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ProMMP-2:TIMP-1 Complexes Identified in Plasma of Healthy Individuals

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Abstract

Activation of MMPs in tissues is an important component of tissue injury. Based on earlier reports that (latent) proMMP-2 is incapable of forming a complex with TIMP-1, we reasoned that the identification of MMP-2:TIMP-1 complexes in blood might serve as a surrogate marker (“smoking gun”) of MMP-2 activation in tissues. Using specific antibodies, we developed a sensitive and specific assay to detect MMP-2:TIMP-1 complexes. We were perplexed to find that approximately 40% of plasma specimens from healthy individuals had detectable levels of the MMP-2:TIMP-1 complexes. Employing recombinant TIMP-1 bound Sepharose beads and Western blots, we demonstrated binding between recombinant proMMP-2 and TIMP-1 proteins. Recombinant MMP-2 lacking the catalytic domain also bound to TIMP-1 coated beads. These data are consistent with TIMP-1 binding to the hemopexin or hinge domain of proMMP-2. The explanation for the presence of plasma proMMP-2:TIMP-1 complexes in selected healthy individuals remains to be determined. In contrast to our immunoassay and bead binding experiments, proMMP-2 failed to bind to immobilized TIMP-1 employing surface plasmon resonance technology. Additional studies are needed to clarify these contrasting results.

Keywords

MMP; TIMP; MMP:TIMP complexes; ELISAs; complex formation; plasma

INTRODUCTION

Proteases, protease inhibitors, and complexes formed between these entities have been identified in plasma and serum and have been reported to be elevated in various diseases. Following the development of specific immunoassays, hundreds of publications have reported the measurements of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) in the blood of patients with cancer, arthritis, and a variety of other diseases (1, 2).

MMPs comprise a large family of zinc dependent proteolytic enzymes that share common functional domain structures and have both distinct and often overlapping substrate

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specificities. The basic structure of secreted MMPs consists of the following homologous domains: a signal peptide which directs MMPs to the secretory pathway, a prodomain that confers latency to the enzymes, a zinc containing catalytic site, a hemopexin domain which mediates protein-protein interactions, and a hinge region which links the catalytic and the hemopexin domains (3, 4). MMPs are collectively able to degrade most extracellular matrix components as well as numerous non-ECM proteins (3, 4). The activity of MMPs is under tight control at several levels; most are expressed in very low amounts and their transcription is tightly regulated by growth factors and cytokines (3, 5). In contrast, MMP-2 is constitutively regulated. Activation of (latent) proMMPs depends on the disruption of the prodomain interaction with the catalytic site, which occurs by conformational changes or proteolytic removal of the prodomain (5). Inhibition of enzyme activity, primarily by TIMPs and alpha-2 macroglobulin, represents the third regulatory mechanism of MMPs (3).

TIMPs comprise a four-member family containing six intrachain disulfide bridges with 44–52% sequence identity (6). TIMP-1 and -2 are soluble proteins and are readily measured in blood by immunoassay (2). TIMPs inhibit activated MMPs by binding to the catalytic domain (3, 4). In addition, the C-terminal domains of TIMP-1 and TIMP-2 (7) form noncovalent complexes with the C-terminal domains of 92 kDa proMMP-9 (8) and 72 kDa proMMP-2 (9), respectively (10). These enzyme-inhibitor complexes are in equilibrium with free MMP and TIMP with the rate of the reaction favoring complex formation (11). The presence of TIMP-2 in complex with proMMP-2 stabilizes but does not prevent enzyme activation (12) and inhibits autodegradation (11). Examination of the crystal structure of the human proMMP-2:TIMP-2 complex confirms an interaction between the hemopexin domain of proMMP-2 and the C-terminal domain of TIMP-2, leaving the catalytic site of MMP-2 and the inhibitory site of TIMP-2 distant and spatially isolated (13). ProMMP-2 and TIMP-2 are often secreted from cells (fibroblasts, tumor cells, and endothelial cells) as a stable complex with 1:1 stoichiometry (9, 12, 14, 15).

