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Exogenously added GPI-anchored tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) displays enhanced and novel biological activities

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Abstract

The family of tissue inhibitors of metalloproteinases (TIMPs) exhibits diverse physiological/biological functions including the inhibition of active matrix metalloproteinases, regulation of proMMP activation, cell growth, and the modulation of angiogenesis. TIMP-1 is a secreted protein that can be detected on the cell surface through its interaction with surface proteins. The diverse biological functions of TIMP-1 are thought to lie, in part, in the kinetics of TIMP-1/MMP/surface protein interactions. Proteins anchored by glycoinositol phospholipids (GPIs), when purified and added to cells *in vitro*, are incorporated into their surface membranes. A GPI anchor was fused to TIMP-1 to generate a reagent that could be added directly to cell membranes and thus focus defined concentrations of TIMP-1 protein on any cell surface independent of protein-protein interaction. Unlike native TIMP-1, exogenously added GPI-anchored TIMP-1 protein effectively blocked release of MMP-2 and MMP-9 from osteosarcoma cells. TIMP-1-GPI was a more effective modulator of migration and proliferation than TIMP-1. While control hTIMP-1 protein did not significantly affect migration of primary microvascular endothelial cells at the concentrations tested, the GPI-anchored TIMP-1 protein showed a pronounced suppression of endothelial cell migration in response to bFGF. In addition, TIMP-1-GPI was more effective at inducing microvascular endothelial proliferation. In contrast, fibroblast proliferation was suppressed by the agent. Reagents based on this method should assist in the dissection of the protease cascades and activities involved in TIMP biology. Membrane-fixed TIMP-1 may represent a more effective version of the protein for use in therapeutic expression.

Keywords: cell painting; GPI anchor; MMP; proliferation; TIMP-1.

Introduction

The matrix metalloproteinase (MMP) superfamily is represented by at least 26 extracellular matrix-degrading metalloendopetidases (Visse and Nagase, 2003). Collectively these enzymes are responsible for the metabolism of extracellular matrix proteins (Visse and Nagase, 2003). MMPs are active during tissue development and differentiation, cellular infiltration, wound healing, and act as moderators of the immune response (Opdenakker et al., 2001; Visse and Nagase, 2003). These enzymes have also been implicated in chronic disease processes such as multiple sclerosis and rheumatoid arthritis as well as in tumor progression and metastasis (Curran and Murray, 2000; Galis and Khatri, 2002; Itoh and Nagase, 2002; Ikeda and Shimada, 2003).

The activity of MMPs is kept under tight biological control (Nagase and Woessner, 1999). MMPs are regulated at the level of gene expression, protein synthesis, compartmentalization of enzyme activity and the processing of MMP pro-enzymes (Nagase and Woessner, 1999; Visse and Nagase, 2003). In addition, in cell membranes and within extracellular spaces MMPs are regulated by the expression/presence of natural MMP inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs) (Gomez et al., 1997; Visse and Nagase, 2003). Four TIMP family members have been identified: TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Brew et al., 2000; Bode and Maskos, 2003). These inhibitors share structural similarities but show different specificity and tissue expression (Brew et al., 2000; Bode and Maskos, 2003). TIMP-1 is the most widely distributed TIMP and, with the exception of MT1-MMP, inhibits the activity of all other MMPs (Brew et al., 2000; Visse and Nagase, 2003). TIMP-1 is a soluble protein, but it can be detected on the cell surface through its interaction with surface-bound structures (Brew et al., 2000; Klier et al., 2001). TIMP-1 has been shown to exhibit growth factor-like activity and has been linked to angiogenesis, cell migration, proliferation, tumor growth and metastasis (Forough et al., 1996; Gomez et al., 1997; Fernandez et al., 1999; Lijnen et al., 1999; Guedez et al., 2001; Bloomston et al., 2002; Lambert et al., 2003).

Protein engineering of cell surfaces is a powerful tool for the study of protein function (Medof et al., 1996). Proteins anchored by glycosylphosphatidylinositol (GPI), when purified and added to cells *in vitro*, are incorporated into their surface membranes (Medof et al., 1996; Premkumar et al., 2001). The incorporability of these molecules into cell surfaces provides a general means for modifying cell surfaces with exogenously added functional determinants. We combined TIMP-1 with a GPI anchor to generate a flexible inhibitory reagent that could be used to selectively moderate cell surface proteolytic environments. This approach provides the basis for the generation of a set of novel reagents to assess TIMP and MMP biology.

