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Expression of matrix metalloproteinase-9 in human platelets: regulation of platelet activation in *in vitro* and *in vivo* studies

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1 The aim of this study was to identify the presence of matrix metalloproteinase-9 (MMP-9) in human platelets and systematically examine its inhibitory mechanisms of platelet activation.

2 In this study, we report on an efficient method for the quantitative analysis of pro-MMP-9 in human platelets using capillary zone electrophoresis (CZE). To elucidate subcellular localization of MMP-9 in human platelets, we investigated intraplatelet MMP-9 by immunogold labeling and visualized it using electron microscopy. In an *in vivo* thrombotic study, platelet thrombus formation was induced by irradiation of mesenteric venules with filtered light in mice pretreated with fluorescein sodium.

3 MMP-9-gold labeling was observed on the plasma membrane, α-granules, open canalicular system, and within the cytoplasma both in resting and activated platelets. Furthermore, activated MMP-9 concentration-dependently (15–90 ng ml^{-1}) inhibited platelet aggregation stimulated by agonists. Activated MMP-9 (21 and 90 ng ml^{-1}) inhibited phosphoinositide breakdown, intracellular Ca^{2+} mobilization, and thromboxane A_2 formation in human platelets stimulated by collagen (1 μg ml^{-1}). In addition, activated MMP-9 (21 and 90 ng ml^{-1}) significantly increased the formation of nitric oxide/cyclic GMP.

4 Rapid phosphorylation of a platelet protein of Mr 47,000 (P47), a marker of protein kinase C activation, was triggered by phorbol-12, 13-dibutyrate (PDBu) (60 nM). This phosphorylation was markedly inhibited by activated MMP-9 (21 and 90 ng ml^{-1}). Activated MMP-9 (1 μg g^{-1}) significantly prolonged the latency period of inducing platelet plug formation in mesenteric venules.

5 These results indicate that the antiplatelet activity of activated MMP-9 may be involved in the following pathways. (1) Activated MMP-9 may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown, protein kinase C activation, and thromboxane A_2 formation, thereby leading to inhibition of intracellular Ca^{2+} mobilization. (2) Activated MMP-9 also activated the formation of nitric oxide/cyclic GMP, resulting in inhibition of platelet aggregation. These results strongly indicate that MMP-9 is a potent inhibitor of aggregation. It may play an important role as a negative feedback regulator during platelet activation.
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Abbreviations: CZE, capillary zone electrophoresis; ECMs, extracellular matrices; IP_3, inositol 1,4,5-trisphosphate; MMPs, matrix metalloproteinases; OCS, open canalicular system; PGE_1, prostaglandin E_1; PRP, platelet-rich platelet; TxB_2, thromboxane B_2

Introduction

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}- and Ca^{2+}-dependent enzymes, which are important in the resorption of extracellular matrices (ECMs) in both normal physiological processes and pathological states (Dollery *et al*., 1995). These enzymes are responsible for the degradation of ECMs such as collagen, laminin, and proteoglycans (Birkedal-Hansen, 1995). Therefore, MMPs have been implicated in the tissue remodeling, which accompanies inflammation, bone resorption, wound healing, thrombosis, atherosclerosis, and the invasion of tumors (Ray & Stetler-Stevenson, 1994). Most MMPs are synthesized and secreted as inactive proenzymes (Lijnen, 2001). MMPs expression appears to be strictly regulated by tissue inhibitors of metalloproteinases (TIMPs) (Ray & Stetler-Stevenson, 1994; Birkedal-Hansen, 1995). Under some pathological conditions such as tumor cell metastasis, inflammation, thrombosis, and atherosclerosis, MMPs expression may be significantly increased over that of the TIMPs.

