Metalloproteinase Inhibition and Erythroid Potentiation Are Independent Activities of Tissue Inhibitor of Metalloproteinases-1

By Louis Chesler, David W. Golde, Noelle Bersch, and Mark D. Johnson

Tissue inhibitor of metalloproteinases-1 (TIMP-1), the major physiological matrix metalloproteinase inhibitor and a potent antimitastatic factor, also stimulates the growth of erythroid progenitors (erythroid-potentiating activity). We analyzed the relationship between the growth factor activity and protease inhibition by preparing purified TIMP-1 "knock-out" proteins lacking in vitro antiproteolytic activity. The growth-stimulatory effect of these N-terminal TIMP-1 point mutants, as tested in an in vitro assay using erythroid precursors (erythroid burst-forming units) was equal to that of unmutated TIMP-1. A fully antiproteolytic C-terminal TIMP-1 truncation also stimulated growth in the erythroid burst-forming unit assay. The results indicate that the influence of TIMP-1 on erythroid precursor growth is independent of its ability to inhibit metalloproteinases. TIMP-1 is analogous to proteins that have both proteolytic and growth factor activity, such as plasmin, thrombin, and urokinase. However, TIMP-1 is novel in this regard because it is a metalloproteinase inhibitor. We show that the antiproteolytic and growth factor activities of the TIMP-1 molecule are physically and functionally distinct.

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From the Department of Pathology, Northwestern University Medical School, Chicago IL; the Division of Hematologic Oncology, Memorial Sloan-Kettering Cancer Center, New York, NY; and the Department of Pediatrics, UCLA School of Medicine, Los Angeles, CA.

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Address reprint requests to Louis Chesler, PhD, Northwestern University Medical School, 333 E Ontario St, #2611B, Chicago, IL 60611.

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Preparation of mutants. Both tagged cDNAs were subcloned into Bluescript, yielding pS2AC128myc and pS2WTmrc, which were used to generate point mutations. For mutagenesis, reactions used the pS2WTmrc template and mutagenic primers consisting of short sequences spanning each target base, synthesized as follows (sense strand listed, altered codon underlined): His7Ala: 5'-GGTGCCTACCCCGCCCCACGAC-3', Gln9Ala: 5'-CCCCACACGCGCGCGGTTC-3', His7AlaGln9Ala: 5'-GTCCACCCGCC-CCCAGCCGCGCTTCCG-3', Tyr35His: 5'-GACACCCATAC ACCAGGGTATG-3'. Script KS or SK were used as complementary primers. Paired mutant strands were then used with script primers to generate four mutant cDNAs, His7Ala, Gln9Ala, His7AlaGln9Ala and Tyr35His, which were sequenced and subcloned into p91023(B), a simian virus 40 (SV40)-dependent mammalian expression vector.

Cloning and preparation of constructs. PCR was used to insert DNA encoding 10 amino acids of the human c-myc into Bluescript with EcoRI. The ΔC128 construct was generated using a 63-mer antisense primer 5'-ttcggagattca CAAGTCCCTCTTCAGAATAGCTTTTGTCAcagcacagcagaggg-3' (Northwestern University Biotechnology Facility, Chicago, IL) containing 21 bases from the 3' TIMP-1 cDNA terminus (italics), was used to add the myc sequence (capitalized), a stop codon, and an EcoRI site (underlined) via PCR. The purified reaction product was subcloned into Bluescript with EcoRI. The ΔC128 construct was used to generate a 63-mer antisense primer 5'-ttcggagattca CAAGTCCCTCTTCAGAATAGCTTTTGTcagcacagcagaggg-3'.

Preparation of clones and expression constructs. (A) Bluescript cloning vector containing the human TIMP-1 cDNA used for PCR addition of myc-epitope tag; primers for the tagging are indicated by 5' and 3': amino acid numbering is shown, starting from the N-terminus of the secreted protein after signal sequence (sig) cleavage; EcoRI site and translation termination site (e) are also shown. (B) myc-epitope- and tagged constructs showing PCR-induced amino-acid change and name used to refer to each construct in the text to the left of the figure; numbering is as in (A). (C) Expression construct, indicating SV40 origin of replication, AdMLP promoter, and C-terminally tagged construct (from B) cloned into the EcoRI site of the plasmid p91023(B).
epitope (EQKLISEEDL) onto the carboxy (C)-terminus of the coding region of a full length recombinant human TIMP-1 cDNA (WT) and at a position corresponding to amino acid 128 of a truncated TIMP-1 cDNA (ΔC128; see Fig 1A). A set of myc-tagged amino-(N) terminal point mutants (His7Ala, Gin9Ala, His7Ala/AlaGin9Ala and Tyr35His) was then generated via PCR using the WT template (Fig 1B). Correct mutagenesis was confirmed by DNA sequence analysis and the constructs were cloned into the expression vector p91023(B), which provides for efficient SV40 large T dependent replication and expression in COS-7 cells (Fig 1C). The mutations are superimposed on a linear model of TIMP-1 structure based on disulfide bonding patterns (Fig 2). The location of the N-terminal mutations within a short stretch (amino acids 3-13) of sequence shared by loops 1 and 3 of TIMP-1 is shown. Numbers refer to the amino acid position relative to the signal peptide cleavage site (cysteine residue at position 1).

