Tissue Inhibitor of Metalloproteinase-2 Protection of Matrix Metalloproteinase-2 from Degradation by Plasmin Is Reversed by Divalent Cation Chelator EDTA and the Bisphosphonate Alendronate

Antonietta R. Farina, Antonella Tacconelli, Anna Teti, Alberto Gulino, and Andrew R. Mackay

Abstract

The degradation of tissue inhibitor of metalloproteinase (TIMP)-free matrix metalloproteinase (MMP)-2 to proteolytically inactive fragments by plasmin was inhibited in equimolar mixtures of purified TIMP-2 and TIMP-free MMP-2 and was not observed in purified MMP-2-TIMP-2 complexes. Divalent cation chelators EDTA and sodium Alendronate did not inhibit plasmin degradation of TIMP-free MMP-2 but reversed the ability of TIMP-2 to protect MMP-2 from degradation by plasmin. Our data confirm a role for plasmin in the clearance of TIMP-free MMP-2, identify a pivotal role for TIMP-2 in regulating MMP-2 longevity in plasmin-containing environments, and highlight a novel therapeutic use for chelators of divalent cations, including the bisphosphonate Alendronate, in the reversal of TIMP-2 protection of MMP-2 from degradation by plasmin. We propose that these observations are relevant to pathologies that are dependent upon plasmin and MMP-2 activity (e.g., tumor invasion and metastasis).

Introduction

Aberrant control of extracellular matrix degradation plays a central role in the loss of basement membrane that accompanies malignant invasive tumor behavior, and it is generally accepted that a proteolytic cascade involving enzymes of the plasmin system and the family of MMPs is involved in the process (1, 2).

Within the MMP family, much attention has focused on the M, 72,000 gelatinase, MMP-2, which exhibits a limited capacity to degrade basement membrane type IV collagen, is overexpressed in malignant tumors, and has been implicated in tumor invasion in vitro (1-4). MMP-2 is secreted in latent form and requires NH2-terminal sequence removal for activity (1). In latent form, it readily complexes with TIMP-2 by way of COOH-terminal sequences (1, 5-7) that regulate enzyme activation and subsequent activity. In TIMP-free form, MMP-2 is activated by organomercurial compounds and by MMPs, including MMP-1, MMP-7, and membrane-type MMPs, but is resistant to activation by plasmin (1, 4, 7, 8). Plasmin, however, can activate MMP-2 indirectly via other plasmin-activated MMPs (7, 9). MMP-2-TIMP-2 complexes are resistant to activation because TIMP-2 inhibits autocatalytic processing. Complexed MMP-2 is, however, susceptible to activation, if TIMP-2 inhibitory sites are saturated by a second active MMP (7).

A further level of complexity in the regulation of MMP-2 activity has been suggested by a recent report demonstrating direct degradation of MMP-2 by plasmin (10). In the latter report, intact cells protected MMP-2 from degradation by plasmin, suggesting that MMP-2 longevity in plasmin-containing cellular environments is dependent upon a balance between degradation and protection against degradation.

The mechanisms that protect MMP-2 from plasmin degradation are unknown at present. The elucidation of such mechanisms would deepen our understanding of the regulation of MMP-2 in pathological processes and may yield potentially novel ways to eliminate MMP-2 from pathological environments in which plasmin is active (e.g., tumor invasion).

In this study, we have investigated a potential role for TIMP-2 in the modulation of MMP-2 degradation by plasmin. We report that TIMP-2 protects MMP-2 from degradation by plasmin, an effect that can be reversed by chelators of divalent cations including the bisphosphonate Alendronate.

Materials and Methods

Reagents. Purified MMP-2 from human A2058 melanoma cells was purchased from Boehringer Mannheim (Monza, Italy). Purified MMP-2 appeared as a M, 72,000-M, 66,000 doublet by SDS-PAGE, with no evidence of TIMP-2 (data not shown). Purified human plasmin was purchased from Sigma Chemical Co. (St Louis, MO) and appeared as a homogeneous M, 66,000 protein by SDS-PAGE and exhibited a single M, 66,000 band of caseinolytic activity in zymograms (data not shown). Aminophenyl mercuric acetate, EDTA, PMSF, leupeptin, bovine gelatin, and casein were purchased from Sigma. Antibodies against human MMP-2 and human TIMP-2 have been described elsewhere (11). Alendronate was kindly provided by Merck, Sharp and Dohme (Rome, Italy).

