Reactive Site Mutations in Tissue Inhibitor of Metalloproteinase-3 Disrupt Inhibition of Matrix Metalloproteinases but Not Tumor Necrosis Factor-α-converting Enzyme*

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Tissue inhibitor of metalloproteinase-3 (TIMP-3) is a dual inhibitor of the matrix metalloproteinases (MMPs) and some adamalysins, two families of extracellular and cell surface metalloproteinases that function in extracellular matrix turnover and the shedding of cell surface proteins. The mechanism of inhibition of MMPs by TIMPs has been well characterized, and since the catalytic domains of MMPs and adamalysins are homologous, it was assumed that the interaction of TIMP-3 with adamalysins is closely similar. Here we report that the inhibition of the extracellular region of ADAM-17 (tumor necrosis factor α-converting enzyme (TACE)) by the inhibitory domain of TIMP-3 (N-TIMP-3) shows positive cooperativity. Also, mutations in the core of the MMP interaction surface of N-TIMP-3 dramatically reduce the binding affinity for MMPs but have little effect on the inhibitory activity for TACE. These results suggest that the mechanism of inhibition of ADAM-17 by TIMP-3 may be distinct from that for MMPs. The mutant proteins are also effective inhibitors of tumor necrosis factor α (TNF-α) release from phorbol ester-stimulated cells, indicating that they provide a lead for engineering TACE-specific inhibitors that may reduce side effects arising from MMP inhibition and are possibly useful for treatment of diseases associated with excessive TNF-α levels such as rheumatoid arthritis.

Two families of zinc endopeptidases, the matrix metalloproteinases (MMPs) and disintegrin-metalloproteinases (ADAMs), catalyze important proteolytic reactions in the extracellular matrix and at the cell surface. The turnover of proteins in the matrix, catalyzed primarily by MMPs, is necessary for morphogenesis, tissue remodeling, blastocyst implantation, wound healing, and many other important physiological processes (1), while ADAMs catalyze the shedding of the ectodomains of cell surface proteins, releasing cytokines, growth factors, cell adhesion molecules, and receptors (2, 3), processes linked to signal transduction, cell growth, and cell-cell and cell-matrix interactions. Enhanced activities of specific MMPs and ADAMs underlie or contribute to many critical human diseases including cancer, rheumatoid arthritis, osteoarthritis, and heart disease (1–3).

MMP activities in the extracellular matrix are regulated by four endogenous inhibitory proteins, tissue inhibitors of metalloproteinases (TIMPs), TIMP-1 to TIMP-4. These are, with few exceptions, broad-spectrum inhibitors of the more than twenty MMPs found in humans (4). In addition, TIMP-3 efficiently inhibits some adamalysins, including ADAM10 (5), ADAM12-S (6), ADAM17/TACE (tumor necrosis factor α-converting enzyme (7)), and certain ADAMs with thrombospondin motifs, such as ADAMTS-4 and ADAMTS-5 (8); TIMP-1 also inhibits ADAM-10 (5).

TIMPs have two domains and exhibit multiple biological activities such as the stimulation of the growth of certain cells, induction or protection from apoptosis, and inhibition of angiogenesis (9, 10). The metalloproteinase inhibitory activity resides in the larger (~120 residue) N-terminal domain whereas the smaller, ~65-residue, C-terminal domain mediates interactions with the hemopexin domains of some pro-MMPs. Mutations in the human TIMP-3 gene that result in X to Cys substitutions and truncations in the C-terminal domain of human TIMP-3 are the cause of Sorsbys fundus dystrophy, an autosomal dominant disorder that produces early onset macular degeneration (11, 12).

