Identification, regulation and role of tissue inhibitor of metalloproteinases-4 (TIMP-4) in human platelets

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1 Matrix metalloproteinase-2 (MMP-2) released during activation of human platelets by aggregating agents and cancer cells is known to stimulate platelet aggregation.

2 The expression, activity and role of tissue inhibitors of metalloproteinases (TIMPs), natural inhibitors of MMPs, in isolated human platelets were investigated.

3 Western blot, reverse zymography, immunogold electron microscopy, aggregometry (collagen-, thrombin and HT-1080 human fibrosarcoma cells-induced aggregation), flow cytometry and the release of ^{14}C-serotonin from labelled platelets recruited to the aggregate were used to characterize the presence and function of platelet TIMPs.

4 TIMP-4 (23 kDa) has been identified as the major MMP inhibitor (12–16 ng per 10^8 platelets) in human platelets. Platelets expressed lower (<1 ng per 10^8 platelets) amounts of TIMP-1. No other TIMPs were detected using Western blot analysis.

5 TIMP-4 co-localized with MMP-2 in resting platelets and was released upon platelet aggregation induced by collagen and thrombin.

6 Collagen resulted also in the release of higher molecular weight (60 kDa) complexes of TIMP-4.

7 The release of TIMP-4 was reduced by prostacyclin and S-nitroso-glutathione (GSNO), an NO donor.

8 Human recombinant TIMP-4 (rTIMP-4), but not human rTIMP-1, inhibited partially both platelet aggregation and recruitment.

9 The recombinant TIMP-4 potentiated the recruitment inhibitor effects of GSNO.

10 TIMP-4 was not released during platelet aggregation induced by HT-1080 cells.

11 Human rTIMP-4 exerted a biphasic effect on HT-1080 cells-induced aggregation.

12 Thus, TIMP-4 is the major intraplatelet MMP inhibitor and it is involved in regulation of platelet aggregation and recruitment.
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Introduction

Matrix metalloproteinases (MMPs), a family of matrix-degrading enzymes (Sternlicht & Werb, 2001), regulate the turnover of the extracellular matrix. Recent evidence suggests that in addition to long-lasting remodelling, MMPs may influence acute cellular reactions including regulation of platelet function (Sawicki *et al.*, 1997; 1998; Fernandez-Patron *et al.*, 1999a; Cheung *et al.*, 2000a; Kazes *et al.*, 2000; Galt *et al.*, 2002; Jurasz *et al.*, 2002), vessel wall reactivity (Fernandez-Patron *et al.*, 1999b, c; 2000a, b), cardiac ischaemia-reperfusion injury (Cheung *et al.*, 2000b) and regulation of chemoattractant properties of monocytes (McQuibban *et al.*, 2000). Indeed, we and others have identified MMP-2 in human platelets, and have shown that platelet aggregation stimulated by aggregating agents such as collagen and thrombin leads to the release of platelet MMP-2 (Sawicki *et al.*, 1997; 1998; Fernandez-Patron *et al.*, 1999a; Kazes *et al.*, 2000). This release mediates a non-ADP, non-thromboxane pathway of platelet aggregation. In contrast to MMP-2, MMP-9 serves as an inhibitor of platelet aggregation (Fernandez-Patron *et al.*, 1999a).

In addition to platelet aggregation induced by classical agonists such as collagen and thrombin, we have recently

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found that MMP-2, when released from platelets, contributes to tumour cell-induced platelet aggregation (TCIPA) (Jurasz *et al.*, 2001a). The process of TCIPA plays an important role in hematogenous spread of cancer facilitating cancer cell embolization in the microvasculature and formation of metastasis (Trousseau, 1865; Gasic *et al.*, 1968; Mehta, 1984; Radomski *et al.*, 1991; Honn *et al.*, 1992; Jurasz *et al.*, 2001b).

Four endogenous tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) control the activity of MMPs and affect matrix breakdown under physiological and pathological conditions (Brew *et al.*, 2000). It has been also shown that pharmacological administration of human recombinant TIMP-2 (rTIMP-2) inhibits platelet aggregation (Sawicki *et al.*, 1997; Kazes *et al.*, 2000).

For these reasons, we have studied protein expression, regulation and function of TIMPs in human platelets.

Methods

Cells

Human washed platelets (2.5×10^8 platelets ml^{-1}) were isolated from blood of healthy volunteers (Radomski & Moncada, 1983). Human fibrosarcoma cells HT-1080 (American Type Culture Collection, Rockville, U.S.A.) were grown in culture as previously described (Jurasz *et al.*, 2001a, b). The cells were harvested and suspended in Tyrode's solution (10^7 cells ml^{-1}).

Platelet aggregation and recruitment

Platelet aggregation was measured in a whole blood ionized calcium lumi aggregometer (Chronolog, Havertown, U.S.A.) following stimulation with collagen (0.5–10 μg ml^{-1}), thrombin (0.01–0.1 u ml^{-1}) or HT-1080 cells (10^4 cells ml^{-1}) (Sawicki *et al.*, 1997; 1998; Jurasz *et al.*, 2001a, b). To study the effects of TIMPs, S-nitroso-glutathione (GSNO) and prostacyclin (PGI$_2$), platelets were pre-incubated with these agents for 1 min prior to addition of maximally effective concentrations of agonists. Aggregation was then monitored for 9 min and analysed using Aggo-Link data reduction system (Chronolog).