Purification of TIMP-2 and MMP-2-TIMP-2 Complexes. MMP-2-TIMP-2 complexes were purified from 72-h serum-free conditioned medium from S-type SK-N-SH cells by gelatin Sepharose affinity chromatography, as described previously (4). Briefly, gelatin-bound proteins, washed extensively in 50 mM Tris, 0.2 mM NaCl, and 5 mM CaCl2, were eluted in the same buffer plus 10% DMSO. Fractions containing MMP-2 and TIMP-2, identified by SDS-PAGE, zymogram, and Western blot and accounting for >95% of the proteins purified, were pooled, dialyzed against 50 mM Tris-0.2 mM NaCl, and concentrated in Centricon 10 concentrators (Amicon, Beverly, MA). TIMP-2 was further purified from the complex by reverse-phase high-performance liquid chromatography, as described previously (5). TIMP-2-containing fractions, identified by SDS-PAGE, were pooled, dialyzed against 50 mM Tris-0.2 mM NaCl, and lyophilized. TIMP-2 identity was confirmed by reverse zymogram and Western blot. Purity of TIMP-2 was assessed to be >95% by silver-stained SDS-PAGE and contained no detectable MMP-2 activity, as judged by gelatin zymogram (data not shown).

Gelatinase Assays. Assays were performed using heat-denatured (30 min at 60°C) rat tail type I collagen, as described previously (11). Briefly, TIMP-free MMP-2 (100 ng) was preincubated with or without human plasmin (5 μg/ml) for the times indicated in figure legends in a buffer containing 0.1 M Tris and 0.2 mM NaCl (pH 8.0). Following preincubation, samples were incu-
TIMP-2 PROTECTION OF MMP-2 DEGRADATION BY PLASMIN

Fig. 1. A, representative gelatin zymograms demonstrating the effect of incubation of purified MMP-2 (100 ng) for 12 h or 24 h in the presence (Lanes +) or absence (Lanes −) of exogenous calcium (CaCl₂, 5 mM), TIMP-2 (35 ng), or EDTA (10 mM). B, effect of incubating MMP-2 (100 ng) for different times with plasmin (5 µg/ml), in the absence of calcium, with (Lanes +) or without (Lanes −) TIMP-2 (35 ng) or EDTA (10 mM).

or absence of MMP-2. Reversal of the capacity of TIMP-2 to protect MMP-2 from degradation by plasmin was observed in the presence of EDTA or sodium Alendronate.

The MMP-2 preparations used in this study contained latent- and active-form enzyme, as judged by zymogram (Fig. 1A). Confirmation of autocatalytic MMP-2 processing by active-form MMP-2 (7, 9) was obtained when pure enzyme was incubated in the presence of exogenous calcium (Fig. 1A). Elimination of calcium from buffers and the addition of TIMP-2 to equimolar concentrations (35 ng/100 ng MMP-2) or of EDTA (10 mM) in the presence of calcium both inhibited autocatalytic MMP-2 processing in reactions incubated for 24 h at 37°C (Fig. 1A). Plasmin degradation of TIMP-free MMP-2 was assessed in the absence of exogenous calcium to avoid confusion with autocatalytic processing.

Plasmin (5 µg/ml) degraded TIMP-free MMP-2 (100 ng/ml) when incubated for 3, 6, and 12 h at 37°C (Fig. 1B) but did not induce the appearance of lower molecular weight active MMP-2 species, as judged by zymogram. Preincubation of MMP-2 (100 ng) with plasmin (5 µg/ml), in the absence of calcium, for 1, 6, 12 and 24 h prior to solution gelatinase assay, in the presence of calcium (5 mM), leupeptin (50 µg/ml), and PMSF (5 mM) significantly reduced MMP-2 gelatin degrading capacity. Preincubation with plasmin for 12 and 24 h reduced constitutive activity to <20% that of MMP-2 preincubated without plasmin (P < 0.001, n = 6; Fig. 2). The plasmin concentration of 5 µg/ml was chosen to represent a physiological concentration, as reported previously (10).

MMP-2 degradation products generated by incubation with plasmin, resolved by Western blot, revealed major fragments approximating M₆, 35,000 (Fig. 3), which were immunoreactive to an anti-MMP-2 antibody raised against a COOH-terminal MMP-2 fusion protein coding for amino acids 364–531 of complete MMP-2 protein (3). No gelatinolytic activity was associated with these fragments (data not shown), consistent with reports that the COOH terminus of MMP-2 is devoid of catalytic activity (6). This would suggest that plasmin had degraded MMP-2 at the NH₂-terminal sites required for enzyme activity. These data extend reports that plasmin degrades (10) but does not directly activate (4, 7, 8) MMP-2.