The structures of complexes of TIMP-1 with the catalytic domain of MMP-3 (13) and of TIMP-2 with a membrane type MMP, MMP-14 (MT1-MMP (14)), show that a structurally contiguous region around the conserved Cys2 to Cys20 disulfide bond of TIMP (TIMP-1 sequence numbering) inserts into the active site groove of the MMP. Cys3 bidentally coordinates the catalytic Zn2+ through its α-amino and carbonyl groups, while the side chain of residue 2 (Thr or Ser) enters into the mouth of the S1’ specificity pocket of the protease. Most (75%) of the interactions with the MMP involve two sections of polypeptide chain of the TIMP around the Cys3 to Cys20 disulfide bond (residues 1–4 and 66–70, see Fig. 1). Blocking the N-terminal α-amino group by carbamylation (15) or acetylation (16), as well as addition of an extra residue (16, 17), inactivates MMP inhibitory activity of TIMPs. Substitutions for key amino acids in the interaction interface, residues 2, 4, or 68, singly and in combination, differentially affect the affinity of N-TIMP-1 for different MMPs (18, 19). This suggests that the specificity of TIMPs can be modified to produce more targeted MMP inhibitors.

TACE (ADAM-17) is a type-1 membrane protein composed of an extracellular multidomain region, a transmembrane segment, and a C-terminal cytoplasmic domain. Within the extracellular region of the active enzyme is a metalloendopeptidase catalytic domain, a disin-
tegrin domain, a cysteine-rich domain, and a crambin-like domain (2, 3). Many previous studies of the structural, catalytic, and inhibitory properties of TACE have focused on the truncated catalytic domain (20–24), but some studies suggest that the non-catalytic domains of the extracellular region have a significant influence on the enzymatic properties such as substrate recognition and zymogen activation (25, 26).

Some ADAMs lack protease activity, but those that are catalytically active share with the MMPs a canonical zinc-binding HExX-HXXGXXH sequence motif and a Met turn in their catalytic domains (www.people.virginia.edu/~jw7g/). However, the ADAMs and MMPs are very divergent in overall sequence and their catalytic domains differ considerably in three-dimensional structure (20), raising the question of whether TIMP-3 binds to and inhibits both protease groups through a similar mechanism. To address this question, we have constructed mutants of N-TIMP-3 that disrupt the interaction interface for MMPs. The properties of these mutants as inhibitors of TACE suggest that the interaction of TIMP-3 with TACE and the mechanism of inhibition are distinct from those for MMPs.

**EXPERIMENTAL PROCEDURES**

**Materials**—The plasmid pET-42b-N-timp-3His₈ containing the gene encoding a C-terminally His-tagged form of the N-terminal domain of TIMP-3 in the pET-42b vector (Novagen) was generated as described previously (8). All reagents, cells, and instruments used for plasmid construction and for the expression, purification, and in vitro folding of N-TIMP-3 mutants were from the same sources as in previous studies (8). Metalloproteinases and substrates used in the kinetic assays were obtained from previously reported sources (19, 27). N-TIMP-1 was expressed in *Escherichia coli* and folded in vitro as described (19, 27), and the synthetic metalloproteinase inhibitor TAPI-2 (HONHCOCH₂CH₂CH₂CH(CH₃)₂–CO-t-butyl-Gly–Ala–NHCH₂C–H₂NH₂) was from Peptides International. Human monocyte THP-1 cells and RPMI 1640 medium were purchased from ATCC, while phorbol 12-myristate 13-acetate (PMA) was from Sigma and the antibodies used for ELISA were from Pharmingen.

**Construction of N-TIMP-3 Mutants**—The plasmid pET-42b-N-timp-3His₈ was used as the template for site-directed mutagenesis by PCR. The forward primers used (the mutated codons are underlined and the restriction sites are shown in italic) were 5’-AAAAACATATGTCGGAGTGCCTGOCAGCCAC-3’ (for T2G) and 5’-AAAAACATATGGCATGTACCATGCTGAGGCAAGGAC-3’ (for-1ALA), The reverse primer was 5’-AAAAAGGCGGGCTTACACCAGAAGAGGATA-3’.