Platelet recruitment was measured by following ^{14}C-serotonin release from the second recruitable population of platelets that were added to collagen-stimulated platelet samples (Freedman *et al.*, 1999). Briefly, platelets in platelet-rich plasma were incubated with ^{14}C-serotonin (1 μM, Amersham, Baie d'Urfe, Canada) for 20 min at room temperature (Holmsen & Dangelmeier, 1989). Under these conditions >95% of ^{14}C-serotonin was incorporated into platelets. The samples of unlabelled platelets (2.5×10^8 ml^{-1}) were placed in the aggregometer, in the presence of 2 μM imipramin, and stimulated with collagen (10 μg ml^{-1}). After 1 min aggregation ^{14}C-labelled platelets (2.5×10^7 platelets ml l^{-1}) were added and the reaction followed for 3 min. The release of ^{14}C-serotonin was arrested by ice-cold formaldehyde in 50 mM EDTA. The samples were then centrifuged ($10,000 \times g$ for 3 min at room temperature) and the ^{14}C-bound radioactivity was measured in the supernatant. To study the effects of TIMPs and NO, human rTIMP-1 (1–100 ng ml^{-1}), rTIMP-4 (1–100 ng ml^{-1}), con-

trol IgG (100 ng ml^{-1}) and GSNO (0.1 μM) were preincubated for 1 min prior to the addition of collagen. The results were expressed as percentage release of ^{14}C-serotonin from platelets.

In some experiments, platelet samples prior and following aggregation were centrifuged at $3200 \times g$ for 10 min at room temperature and the resultant pellet and platelet releasate used for Western blotting, reverse zymography, flow cytometry and immunogold electron microscopy.

Western blotting

Immunoblot analysis was performed as previously described (Sawicki *et al.*, 1997; 1998; Mayers *et al.*, 2001). Briefly, to study TIMP-1, TIMP-2, TIMP-3 and TIMP-4 expression in unstimulated platelets, the samples were homogenized in homogenization buffer (containing 320 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, 1 mM DL-dithiothreitol, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} soybean trypsin inhibitor and 2 μg ml^{-1} aprotinin) sonicated, centrifuged at $10,000 \times g$ for 20 min at 4°C and the resultant supernatants (65 μg protein per lane) were subjected to 12% SDS–PAGE. To study TIMP-4 during TCIPA, 10 μg of protein was loaded per lane.

To study TIMP-4 release during platelet aggregation both aggregated platelets and releasates were used. The pellets of aggregated platelets were sonicated, centrifuged ($10,000 \times g$ for 20 min at 4°C) and the resultant supernatant was diluted 10 times. Ten microliters of samples were loaded per lane. Following electrophoresis and transfer of samples onto PVDF membranes (Bio-Rad, Hercules, U.S.A.), the blots were blocked overnight in blocking buffer and then incubated with the primary antibodies (0.1–0.2 μg ml^{-1}). The following antibodies were used: rabbit polyclonal anti-TIMP-1 and mouse monoclonal anti-TIMP-2 were obtained from Oncogene, Cambridge, U.S.A., while rabbit polyclonal anti-TIMP-3 and anti-TIMP-4 antibodies were from Chemicon, Temecula, U.S.A. The immunoreactive bands were visualized with ECL kit. The intensity of bands was measured by densitometry. Human recombinant TIMP standards (except human rTIMP-4 standard that was prepared as described by Bigg *et al.*, 1997) were obtained from respective antibody manufacturers.

Reverse zymography

This was performed as described by Oliver *et al.* (1997). Briefly, 12% separating SDS gels were copolymerized with 2 mg ml^{-1} gelatin containing 1% SDS and pro MMP-2 (160 ng ml^{-1}) (Chemicon). The samples of platelet homogenate (21.5 μg protein) releasate (10 μl), and human recombinant TIMP-1, TIMP-2, TIMP-3, and TIMP-4 standards (0.5–5.0 ng) were loaded and subjected to electrophoresis. TIMPs were identified by inhibition of gelatinolysis when compared with standards.

Flow cytometry

Flow cytometry analysis of TIMP-4 was performed essentially as described by Jurasz *et al.* (2001b). Briefly, platelets were incubated at room temperature in the presence or absence of 3 μg ml^{-1} collagen for 5 min. To minimize the presence of aggregates in samples, platelets were diluted 10 fold using PBS. Following, platelet samples were incubated with sheep anti-human TIMP-4 antibodies (Oncogene) or

sheep IgG as control for 15 min. Next, samples were incubated with FITC-labelled donkey anti-sheep antibodies for 15 min. Flow cytometry was performed using a Becton Dickinson flow cytometer (FACSCalibur, New Jersey, U.S.A.) equipped with a 488 nm wavelength argon laser and with Cell Quest software (Becton Dickinson). Platelets were identified by forward and side scatter signals. Ten thousand platelet specific events were initially analysed by the cytometer, and activated and non-activated platelets were gated so as not to analyse platelet aggregates and microparticles. The gates were then analysed for mean fluorescence. Subsequently, platelet-anti-TMP-4 or platelet-IgG samples were subtracted from their corresponding samples containing FITC-labelled antibodies to account for autofluorescence. Furthermore, platelet-sheep IgG-FITC labelled samples were subtracted from platelet-anti-TIMP-4-FITC labelled samples to account for non-specific binding.