Synthesis and secretion of proteins. Constructs were
then transiently transfected into COS-7 cells. To verify adequate transcription, total RNA was collected 24 hours post-transfection and analyzed by Northern blot using a human TIMP-1 cDNA probe (Fig 3A). The clones were found to express relatively equal amounts of TIMP-1 mRNA. To confirm that this high level transcription was reflected by an increase in secreted protein, duplicate transfections were metabolically labeled with [35S]methionine/cysteine, and the secreted media were analyzed by SDS-PAGE and autoradiography (Fig 3B). The WT, ΔC128, and Tyr35His mutants were efficiently secreted, constituting the major band of labeled protein, but the His7Ala, Gln9Ala, and His7Ala Gln9Ala mutants were not detectable. To confirm that immunoreactive protein was being produced in these clones, concentrated conditioned medium derived from these three transfections was immunoprecipitated with MoAb 9E10, directed against the myc-epitope tag (Fig 3C). The analysis shows that His7Ala and Gln9Ala, as well as the double mutant (not shown), are expressed at approximately 1% of the WT level, because 100-fold equivalents of conditioned medium from these clones were required to immunoprecipitate specific bands of approximately equal intensity to those in the WT lane.

Purification of mutant proteins. Having verified the synthesis and secretion of myc-tagged protein in the transfections, purification from secreted media was accomplished using a two step procedure combining myc-epitope immunoaffinity chromatography with fast-protein liquid chromatography (FPLC) size fractionation. For the abundantly expressed WT and ΔC128 proteins, 10-fold concentrates of 200-mL batches of medium were applied to a MoAb 9E10 α-myc immunaffinity column. Figure 4A shows the column profile for loading and elution of the WT protein. The eluant (Fig 4A, peak 1) obtained on treatment of column-bound protein with 100 mmol/L glycine pH 2.5 was analyzed by silver-stained SDS-PAGE and was shown to consist of a major 31-kD band and a minor 45-kD band (Fig 4A, silver-stained SDS-PAGE profile shown within peak 1). Peak 1 was concentrated, applied to an FPLC gel filtration column, and resolved into two further peaks, ie, peak 2, containing the 45-kD band, and peak 3, containing a single 31-kD band (Fig 4B, silver-stained profile within the peak). Peak 3 was immunoreactive on Western blots using MoAb 9E10 α-myc (Fig 4C, right panel). Figure 4C summarizes the silver-staining profile (left panel) and 9E10 α-myc immunoblotting pattern (right panel) of duplicate samples from each purification fraction. Typical purification yields for the WT and His7Ala proteins are summarized in Table 1, showing that expression of the N-terminal mutants varied from 1% to 5% of the level of WT, ΔC128, and Tyr35His. Approximately 70 to 100 pg WT was obtained from 200 mL of COS-7-conditioned media.
medium, whereas 20 to 30 μg of His7Ala was obtained from 2 to 3 L of medium. To increase yields of the three N-terminal point mutations in subsequent purifications, an additional immunoaffinity chromatography step was added. An initial 6B11 antihuman TIMP-1 MoAb column was used to enrich specific protein before application to the 9E10 column, which somewhat improved TIMP-1 protein yields.

Qualitative assay of gelatinase inhibition. Gelatin inhibitor zymography was used to confirm that the purified proteins had retained the ability to inhibit MMP, and to provide a general estimate of gelatinase inhibition. Figure 5 shows a 9E10 α-myc immunoblot (upper panel), confirming equal loading of purified, α-myc-tagged TIMP-1 mutants by immunoreactivity. A duplicate gelatin inhibitor zymogram (Fig 5, lower panel) indicates that WT, ΔC128, and Tyr35His show equal levels of inhibition but that His7Ala fails to show inhibition in this assay (as well as Gln9Ala and His7Ala/Gln9Ala; data not shown). In the assay, gelatin and a mixture of proteases derived from rabbit epithelial explant cultures, are copolymerized into the running gel of a 15% SDS-PAGE gel (see Materials and Methods). After Coomasie Blue staining, inhibitory activity is visualized as darkly stained areas on a lighter background. Controls confirmed that these areas did not correspond to stained protein bands but to actual gelatinase activity (not shown). Additionally, α-myc-tagged WT protein, a recombinant bacterially purified TIMP-1 (kindly provided by Dr. David Carmichael, Synergen, Inc. Boulder, CO), or untagged TIMP-1 show the same level of activity in the assay (data not shown).