Reactions were carried out for 35 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min after a hot start at 94 °C for 3 min in a PCR Sprint HYBAID system using the Vent PCR kit (New England Biolabs). PCR products were cloned back into the pET-42b vector using the Ndel and NotI sites (both enzymes were from New England Biolabs) and confirmed by automatic DNA sequencing using T7 promoter primer.

**Expression, Purification, and in Vitro Folding of N-TIMP-3 and Mutants**—N-TIMP-3 and its mutants were expressed in *E. coli* BL21(DE3) cells as inclusion bodies. The proteins were extracted with 6 M guanidine HCl and purified by Ni²⁺–chelate chromatography in 6 M guanidine as described previously (8). Purified proteins were treated with cystamine and were folded in vitro by removing the denaturant by dialysis in the presence of 5 mM β-mercaptoethanol and 1 mM 2-hydroxyethyl disulfide essentially as described (8) except that 1 M NaCl was included to enhance protein solubility during the folding process. The folded proteins were subsequently loaded to a 5-ml Ni²⁺-NTA column previously equilibrated with 20 mM Tris-HCl (pH 7.0), 1 M NaCl and 20% glycerol and eluted with the same buffer containing 200 mM imidazole.

**Enzyme Inhibition Kinetic Studies**—Inhibition kinetic studies for MMPs and TACE were carried out as described previously (19, 27) with modifications. Purified N-TIMP-3 and mutants were dialyzed against 20 mM Tris-HCl (pH 7.0), 50 mM NaCl containing 20% glycerol and centrifuged at 14,000 rpm for 10 min to remove any precipitate, and protein concentration was re-measured before conducting inhibition assays. Since NaCl inhibits the activity of the TACE ectodomain in vitro (28), we adjusted the final concentration of NaCl to 5 mM in all assays with TACE. The same dialysis buffer was used to make serial dilutions of N-TIMP-3 and mutants, and equal volumes (10% of total assay volume) were added to TACE assays, resulting in a final pH of 8.8 and NaCl concentration of 5 mM. Inhibition data were analyzed by fitting to the following equations as appropriate: tight binding inhibition (Equation 1),

\[
\frac{v}{v_0} = \frac{E - I - K + ((E - I - K)^2 + 4EK)^{0.5}}{2E}
\]  

(Eq. 1)

normal inhibition (Equation 2),

\[
v = \frac{v_0}{1 + I/K}
\]  

(Eq. 2)

and cooperative Inhibition (Equation 3) (29),

\[
v = \frac{v_0}{1 + ((I/K)^h)}
\]  

(Eq. 3)

where \(v\) is the experimentally determined reaction velocity, \(v_0\) is the uninhibited activity, \(E\) is enzyme concentration, \(I\) is inhibitor concentration, \(K\) is the apparent inhibition constant (\(K_{i(app)}\)), and \(h\) is the Hill coefficient.

**Inhibition of TNF-α Shedding from THP-1 Cells**—All TIMP solutions were dialyzed against 20 mM Tris-HCl (pH 7.0), 150 mM NaCl, and 20% glycerol before use. Human monocyte THP-1 cells cultured in RPMI 1640 medium supplemented with 5% fetal calf serum were harvested, extensively washed, and resedated into serum-free medium at 2.5 × 10⁶ cells/ml. Shedding was stimulated by adding PMA to a final concentration of 100 ng/ml, and cells were incubated at 37 °C with 5% CO₂ for 20
In vitro folding procedure. This significantly increased the yield of N-TIMP-3 and mutants (data not shown).

**Inhibitory Properties of Mutants with Purified Metalloproteinases—** The inhibitory activities of wild-type N-TIMP-3 and the two mutants were determined with MMPs representing four different subgroups: full-length collagenase 1 (MMP-1), gelatinase A (MMP-2), and the catalytic domains of stromelysin 1 (MMP-3(C)) and membrane-type 1 MMP (MMP-14). As previously reported for the corresponding mutants of N-TIMP-1 and TIMP-2 (17, 18), both mutations in N-TIMP-3 reduced the inhibitory activity toward the four MMPs by 2–3 orders of magnitude (TABLE ONE). Fig. 2A highlights the difference in inhibition of MMP-14(CD) by wild-type and mutated N-TIMP-3.