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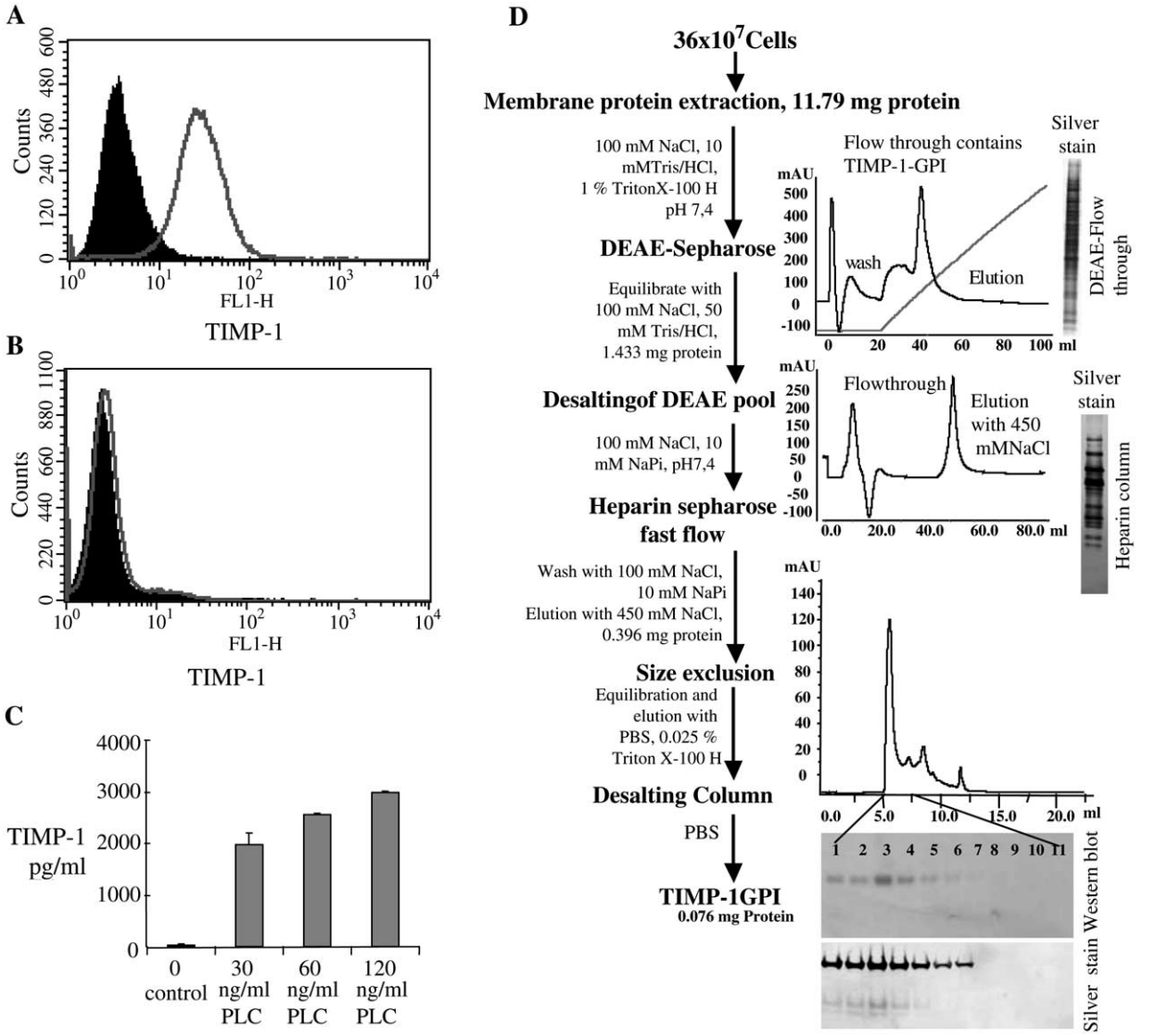

Figure 1 Generation and purification of GPI-anchored TIMP-1.
(A) The GPI-anchored TIMP-1 was overexpressed in CHO cells. The FACS plot of stably transfected CHO cells using an anti-human TIMP-1 monoclonal antibody is shown. (B) In parallel to the experiment shown in (A), TIMP-1-GPI expressing CHO cells were treated with 60 ng/ml phosphatidylinositol-specific phospholipase C for 30 min before FACS analysis using the anti-human TIMP-1 antibody. (C) The GPI-anchored TIMP-1 CHO cells were treated with 0, 30, 60, 120 ng/ml phosphatidylinositol-specific phospholipase C in serum-free media for 30 min at 37°C. TIMP-1 was measured in the supernatant using a human TIMP-1-specific ELISA kit. Data represent the mean of n=3 experiments. (D) Outline of the procedure used to purify the TIMP-1-GPI protein from the CHO overexpressing cells using fast protein liquid chromatography (FPLC). Protein was detected by silver stain and human TIMP-1 protein by Western blotting using an anti-human TIMP-1 monoclonal antibody. The fraction numbers listed for the silver stain and Western blot each represent 0.25 ml of the size exclusion column from 5.25 to 7.5 ml.

Results

Construction and purification of the TIMP-1-GPI fusion protein

The human TIMP-1 gene was cloned from cDNA using hTIMP-1-specific primers (see materials and methods section). The TIMP-1 cDNA sequence (without a translation stop codon) was fused to a GPI signal sequence cloned from LFA-3 cDNA (Kirby et al., 1995; Medof et al., 1996). The resulting fusion construct was then cloned into pEF-DHFR (Mack et al., 1995), and stably introduced into DHFR-deficient Chinese hamster ovary (CHO) cells. Stable transfectants were selected as described (Mack et al., 1995). Surface TIMP-1 expression was determined by fluorescence-activated cell sorting (FACS) analysis using a hTIMP-1-specific antibody (Klier et al., 2001) (Figure 1A). GPI anchorage was confirmed using TIMP-1 FACS analysis (Figure 1B) and a TIMP-1-specific ELISA of the growth media (Figure 1C) following 30 min PI-PCL (phosphatidylinositol-specific phospholipase) digestion.

TIMP-1-GPI-fusion protein was purified from the transfected cells by Triton X-100 (hydrogenated) detergent extraction followed by column purification using DEAE, heparin Sepharose and size exclusion chromatography (Medof et al., 1996). The general purification scheme is outlined in Figure 1D (and the materials and methods section). The resultant protein was identified by Western blot analysis and silver staining (Figure 1D). Quantification of the protein concentration using Bradford protein

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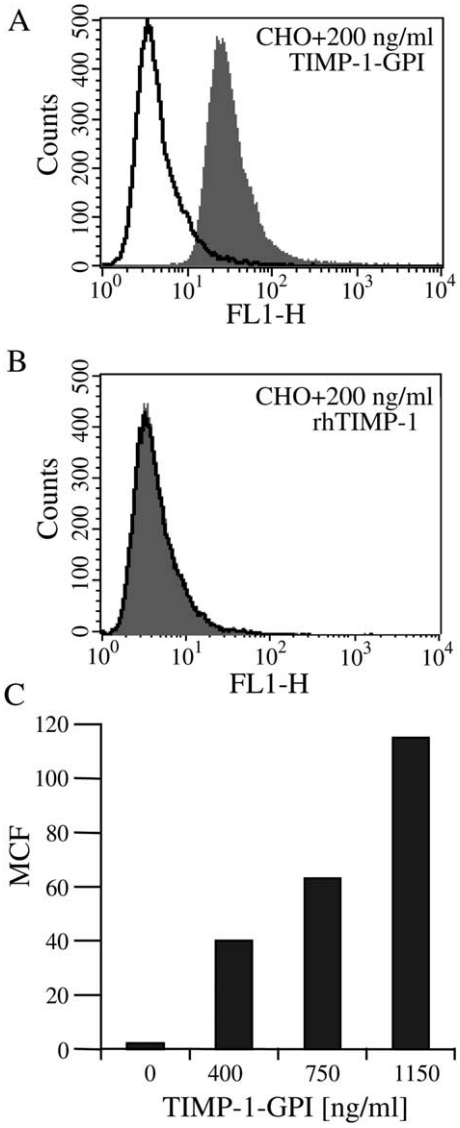