Based on the premise that plasma measurements of MMP-2:TIMP-1 complexes in disease might provide a unique approach to identify the “smoking gun” of MMP-2 activation in tissues, we have developed a specific enzyme-linked immunosorbent assay (ELISA) to detect this complex in plasma. Following standardization of this immunoassay, we were bewildered to find that plasma levels of MMP-2:TIMP-1 complexes were readily identified in approximately half of healthy individuals. This observation has led us to reexamine the dogma that TIMP-1 binds only to (mature) MMP-2, but not to proMMP-2.

METHODS

Specimen Collection and Patient Populations

Anticoagulated blood was collected from subjects using standard clinical procedures. The clinical studies were approved by the Institutional Review Board at each participating institution; all patients signed informed consent. Blood samples were collected and plasma was prepared on four separate occasions over a three year period from 20 healthy women ranging in age between 20–62 (70% black, 20% latina, 10% caucasian) who were HIV uninfected control subjects participating in a longitudinal observational study of patients with HIV/AIDS (pubmed/9504278) (16). Plasma samples were also isolated from 40 healthy hospital employees (28 males and 12 females ranging in age between 22 to 60 years). Samples were stored at –80°C until assayed.

Immunoassays and Gelatin Substrate Zymography

Plasma levels of MMP-2:TIMP-1 complexes (see details below), MMP-2, TIMP-1, MMP-9, and MMP2:TIMP-2 complexes were measured as per recommendations of the manufacturer (R&D Systems, Inc.).

Gelatin substrate zymography was performed as previously described (17).

ELISA procedure for human MMP-2:TIMP-1 complex (DuoSet, R&D Systems, Minneapolis, MN)—Goat anti-human MMP-2 antibodies (800 ng/ml) were coated overnight on 96 well microplates as capture antibody. Wells were washed thoroughly and plates were blocked for 1 hour with block buffer (1% bovine serum albumin, 5% sucrose in phosphate buffered saline, with 0.05% NaN₃) and then washed thoroughly. 100 μ l of samples (plasma) or standard in diluent (50 mM TRIS, 10 mM CaCl₂, 150 mM NaCl, 0.05% Brij 35, pH 7.5 [TCN buffer]) were added to each well for 2 hours. After aspiration and washing, 100 μ l of biotinylated goat anti-human TIMP-1 detection antibody (50 ng/ml) in diluent was added to each well and incubated for 2 hours. After washing, Streptavidin-HRP reagent diluted 1:200 was added to each well for 20 minutes followed by washing and addition of Substrate solution (mixture of H₂O₂ and tetramethylbenzidine). Stop solution (2N H₂SO₄) was added and optical density of each well was determined in a microplate reader set to 450 nm with wave length correction at 570 nm. Samples were run in duplicate.

The MMP-2:TIMP-1 complex standard (see below) was serially diluted to produce a 7 point standard curve. Samples were quantified based on extrapolation from this curve as described by the manufacturer. Regression plot analysis revealed an excellent correlation coefficient ($r=0.994$) on a log-log plot at concentrations ranging between 0.156–10.00 ng/ml. Cross reactivity in this ELISA was minimal with individual components of the complex (recombinant human MMP-2 or TIMP-1). Complexes of recombinant MMP-2:TIMP-2, MMP-2:TIMP-3, MMP-2:TIMP-4, and MMP-9:TIMP-1 do not cross-react in the MMP-2:TIMP-1 assay. In contrast to the MMP-9 ELISA (2), MMP-2:TIMP-1 complex measurements in serum were comparable to plasma anticoagulated with heparin or citrate.