Immunogold electron microscopy

Platelets were fixed and sections prepared as previously described (Sawicki *et al.*, 1998). Briefly, platelets were fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 30 min at 21°C. Samples were then embedded in LR gold resin (Polysciences Inc, Warrington, U.S.A.) and thin sections were cut and mounted on uncoated nickel grids. For cytochemical staining the following antibodies were used: monoclonal anti-MMP-2, (Oncogene), polyclonal anti-TIMP-4 (Chemicon) and polyclonal anti MMP-9 antibodies (Fernandez-Patron *et al.*, 1999a). Following double labelling (anti-MMP-2 or anti-MMP-9, and anti-TIMP-4 antibodies, each diluted 1:20 v v^{-1}), the samples were treated with mouse (MMP-2 immunodetection) and rabbit (TIMP-4 and MMP-9 immunodetection) anti-IgG-gold (5 and 15 nm, particle size). In control experiments the primary antibodies were omitted. Platelets were examined with a Philips 300 electron microscope.

Statistical analysis

Statistics were performed using Graph Pad Software Prism 3.0 (San Diego, U.S.A.). All means are reported with standard deviation. One-way analysis of variance (ANOVA) and repeated measures ANOVA were performed where appropriate, and a *P*-value of less than 0.05 was considered as significant.

Reagents

Collagen and thrombin were obtained from Chronolog. If not otherwise indicated all remaining reagents were purchased from Sigma (Oakville, Canada)

Results

TIMPs in resting platelets

In resting platelets, Western blot analysis using specific anti-TIMP-4 antibodies revealed the presence of major TIMP-4 immunoreactive protein migrating at 23 kDa (Figure 1a). A weak TIMP-4-related immunoreactivity band

migrating at 29 kDa was also detected. The amounts of platelet TIMP-4, as calculated using TIMP-4 standard (Figure 1b), were in the range of 12–16 ng per 10^8 platelets ($n = 6$). In addition to TIMP-4, a 28 kDa immunoreactivity band corresponding to TIMP-1 (Figure 1c) was identified; however, TIMP-1 immunoreactivity in platelets was lower than that of TIMP-4 (< 1 ng per 10^8 platelets). Under experimental conditions used, no TIMP-2 or TIMP-3-related immunoreactivity was found in platelets (data not shown, $n = 6$).

Release of TIMP-4 during platelet aggregation

Reverse zymography confirmed the presence of TIMP-4 in platelets and showed a single activity band corresponding to
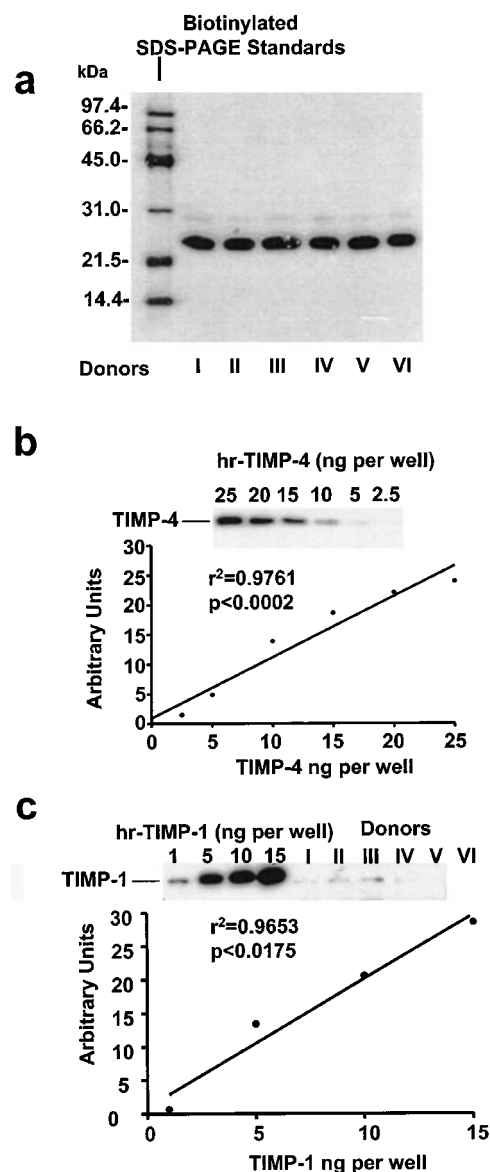

Figure 1 Western blot analysis of TIMPs in resting human platelets. (A and B) show the identification and quantification of TIMP-4, while (C) those of TIMP-1. Molecular weight of TIMP-4 immunoreactivity (23 kDa) was determined using biotinylated SDS–PAGE standards. The amounts of TIMP-4 and TIMP-1 in platelets were measured from regression analysis of TIMP standards.