Figure 2 TIMP-1-GPI incorporation.
To demonstrate reincorporation of the GPI-TIMP-1 protein into cell membranes, the purified TIMP-1-GPI protein or recombinant human TIMP-1 was added to native CHO cells and human TIMP-1 detected on the cell surface by FACS analysis. (A) Example FACS after addition of 200 ng/ml TIMP-GPI or (B) 200 ng/ml rhTIMP-1 protein. (C) The plot of mean channel fluorescence vs. concentration of TIMP-1-GPI used demonstrates a dose-dependent localization of the GPI-anchored protein on the cell surface.

assay as compared to the level of TIMP-1 protein determined using TIMP-1 ELISA showed the protein to be over 90% pure (data not shown).

Exogenously added TIMP-1-GPI-anchored protein is incorporated into cell membranes in a dose-dependent manner

To demonstrate reincorporation of the GPI-TIMP-1 protein into cell membranes, 200 ng/ml of purified TIMP-1-GPI or rhTIMP-1 protein was added to CHO cells and TIMP-1 protein was detected on the cell surface using FACS (Figure 2A and B). Addition of increasing concentrations of TIMP-1-GPI shows a dose dependent incorporation of the GPI-anchored protein into the cell surface (Figure 2C).

Human TIMP-1 ELISA was used to determine the amount of protein associated with the CHO cells following incubation with 200 ng/ml or 700 ng/ml of either TIMP-1-GPI or rTIMP-1 protein. Cell supernatant was harvested and subjected to analysis. The TIMP-1-GPI protein efficiently bound to the CHO cells (approximately 90% of starting material associated with the CHO cells) while control rTIMP-1 protein showed no binding to the CHO cells (Table 1).

The TIMP-1-GPI protein blocks release of MMP-2 and MMP-9 in the U-2 OS osteosarcoma cell line following incorporation into the cell membrane

Native TIMP-1 is a soluble protein but it can be detected on the surface of cells through its association with other proteins, such as proteases, that are cell surface associated. The osteosarcoma cell line U-2 OS secretes both MMP-2 and MMP-9 constitutively (Figure 3B). We tested the effect of surface incorporated TIMP-1 on the constitutive secretion of these proteins using gelatinase zymography. The osteosarcoma cell line was treated with increasing concentrations of TIMP-1-GPI. FACS analysis showed a shift corresponding to an increasing incorporation of the GPI-anchored protein (Figure 3A). Subsequently the secretion of MMP-2 and MMP-9 into the culture supernatant was assessed. The results show that recombinant hTIMP-1 protein at 600 or 1200 ng/ml had no effect on the secretion of MMP-2 or MMP-9. In contrast, at 10 ng/ml TIMP-1-GPI demonstrates a significant reduction in the release of both MMP-2 and MMP-9 into the growth media. Increasing TIMP-1-GPI resulted in a dose-dependent reduction in MMP-2 and MMP-9 secretion. At TIMP-1-GPI levels above 240 ng/ml no MMP-2 or MMP-9 could be detected in the growth media (Figure 3B).

This effect appears to be due at least in part to sequestration of the proteases on the cell surface by membrane-bound TIMP-1. Following incubation of U-2 OS cells with 700 ng/ml of purified hTIMP-1-GPI protein for 24 h, FACS analyses using MMP-2- and MMP-9-specific antibodies showed an increase in mean channel fluorescence signal for TIMP-1, MMP-2 and MMP-9 from (Figure 3C). While these results show an increase of MMP-2 and MMP-9 protein on the cell surface, they do not rule out additional effects on signal transduction, mRNA transcription or protein expression.

Substrate assays for MMP-2 and MMP-9 enzymatic activity demonstrate that GPI-anchored TIMP-1 is functionally equivalent to recombinant TIMP-1

Fusion of a GPI anchor to the carboxy-terminus of TIMP-1 could influence the ability of the resultant fusion protein to inhibit protease activity. To address this, a commercial gelatinase substrate assay system (Molecular Probes E-12055) was used to compare and contrast the inhibitory properties of hTIMP-1-GPI to rhTIMP-1 to the suppression of recombinant MMP-2 and MMP-9 enzymatic activity.

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The assay uses a quenched, fluorescently labeled gelatin (see materials and methods). Two μmol of recombinant MMP-2 or MMP-9 in 100 μl were used to establish an arbitrary 100% fluorescence signal linked to digestion of the substrate for each enzyme. Increasing levels of TIMP-1-GPI or rTIMP-1 inhibitors (0.5–30 μmol) were added to parallel wells at time zero and fluorescence measured every 30 min over 120 min. The resulting data showed similar results for the rhTIMP-1 and TIMP-1-GPI proteins with respect to their ability to suppress MMP-2 and MMP-9 activities (Figure 4A and B). Addition of the GPI anchor does not appear to significantly diminish or enhance the function of TIMP-1 in a soluble substrate assay.

The GPI-anchored TIMP-1 protein is significantly more potent at stimulating endothelial cell proliferation than rhTIMP-1

TIMP-1-GPI can integrate into cell membranes and appears functionally active and should therefore focus TIMP-1 activity on the cell surface. This may result in a more pronounced effect on those biologic parameters dependent on cell surface protease activity. TIMP-1 has been shown to mediate angiogenesis (Ikenaka et al., 2003; Reed et al., 2003; Visse and Nagase, 2003). The effect of TIMP-1-GPI on the proliferation of primary human microvascular endothelial cells was compared to soluble TIMP-1 at 24, 48 and 72 hs using a MTT cell proliferation assay (Figure 5A).