Quantitative assay of MMP3 inhibition. The ability of each purified α-myc-tagged TIMP-1 protein to inhibit an MMP target was analyzed using a soluble radiolabeled substrate cleavage assay. In this assay, proteinase activity is detected by release of radioactivity from a [3H] carboxymethylated transferrin substrate. As shown in Fig 6, WT, ΔC128, and Tyr35His show indistinguishable inhibition curves for tMMP3, a truncated stromelysin enzyme (kindly supplied by Dr Alice Marcy, Merck Research Laboratories, Rahway, NJ). WT TIMP-1 showed complete inhibition at an inhibitor:enzyme molar ratio of 3:1 with a 50% inhibition concentration (IC50) of approximately 1.5:1 (Fig 6). Partial inhibition by His7Ala, Gln9Ala, and the double mutant was detected in this assay only at greater than 7:1 molar ratios of inhibitor:enzyme (Fig 6, □, ▢, and ▴). The results divide the TIMP-1 proteins into two groups, those that retain MMP inhibition (WT, ΔC128, and Tyr35His) and those that have effectively lost the capacity to inhibit MMP (His7Ala, Gln9Ala and the double mutation). The activities of the various tested proteins are summarized in Table 2.

Binding of TIMP-1 mutants to MMP3. The ability of each purified TIMP-1 mutant to bind to tMMP3 was assayed via ELISA, using a biotinylated tMMP3 target protease (btMMP3). In this assay, TIMP-1/btMMP3 complexes were preformed for 1 hour in binding buffer and then applied to ELISA plates coated with MoAb 6B11 α-εPA (α human TIMP protein MoAb). After binding to the immobilized antibody, residual protein was removed by washing. Immobilized, TIMP-bound btMMP3 was quantitated by incubation with a preformed mixture of avidin and biotinylated alkaline

<table>
<thead>
<tr>
<th>Table 1. Purification of myc Tagged TIMP-1s</th>
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<tr>
<td>Purification Step</td>
</tr>
<tr>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>Purification of WT</td>
</tr>
<tr>
<td>1 Starting COS-7 CM</td>
</tr>
<tr>
<td>2 N2 concentration</td>
</tr>
<tr>
<td>3 MoAb 6B11 affinity</td>
</tr>
<tr>
<td>4 MoAb 9E10 affinity</td>
</tr>
<tr>
<td>5 Superdex HR-75 FPLC</td>
</tr>
<tr>
<td>6 Ultraconcentration</td>
</tr>
<tr>
<td>Purification of His7Ala</td>
</tr>
<tr>
<td>1 Starting COS-7 CM</td>
</tr>
<tr>
<td>2 N2 concentration</td>
</tr>
<tr>
<td>3 MoAb 6B11 affinity</td>
</tr>
<tr>
<td>4 MoAb 9E10 affinity</td>
</tr>
<tr>
<td>5 Superdex HR-75 FPLC</td>
</tr>
<tr>
<td>6 Ultraconcentration</td>
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* Bio-Rad dye binding assay. † 9E10 α-myc MoAb immunoblot.
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Fig 6. MMP-inhibitory activity of TIMP-1 mutants. Increasing amounts of purified myc-tagged TIMP-1 mutants were incubated with a constant amount of truncated stromelysin (tMMP3) enzyme, and a [3H]-labeled transferrin substrate. Numbers on the y-axis represent radioactivity cleaved from substrate (averages of 4 determinations). Background represents the average amount of radioactivity present in enzyme blank control reactions. Briefly, activity is assayed by cleavage of radiolabeled substrates using a purified truncated enzyme (tMMP3) kindly provided by Alice Marcy (Merck) and [3H]carboxymethyl-transferrin. Purified inhibitors are precomplexed with 30 ng tMMP3 in MMP3 buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L CaCl2, 0.05% Brij-35, and 0.02% Na-azide) for 1 hour at 4°C. Substrate is added, and reactions are incubated for 8 hours at 37°C. After incubation, 3.3% TCA and centrifugation is used to precipitate substrate. Aliquots of supernatant are counted via scintillation.