MMP-9, also known as gelatinase B, has a broad range of substrate specificity for different native collagens (types IV, V, VII, and X) as well as denatured collagens (gelatin) and elastin (Ray & Stetler-Stevenson, 1994; Birkedal-Hansen, 1995). MMP-9 is secreted as a 92-kDa proenzyme and can

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be activated to be an 86-kDa active form (Birkedal-Hansen, 1995). Vascular smooth muscle and endothelial cells are known to synthesize and release MMPs including MMP-9 (Ray & Stetler-Stevenson, 1994; Sawicki *et al*., 1997), suggesting that this protein may be involved in the process of hemostasis and/or thrombosis. Intravascular thrombosis is one of the generators of a wide variety of cardiovascular diseases. Initiation of intraluminal thrombosis is believed to involve platelet adherence and aggregation. Thus, platelet aggregation may play a crucial role in atherothrombotic processes.

Fernandez-Patron *et al*. (1999) demonstrated that human platelets release MMP-9, and that it significantly inhibited platelet aggregation stimulated by thrombin. However, no data are available concerning the effect of MMP-9 on platelet activation. The detailed inhibitory mechanisms of MMP-9 underlying the signaling pathways in platelets still remain obscure. We therefore systematically examined the influence of MMP-9 on human platelets, and utilized the findings to characterize the mechanisms involved in this influence. Furthermore, the release of MMP-9 from human platelets has been demonstrated (Fernandez-Patron *et al*., 1999). However, MMP-9 subcellular localization within the platelet is still unknown. Therefore, we performed immunogold labeling of MMP-9 on London Resin (LR) White-sections of resting and collagen-activated platelets. In addition, we previously reported that platelet thrombi were induced by irradiation with filtered light in the microvasculature of mice pretreated with fluorescein sodium (Sheu *et al*., 1994). Therefore, we used this model to further evaluate the inhibitory effect of platelet plug formation by MMP-9 *in vivo*. Moreover, the amount of platelet-derived pro-MMP-9 was also determined by capillary zone electrophoresis (CZE) in this study.

Methods

Materials

Pro-MMP-9 was purchased from Calbiochem (San Diego, CA, U.S.A.). Collagen (type I, bovine achilles tendon), U46619, luciferin-luciferase, fluorescein sodium, Dowex-1 (100–200 mesh; X_8, chloride form), myoinositol, prostaglandin E_1 (PGE_1), arachidonic acid, phorbol-12, 13-dibutyrate (PDBu), apyrase, osmium tetroxide, *p*-aminophenylmercuric acetate (APMA), gold-conjugated anti-mouse IgG, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), and thrombin were purchased from Sigma Chem. (St Louis, MO, U.S.A.). Fura 2-AM was purchased from Molecular Probe (Eugene, OR, U.S.A.). LR White resin was purchased from London Resin Co. (Reading, U.K.). Anti-MMP-9 antibody was obtained from Oncogene (MA, U.S.A.). Myo-2-[^{3}H]inositol was purchased from Amersham (Buckinghamshire, HP, U.K.). Thromboxane B_2 (TxB_2), cyclic AMP, and cyclic GMP EIA kits were purchased from Cayman (Ann Arbor, MI, U.S.A.).

Preparation of human platelet suspensions

Human platelet suspensions were prepared as previously described (Sheu *et al*., 1999c). In this study, human volunteers gave informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 weeks, and was mixed with acid/citrate/glucose (9 : 1, vol vol^{-1}). After centrifugation at 120 *g* for 10 min at room temperature, the supernatant (platelet-rich plasma; PRP) was supplemented with PGE_1 (0.5 μM) and heparin (6.4 IU ml^{-1}), then incubated for 10 min at 37°C and centrifuged at 500 *g* for 10 min. The washed platelets were finally suspended in Tyrode's solution containing BSA (3.5 mg ml^{-1}) and adjusted to about 4.5×10^8 platelets ml^{-1}. The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

Analysis of pro-MMP-9 in human platelets by capillary zone electrophoresis

Human platelets were homogenized by ultrasound sonication for 30 s at 4°C in lysis buffer (1% SDS, 0.1%. Triton X-100, and 10 mM Tris-HCl, pH 7.4) containing protease inhibitors (PMSF, aprotinin, and leupeptin) and centrifuged at 30,000 *g* for 60 min at 4°C. Supernatant was collected and stored at −85°C.