TIMP-4 in the releasate of platelets aggregated with collagen (Figure 2a).

In addition, collagen-induced platelet aggregation led to a reduction of intraplatelet content of TIMP-4 with the corresponding liberation of this inhibitor into platelet releasate, as shown by Western blot (Figure 2b). The effects of collagen were concentration-dependent. We also found in the releasate a TIMP-4-related immunoreactivity band, migrating at approximately 60 kDa. The presence of this band was increased by platelet aggregation.

In contrast, TIMP-4 could not be detected on the platelet surface using flow cytometry (Figure 2c).

The release of TIMP-4 during platelet aggregation induced by collagen (1 and 10 μg ml^{-1}) was inhibited by PGI$_2$ (0.6 – 30 nM) and GSNO (0.1 – 3 μM) (Figure 3a,b).
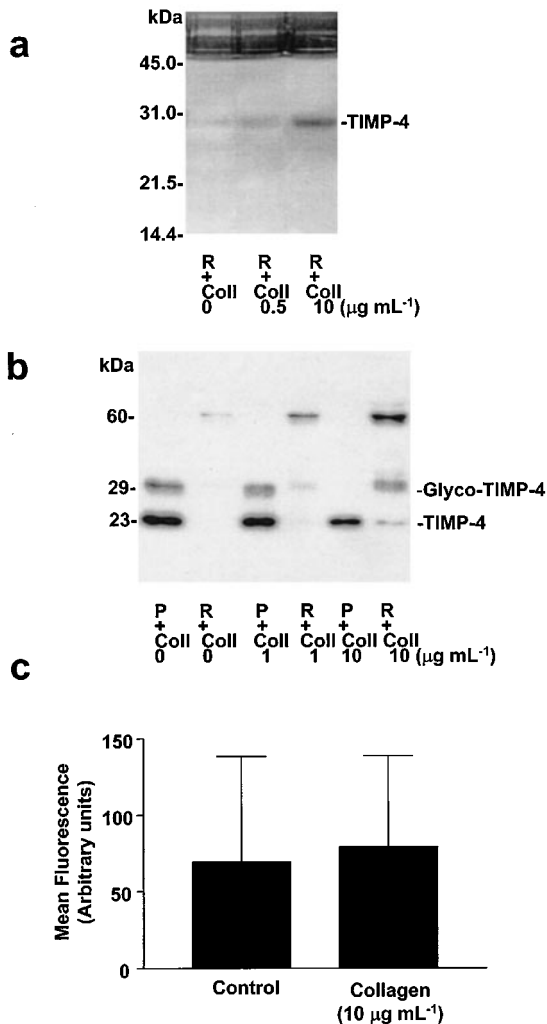

Figure 2 The release of TIMP-4 from platelets aggregated with collagen. Reverse zymography of platelet releasates (A) showing single band of inhibition of MMP-2-induced gelatinolysis. Western blot showing TIMP-4-related immunoreactivity in platelets and platelet releasates (B). Platelet aggregation led to reduction of intraplatelet TIMP-4 and the release of TIMP-4 immunoreactivity migrating at 60 kDa. (C) Flow cytometry analysis of TIMP-4 on platelet surface membranes. Neither resting nor collagen-aggregated platelets showed TIMP-4-related immunoreactivity on the surface. P: platelet pellets, R: platelet releasates and Coll: collagen. Representative reverse zymography and Western gels from four similar experiments. Data shown on C are mean$\pm$s.d., $n=3$.

Immunolocalization of TIMP-4, MMP-2 and MMP-9 in platelets

Immunogold electron microscopy of TIMP-4 and MMP-2 demonstrated that these two proteins closely co-localized in resting platelets (Figure 4a). In contrast to MMP-2 that readily localizes to the platelet surface membranes during collagen-induced aggregation (Sawicki *et al.*, 1998), the surface localization of TIMP-4 upon aggregation was not detectable. Co-localization of TIMP-4 with MMP-9 was less apparent (Figure 4b). TIMP-1, TIMP-2 and TIMP-3 were not detected in platelets using this method (data not shown). Control experiments did not show any non-specific immunogold reactivity in platelets (Figure 4a, upper inset).