Production of MMP-2:TIMP-1 protein standard for use in ELISA—To produce a protein standard for the ELISA kit, recombinant human proMMP-2 (100 μ g/ml, R&D Systems) was activated by incubation with 1 mM aminophenylmercuric acetate (APMA) at 37 °C for 1 hour. Proteolytic activity of MMP-2 was monitored by the cleavage of a fluorogenic peptide substrate, ES001 (R&D Systems). MMP-2 (10 μ g/ml) was incubated with recombinant TIMP-1 (R&D Systems) at 10-fold molar excess overnight at 4 °C. The lack of free MMP-2 in the mixture after incubation was shown by the lack of ES001 cleavage, indicating all MMP-2 was in the complex with TIMP-1. The MMP-2:TIMP-1 complex was stored at –20 °C until use. The concentration of MMP-2:TIMP-1 in the complex was arbitrarily set at 10 μ g/ml, which was diluted to 10 ng/ml as the highest standard for immunoassay.

TIMP-1 Binding of proMMP-2 on Beads via the C-terminal Domain of MMP-2—

Six proteins were tested for their binding ability to TIMP-1 conjugated to Sepharose beads. The proteins were: pro-MMP-2 and (mature) MMP-2, pro-MMP-9 and MMP-9, the functionally inactive C-terminal portion of MMP-2 lacking amino acids 1–414 (C-MMP-2), and bovine serum albumin (BSA). The TIMP-1 beads, proMMP-2, proMMP-9 and BSA were obtained from R&D Systems; C-MMP-2 was described previously (17). (mature) MMP-2 and MMP-9 were obtained following APMA activation of the proMMPs. Each of the six proteins at 1 μ g/mL was mixed with the TIMP-1 beads (100 μ L of 50 % slurry) in a final volume of 1 ml containing TCN buffer. A broad-spectrum synthetic MMP inhibitor at

200 μ M (R&D Systems) was included in the proMMP mixtures to block the catalytic site and prevent auto-activation. The mixtures were incubated on a shaker overnight at 4 °C. The mixtures were centrifuged at 13,000 g for 5 minutes, and the supernatants (unbound) were removed and saved. Subsequently, the TIMP-1 beads were washed twice with 1 mL of TCNB buffer. The bound proteins were eluted from the TIMP-1 beads twice with 70 μ L of SDS PAGE sample buffer containing 15 mM EDTA for 5 min at 95 °C. Samples (20 μ l each) from each step with exception of the elution (10 μ l each) were separated on 12% gels by SDS-PAGE and followed by Western blots using goat antihuman MMP-2 and MMP-9 polyclonal antibodies (R&D Systems) and rabbit anti-BSA antibody (Bethyl Laboratories).

Immobilization of TIMP-1 and TIMP-2 (ligands) to Sensor Chip—Stock solutions of purified TIMP-1 (1.090 mg/mL) and purified TIMP-2 (0.599 mg/mL) were prepared in 10 mM sodium acetate buffer, pH 6.0, and individually immobilized at 1:100 dilution to a CM5 sensor chip using the amine coupling reaction as described by the manufacturer (Pharmacia Biosensor AB, Uppsala, Sweden). Briefly, the surface of the chip consisting of flow cell (FC)-1, 2 and 3 was activated by exposing them to a mixture of 200 mM *N*-ethyl-*N*'-dimethylaminopropyl carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) for 10 min. FC-1 was used as a reference surface and was directly deactivated by injecting 1M ethanolamine at pH 8.5 for 10 min. The other two flow cells were injected with TIMP-1 and TIMP-2, respectively, followed by injecting 1M ethanolamine to block the unreacted groups on the surface.

Determination of Kinetic and Equilibrium Constants by Surface Plasmon Resonance (SPR)—Broad spectrum hydroxamic acid-derived inhibitors of MMPs (1 μ M CT1746) were added to purified proMMP-2 and purified proMMP-2^{E→A} (used as analytes) (18) to prevent binding of TIMP-1 and TIMP-2 to pre-activated MMP-2. Binding assays were performed at 25 °C in HBS buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Surfactant P20) at a flow rate of 50 μ l/min, as described previously (19). Injection times in the HBS buffer were 4 min followed by 4 min of dissociation. Regeneration of the ligands after each binding cycle was performed using 1 min pulse of 100 mM acetic acid containing 2.0 M NaCl. Each cycle consisted of a 1 min waiting period. Association and dissociation rate constants were calculated using the BIAEVALUATION 3.1 software.