CZE was performed on a P/ACE System Model 2100 (Beckman, Fullerton, CA, U.S.A.) without coatings. Pro-MMP-9 was monitored with a fixed-wavelength detector at 214 nm. Beckman System Gold software was used for data processing. Separations were performed at 25°C and 10 kV for 20 min. The authentic pro-MMP-9 (0.1 mg ml^{-1}) and supernatants were dissolved in a Tris-HCl solution (50 mM, pH 7.0) and subjected to CZE analysis. All samples were injected to P/ACE by positive pressure (0.5 psi for 10 s). The running buffer used was a boric acid-based electrolyte buffer solution (150 mM, pH 7.0). All separations were repeated at least four times to ensure reproducibility.

Postembedding immunolabeling for MMP-9

Resting and collagen (1 μg ml^{-1})-activated human platelets were fixed with 2% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) for 30 min. After rinsing with cacodylate buffer, platelets were further postfixed with 1% osmium tetroxide for 30 min. After dehydration in a graded series of ethanol, samples were embedded in LR White resin. Ultrathin sections were mounted on Formvar-coated nickel grids, and were blocked for 20 min with 1% BSA in cacodylate buffer. Sections were incubated for 1 h with mouse monoclonal anti-MMP-9 antibody (1 : 1000), which raised against pro-MMP-9 and/or activated MMP-9 or control mouse IgG (0.05 μg ml^{-1}) with cacodylate buffer. After rinsing with cacodylate buffer, sections were incubated for 1 h with 10 nm of gold-conjugated anti-mouse IgG (1 : 100) with cacodylate buffer. Sections were treated with 2% uranyl acetate and lead citrate, and were examined with a Hitachi H-600 electron microscope.

Platelet aggregation

The turbidimetric method was applied to measure platelet aggregation (Born & Cross, 1963), using a Lumi-Aggregometer (Payton, Canada). Pro-MMP-9 was activated by AMPA (10 μM) as described by Marcy *et al*. (1991). Platelet suspensions (4.5×10^8 platelets ml^{-1}, 0.4 ml) were prewarmed to 37°C for 2 min, activated MMP-9 (15–90 ng ml^{-1}) and a solvent control (10 μM AMPA) were added 3 min before the

addition of agonists. The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed in light-transmission units. While measuring ATP release, 20 μl of a luciferin/luciferase mixture was added 1 min before the addition of agonists, and ATP release was compared with that of the control.

Labeling of membrane phospholipids and measurement of the production of [^{3}H]inositol phosphates

The method was carried out as previously described (Sheu *et al*., 1999c). Briefly, citrated human PRP was centrifuged, and the pellets were suspended in Tyrode's solution containing [^{3}H]inositol (75 μCi ml^{-1}). Platelets were incubated for 2 h followed by centrifugation, and were finally resuspended in Ca^{2+}-free Tyrode's solution (5×10^8 ml^{-1}). Activated MMP-9 (21 and 90 ng ml^{-1}) was preincubated with 1 ml of loaded platelets for 3 min, and collagen (1 μg ml^{-1}) was then added to trigger aggregation. The reaction was stopped, and samples were centrifuged for 4 min. The inositol phosphates of the supernatants were separated in a Dowex-1 anion exchange column. Only [^{3}H]inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

Measurement of platelet [Ca^{2+}]i mobilization by fura 2-AM fluorescence

Citrated whole blood was centrifuged at 120 *g* for 10 min. The supernatant was incubated with Fura 2-AM (5 μM) for 1 h. Human platelets were then prepared as described above. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 mM. The $[Ca^{2+}]$i rise was measured using a fluorescence spectrophotometer (CAF 110, Jasco, Tokyo, Japan) with excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm. The $[Ca^{2+}]$i was calculated from the fluorescence measured using 224 nM as the Ca^{2+}-Fura 2 dissociation constant (Grynkiewicz *et al*., 1985).

