1 cells, which contain low levels of endogenous TIMP-2, exhibit enhanced pro-MMP-2 activation upon administration of the MMPIs. In this case, enhanced activation is the result of the inhibition of MT1-MMP autocatalysis and ternary complex formation is not a limiting step. The result with the BS-C-1 cells also suggests that invasive tumor cells equipped with both MT1-MMP and TIMP-2 may be subject to similar synergistic effects of synthetic MMPIs on MT1-MMP activity under the right conditions.

The relationship between inhibition of MT1-MMP autocatalysis and ternary complex formation was also demonstrated in the experiments in which the Timp2 (−/−) mutant cells were treated with marimastat followed by administration of Δ-TIMP-2 or TIMP-1, in which case pro-MMP-2 activation was not observed. Furthermore, the Ala + TIMP-2 mutant, devoid of inhibitory activity, failed to support pro-MMP-2 activation in the BS-C-1 cells in the presence of endogenous TIMP-2 due to its inability to inhibit MT1-MMP turnover. Likewise, pretreatment of the Timp2 (−/−) cells with marimastat had no effect on pro-MMP-2 activation without subsequent addition of TIMP-2 revealing that accumulation of active MT1-MMP alone is not sufficient for pro-MMP-2 activation and requires a functional full-length TIMP-2 to generate the ternary complex. This was also demonstrated by the results with TIMP-4, which, despite its ability to inhibit MT1-MMP activity as found by Bigg et al.2 and to bind pro-MMP-2 (68), was unable to promote MT1-MMP-dependent activation of pro-MMP-2 or to act synergistically with TIMP-2 in this process. Recent studies from Overall’s laboratory2 also show that TIMP-4 cannot form a ternary complex in a purified system and, if administered with TIMP-2, inhibits pro-MMP-2 activation by MT1-MMP in Timp2 mutant cells. Here we have shown that TIMP-4, like other MMPIs, induces accumulation of the 57-kDa form of MT1-MMP, consistent with its inhibitory activity, but fails to act synergistically with TIMP-2 in the promotion of pro-MMP-2 activation. The reason for this puzzling result is yet unknown but may be related to differences in affinity between these inhibitors for MT1-MMP and pro-MMP-2. Indeed, TIMP-4 exhibits a lower affinity for pro-MMP-2 when compared with TIMP-2.2 Also, a potential rapid internalization of the putative MT1-MMP/TIMP-4 complex, although yet unproven, may play a role. Further enzymatic and biochemical studies are required to understand the dynamics of TIMP-4 and TIMP-2 inhibitory activities in relation to MT1-MMP functions. Nevertheless, these studies suggest that the effects of TIMP-4 on TIMP-2 may represent a natural and unique regulatory mechanism of MMP-dependent proteolysis on the cell surface in which TIMP-4 may play a counter role to that of TIMP-2, physiologically, by binding to active MT1-MMP with high affinity. We therefore propose that the long held view of a balance between MMPs and TIMPs as a key determinant of proteolytic activity and tumor progression (73) may well include a balance of TIMP-2 and TIMP-4 as a major determining factor for MT1-MMP-dependent proteolysis in cancer tissues where both inhibitors may be present.

The results presented here demonstrate that pro-MMP-2 activation by MT1-MMP at the cell surface is the result of a...
highly regulated enzymatic process that involves two independent events, which under certain conditions may work synergistically to enhance MT1-MMP-dependent activation of pro-MMP-2. It should be noted that this might not be the case in all circumstances or with different MT1-MMP substrates. For example, for pro-MMP-2, our data show that a short (5-min) exposure to TIMP-2 followed by a 15-min incubation with pro-MMP-2 was sufficient to rapidly activate pro-MMP-2 without detectable accumulation of active (57-kDa) MT1-MMP. The reason for the lack of detection of active enzyme under this condition is unclear but may be related to the detection method (immunoblotting), rapid enzyme turnover, and/or the internalization and turnover of the MT1-MMP (57 kDa)/TIMP-2 complex as recently reported (72). Under conditions of substoichiometric TIMP-2 molar concentrations relative to MT1-MMP, the efficient binding of TIMP-2 and the catalytic efficiency of MT1-MMP for its substrate result in optimal pro-MMP-2 activation (39). Thus, while rapid bursts of TIMP-2 expression will be sufficient to generate ternary complex and consequently activate pro-MMP-2 in the absence of a significant and detectable accumulation of active MT1-MMP, chronic exposure to TIMP-2 or MMPIs would maintain a steady level of MT1-MMP on the cell surface due to inhibition of autocatalysis. For other MT1-MMP substrates such as ECM components, which do not require ternary complex formation to be hydrolyzed by MT1-MMP, sustained TIMP-2 expression and/or the presence of synthetic MMPIs may indirectly enhance catalytic activity, as demonstrated here using pro-MMP-2 as a target substrate.