Table 2. Summary of TIMP-1 Mutant Activities

<table>
<thead>
<tr>
<th>Protein</th>
<th>Domain Affected</th>
<th>Relative tMMP3 Inhibition* (%)</th>
<th>Relative Binding†</th>
<th>Relative EPA‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT untagged</td>
<td>None</td>
<td>100</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>WT</td>
<td>None</td>
<td>100</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>ΔC128</td>
<td>C terminal 3 loops</td>
<td>100</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>His7Ala</td>
<td>N terminal AAs 3-13</td>
<td>&lt;10</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Gln9Ala</td>
<td>N terminal AAs 3-13</td>
<td>&lt;12</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>His7AlaGln9Ala</td>
<td>N terminal AAs 3-13</td>
<td>&lt;10</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Tyr35His</td>
<td>Loop 1</td>
<td>100</td>
<td>++</td>
<td>NT</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.
* tMMP3 soluble enzyme assay.
† Biotin tMMP3 binding assay.
‡ BFU-E colony formation assay.

phosphatase and spectrophotometric detection at 405 nm (Fig 7, solid lines). Specificity of complex formation was confirmed by competing parallel reactions with a 1,000-fold excess of unbiotinylated tMMP3, which reduced binding to background levels (Fig 7, dotted lines). Figure 7 shows that the ability of the TIMP-1 point mutants to bind tMMP3 is equal to that of either the WT or Δ128 proteins.

EPA activity of TIMP-1 mutants and synthetic inhibitors. The mitogenic potential of COS-7 TIMP-1 expressing conditioned media and purified myc-tagged proteins was measured as the ability to stimulate BFU-E colony formation in microwell assays. This EPA typically stimulates a twofold to threefold maximal increase in the number of erythroid colonies per well and generally increases individual colony size. Data in this assay are presented as potentiation above background, the number of colonies observed using equivalent amounts of mock-transfected COS-7 conditioned medium. These values did not differ significantly from those of control buffer-treated microwells. Media containing untagged WT protein shows this activity (Fig 8A, ○), and media containing myc-tagged WT protein shows a similar profile (Fig 8A, ●). Purified, myc-tagged proteins were tested in the same assay (Fig 8B) and again showed EPA profiles similar to those shown in the assay of conditioned media. Therefore, myc-tagging does not appear to influence the EPA of these proteins. It is significant that the EPA profiles obtained for both WT and mutated TIMP-1 proteins were approximately equal. In some cases, EPA is observed over a slightly wider dose range. Generally, however, the magnitude and concentration range of the EPA effect observed here are consistent with previous descriptions of this activity. It is also of interest to note that the purified proteins show EPA in the picomolar concentration range, well below the nanomolar affinity constants reported for TIMP-1 MMP complex formation.

Finally, an attempt was made to assess the effects of direct manipulation of protease activity on EPA in the in vitro BFU-E assay. For this purpose synthetic, hydroxamate-based MMP inhibitors (kindly provided by Dr Jamie Conway, Glaxo) were used. These small compounds exert potent inhibition by a direct interaction within the MMP active site. One of these inhibitors, GI213, is identical to BB94, a synthetic inhibitor that has shown some promise in clinical testing as an inhibitor of ovarian carcinoma metastasis. Although all of these inhibitors showed potent inhibition in the soluble
substrate cleavage assay (described above), none of the inhibitors elicited any EPA when tested over a wide dose range (1 pmoVL to 10 mmol/L) in this assay (data not shown).

**DISCUSSION**

A system for the generation and rapid purification of TIMP-1 WT and mutant proteins from mammalian cells was established and used to analyze the interdependence of the two major activities of TIMP-1, MMP inhibition and EPA. TIMP-1 point mutants lacking MMP-inhibitory activity were assayed for the ability to bind to a protease target and to stimulate erythroid colony formation in EPA assays (Table 2). The N-terminal point mutations of TIMP-1 at His7, Gln9, and a double His7/Gln9 were found to (1) lose MMP inhibitory capacity, (2) maintain the ability to complex with activated MMP, and (3) fully retain EPA. A C-terminal truncation was indistinguishable from WT TIMP-1 in the same assays. Finally, two high-affinity, synthetic MMP inhibitors were found not to show EPA over a wide range of test doses. The EPA profile reported here for TIMP-1 is consistent with previous analyses that showed an approximately twofold to threefold stimulation of erythroid colony formation over a narrow, picomolar dose range and serves as further confirmation of this activity for TIMP-1 protein.