Measurement of thromboxane B_2 formation

Platelet suspensions (4.5×10^8 ml^{-1}) were preincubated for 3 min in the presence or absence of activated MMP-9 (21 and 90 ng ml^{-1}) before the addition of collagen (1 μg ml^{-1}). At 6 min after the addition of agonists, 2 mM EDTA and 50 μM indomethacin were added to the reaction suspensions. The vials were then centrifuged for 3 min at 15,000 *g*. The TxB_2 levels of the supernatants were measured using an EIA kit according to the instructions of the manufacturer.

Estimation of nitrate, cyclic AMP, and cyclic GMP formations

NO was assayed in platelet suspensions as previously described (Sheu *et al*., 1999a). In brief, platelet suspensions (1×10^9 ml^{-1}) were preincubated with collagen (1 μg ml^{-1}) or activated MMP-9 (21 and 90 ng ml^{-1}) for 6 min, respectively, followed by centrifugation. The amount of nitrate in the platelet suspensions (10 μl) was measured by adding a reducing agent (0.8% VCl_3 in 1 M HCl) to the purge vessel to convert nitrate to NO, which was stripped from the platelet suspensions by a helium purge gas. The NO was then drawn into a Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Sievers Inc., Boulder, CO, U.S.A.). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate.

The method of Karniguian *et al*. (1982) for measurement of cyclic AMP and cyclic GMP was followed. In brief, platelet suspensions were incubated with nitroglycerin (10 μM) and activated MMP-9 (21 and 90 ng ml^{-1}) for 6 min. The incubation was stopped, and the solution was immediately boiled for 5 min. In total, 50 μl of supernatant was used to determine the cyclic AMP and cyclic GMP contents with EIA kits following acetylation of the samples as described by the manufacturer.

Measurement of protein kinase C activity

Washed platelets (2×10^9 ml^{-1}) were incubated for 60 min with phosphorus-32 (0.5 mCi ml^{-1}). The [^{32}P]labeled platelets were incubated with activated MMP-9 (21 and 90 ng ml^{-1}) for 3 min, then PDBu (60 nM) was added for 1 min to trigger protein kinase C activation. Activation was terminated by the addition of Laemmli sample buffer, and analyzed by electrophoresis (12.5%; wt vol^{-1}) as described previously (Grabarek *et al*., 1992). The gels were dried, and the relative intensities of the radioactive bands were analyzed using a Bio-imaging analyzer system (FAL 2000, Fuji, Tokyo, Japan), and expressed as PSL mm^{-2} (PSL, photostimulated luminescence).

Fluorescein sodium-induced platelet thrombi in mesenteric microvessels of mice

As we previously described (Sheu *et al*., 1994), mice were anesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.). After a tracheotomy was performed, an external jugular vein was cannulated with polyethylene tubing (PE-10) for administration of the dye and drug (by an i.v. bolus). A segment of the small intestine with its mesentery attached was loosely exteriorized through a midline incision in the abdominal wall and was placed onto a transparent culture dish for microscopic observation. Venules with diameters of 30–40 μm were selected for irradiation to produce a microthrombus. Filtering the light eliminated wavelengths below 520 nm, and this light was used to irradiate a microvessel (the area of irradiation was about 100 μm in diameter on the focal plane) through an objective lens ($\times$20). The dose of fluorescein sodium used was 15 μg kg^{-1}. Various doses of activated MMP-9 (0.5 and 1.0 μg g^{-1}), aspirin (150 and 250 μg g^{-1}) or solvent control (10 μM AMPA) were administered 1 min after fluorescein sodium addition. The time lapse for inducing thrombus formation leading to cessation of blood flow was measured.

Statistical analysis

The experimental results are expressed as the means $\pm$ s.e.m. and are accompanied by the number of observations. Data were assessed using analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman–Keuls method. A P-value < 0.05 was considered statistically significant.

Results

Quantification of pro-MMP-9 in washed human platelets

Accurate quantification of pro-MMP-9 was extremely important to this study. This study is the first to report a fast, simple, efficient method for analysis of pro-MMP-9 using CZE. This technique has a higher peak capacity and resolution, and improved reproducibility compared with HPLC. The relative migration time of the pro-MMP-9 peak was about 3.3 min (indicated by an arrow) (Figure 1a). Identification of the peak, corresponding to the pro-MMP-9 compound, was based on the use of authentic pro-MMP-9 and anti-MMP-9 antibody. For example, authentic pro-MMP-9 compound and anti-MMP-9 antibody were mixed with sample, and then applied to CZE under the same conditions, we found that the peak eluted at 3.3 min were superimposed and disappeared, respectively, indicating that this peak corresponded to the pro-MMP-9 compound (data not shown).