Exogenously added TIMP-1-GPI protein elicited a dose-dependent increase in the proliferation of microvascular endothelial cells with a maximal effect seen at 600 ng/ml. At approx. the same concentration, rTIMP-1 showed a substantially lower effect and that treated TIMP-1-GPI had no effect (Figure 5A). The optimal stimulatory effect was seen at 24 h after addition of the TIMP proteins. This suggests that by focusing the bioactivity of TIMP-1 onto the cell surface it is approx. 3- to 4-fold more effective in increasing the proliferation of microvascular endothelial cells.

The proliferative effects of TIMP-1 have been shown to be dependent, to some degree, on the cell type used (Hayakawa et al., 1992). To evaluate the general effect of TIMP-1-GPI on cellular proliferation, the effect on primary human dermal fibroblasts was then determined. Here the results were quite different. Both the GPI anchored and recombinant TIMP-1 protein showed a slight suppression of cell proliferation after 24 h at similar protein concentrations, but a pronounced suppression of proliferation was seen with the GPI-anchored protein by the 72 h time point (Figure 5B).

The TIMP-1-GPI protein is significantly more effective at inhibiting microvascular endothelial cell migration than is rhTIMP-1

Proteolysis of vascular basement membranes and the surrounding extracellular matrix is a critical early step in neovascularization (Nguyen et al., 2001). It requires alteration in the balance between MMPs and TIMPs (Gomez et al., 1997; Nguyen et al., 2001). TIMP-1 has been previously shown to have both positive and negative effects on neovascularization (Collen et al., 2003; Ikenaka et al., 2003; Reed et al., 2003). We studied the effect of the GPI-anchored TIMP-1 protein on the migration of human microvascular endothelial cells (huMVECs) driven by bFGF. A potent suppression of migration was seen following treatment with the TIMP-1-GPI agent (Figure 6). The addition of 540 ng/ml recombinant TIMP-1 resulted in an approx. 50% decrease in huMVEC migration. By contrast, the addition of 10 ng/ml of TIMP-1-GPI dramatically suppressed huMVEC migration, and at 270 ng/ml a complete suppression of migration was seen (Figure 6). In additional control experiments, the effect of 600 ng/ml heat-treated TIMP-1-GPI was compared to the same concentration of rhTIMP-1, or TIMP-1-GPI, in the suppression of bFGF induced endothelial cell migration at the four h time point. The heat-treated protein or recombinant protein showed no significant reduction in migration while the TIMP-1-GPI anchored protein resulted in a 90% suppression of migration (data not shown).

Discussion

TIMP-1, a 184-residue protein, was initially identified as an erythroid potentiating activity that was later shown to either promote or inhibit cell proliferation and migration depending upon the biologic settings (Dean and Woessner, 1984; Gasson et al., 1985; Bertaux et al., 1991; Hayakawa et al., 1992; Yamada et al., 2001). The protein was subsequently found to moderate MMP activity and to be a member of the TIMP family of MMP inhibitors (Dean and Woessner, 1984; Hayakawa et al., 1992; Brew et al., 2000). TIMP-1 is normally secreted, but it can also be found on cell surfaces when complexed with surface-associated proteins. We sought to combine TIMP-1 with

Table 1 Determination of TIMP-1 incorporation by ELISA.

	Protein added to 2.25×10^6 CHO cells (ng/ml)	Total protein (ng)	TIMP-1 protein/10^5 cells (ng)	Total TIMP-1 protein in supernatant (ng)	Calculated TIMP-1 protein bound per 10^5 cells (ng)	TIMP-1 incorporated into cells (%)
1	Control	0	0	0	0	0
2	200 TIMP-1-GPI	60	2.66	3±0.3	2.53±0.87	95
3	700 TIMP-1-GPI	210	9.33	22.5±0.01	7.35±0.01	89
4	200 rhTIMP-1	60	2.66	60±1.3	0	0
5	700 rhTIMP-1	210	9.33	210±3.8	0	0

Following a 2 h incubation the efficiency of incorporation TIMP-1-GPI as compared to rTIMP-1 into CHO cells was determined using human TIMP-1-specific ELISA. The protein bound to CHO was calculated by subtracting protein detected in the cell supernatant and wash supernatant from the starting concentration.