RESULTS

MMP and TIMP Measurements in Blood of Healthy Individuals

MMP-2, MMP-9, TIMP-1, MMP-2:TIMP-2, and MMP-2:TIMP-1 complexes were measured in plasma samples collected on 4 separate occasions from 20 healthy women who were HIV-uninfected controls. Levels of MMP-2, MMP-9, and TIMP-1 ranged between 73–211 ng/ml, 8–76 ng/ml, and 37–96 ng/ml, respectively. Plasma MMP-2:TIMP-2 complexes ranged between 24–103 ng/ml in this population. All subjects, including those with undetectable plasma levels of MMP-2-TIMP-1 complexes, had readily detectable plasma levels of MMP-2:TIMP-2. In 12 of these 20 healthy control individuals (60%), plasma levels of the complex of MMP-2:TIMP-1 were undetectable (below the sensitivity of the assay; <2 ng/ml). However in the remaining 8 individuals (40%), MMP-2:TIMP-1 complex levels ranged between 15–353 ng/ml (Figures 1 and 2). Minimal variation was noted in levels of MMP-2:TIMP-1 complexes in plasma specimens from single individuals collected at different time points over 3 years (intraclass correlation coefficient = 0.86, 95% confidence interval 0.77, 0.95). A low level but significant correlation was noted between MMP-2:TIMP-1 and MMP-2:TIMP-2 complexes ($r = 0.3893$, $p < 0.05$). Significant

correlations between plasma MMP-9, MMP-2, TIMP-1, or TIMP-2 versus MMP-2:TIMP-1 complexes were not observed.

ELISAs were also performed on fresh blood specimens obtained from healthy hospital employees. In 23 of the 40 individuals (57%), plasma levels of the proMMP-2:TIMP-1 were undetectable. The remaining 17 (43%), showed MMP-2:TIMP-1 complex levels ranging between 3–103 ng/ml (Figure 2). Of note, the absolute levels of MMP-2:TIMP-1 complexes noted in the two populations of healthy individuals differed perhaps related to the anticoagulant employed in blood collection or the duration of storage of plasma specimens before assay. Future studies will need to address these issues.

Gelatin zymograms on all plasma samples displayed distinct 72 kDa proMMP-2 and variable, but lower amounts of 92 kDa proMMP-9 gelatinolytic bands; 82 kDa (MMP-9), 64 kDa or 62 kDa gelatinolytic bands (MMP-2) were not detected (Figure 3 displays typical examples).

Specificity of Binding to Anti-MMP-2 Antibodies in ELISA

To assess the possibility that MMP-9 might surreptitiously bind to the anti-MMP-2 coated ELISA plate, plasma samples with high and undetectable levels of MMP-2:TIMP-1 complexes were added to the anti-MMP-2 antibody coated plate. As demonstrated by gelatin substrate zymography, proMMP-2 was present in the untreated plasma and was efficiently eluted with SDS buffer from the antibody-coated plate (Figure 3). ProMMP-9, which was also identified in untreated plasma, was present in the unbound fraction, and was absent from the eluate. This result excludes the possibility that the TIMP-1 identified in the MMP-2:TIMP-1 assay, might be a component of a proMMP-9:TIMP-1 complex.

Recombinant Human MMP-2 and TIMP-1 Form Complexes During Prolonged Incubation *in Vitro*: TIMP-1 Binds to the C-terminal domain of proMMP-2

To examine the formation of protein complexes *in vitro*, recombinant TIMP-1 conjugated Sepharose beads were mixed overnight with recombinant proMMP-2 and with recombinant C-terminal domain of MMP-2 (lacking the catalytic domain). For comparison, the TIMP-1 beads were also mixed with recombinant proMMP-9 and MMP-2 and MMP-9. After incubation, centrifugation, and washing, bound proteins were eluted from the beads and analyzed by Western blots. As depicted in Figure 4, proMMP-2, MMP-2, and the C-terminal domain of MMP-2 bound to the TIMP-1 laden beads. As expected, proMMP-9 and MMP-9 also bound to the TIMP-1 beads whereas BSA did not.