The inhibitory activities of the mutants were also compared with that of wild-type N-TIMP-3 against a soluble form of TACE in which the transmembrane and C-terminal cytoplasmic domains are deleted (TACE R651 (28)). These assays were carried out at pH 8.8 and low ionic strength, because the activity of TACE is optimal at higher pH (7) and the protocol from R&D Systems) and is strongly inhibited by salt (28).

![TABLE ONE](image)

| $K_{app}$ (nM) of wild-type and mutant N-TIMP-3 with some MMPs | MMP-1 | MMP-2 | MMP-3(C) | MMP-14(CD) |
|---------------------------------------------------------------|-------|-------|----------|------------|
| Wild-type                                                    | 1.2 ± 0.5 | 4.3 ± 0.5 | 67 ± 2.8 | 0.8 ± 0.03 |
| T2G                                                          | 547 ± 100 | 4.2 ± 10        | >1 × 10^7 | 2.3 ± 10^7 |
| -1A                                                          | 1.3 ± 10^4 | 614 ± 32       | 3.3 × 10^7 | 941 ± 206 |

*Data taken from Ref. 8.

These mutants, as well as wild-type inhibitor, were expressed in bacteria as inclusion bodies, purified, and folded in vivo. A high salt concentration was found to increase the solubility of N-TIMP-3; therefore we included 1 M NaCl throughout the in vitro folding procedure. This significantly increased the yield of N-TIMP-3 and mutants (data not shown).

**RESULTS**

**Design and Production of N-TIMP-3 Mutants—** Mutations in N-TIMP-3 were designed to disrupt inhibitory activity toward MMPs based on the known structures of the TIMP-1-MMP-3 complex and the TIMP-2-MT1-MMP complex (13, 14) and previous mutational studies with TIMPs (17, 18). The specific mutations are: (i) The addition of an N-terminal alanine extension (-1A) to perturb the interaction of Cys1 with the active site Zn2+; this mutation in N-TIMP-1 and TIMP-2 (17) drastically curtailed inhibitory activity for MMPs. (ii) A Thr2 to Gly (T2G) mutation, which removes the side chain of residue 2; this residue interacts with the S1 specificity pocket of MMPs and this mutation in N-TIMP-1 reduces the affinity for MMP-1, -2, and -3 about 1,000-fold (18).

![FIGURE 2. Inhibition of MMP and TACE by N-TIMP-3 and its mutants](image)

A. inhibition of MMP-14(CD) by wild-type and mutated N-TIMP-3. Open circles, wild-type N-TIMP-3; closed circles, T2G; and open squares, -1A. B. comparison of the inhibition of TACE by wild-type N-TIMP-3, N-TIMP-1, and TAPI-2. The inhibitors were incubated with 0.5 nM TACE for 3 h at room temperature, and the residual enzyme activity was measured with 10 μM Substrate III (R&D Systems). The assays were performed at pH 8.8 at a final NaCl concentration of 5 mM. Open circles, N-TIMP-3; closed circles, TAPI-2; and open squares, N-TIMP-1. C. inhibition of TACE (0.5 nM) by wild-type and mutated N-TIMP-3. Open circles, wild-type inhibitor; closed circles, T2G; and open squares, -1A.

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TABLE TWO
Comparison of inhibition parameters for TACE of N-TIMP-3 and its mutants with TAPI-2

|                | $K_{\text{app}}$ | $h$  |
|----------------|------------------|------|
| Wild-type      | $13.7 \pm 0.2$   | 3.59 ± 0.16 |
| T2G            | $35.6 \pm 1.9$   | 2.54 ± 0.25  |
| -1A            | $33.9 \pm 2.8$   | 1.9 ± 0.22   |
| TAPI-2         | $4.28 \pm 0.001$ | 1    |

FIGURE 3. Effects of mutations in N-TIMP-3 on inhibition of cellular shedding of TNF-α. THP-1 cells (2.5 × 10⁶/ml) growing in serum-free RPMI 1640 medium were stimulated with 100 ng/ml PMA for 20 min before adding various concentrations of N-TIMP-3 (wild-type and mutants). Cells were allowed to grow for another 6 h, and conditioned media were collected for the ELISA assays.