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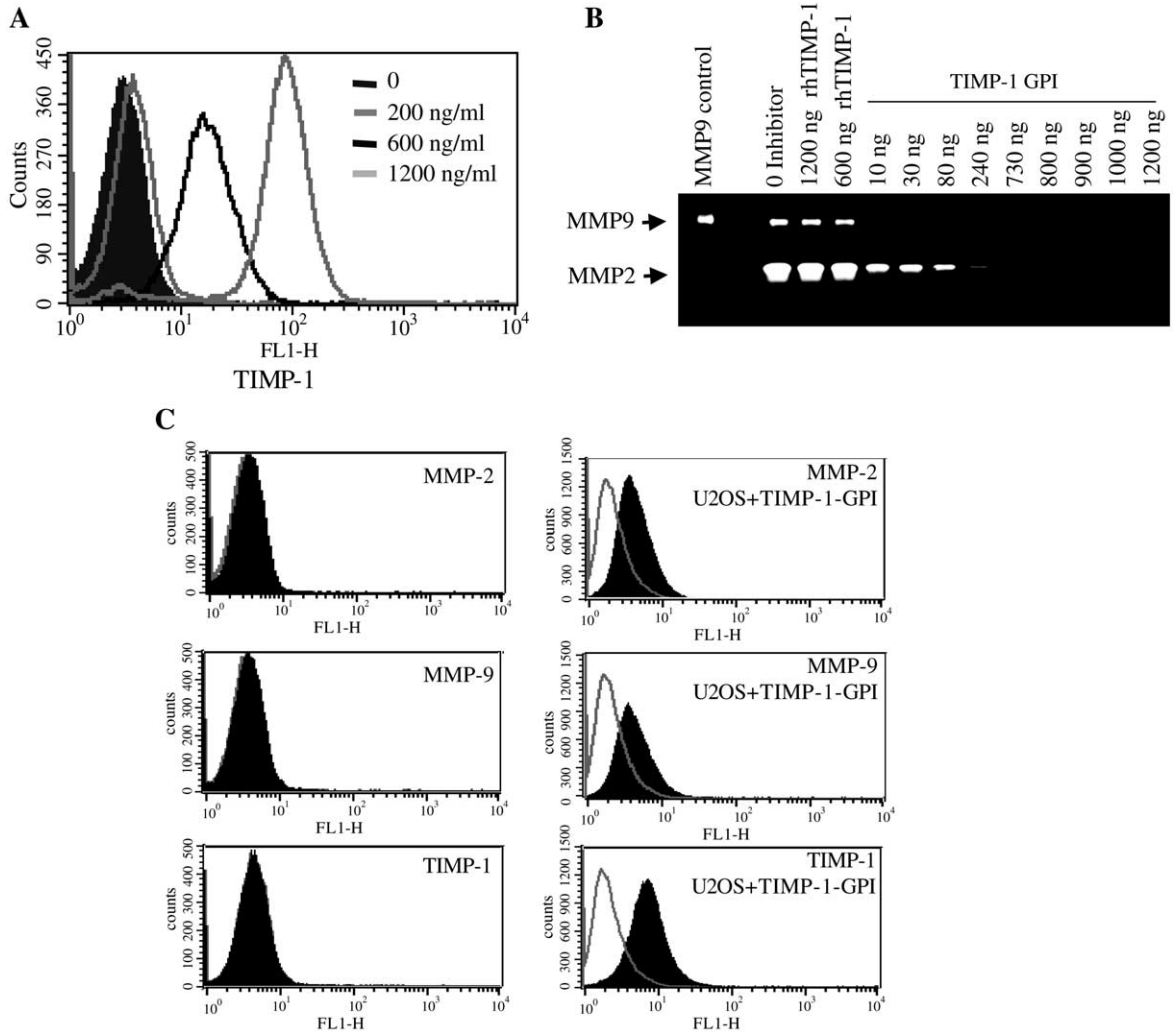

Figure 3 TIMP-1-GPI modulation of MMP-2 and MMP-9 release from U-2 OS cells.
(A) The purified hTIMP-1-GPI protein is integrated into the membrane of the osteosarcoma cell line U-2 OS and shows an increase in surface expression by TIMP-1 FACS with increasing concentrations of protein used. (B) TIMP-1-GPI protein incorporated into the U-2 OS osteosarcoma cell line leads to a reduction in MMP-2 and MMP-9 release into the growth media. Zymography was used to study the secretion of MMP-2 and MMP-9 from the cell line. 50 000 cells were placed into a 24-well plate and increasing TIMP-GPI or control rTIMP-1 was added. After 48 h the culture supernatant was removed and analyzed. (C) FACS analysis following incubation of U-2 OS cells with 700 ng/ml for 24 h showed an increase in surface staining for MMP-2, MMP-9 as well as TIMP-1.

a GPI anchor to generate a flexible and efficient reagent to characterize the biologic effect of focusing the inhibitor on the cell surface irrespective of protein-protein interactions.

Reincorporation of purified GPI-anchored TIMP-1 protein into cells was efficient (approx. 90% of protein bound to CHO cells) while exogenously added rTIMP-1 was not detectable on the CHO cells. Incorporation of purified TIMP-1-GPI into osteosarcoma cells was found to block, in a dose-dependent manner, the release of proMMP-2 and proMMP-9 into the growth medium. Parallel FACS analysis showed an increase in surface staining for MMP-2 and MMP-9 antigens following incorporation of TIMP-1-GPI protein. This suggests that the reduction in the release of these proteins may be due in part to a sequestering of the proteases on the cell surface by the membrane bound TIMP-1. Interestingly, while TIMP-1 can suppress MMP-2 activity, it has not been shown to bind efficiently to the pro-enzyme form of MMP-2 (Bode et al., 1999; Nagase and Woessner, 1999). Modification of TIMP-1 with a GPI anchor appears may alter or enhance the ability of the TIMP-1 protein to bind proMMP-2.

Endothelial cell invasion is an essential event during angiogenesis (formation of new blood vessels) (Brew et al., 2000; Nguyen et al., 2001; Reed et al., 2003). The process involves degradation of the basement membrane and the underlying interstitium. MMP-2 and MMP-9 have both been shown to play roles in angiogenesis (Collen et al., 2003). Latent MMP-9 can be secreted from endothelial cells and will accumulate in the cytosol as an active enzyme, free of TIMP-1. Latent MMP-2 is constitutively secreted from the cells (Collen et al., 2003).

We assessed the effect of exogenously added TIMP-1-GPI on primary microvascular endothelial cell migration and proliferation. While control rhTIMP-1 protein did not significantly effect migration at the concentrations tested, the GPI-anchored TIMP-1 protein showed a pronounced suppression of endothelial cell migration in response to bFGF. GPI-anchored TIMP-1 was found to dramatically stimulate endothelial cell proliferation, while rhTIMP-1 had little or no effect. TIMP-1 is a stable protein (Dean

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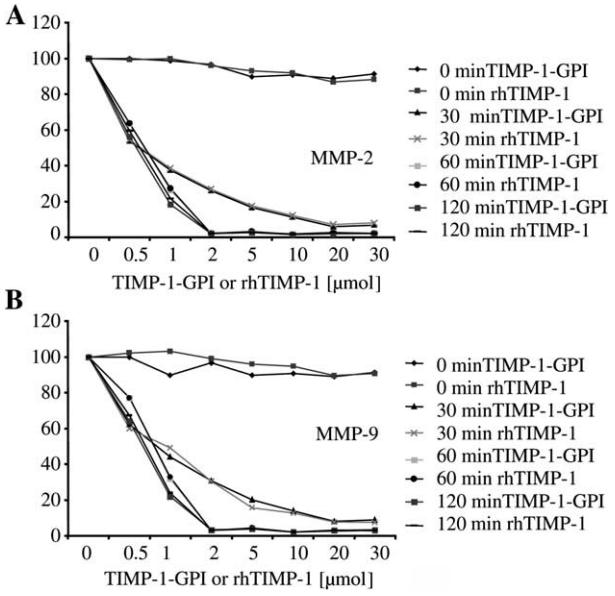

Figure 4 Functional *in vitro* assay of TIMP-1 activity.
This assay was used to contrast the inhibitory activity of GPI-anchored TIMP-1 with recombinant TIMP-1. A gelatinase substrate assay system and recombinant MMP-2 (A) and MMP-9 (B) was used to compare the inhibition of gelatinase activity. Recombinant hTIMP-1 and TIMP-1-GPI show essentially equivalent suppression of gelatinase activity. Data represent the mean of n=6 experiments.

and Woessner, 1984). In the experiments detailed here, heating the TIMP-1-GPI fusion protein at 90°C for 30 min was found to be sufficient to disrupt the enhanced activity associated with addition of the GPI anchor.