That TIMPs possess growth factor-like activity is remarkable, because they are primarily considered to be tumor suppressors. TIMPs are thought to prevent growth of the primary tumor mass and metastatic foci by inhibiting tumor-induced angiogenesis, as surmised from the strongly inhibitory effects of purified TIMPs in in vitro assays of bFGF-induced endothelial cell migration (Boyden chamber assays), rat corneal neovascularization, and chick chorioallantoic membrane assays. TIMPs may also inhibit tumor cell metastasis and basement membrane invasion by direct binding and inactivation of tumor cell-secreted metalloproteinases, as observed in vivo tumor cell basement membrane invasion assays and in nude mouse models for hematogenous lung metastasis of B16 melanoma cells.

Several other molecules, such as thrombin, plasmin, plasminogen activators, trypsin inhibitor and hepsin (reviewed in Scott) show effects on both proteolysis and cell growth. These bifunctional proteins have pleiotropic roles in the regulation of the clotting cascade, proenzyme activation, and cell growth and are primarily serine proteinases or their inhibitors. In each case, a varying dependence of mitogenesis on proteolytic capacity and a variable degree of domainal separation of the two functions is shown. Urokinase, for example, contains a serine protease domain and a separate growth factor (EGF-like) domain, the “GFD” that appears to function mitogenically, independent of proteolysis, through cell surface binding. With thrombin, which has a serine-protease domain but no distinct growth factor-like domain, protease-dependent mitogenesis is effected (1) by release of bFGF from proteolyzed matrix and (2) by binding to and proteolysis of specific cell surface thrombin receptors. However, thrombin also elicits protease-independent growth stimulation mediated by a structurally distinct “loop B” domain. Finally, hepatocyte growth factor/scatter factor and macrophage-stimulating protein, which are mitogenic for endothelial/epithelial cells and macrophages, respectively, both contain a nonproteolytic serine-protease-like domain that apparently mediates the growth-related effects of these two molecules. Mutation of this domain in hepatocyte growth factor/scatter factor eliminates mitogenesis. Human pancreatic secretory trypsin inhibitor is an example of a mitogenic protease inhibitor that stimulates DNA synthesis and fibroblast growth at doses below the range of its protease interactions, but inhibits growth at doses within this range.
The current study supports the concept that the growth-related activity of TIMP-1 is independent of its ability to inhibit metalloproteinases. Previously, this activity has been proposed to indirectly result from the effects of matrix proteolysis on the release of either growth-regulating matrix subfragments or matrix-sequestered growth factors. This explanation has been satisfying in that it accounts for discrepancies in reports of the stimulatory versus inhibitory effects of TIMP-1 on the proliferation of various cell types. However, several lines of evidence support the notion that EPA and MMP are functionally discrete, such as the specific binding of TIMPs to cell surfaces, the persistence of growth stimulation after reduction/alkylation (which eliminates protease inhibition), and the evidence obtained here, primarily that (1) TIMP-1 mutants structurally altered to lose MMP inhibitory activity retain EPA and (2) that synthetic MMP inhibitors including BB94 do not elicit EPA, indicating that alteration of MMP activity through active site interaction does not affect the EPA response. Finally, the picomolar concentration range observed for the EPA response is significantly lower than that reported for the affinities of TIMP/MMP interactions.

This last point highlights a difference in reports of the dose ranges evoking growth-related responses with erythroid cells as opposed to other cell types. Nonerythroid cell stimulation has typically been reported to occur in the nanomolar range, although inhibition of cell growth by TIMP-2 (not TIMP-1) also occurs at this level. This heterogeneity may describe a biphasic, dose-sensitive response similar to that of human pancreatic secretory trypsin inhibitor. Alternatively, it may resemble other growth factor-like responses that show highly variable stimulatory versus inhibitory effects depending on the assay system used, the supporting matrix environment, or the target cell type, as is the case with factors such as transforming growth factor β (TGFβ). However, for TIMP-1, a positive response in the EPA assay correlates well with the in vivo erythroid potentiation elicited by this protein on peripheral injection into the bloodstream of anemic mice, in which a strong stimulation of circulating erythrocytes is observed. The significance of the growth stimulation/inhibition of nonerythroid cell types is not yet clear.

These preliminary attempts to map EPA to the TIMP-1 structure show that the growth-related and antiproteolytic activities of TIMP-1 are functionally distinct and, perhaps, structurally distinct as well. The implication here is that EPA is localized within the TIMP-1 N-terminus, although more specific mapping will be required to confirm this idea and to locate residues critical to this activity. The identification of N terminal TIMP-1 mutants lacking EPA is a focus of current work and would be particularly useful in our continuing efforts to localize a specific cell surface TIMP-1 receptor and to define a receptor-mediated mechanism for the growth-stimulatory effect.

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