Based on the fact that plasma samples did not display (mature) MMP-2 by gelatin substrate zymography (Figure 3), we propose that the MMP-2:TIMP-1 complex ELISA employed herein detected proMMP-2 complexed to TIMP-1 in the plasma of healthy subjects. This proposal is supported by our demonstration that proMMP-2 is able to bind to bead-immobilized TIMP-1.

Surface Plasmon Resonance Analysis of MMP-2, MMP-2^{E→A} and TIMP Interactions

Purified human recombinant proMMP-2, proMMP-2^{E→A}, TIMP-1 and TIMP-2 were subjected to surface plasmon resonance using a Biacore2000™ instrument; kinetic and equilibrium constants for TIMP-1 and TIMP-2 binding were determined (Table I). proMMP-2 and proMMP-2^{E→A} (analytes) were incubated with a broad spectrum hydroxamic acid-derived inhibitor of MMPs (1 μM CT1746) to prevent TIMP-1 and TIMP-2 binding to (inadvertently-activated) MMP-2. Dilute solutions of analytes were allowed to bind to immobilized TIMP-1 and TIMP-2. As shown in Table I, proMMP-2 and

proMMP-2^{E→A} bound avidly to TIMP-2, but not to TIMP-1. These data concur with previous results from Olson et al. (20).

DISCUSSION

The development of precise immunoassays to measure circulating levels of MMPs and TIMPs in blood has fostered numerous reports describing altered levels of these proteinases and inhibitors in a variety of diseases (2). Plasma levels of MMP-2, the most prominent MMP in blood, have been reported to be increased in cancer, atherosclerosis, and inflammatory diseases. Elevated plasma levels of MMP-9, MMP-1, and MMP-3 have been reported in the same disease entities. Plasma levels of TIMP-1 and TIMP-2 have been reported to be increased in cancer and cirrhosis (2).

Activation of proMMPs in tissues is considered to be a common component of tissue injury and repair (2). Numerous investigators have employed gelatin substrate zymography to evaluate the molecular weight of gelatin degrading bands in human tissues and plasma samples as a means of distinguishing between (latent) proMMP-2/proMMP-9 and (mature) MMP-2/MMP-9. In contrast to tissue and cell conditioned media, MMP-2 or MMP-9 have not generally been observed in the blood of patients (2, 21).

Based on reports that proMMP-2 is incapable of forming a complex with TIMP-1 (10, 11), we reasoned that the identification of MMP-2:TIMP-1 complexes in peripheral blood might serve as a surrogate marker of MMP-2 activation in tissues (2). Using specific antibodies, we have developed a sensitive, specific, and highly reproducible assay to detect MMP-2:TIMP-1 complexes in tissues and blood. Repeated measurement of plasma MMP-2:TIMP-1 complexes over time revealed the stability of complex levels in blood.

On field testing of the MMP-2:TIMP-1 complex immunoassay, we were perplexed to find that approximately 40% of healthy individuals had readily detectable levels of this complex in plasma samples. Of note, in some patients the combined levels of plasma MMP-2:TIMP-2 and MMP-2:TIMP-1 complexes exceeded the MMP-2 level, which should encompass both free and complexed forms of MMP-2. This discrepancy is presumably due to differences in signal detection between free and complexed recombinant MMP-2 employed in standard curve determinations. Thus, the MMP-2 ELISA may underestimate MMP-2 levels if MMP-2 molecules complexed with TIMP-1 or TIMP-2 are not detected with the same intensity as the native protein.