Effects of TIMP-4 and TIMP-1 on platelet aggregation and recruitment

Human rTIMP-4 inhibited platelet aggregation induced by collagen (5 μg ml^{-1}) and thrombin (0.1 u ml^{-1}) (Figure 5a,b)
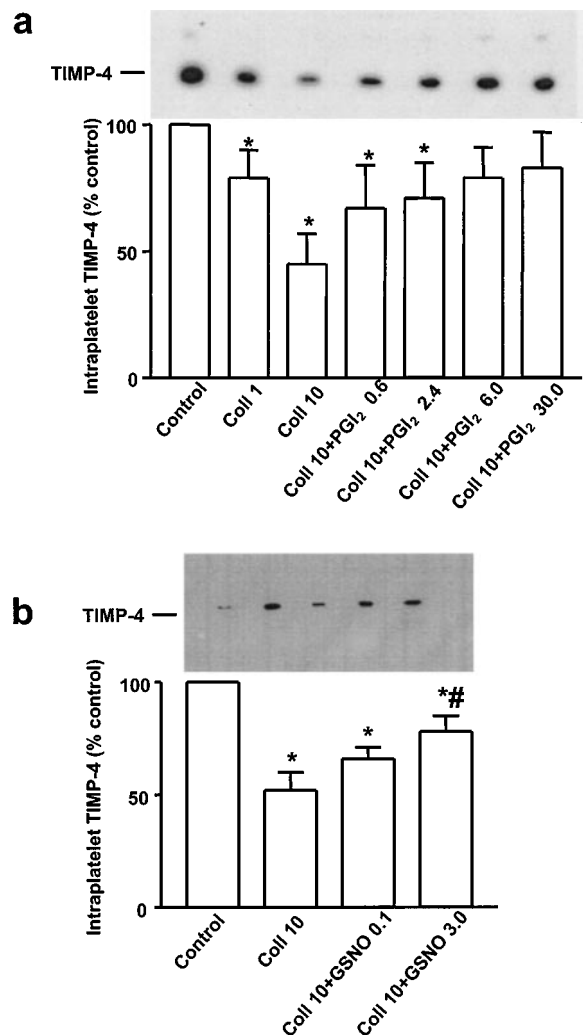

Figure 3 Regulation of collagen-stimulated TIMP-4 release from platelets by PGI$_2$ and NO. Western blot (densitometry and representative insert) showing inhibition of TIMP-4 release by PGI$_2$ (A) and GSNO (B). Data are mean$\pm$s.d., $n=6$. *$P<0.05$ treatments versus control. #$P<0.05$ Coll 10 versus Coll 10+GSNO 3. Control: unstimulated platelets; Coll: μg ml^{-1}, PGI$_2$ (nM) and GSNO (μM).
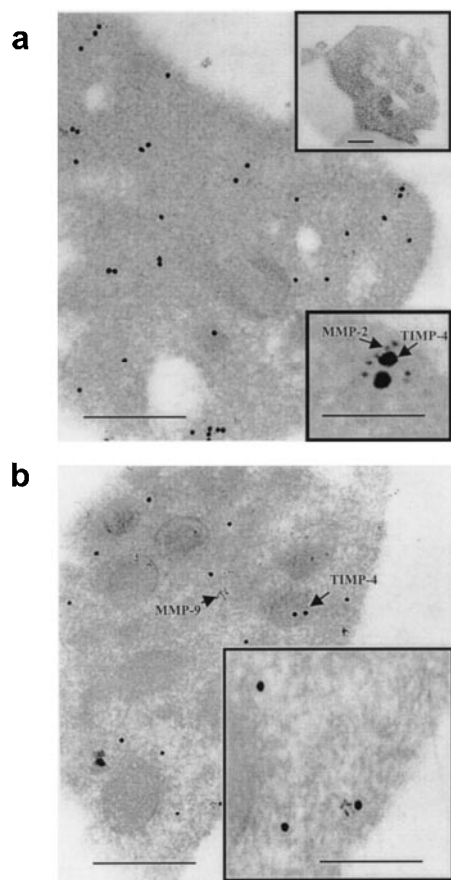
Figure 4 Immunogold electron microscopy examination of TIMP-4, MMP-2 and MMP-9 in non-aggregated platelets. Large gold particles (15 nm) label TIMP-4, while small particles (5 nm) label MMP-2 (A) or MMP-9 (B). TIMP-4 and MMP-2 co-localize as shown by arrows (A, lower inset). No IgG-related immunoreactivity was detected in control experiment (A, upper inset). Co-localization of TIMP-4 and MMP-9 is less apparent. Bar: 0.5 μm, inset bar: 0.1 μm.

Figure 5 Differential effects of TIMP-4 and TIMP-1 on platelet aggregation. (A and B) show inhibition of aggregation induced by collagen and thrombin. (C) shows that TIMP-1 exerted no significant effects on collagen-induced aggregation. (A and C) superimposed representative traces of four similar experiments. Data in (B) are mean $\pm$ s.d., $n = 4$.

with maximal effect at 10 ng ml^{-1} (Figure 5a). In contrast, collagen-induced platelet aggregation was not inhibited by rTIMP-1 at concentrations up to 100 ng ml^{-1} (Figure 5c).

Prostacyclin (10 nM) was a strong inhibitor of platelet recruitment to the aggregate (Figure 6a). Human rTIMP-4 (1 – 10 ng ml^{-1}), but not control IgG, significantly reduced collagen-stimulated platelet recruitment; however, its effects were weaker when compared to PGI$_2$. Moreover, the effects of higher concentrations of rTIMP-4 (>30 ng ml^{-1}) were less pronounced than lower (1 – 10 ng ml^{-1}). Human rTIMP-1 did not exert any significant effects on recruitment (Figure 6a).