TAPI-2, effectively inhibited the activity of TACE; in contrast, wild-type N-TIMP-1 had minimal inhibitory activity under the same condition (Fig. 2B). The inhibition curve of TACE by wild-type N-TIMP-3 is sigmoid, in striking contrast with the inhibition by TAPI-2 and with the inhibition of MMPs by N-TIMP-3 and N-TIMP-1 (Fig. 2, A and B (31)). Sigmoid inhibition curves were also obtained for TACE with the T2G and -1A mutants of N-TIMP-3 (Fig. 2C). These mutations, which severely reduced activity against MMPs, had little effect on the inhibition of TACE. The inhibition data obtained with N-TIMP-3 and its mutants did not fit well with Equations 1 or 2 for tight binding or weak to moderate inhibitors or to other equations describing multsite binding (data not shown) but fit well to Equation 3 for positively cooperative binding. The results indicate that the mutations have only a minor effect on the apparent inhibition constant ($K_{\text{app}}$) but also reduce the Hill coefficient, $h$ (TABLE TWO).

The conditions used for TACE and MMP activity measurements differ in pH and ionic strength. To determine whether this could influence our results, we first investigated the inhibitory activities of wild-type N-TIMP-3 and the T2G mutant against TACE at pH 7.5, since MMP inhibition measurements were conducted at this pH. Sigmoid inhibition curves were obtained for both proteins and $K$ values were calculated to be $26 \pm 3$ and $46 \pm 2$ nM, respectively (data not shown). Second, although it was not possible to conduct TACE activity measurements at higher NaCl concentrations because of the strong inhibitory effects of ionic strength on activity, we also quantified the inhibition of MMP-1 by N-TIMP-3 and the -1A mutant under the conditions used for TACE activity measurements. Both proteins showed normal hyperbolic inhibition profiles with $K_i$ values of 1.6 and 412 nM, respectively (data not shown). Thus, these results show that binding of the wild-type inhibitor was not significantly affected at the higher pH and low ionic strength and the mutation strongly disrupts inhibitory activity against MMP-1 under these conditions.

Effects of Mutations in N-TIMP-3 on Inhibition of Cellular Shedding of TNF-α—The ectodomains of many cell surface proteins are released in soluble forms through processing catalyzed by cell surface “sheddases.” Both TACE/ADAM17 and ADAM10 have been found to be active as sheddases, TACE being particularly important for the release of the cytokine TNF-α from its cell surface precursor (32). The release of TNF-α from monocytes is a key for inflammation and immunity, making TACE an interesting target for anti-proteolytic therapies. We investigated the abilities of N-TIMP-3 and mutants to inhibit TNF-α shedding from human monocyte THP-1 cells, where TACE, but not other sheddases, was shown to be the major enzyme responsible for releasing TNF-α from cell surface (33). In cell culture systems, higher inhibitor concentrations are required than for the inhibition of purified enzyme in vitro; nevertheless N-TIMP-3, at concentrations of 50–500 nM, effectively inhibited the PMA-stimulated release of TNF-α, whereas N-TIMP-1 had no effect. As in the studies with pure enzyme shown in Fig. 2C, the T2G and -1A mutations in N-TIMP-3 exhibited only slightly reduced inhibitory activity for TNF-α release (Fig. 3).