The possibility of protein aggregate formation in plasma between proMMP-9:TIMP-1 complexes and proMMP-2 leading to an artifact in the MMP-2:TIMP-1 ELISA was ruled out by demonstrating the absence of MMP-9 binding to immobilized anti-MMP-2 antibodies. An alternative untested hypothesis is that an unidentified factor in the plasma of selected individuals binds to both MMP-2 and TIMP-1, thus serving as a bridge connecting MMP-2 and TIMP-1.

To address this possibility, we turned to an *in vitro* approach to examine complex formation. Employing recombinant TIMP-1 bound Sepharose beads, we demonstrated binding between proMMP-2 and TIMP-1 *in vitro*. The presence of a synthetic broad-spectrum hydroxamic acid-based inhibitor of MMPs did not alter binding between proMMP-2 and TIMP-1 *in vitro*, negating the likelihood of TIMP-1 binding to MMP-2. The binding of recombinant MMP-2 lacking the catalytic domain to TIMP-1 coated beads is consistent with TIMP-1 binding to the hemopexin or the hinge domain of proMMP-2.

As a result of our current findings that proMMP-2:TIMP-1 complexes are present in the plasma of many healthy individuals and our demonstration of *in vitro* complex formation

between proMMP-2 and TIMP-1, we need to discuss earlier reports describing the absence of *in vitro* complex formation between purified human proMMP-2 and TIMP-1. Using proteins purified from fibroblast conditioned media, Ward et al. (15) reported that ¹²⁵I-labelled TIMP-1 formed a complex with proMMP-9, but not with proMMP-2. Goldberg et al. (12), Kleiner et al. (11) and Fridman et al. (7), employing protein purification and recombinant technologies, also failed to demonstrate complex formation between purified human proMMP-2 and TIMP-1. In this report, employing surface plasmon resonance technology, we confirmed the findings of Olson et al. (20) that proMMP-2 bound avidly to TIMP-2, but not to TIMP-1.

How then can we reconcile these apparent discrepancies in proMMP-2:TIMP-1 complex formation *in vitro*? One possibility is that differences in the immobilization of proMMP-2 on an antibody-coated plate (ELISA) or TIMP-1 conjugated to Sepharose beads versus immobilization of TIMPs on a Biacore sensor chip leads to alterations in the binding properties of each protein, hence resulting in differences in complex formation on the addition of the companion protein. Another technical consideration is that the ELISA and bead binding experiment involve prolonged co-incubation (2–16 hours) of proMMP-2 and TIMP-1 proteins, whereas surface plasmon resonance measurements take place in a fraction of a minute.

The second issue raised in this report deals with the observation that ~40% of healthy individuals have readily detectable levels of (pro)MMP-2:TIMP-1 complexes in their plasma, but these complexes are not detected in the remaining ~60%. One possibility is the existence of two species of human proMMP-2 molecules, only one of which is able to form a complex with TIMP-1. Price et al. (22) have described allele frequency of the human MMP-2 gene in healthy individuals; 15 novel sequence variants were identified. The majority of coding region polymorphisms resulted in synonymous substitutions, however a G->A transition at cDNA position 1646 caused a non conservative amino acid change from glycine to serine (G456S) in the hinge region, but at a low frequency of 0.02. The authors speculated that substitution of a small neutral residue with a larger hydrophobic amino acid may impact enzymatic activity/substrate specificity. Another allele substitution G->A at nucleotide 1660 was synonymous and did not affect threonine at amino acid 460 in proMMP-2. Relevant to our data, the frequency of this variation was 0.48. Based on evidence that single nucleotide variation at synonymous sites can give rise to allele-specific differences in structural folds of mRNA resulting in proteins that possess subtle differences in biologic function (23–25), the possibility of conformational differences in pro-MMP-2 molecules in healthy individuals merits consideration. Theoretically, protein structural differences in the hemopexin domain of proMMP-2 may facilitate formation of a complex with TIMP-1. In proMMP-2, the main cluster of hydrophobic interactions with TIMP-2 is recognized by Ala-583, Tyr-607, Leu-609, Val-619, and Phe-621 (26). Testing of this hypothesis will require detailed analysis of the function of MMP-2 alleles. Allelic differences in TIMP-1 also need consideration.