MMP-2/TIMP-2 complexes purified from S-type SK-N-SH cells (Fig. 4) were used for the purification of TIMP-2 (11). Purified

**Results and Discussion**

Here, we report that TIMP-2 protects MMP-2 from degradation by plasmin to nonactive fragments. Protection occurred in equimolar mixtures of TIMP-2 with TIMP-free MMP-2 or in purified MMP-2-TIMP-2 complexes. Plasmin did not degrade TIMP-2 in the presence
TIMP-2 (1, 10, and 35 ng) inhibited plasmin degradation of TIMP-free MMP-2 (100 ng) in a dose-dependent manner, with almost complete inhibition of degradation observed at the TIMP-2 concentration of 35 ng per 100 ng of MMP-2, which approximately represented equimolar stoichiometry (Figs. 1 and 3; data displayed for 35 ng of TIMP-2 only). Similar TIMP-2 protection of MMP-2 from degradation by plasmin was observed in the presence of exogenous calcium (data not displayed). EDTA (10 mM) did not inhibit plasmin degradation of MMP-2 in the absence (Fig. 1) or presence (data not shown) of calcium. Plasmin (up to 10 µg/ml) also failed to degrade MMP-2 present in purified MMP-2-TIMP-2 complexes (100 ng) assayed for 24 h at 37°C in the absence (Fig. 4) or presence of exogenous calcium (data not shown). The data suggest that protection of MMP-2 by TIMP-2 was not dependent upon the capacity of TIMP-2 to inhibit MMP activity but possibly involved direct physical protection of plasmin-sensitive MMP-2 sequences subsequent to MMP-2-TIMP-2 complex formation.

The addition of EDTA (10 mM) or sodium Alendronate (0.1 and 1 mM) to reactions containing plasmin and equimolar TIMP-2-MMP-2 mixtures (35 and 100 ng, respectively) or purified MMP-2-TIMP-2 complexes (100 ng), rendered MMP-2 sensitive, once again, to degradation by plasmin (5 µg/ml; Figs. 1, 3, and 4) and resulted in the generation of MMP-2 fragments that were immunoreactive to anti-CTMMP-2 antibody and devoid of enzyme activity, similar to those generated from TIMP-free MMP-2 (Figs. 3 and 4). Plasmin did not degrade TIMP-2, as judged by reverse zymogram (data not shown), suggesting that TIMP-2 protection of MMP-2 was divalent cation dependent. The capacity of TIMP-2 to protect MMP-2 from degradation by plasmin in the absence or presence of calcium suggests a critical role for the MMP-2-zinc ion (1) in the maintenance of the molecular conformation required for TIMP-2 protection by TIMP-2.

The bisphosphonate Alendronate is used clinically to inhibit bone resorption associated with metastatic bone disease (13, 14) and reaches bioavailable concentrations within the range of those used in this study (14, 15). Because both physiological and pathological bone resorption may involve MMP-2 activity (16, 17), the capacity of Alendronate to reverse TIMP-2 protection of MMP-2 at doses within the bioavailable range suggests a novel mechanism of drug action, in addition to direct inhibition of MMP activity (15). This may be of relevance to the capacity of Alendronate to inhibit bone resorption (13–15).

In conclusion, we propose that TIMP-2 regulates MMP-2 longevity in plasmin-containing environments by protecting MMP-2 from direct degradation. This mechanism may subsequently influence net MMP-2 activity by maintaining a reservoir of latent MMP-2-TIMP-2 complexes available for activation upon saturation of TIMP-2-inhibitory sites (7) or by other mechanisms (10). This represents a novel function for TIMP-2, which is of potential relevance to pathological process dependent upon plasmin and MMP-2 activity (e.g., tumor invasion). Such a mechanism would require sufficient levels of TIMP-2 expression and may, therefore, help to explain reports of elevated TIMP-2 expression in malignant invasive human tumors with poor prognosis (18, 19) and may also help to explain cell mediated protection of MMP-2 against plasmin degradation (10) because cell surface MMP-2 interactions may be mediated by TIMP-2 (20). Finally, the capacity of divergent cation chelators EDTA and the bisphosphonate Alendronate to override TIMP-2 protection of MMP-2 against degradation by plasmin suggests a novel therapeutic use for these agents in promoting MMP-2 clearance from pathological environments in which plasmin is active. We are further investigating this possibility and whether TIMPs other than TIMP-2 also protect MMP-2 from degradation by plasmin.

References
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