Recent accomplishments in drug design have resulted in the generation of a variety of novel MMPIs with effective anti-tumor and anti-angiogenic activities in animal models of cancer (3, 4, 33, 46, 47). These encouraging results have brought some of these compounds, such as marimastat and batimastat, to human clinical trials. The majority of the compounds undergoing testing in humans, however, lack specificity toward the various MMP families. The hydroxamates, for instance, inhibit a wide spectrum of MMPs, including MT1-MMP as herein demonstrated, often with similar affinities (70, 71). The complex outcome of MT1-MMP inhibition on catalytic activity demonstrated here raises important issues regarding the potential consequences of inhibiting MT1-MMP. The intermolecular autocatalytic turnover of MT1-MMP on the cell surface may represent an important regulatory step aimed at controlling peripheral proteolysis, a process that is likely to be favored by lateral diffusion and clustering of MT1-MMP molecules in the cell surface (74, 75). Thus, reversible inhibition of MT1-MMP activity would play a role in preventing excessive enzyme clearance from the cell surface and indirectly favor proteolysis. Such an effect by synthetic MMPIs would depend on the spectrum of activity (Ki values) elicited by each particular inhibitor against the different members of the MMP family and on their pharmacokinetics and dosing regime. The MMPIs tested here exhibit different Ki values for the catalytic domain of MT1-MMP, which correlated well with their efficacy in promoting pro-MMP-2 activation with TIMP-2. We recently described the first example of a mechanism-based inhibitor for MMPs (42). This inhibitor, SB-3CT, is highly specific for inhibition of gelatinases, enzymes that were inhibited covalently by this inhibitor. SB-3CT does not pursue the metal chelation strategy for its inhibition, in contrast to the case of the existing inhibitors. We have shown here that SB-3CT is substantially less effective in inhibition of MT1-MMP, for which it was not designed, and simply behaves as a simple linear competitive inhibitor, in contrast to the case of gelatinases (42). Again in contrast to marimastat and batimastat, SB-3CT did not show any ability to stimulate activation of pro-MMP-2 induced by TIMP-2. Thus, the design of highly specific MMPIs will minimize potential adverse effects in conditions where inhibition of the MT1-MMP system is a therapeutic goal.

The complex regulation of MT1-MMP activity by TIMP-2 may provide a biochemical framework for understanding several intriguing observations in human tumors and experimental models of metastasis using synthetic MMP inhibitors. High levels of TIMP-2 expression were found in various human cancers, which positively correlated with metastasis and poor survival (21, 76–79). A recent study reported that treatment of tumor-bearing mice with batimatost significantly inhibited tumor growth but promoted tumor cell invasion into the liver of a variety of human cancer cells (80). However, the mechanism for such effect was not reported. Finally, recent tumorigenicity studies with the heterozygous and homozygous Timp-2 mutant cells indicate a higher incidence of tumor formation and metastasis in the heterozygous cells, suggesting a role for TIMP-2 in promotion of tumor progression.5 Our findings disclosed in this report provide one plausible explanation for these observations, that by binding to active MT1-MMP, both natural and synthetic MMP inhibitors may produce a “pool” of active MT1-MMP available to degrade ECM components and to activate pro-MMP-2. While this may represent an undesired effect of some strategies for anti-MMP therapies in cancer that are being investigated, this effect may be beneficial in pathological conditions characterized by excessive deposition of collagen such as fibrosis and connective tissue disorders where increased MMP activity might be desired. These examples and the studies presented herein emphasize the importance of a rational approach for the design of specific MMP inhibitor, which should also be based on an understanding of the regulation of MT1-MMP and likely other members of the MT-MMP subfamily by TIMPs and MMPIs at the cell surface.

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