In conclusion, the detection of MMP-2:TIMP-1 complexes in the plasma of healthy individuals raises the possibility these measurements may be useful in diseases such as cancer and inflammatory states. Based on numerous reports describing elevated individual levels of plasma MMP-2 and TIMP-1 in cancer (2), it remains to be determined whether the MMP-2:TIMP-1 complex assay will be useful in diagnosis and prediction of clinical outcome.

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Figure 1.

Plasma ELISAs of MMP-2, TIMP-1, MMP-2:TIMP-1 complexes and MMP-2:TIMP-2 complexes in 6 healthy HIV/AIDS negative control subjects; comparison between individuals with or without circulating MMP-2:TIMP-1 complexes. Data represent the mean $\pm$ standard error of 4 plasma samples collected over 3 years.

MMP-2:TIMP-1 Complex

Figure 2.

MMP-2:TIMP-1 complexes identified in the plasma of healthy HIV/AIDS negative control subjects (citrate anticoagulant) and healthy hospital employees (EDTA anticoagulant). Non-measurable levels are denoted as blank spaces.

Figure 3.

Plasma MMP-9 Does Not Bind to Anti-MMP-2 Antibody Coated Plates. Two plasma samples (identified as “untreated”) with detectable (lower panel) and two samples with non detectable levels (upper panel) of MMP-2:TIMP-1 complexes were added to anti-MMP-2 antibody coated plates. SDS detergent (1%) in buffer was used to elute plasma proteins bound to solid state anti-MMP-2 antibodies. Gelatin substrate zymography was performed to identify proMMP-2 (72 kDa) and proMMP-9 in bound (present in eluate) and unbound fractions. As noted, discernable proMMP-9 bands were not identified in many plasma specimens. A positive control consisting of HT1080 fibrosarcoma cell conditioned media (upper panel, lane 1) displays proMMP-9 (92 kDa) and activated MMP-2 (62 Kda).

Figure 4.

TIMP-1 binding of proMMP-2 occurs via the C-terminal domain of MMP-2. TIMP-1 conjugated Sepharose beads were mixed with: (A) pro- and mature (mat) MMP-2, (B) pro- and mature MMP-9, (C) the C-terminal domain of MMP-2 (C-MMP-2) and (D) BSA. The antibodies used for Western blots were: goat anti-human MMP-2 polyclonal antibody (A and C), goat anti-human MMP-9 antibody (B), and rabbit anti-BSA antibody (D). Lane 1: starting material; Lane 2: unbound; Lane 3 and 4: the first and second wash, respectively; Lane 5: elution. Molecular weight standards are listed on the left of each panel.

TABLE I
Interactions of proMMP-2 and proMMP-2^{E→A} with TIMP-1 and TIMP-2, as evaluated by SPR

The errors for the k_a and k_d rate constants are expressed as the standard deviation of the slope and the standard deviation of 4 analyte concentrations, respectively, from two separate experiments. The errors for the K_d values represent the sum of the errors from the k_a and k_d values. K_d values were calculated using the formula: $K_d = k_d / k_a$.

Analytes	k_a (1/Ms) ($\times 10^{-4}$)	k_d (1/s) ($\times 10^{-5}$)	RU ^a ($\times 10^3$)	K_d (M) ($\times 10^{-9}$)
TIMP-1				
proMMP-2	NM ^b	NM		
proMMP2 ^{E→A}	NM	NM		
TIMP-2				
proMMP-2	1.67 ± 0.37	4.22 ± 0.53	4.88 ± 0.65	2.53 ± 0.90
proMMP-2 ^{E→A}	1.62 ± 0.11	2.85 ± 0.47	4.67 ± 0.52	1.75 ± 0.58

^aRU, Resonance units

^bNM, not measurable