Figure 1b shows the regression line of cell number vs total amount of pro-MMP-9 in human platelets. A correlation coefficient (r^2) of about 0.94 was obtained form this line. This result reveals that no obvious variation in the ratio of cells associated with the amount of pro-MMP-9 was observed. The amount of pro-MMP-9 in human platelets was calculated to be about 8.8 ± 0.7 ng 10^{-6} cells ($n = 4$).

Distribution of MMP-9 in resting and activated platelets

Immunogold labeling on LR White-sections of resting platelets revealed that the cytoplasma was the main storage compartment for MMP-9 (Figure 2b). Some gold particles were labeled on the plasma membrane and α-granules, whereas there is only a little labeling on open canalicular system (OCS). To determine whether platelet activation might cause changes in MMP-9 labeling, we processed collagen-activated platelets in the same way, and they showed a marked increase in labeling of MMP-9 in the cytoplasma, on the plasma membrane, and on the OCS, but with only a little labeling on α-granules (Figure 2d). On the other hand, no labeling reaction was visible on cytoplasma, OCS, or α-granules in control preparations incubated with normal nonimmune mouse IgG in resting and activated platelets, respectively (Figure 2a and c).

Effect of activated MMP-9 on platelet aggregation in human platelets

Zymographic analysis confirmed that pro-MMP-9 (92-kDa) was activated by AMPA (10 μM) to form an 86-kDa activated MMP-9, as shown by gelatinolytic activities (data not shown). In subsequent experiments, we used this activated MMP-9 to investigate antiplatelet activity. Activated MMP-9 (15–90 ng ml^{-1}) concentration-dependently inhibited platelet aggregation stimulated by collagen (1 μg ml^{-1}), thrombin (0.01 U ml^{-1}), arachidonic acid (60 μM), and U46619 (1 μM), a prostaglandin endoperoxide analogue in washed human platelets (Figure 3) and PRP (data not shown). It similarly inhibited ADP (20 μM)-induced platelet aggregation in the presence of fibrinogen (200 μg ml^{-1}). Furthermore, activated MMP-9 also inhibited the ATP-release reaction when stimulated by agonists (i.e. collagen) (Figure 3a). IC_{50} values of activated MMP-9 for platelet aggregation induced by collagen, thrombin, ADP, U46619, and arachidonic acid were estimated to be approximately 21, 54, 64, 49, and 5.4 ng ml^{-1}, respectively. Furthermore, platelets were preincubated with a higher concentration of activated MMP-9 (150 ng ml^{-1}) or normal saline for 10 min, followed by two washes with Tyrode's solution, we found that there were no significant differences between the aggregation curves of either platelet preparations stimulated by collagen (1 μg ml^{-1}), indicating that the effect of activated MMP-9 on inhibition of platelet aggregation occurs in a reversible manner (data not shown). In contrast to activated MMP-9, neither pro-MMP-9 (10 μg ml^{-1}) nor AMPA (10 μM) significantly inhibited collagen (1 μg ml^{-1})- or thrombin (0.01 U ml^{-1})-induced platelet aggregation (data not shown). In subsequent experiments, we used collagen as an agonist to explore the inhibitory mechanisms of activated MMP-9 in platelet aggregation.