TIMP-1 has been shown to have diverse biologic effects and the effects can differ from cell type to cell type. This dichotomy of TIMP-1 bioactivity was further demonstrated when the effect of the GPI-anchored protein was assessed on fibroblast proliferation. Unlike the induction of proliferation seen in the primary microvascular endothelial cells, TIMP-1-GPI was found to suppress proliferation of primary dermal fibroblasts after incubation for 72 h.

TIMP-1 is a multifunctional protein (Sobue et al., 2001). TIMP-1 confers resistance to Fas-ligand-dependent and -independent apoptosis in Burkitt's lymphoma (Guedez et al., 1998). The overall role of TIMP-1 in cancer biology remains the subject of conflicting reports with both an anti-tumor activity and a tumor growth-stimulation activity by several mechanisms (Hayakawa et al., 1992; Guedez et al., 2001; Bloomston et al., 2002; Ikenaka et al., 2003; Seiki, 2003). TIMPs possess both proteolytic inhibitory activities and other functions. Their biology is being exploited in the search for novel therapeutic strategies with the hope that, by altering the MMP/TIMP balance, disease progression can be modulated. To this end an increase in the local concentration of TIMPs by recombinant protein administration or gene transfer is an option that has shown efficacy in animal models and may have broad clinical potential.

Studies of the structural basis of the interactions of TIMPs and MMPs are providing clues for tuning the biology of TIMP-1 and other TIMPs for potential therapeutic use. Combinations of mutations in the major MMP-binding region that enhance the specificity of TIMP-1 show improved selectivity or binding affinity to specific MMPs (Nagase and Brew, 2002; Wei et al., 2003). By exploiting point mutations it has been possible to uncouple the erythroid precursor growth stimulation activity from protease inhibition (Wei et al., 2003). The addition of a GPI anchor represents an additional modification of the native TIMP-1 protein that when combined with site-specific mutations will result in a dramatic biologic effect.

Materials and methods

Cell lines and cell culture

DHFR-deficient Chinese hamster ovary cells (CHO/dhfr$^-$) (ATCC, Rockville, USA; no. CRL.9096) were cultured in complete α-medium (GIBCO BRL, Life Technologies GmbH, Eggenstein, Germany) supplemented with 10% heat-inactivated dialyzed FCS (GIBCO BRL, Life Technologies GmbH) and HT supplement (GIBCO BRL, Life Technologies GmbH). U-2 OS cells (ATCC; no. HTB 96) were cultured in RPMI 1640 medium (GIBCO BRL, Life Technologies GmbH) supplemented with 10% heat-inactivated FCS (Biochrom KG, Berlin, Germany). Primary human dermal fibroblast cells NHDF-c (PromoCell, Heidelberg, Germany) were cultured in DULBECCO'S MEM with Glutamax-1 (GIBCO BRL, Life Technologies GmbH) supplemented with 10% heat-inactivated FCS. Human microvascular endothelial cells (PromoCell) were cultured in Endothelial Cell Growth Medium (PromoCell), supplemented with 10% heat-inactivated FCS.

Plasmid construction and stable expression in CHO (dhfr$^-$ CRL)

A DNA fragment encoding the signal for GPI-anchor attachment of LAF3 was amplified by PCR and cloned into the pEF-DHFR vector (Mack et al., 1995). The sense primer corresponded to nucleotides 616–633 and contains an *Xba*I restriction site (underlined): 5′-TCT TTG GAG ARG AGC TCT AGA ACA ACC TGT ATC CCA AGC AG-3′. The antisense primer corresponded to nucleotides 860–877 and contains a *Sal*I site (underlined): (5′-TCC CGC GGC CGC TAT TGG CCG ACG TCG ACT CAT AAT ACA TTC ATA TAC AGC ACA ATA CAT GTT G-3′. A DNA fragment encoding hTIMP-1 but excluding the stop codon was amplified by PCR from cDNA. The sense primer corresponded to nucleotides 36–44, including the initiation codon for human TIMP-1 with a modification to include an *Eco*RI restriction site (underlined): 5′-GAA CCC ACC GAA TTC ATG GCC CCC T-3′. The antisense primer corresponded to nucleotides 653–666 of human TIMP-1 with the introduction of an *Xba*I site (underlined): 5′-GGC GCC TCT AGA GGC TAT CTG GGA CC-3′. The GPI anchor signal sequence and fusion contributed nine additional amino acids to the carboxyl end of TMP-1 (MSRTTCIPS). The amplified gene sequences were subcloned sequentially into the pEF-DHFR vector and introduced into CHO (dhfr$^-$) using electroporation and selected as described (Mack et al., 1995).