The recruitment-inhibitor effects of rTIMP-4 were potentiated by GSNO (Figure 6b).

Effects of TIMP-4 on tumour cell-induced platelet aggregation

Human fibrosarcoma HT-1080 cells expressed TIMP-4 and released small amounts of this protein during incubation (Figure 7a). TIMP-4 was not released during platelet aggregation induced by HT-1080 cells (Figure 7a). Human rTIMP-4 inhibited HT-1080-induced platelet aggregation
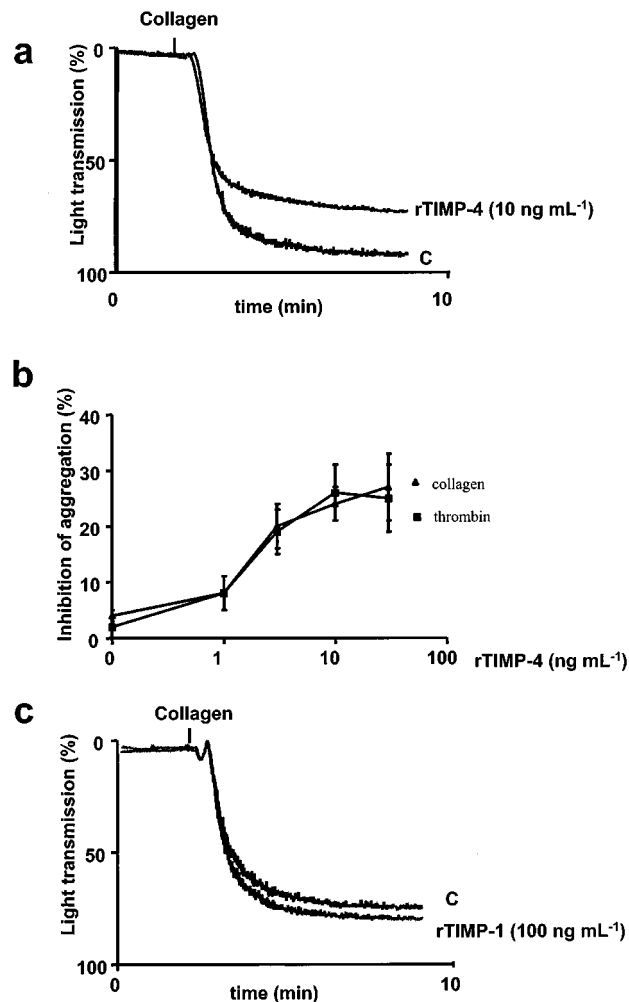
(Figure 7b). This effect was biphasic and detected at lower (5 and 50 ng ml^{-1}), but not higher (500 ng ml^{-1}) concentrations of rTIMP-4 (Figure 7b).

Discussion

Cooper *et al*. (1985) first reported that purified human platelets contain a collagenase inhibitor that appeared to be identical to that produced by human skin fibroblasts. They speculated that this inhibitor might be a significant modulator of collagenase activity following acute tissue injury. However, the molecular identity of this inhibitor was not elucidated. More recently, Murate *et al*. (1997) found a correlation between platelet count and the levels of TIMP-1 and TIMP-2 in human serum from patients with disorders associated with alterations of platelet levels. In addition, immunohistochemistry of megakaryocytes showed TIMP-1 and TIMP-2 staining in these cells. Moreover, another study found the expression of TIMP-2 mRNA transcripts in
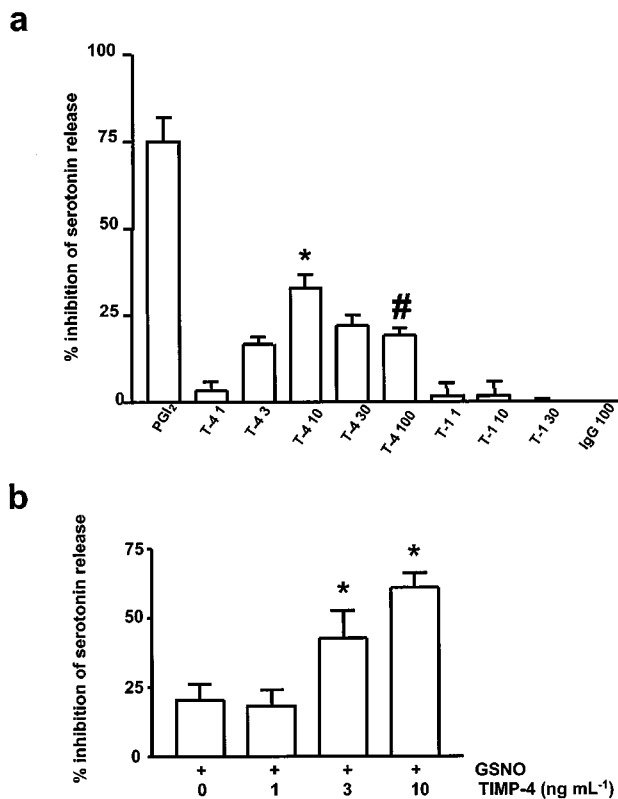
a