DISCUSSION

Among the four mammalian TIMPs, TIMP-3 has the broadest range as a metallopeptinase inhibitor that includes both the MMPs and disintegrin-metalloproteinases. The latter are complex multi-domain enzymes that share only catalytic and prodomains with the MMPs. Although the ADAM and MMP catalytic domains are homologous, their levels of sequence identity are low and the crystallographic structure of the TACE catalytic domain indicates that they differ in tertiary structure (20); the root mean square deviation of ~120 Cα atoms that are topologically equivalent between the TACE and MMP structures is 1.6 Å. ADAMs have unique structural features including an additional $\alpha$-helix and a multiple-turn loop, but lack the structural zinc and calcium ions shared by the MMPs (20). Although TACE and MMPs have generally similar active site structures, that of TACE differs in having a deep S3’ pocket merging with the hydrophobic S1’ specificity pocket. Much previous work has focused on the truncated catalytic domain of TACE including structural studies (20) and inhibitory studies using N-TIMP-3 and their mutants (21–24). In the absence of a structure of a TIMP-3-TACE complex, Lee et al. (34) modeled the structure of TIMP-3 using the known structures of TIMP-1 and TIMP-2 and were able to dock this with the catalytic domain of TACE in a manner similar to that in the two known inhibitory TIMP-MMP complexes. This suggests that the mechanism of TIMP-3 inhibition of TACE could be similar to that for MMPs. However, there is a significant difference in susceptibility to TIMP-3 inhibition between the truncated catalytic domain of TACE and longer forms similar to that used here that contain the disintegrin, cysteine-rich, and the crambin-like domains (35). Non-catalytic domains have been shown to influence substrate specificity in TACE and other ADAMs (25, 36).

The present study identifies significant differences between the inhibition of the long form of TACE and MMPs by TIMP-3. First, the inhibition of TACE by wild-type N-TIMP-3 and two mutants displays positive cooperativity with Hill coefficients of 1.9–3.5. This observation was unexpected but has been confirmed with different preparations of TACE and also at a lower pH (7.5). Positive cooperativity arises from the presence of multiple interacting binding sites and alternative conformational states, and its structural basis in TACE is currently unknown.
However, positive cooperativity has been previously described for the hydrolysis of a synthetic peptide substrate by a similar form of TACE (37). Cooperativity was only observed with a peptide substrate derivatized at the N and C termini, whereas untagged peptides showed normal hyperbolic saturation curves (37). This apparent allosteric behavior could have important implications for the regulation of TACE activity; studies are in progress to investigate the possible roles of non-catalytic domains in cooperativity.

A second major difference in N-TIMP-3 inhibition is the observation that both the T2G and -1A mutants of N-TIMP-3 are potent inhibitors of TACE but are extremely weak inhibitors of the four representative MMPs (collagenase 1, gelatinase A, stromelysin 1, and membrane-type 1 MMP) and are likely also to be weak inhibitors of other MMPs. The presence of any extension N-terminal to the α-amino group in TIMPs has been shown to drastically reduce inhibitory activity for MMPs (15–17), presumably because such extensions prevent the interaction of Cys with the catalytic Zn$^{2+}$. The fact that the -1A mutant of N-TIMP-3 is an effective inhibitor of TACE but not MMPs suggests that the interaction of the inhibitor with the active site Zn$^{2+}$ may be relatively unimportant for the strength of binding to TACE. This appears to be consistent with previous studies of TACE inhibition by its own prodomain in which it was found that a bacterially expressed form of the isolated prodomain (residues 22–214) inhibits both the catalytic domain and the full-length soluble form of TACE. Mutation of Cys$^{184}$ of the cysteine switch region in the isolated prodomain, which in MMPs interacts with the catalytic Zn$^{2+}$ of the metalloproteinase domain, had no significant effect on prodomain inhibition (26).