Fluorescence-activated cell sorting (FACS) analysis

Human TIMP-1, MMP-2 and MMP-9 were identified on the cell surface of CHO and U-2 OS by FACS analysis. Cells were detached with 1.5 mM EDTA (Biochrom) in 1× PBS and incubated for 60 min on ice with the TIMP-1 antibody (Calbiochem/Merck, Darmstadt, Germany), anti-human MMP-2 antibody (Calbiochem/Merck) (5 µg/ml), anti-human MMP-9 antibody (Oncogene, Bad Soden, Germany) (5 µg/ml) or IgG1κ (Sigma-Aldrich, Taufkirchen, Germany) (5 µg/ml) isotype control. The cells were

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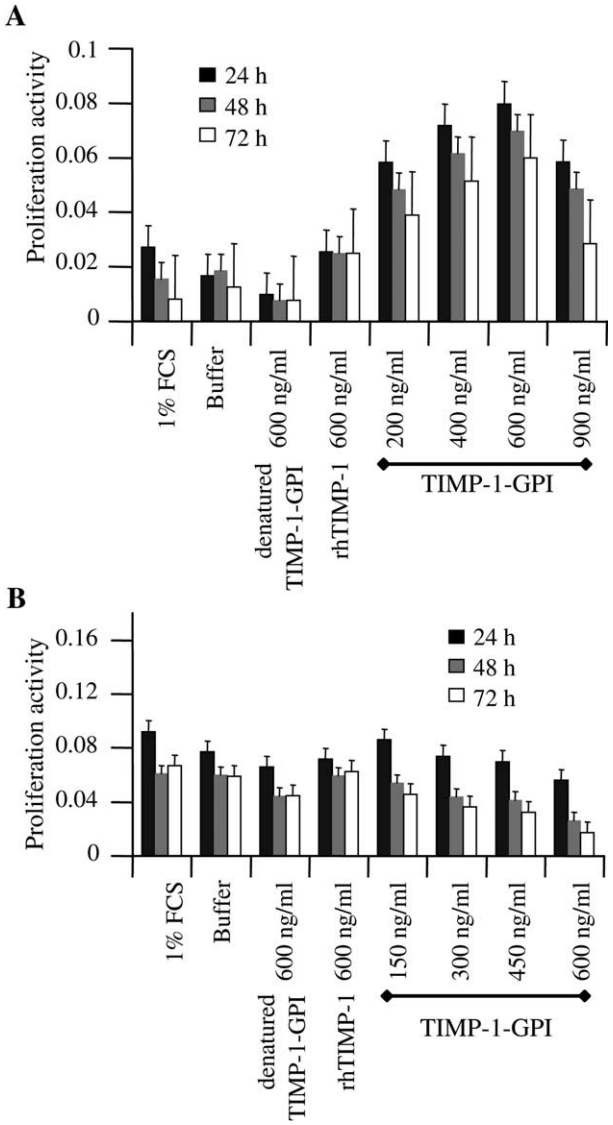

Figure 5 Effect of rTIMP-1 and TIMP-1-GPI protein on the proliferation of human dermal microvascular endothelial cells and primary human dermal fibroblasts.
(A) The TIMP-1-GPI protein is more effective in inducing proliferation of the microvascular endothelial cells. (B) In contrast, both rTIMP-1 and TIMP-1-GPI moderately suppress the proliferation of primary dermal fibroblast cells.

washed three times with 1× PBS and incubated with a FTIC-conjugated donkey anti-mouse (Dako A/S, Glostrup, Denmark) IgG for 45 min on ice. The cells were washed three times with 1× PBS and analyzed on a flow cytometer (FACS Calibur, Becton, Dickinson, San Jose, USA) using CellQuest analysis software.

Purification of TIMP-1-GPI protein

The cells were washed three times with cold 1× PBS and detached with 1.5 mM EDTA. The cells were rotated 1 h at 4°C with hypertonic lysis buffer (5 mM Tris, 2 mM MgCl, 0.1 mM EDTA, 1 mM PMSF (Roche, Basel, Switzerland), 2 μg/ml aprotinin (Roche), 2 μg/ml leupeptin (Sigma), 1 μg/ml pepstatin A (Roche), pH 7.4. Cell membranes were isolated with extraction buffer [100 mM NaCl, 1% Triton X-100 Hydrogenated (Triton X-100 H; Calbiochem/Merck), 10 mM Tris, 5 mM EDTA, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, pH 7.4] and were rotated for 1 h at 4°C followed by centrifugation at 11 000 *g* for 20 min at 4°C. The supernatant was used for further purification by FPLC.

DEAE resin (DEAE Sepharose Fast Flow, Amersham Biosciences, Uppsala, Sweden) was used for the initial column purification step. The column was equilibrated with 100 mM NaCl, 50 mM Tris/HCl, 0.1% Triton X-100 H, pH 7.4. The flow-through fractions containing TIMP-1-GPI were pooled and the buffer changed using a desalting column (HiTrap Desalting, Amersham Biosciences) equilibrated with 100 mM NaCl, 0.1% Triton X-100 H and 10 mM sodium phosphate, pH 7.4. Heparin affinity chromatography (heparin Sepharose Fast Flow, Amersham Biosciences) was then used. The column was equilibrated with 100 mM NaCl, 10 mM sodium phosphate, 0.1% Triton X-100 H pH 7.4 and the TIMP-1-GPI protein was eluted from the heparin column with 450 mM NaCl, 0.1% Triton X-100 H, 10 mM sodium phosphate, pH 7.4. Eluted fractions were pooled and 20-fold concentrated using a Centricon concentrator Amicon Ultra with pore size of 10 000 MWCO (Millipore, Eschborn, Germany). The eluted concentrate was then purified by gel filtration using a TSK G3000SWXL (TOSOH Corporation, Tokyo, Japan) column using 1× PBS and 0.025% Triton X-100 H. The final step used a desalting column equilibrated with 1× PBS to decrease the concentration of Triton X-100 H.

For negative controls the TIMP-1-GPI fusion protein was treated by heat at 90°C for 30 min. While recombinant TIMP-1 is a very stable protein (Dean and Woessner, 1984) this treatment was sufficient to block the 'enhanced' activity associated with the addition of a GPI anchor.

Protein concentration levels were routinely determined using the Quick Start Bradford Assay Kit (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's directions.

Western blot analysis

The purified TIMP-1-GPI was detected using a commercial Western blot analysis kit (Chemiluminescent Immunodetection System; Invitrogen, Groningen, The Netherlands) and an anti-human TIMP-1 (Calbiochem/Merck). Control rhTIMP-1 was obtained from R&D Systems (Minneapolis, USA).