b

Figure 6 Inhibition of the secondary platelet recruitment by rTIMP-4 and its interactions with NO. (A) shows inhibition of platelet recruitment by rTIMP-4, but not rTIMP-1. (B) shows potentiation of recruitment-inhibitor effects of GSNO by TIMP-4. Human rTIMP-4 (T-4, ng ml^{-1}), rTIMP-1 (T-1, ng ml^{-1}), PGI$_2$: 10 nM, GSNO: 0.1 μM, IgG: 100 ng ml^{-1}. (A) *$P < 0.01$ T-4 10 versus PGI$_2$; #$P < 0.05$ T-4 100 versus T-4 10. (B) *$P < 0.05$ rTIMP-4 + GSNO versus GSNO.
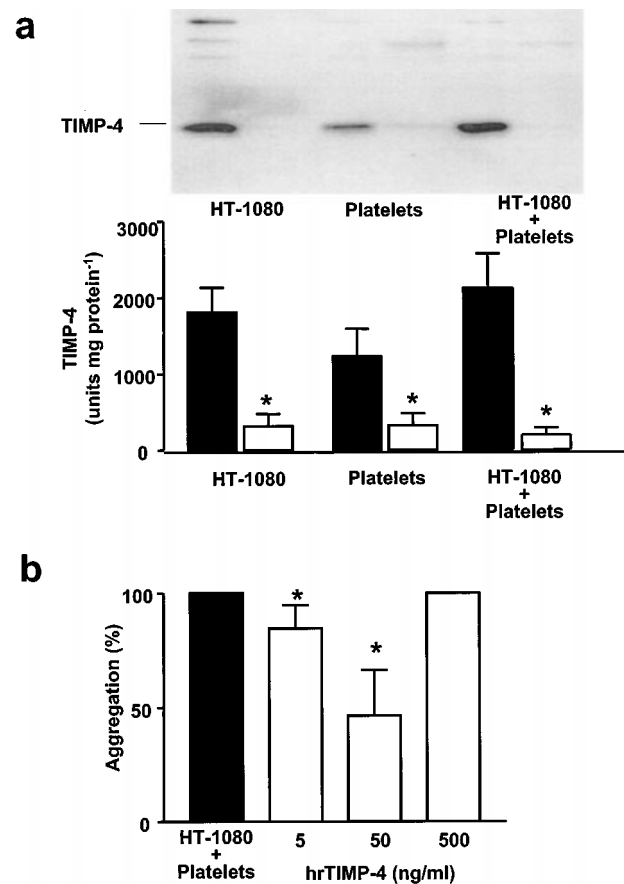
a

b

Figure 7 TIMP-4 in tumour cell-induced platelet aggregation. (A) shows Western blot analysis of homogenates of TIMP-4 content in HT-1080 cells and platelets (closed bars) and their respective releasates (open bars) during tumour cell-induced platelet aggregation. Inset shows a representative blot. The results indicate that TIMP-4 was not released during platelet aggregation by HT-1080 cells. Data are mean ± s.d., $n = 4$. A biphasic effect of rTIMP-4 on aggregation induced by HT-1080 cells is shown in B. Data are mean ± s.d., $n = 4$. *$P < 0.05$ treatments versus control.

isolated human platelets, however, the authors could not detect any TIMP-2 immunoreactivity using Western blot analysis (Kazes et al., 2000).

We have used Western immunoblots, immunogold electron microscopy, and reverse zymography to study the expression of TIMP proteins and their activities in isolated human platelets. Using Western blot we could not detect a significant immunoreactivity of TIMP-2 or TIMP-3 in platelets. In contrast, we found small, but detectable, expression of TIMP-1 (<1 ng per 10^8 platelets) and a significant expression of TIMP-4 (>10 ng per 10^8 platelets). These results were subsequently confirmed by immunogold electron microscopy and using reverse zymography that showed a single band of TIMP activity co-migrating with TIMP-4 standard. These data suggest that TIMP-4 is the major TIMP in human platelets.

The TIMP-4 gene was first identified using the expressed sequence tag sequencing approach (Greene et al., 1996). This gene was localized to human chromosome 3p25 (Olson et al., 1998). The analysis of distribution of TIMP-4 cDNA in mice showed the presence of transcripts in various murine tissues including brain, heart, ovary and skeletal muscle (Leco et al., 1997). Human recombinant TIMP-4 inhibits a number of MMPs including MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-14 (Liu et al., 1997; Bigg et al., 1997; 2001) and there is evidence for specific,

high affinity binding of TIMP-4 to the COOH-terminal hemopexin-like domain of human MMP-2 (Bigg et al., 1997; 2001).