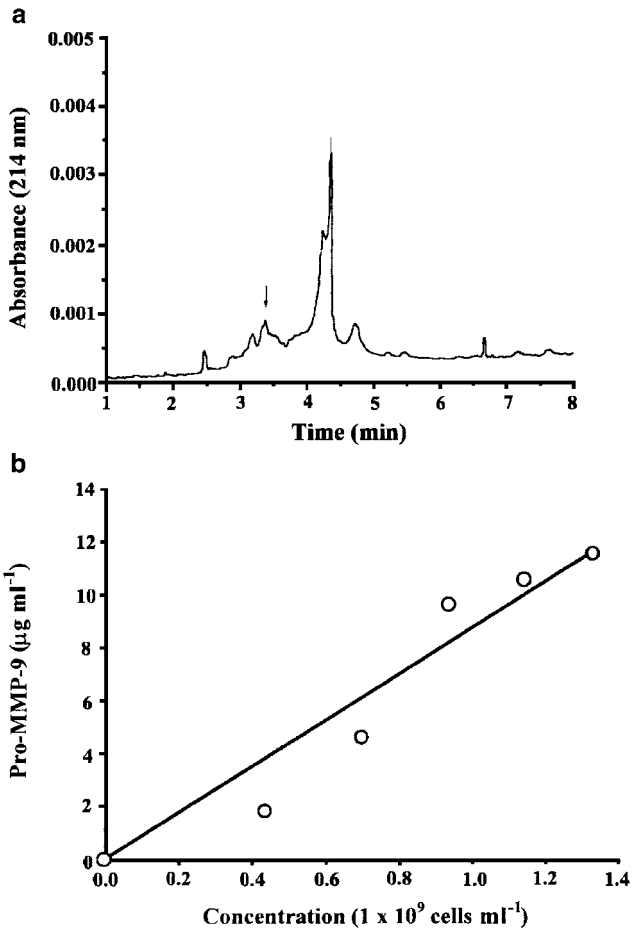

Figure 1 Various concentrations of human platelets were homogenized by ultrasound sonication in lysis buffer followed by centrifugation. The supernatants were applied into the capillary zone electrophoresis (CZE) system for the quantitative analysis of pro-MMP-9 as described in 'Methods'. (a) Profile of pro-MMP-9 using the CZE system performed. One peak of the pro-MMP-9 compound was eluted at about 3.3 min (arrow). (b) Linear regression line of the amount of pro-MMP-9 vs various concentrations of human platelets. The line is a representative example for four similar experiments.