ELISA

A human TIMP-1 ELISA system was used to monitor levels of TIMP-1 protein in solution. Coating anti-human TIMP-1 monoclonal antibody (MAB970), biotinylated anti-human TIMP-1 detection antibody (BAF970) and recombinant human TIMP-1

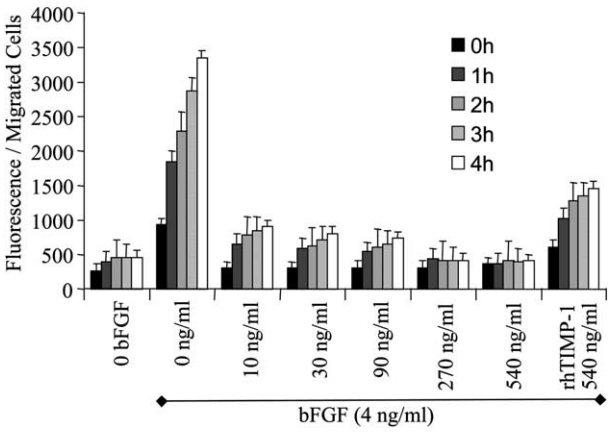

Figure 6 Effect of the GPI-anchored TIMP-1 protein on microvascular endothelial cell migration.
The Figure shows a significant suppression of migration in response to 4 ng/ml bFGF. In contrast, rTIMP-1 shows a less pronounced effect on migration.

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protein (970-TM) were all purchased from R&D Systems GmbH (Wiesbaden, Germany). The agents were used to construct a human TIMP-1 specific ELISA using the protocol described in information sheet catalog number MAB970 (R&D Systems).

GPI anchor cleavage

The GPI-anchored TIMP-1 expressing CHO cells were treated with 30, 60 and 120 ng/ml phosphatidylinositol-specific phospholipase C (Sigma-Aldrich) in serum-free medium for 30 min at 37°C. Supernatants were harvested, and released human TIMP-1 protein was determined by ELISA.

Incorporation of TIMP-1-GPI into cell membranes

Detached CHO cells or osteosarcoma cells (5–10$\times$10^6 cells/ml) were incubated with various concentrations between 10 and 1200 ng/ml of purified hTIMP-1-GPI for 1 h at 37°C. The cells were then washed three times with cold PBS and then analyzed by FACS or zymography as described above, or ELISA was performed on the pooled supernatants.

Proliferation

To assess the proliferation activity of microvascular endothelial cells and fibroblasts MTT (Sigma-Aldrich) assays were performed (Banas et al., 2002). 3$\times$10^4 cells in 100 μl medium were cultured in 96-well microtiter plates for 24 h under standard conditions to yield firmly attached and stably growing cells. After discarding supernatants, 50 μl of medium containing native TIMP-1-GPI, heat-treated TIMP-1-GPI, buffer or rhTIMP-1 were added and the cells were incubated from 24 to 72 h. Then 50 μl of a 1 mg/ml solution of MTT was added. After 3 h incubation at 37°C formazan crystals were dissolved by addition 100 μl isopropanol and 0.04 N HCl. Absorbance was measured at 550 nm using Spectra Fluor and a Tecan ELISA reader. For each experiment at least 6 wells analyzed per experimental condition and time point.

Endothelial cell migration assay

Human microvascular endothelial cells were cultured in FCS-free medium 12 h before the start of the experiment. The cells were detached and labeled with calcein AM (5 μg/ml) (Molecular Probes, Eugene, USA) for 1 h before migration. The assay medium [FCS-free medium with 1% BSA (Gibco-BRL, Life Technologies GmbH)] was used to dilute the TIMP inhibitors. Six hundred μl of assay medium containing 4 ng/ml bFGF (PromoCell) or without bFGF were placed in the bottom chamber of a BD FluoroBlok Insert System with pore size 3 μm (Becton Dickinson, San Jose, USA) and 50 000 calcein-labeled cells with and without TIMP-1-GPI in a final volume of 100 μl were placed in the wells and measured by detection of the fluorescence of cells migrated through the bottom chamber with an Spectra Fluor plus Tecan reader at 485 nm excitation and 515 nm emission. Data represent the mean of n=3 inserts.

Zymography

U-2 OS HTP 96 cells were cultured in 24-well plates (50 000 cells in each well). The medium was changed after 24 h with serum-free medium containing the TIMP-1-GPI and rhTIMP-1 and incubated for 24 h and 48 h.

Cell supernatants were analyzed by zymography using 10% SDS-polyacrylamide gels (Invitrogen) containing 0.1% gelatin loaded with cell supernatant in a 1:2 dilution with SDS sample buffer (Invitrogen). MMP-9 enzyme (Amersham Biosciences) was used as positive control. Following electrophoresis at 130 V for 1.5 h the gels were denatured for 30 min and subsequently developed. The reaction was stopped after 96 h of incubation at 37°C. Gels were then stained with Coomassie Brilliant Blue and destained with deionized Water.

Assay of TIMP-1 activity

The inhibitory activity of the TIMP-1-GPI protein was measured using the EnChek Gelatinase Assay Kit (Molecular Probes). MMP-9 (Amersham Bioscience) and MMP-2 (Sigma-Aldrich) were used to compare the inhibitory properties of hTIMP-1-GPI and rhTIMP-1. The Kit consists of highly quenched, fluorescence labeled gelatin. Upon proteolytic digestion, green fluorescence is revealed and monitored with a excitation 485 nm and emission 515 nm using a Spectra Fluor plus Tecan ELISA reader. Two μmol of each enzyme (MMP-2 and MMP-9) were placed in a 96-well microplate. Twenty μl of substrate (1 mg/ml) were added into each well and increasing concentrations of the inhibitors (0.5–30 μmol) were added to the wells. The samples were incubated at room temperature protected from light for 120 min and fluorescence was measured every 30 min. The resulting data from 30 to 120 min were essentially equivalent. The no-MMP control was used to detect the zero point of fluorescence for the conjugated substrate and the no-inhibitor point was used to determine the fluorescence quench of the substrate with MMP but without inhibitor.

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