Another key feature of the interaction of TIMPs with MMPs is the extension of the side chain of residue 2 of the TIMPs into the S1′ specificity pocket of the MMPs. The corresponding residue has been proposed to have a similar role in the model of TIMP-3-TACE complex (34). As compared with most MMPs, the S1′ pocket of TACE is deep and very hydrophobic. However, substitution of Thr$^2$ of N-TIMP-3 by residues with larger hydrophobic side chains that should fit better into the S1′ site of TACE failed to improve the binding of the inhibitor to this enzyme (21). Mutation of this residue into glycine, which lacks a side chain for potential interaction with the S1′ pocket of the protease, results in a major reduction in the affinity for MMPs but has little effect on the inhibition of TACE. This suggests that this site of interaction also contributes little to the free energy of binding. We also cannot rule out the possibility that TIMP-3 is oriented in a different way in the complex with TACE than with MMPs, so that Thr$^2$ is not even in contact with the S1′ pocket of the enzyme.

The long form of TACE, used in the present work, differs from the catalytic domain in responses to inhibitors. It is more than 30-fold less sensitive to inhibition by the TACE prodomain (26) and also more weakly inhibited by N-TIMP-3 (35). Furthermore, several mutations that enhance N-TIMP-3 binding to the TACE catalytic domain were found to have little effect on binding to the longer form of the enzyme (35). Murphy and co-workers (22) have suggested that the cysteine-rich domain of TACE may act to inhibit TIMP-3 binding to the catalytic domain and reported that mutation of lysines distant from the MMP reactive site produces inhibitors that are more effective with longer enzyme forms. These results suggest that the non-catalytic domains modulate the properties of the catalytic domain and emphasize the importance of considering the inhibitory properties of the longer enzyme forms in developing specific inhibitors for possible use in vivo.

Soluble TNF-α is released from cultured cells or tissues by several proteases besides TACE/ADAM17, including ADAM10, ADAM19, MMP-7, and the leukocyte serine protease, protease 3 (38–41). Although ADAM10, purified from the membrane extract of THP-1 cells, was shown to process pro-TNF-α in vitro (42), studies with antisense oligonucleotides specifically targeting different ADAM mRNAs suggest that TACE, but not ADAM10, is the major sheddase for TNF-α in this cell line (33). This agrees with our finding that N-TIMP-3 efficiently inhibits the shedding of TNF-α in THP-1 cells, whereas the inhibitory domain of TIMP-1, a potent inhibitor of ADAM10, has no effect. The fact that N-TIMP-3 mutants that do not efficiently inhibit MMPs have similar effects to the wild-type inhibitor effectively rules out the possibility that MMPs make a major contribution to the shedding activity in these cells. These mutants provide useful tools for differentiating the activities of MMPs from that of TACE and possibly other ADAMS in biological systems. In the latter regard it is interesting to find out how these mutations affect the inhibitory activity of TIMP-3 for other disintegrin-metalloproteinases such as ADAM10.

The direct involvement of TIMP-3 in the inhibition of TNF-α shedding in vivo was demonstrated recently in a mouse model, where elimination of the TIMP-3 gene results in excessive TACE activity, elevated levels of soluble TNF-α, and severe inflammation in the liver (43). This observation further validates the feasibility of using TIMP-3 in the therapy of inflammatory diseases that involve unregulated TNF-α levels including rheumatoid arthritis and Crohn disease. However, although a series of MMPs are overexpressed in arthritis (44), the lack of MMP activities has been blamed for joint and bone abnormality. For example, MT1-MMP is indispensable for maintenance of a stable pool of osteocytes and normal development of bones (45), and mice with deficiency in the gene encoding MT1-MMP develop osteopenia and arthritis (46). Furthermore, two mutations in the MMP-2 gene, identified in a number of consanguineous Saudi Arabian families, result in loss of MMP-2 activity and may be the cause of an autosomal recessive form of multicentric osteolyis and arthritis in affected family members (47). These observations suggest that MMPs may have important protective effects against arthritis. Since the N-terminal domain of TIMP-3 is a potent inhibitor of both MMP-2 and MT1-MMP (27), the outcome of the potential therapy using the wild-type inhibitor is unpredictable. The N-TIMP-3 mutants described here may have an advantage over the wild-type inhibitor in clinical applications, since they essentially spare the MMPs, a large family of proteases that have important roles in normal physiological processes.

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