Using immunogold electron microscopy, we have previously shown that MMP-2 is randomly distributed in platelets without apparent association with granules (Sawicki et al., 1998). This study showed that in non-stimulated platelets TIMP-4 co-localizes with MMP-2 in platelet cytoplasm suggesting that TIMP-4 is in complex with MMP-2. Collagen-induced platelet aggregation led to a reduction of intraplatelet levels of TIMP-4 due to its liberation to the platelet releasate. The release was clearly associated with aggregation since this was inhibited by prostacyclin and GSNO, potent inhibitors of this process (Moncada et al., 1976; Radomski et al., 1992). Interestingly, we could not detect the presence of TIMP-4 on the platelet surface during aggregation, as shown by flow cytometry. This is in contrast to MMP-2 release, which leads to the association of this protein with the platelet surface (Sawicki et al., 1997). We suggest that the dissociation of TIMP-4 from its complex with MMP-2 may facilitate the interactions of MMP-2 with platelet integrin GPIb and GPIIb/IIIa

(Radomski *et al*., 2001; Martinez *et al*., 2001) and stimulate aggregation.

In addition to inhibition of MMPs activity, TIMP-4 appears to be involved in regulation of activation of latent proMMPs to MMPs by inhibiting MT1-MMP-stimulated activation of MMP-2 (Bigg *et al*., 2001; Hernandez-Barrantes *et al*., 2001). This is in contrast to TIMP-2 that is known to form a trimolecular complex with cell surface MT1-MMP and proMMP-2, thus activating the latent enzyme (Sato & Saiki, 1996). Recently, Kazes *et al*. (2000) identified MT1-MMP in human platelet membranes. In addition, we have previously shown that the activated, but not latent, MMP-2 aggregates platelets (Sawicki *et al*., 1997). However, since we did not identify TIMP-2 in platelets and TIMP-4 appears not to be associated with platelet surface during platelet aggregation *in vitro*, it is likely that activation of proMMP-2 is not controlled by TIMP-2 under these conditions. Interestingly, Morrison *et al*. (2001) has recently shown that MMP-2 could be activated by MT2-MMP *via* a TIMP-2-independent pathway. This, of course, does not preclude a possibility that TIMP-2 and TIMP-4 play an important role in regulation of MMP-2 activation *in vivo* since both TIMPs are readily detected in the vasculature (Galis *et al*., 1997; Dollery *et al*., 1999; Kranzhofer *et al*., 1999). Finally, we found that the release of TIMP-4 during collagen-induced platelet aggregation was associated with increased formation of TIMP-4-related immunoreactivity bands migrating at approximately 60 kDa. The nature of these bands is currently under investigation.

In addition to agonist-induced release of TIMP-4 from platelets, we have also investigated the effects of tumour cells HT-1080 on the release of this inhibitor during TCIPA. Interestingly, TCIPA was not associated with the release of TIMP-4. This is likely to leave the proaggregatory effects of MMP-2 released from cancer cells largely unopposed, thus promoting formation of tumour cell-platelet thrombi and metastasis (Jurasz *et al*., 2001a, b).

The results of previous experiments showed that human recombinant TIMP-2 is an effective pharmacological agent that reduced platelet aggregation stimulated by aggregating agents (Sawicki *et al*., 1997; Kazes *et al*., 2000). In contrast, TIMP-1 appears to be devoid of ability to inhibit platelet activation. TIMP-4 exerted significant inhibitory effects of collagen- and thrombin-induced platelet aggregation, although its effectiveness was lower than that of TIMP-2. Moroever,

TIMP-4 also decreased the secondary platelet recruitment to the aggregate. Interestingly, the effects of TIMP-4 on TCIPA and the secondary platelet recruitment were biphasic: lower concentrations inhibited, while higher were less effective in inhibiting platelet activation. A number of studies have evidenced that TIMPs, including TIMP-4, have also biological actions that are independent of their MMP-inhibitory effects (Sang, 1998; Tummalapalli *et al*., 2001) including growth modulating and apoptosis-regulating properties. Therefore, the net pharmacological and probably biological effects of TIMPs on platelets may depend on the balance between the MMP-inhibitory and MMP-unrelated effects.

We have previously shown that inhibition of MMP activity with phenanthroline, a synthetic inhibitor of MMP activity, greatly enhances the platelet adhesion-inhibitor effects of NO (Martinez *et al*., 2001). Similar interactions have been detected between rTIMP-4 and GSNO in relation to their ability to reduce the secondary platelet recruitment to the aggregate. Thus, the simultaneous administration of NO donors and MMP inhibitors may potentially provide a powerful pharmacological strategy to limit vascular disease-induced platelet activation and thrombosis.

In conclusion, our findings are of significance for vascular haemostasis and disease. Under physiological conditions TIMP-4 appears to be the major intraplatelet TIMP controlling the activity of MMP-2-dependent pathway of aggregation. Interestingly, the expression of TIMP-4 in the vascular wall may control the rate of smooth muscle migration and collagen accumulation during experimental rat balloon angioplasty restenosis (Dollery *et al*., 1999). We postulate that, similar to the heart (Mayers *et al*., 2001), in platelets, an imbalance between MMP-2 and TIMP-4 activities may contribute to thrombotic complications of vascular disorders, as well as to hematogenous cancer metastasis.

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