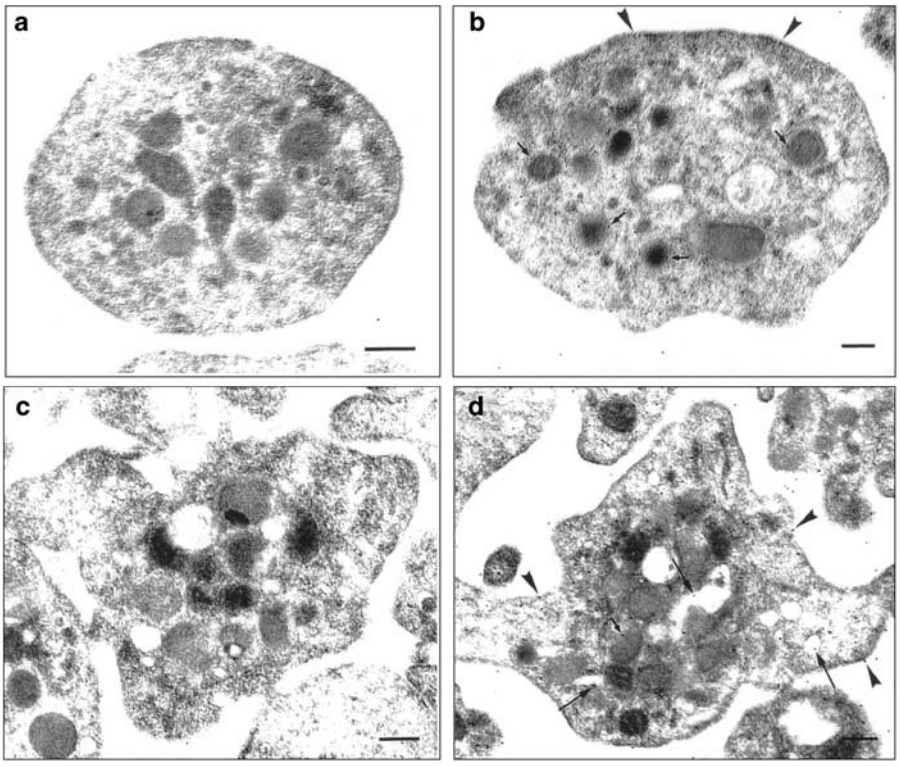

Figure 2 Immunogold labeling for MMP-9 on LR White-sections in resting and collagen-activated human platelets. Resting (a, b) and collagen (1 μg ml^{-1})-activated platelets (c, d) were fixed followed by the addition of control mouse IgG (a, c) or monoclonal anti-MMP-9 antibody (b, d). The illustrated sections show that gold particles were observed on the plasma membrane surface (arrowheads), on the open canalicular system (OCS) (large arrows), and on the α-granules (small arrows) in both resting (b) and activated platelets (d). Bars: 0.2 μm. The figures are representative examples of four similar experiments.

Effect of activated MMP-9 on phosphoinositide breakdown in human platelets

Phosphoinositide breakdown occurs in platelets activated by many different agonists (Broekman *et al*., 1980). In this study, we found that collagen (1 μg ml^{-1}) induced the rapid formation of radioactive IP, IP_2, and IP_3 in human platelets loaded with [^{3}H]inositol. We only measured [^{3}H]IP formation as an index of total inositol phosphate formation. As shown in Figure 4, the addition of collagen (1 μg ml^{-1}) resulted in a rise of IP formation of about 2.1-fold compared to that in resting platelets ([26.0$\pm$0.3 vs 12.5$\pm$0.5] $\times 10^3$ cpm). In the presence of activated MMP-9 (21 and 90 ng ml^{-1}), the radioactivity of IP formation in collagen-stimulated human platelets markedly decreased. In addition, we also found that activated MMP-9 (90 ng ml^{-1}) also significantly inhibited the IP formation in thrombin (0.5 U ml^{-1})-stimulated human platelets (data not shown).

Effect of activated MMP-9 on [Ca^{2+}]i mobilization

Free cytoplasmic Ca^{2+} concentrations in human platelets were measured by the Fura 2-AM loading method. As shown in Figure 5, collagen (1 μg ml^{-1}) evoked an increase in [Ca^{2+}]i of from 29.8$\pm$3.4 to 356.7$\pm$34.2 nM. This collagen-evoked increase in [Ca^{2+}]i was markedly inhibited in the presence of activated MMP-9 (21 ng ml^{-1}, 75.3$\pm$8.0%; 90 ng ml^{-1}, 91.2$\pm$3.6%) (Figure 5).

Effect of activated MMP-9 on thromboxane B_2, nitrate, cyclic AMP, and cyclic GMP formations in human platelets

As shown in Table 1, resting platelets produced relatively little TxB_2 compared with collagen-activated platelets. Activated MMP-9 (21 and 90 ng ml^{-1}) significantly inhibited TxB_2 formation in platelets stimulated by collagen (1 μg ml^{-1}).

Furthermore, NO was quantified using a sensitive and specific ozone redox-chemiluminescence detector. As shown in Table 1, collagen (1 μg ml^{-1}) caused about a 3.8-fold rise in nitrate formation, compared to that in resting platelets. In the presence of activated MMP-9 (21 and 90 ng ml^{-1}), nitrate production significantly increased after incubation with washed platelets. In addition, the level of cyclic GMP in resting platelets was about 5.7$\pm$0.2 pmol ml^{-1}. The addition of nitroglycerin (10 μM) increased the cyclic GMP level to 12.6$\pm$1.6 pmol ml^{-1} (Table 1). The addition of activated MMP-9 (21 and 90 ng ml^{-1}) resulted in significant increases in platelet cyclic GMP levels (Table 1). On the other hand, activated MMP-9 (21 and 90 ng ml^{-1}) did not significantly increase the cyclic AMP levels in human platelets (data not shown).

Effect of activated MMP-9 on phorbol-12, 13-dibutyrate-stimulated phosphorylation of the 47-kDa protein

Stimulation of platelets with a number of different agonists, and PDBu in particular, induces activation of protein kinase

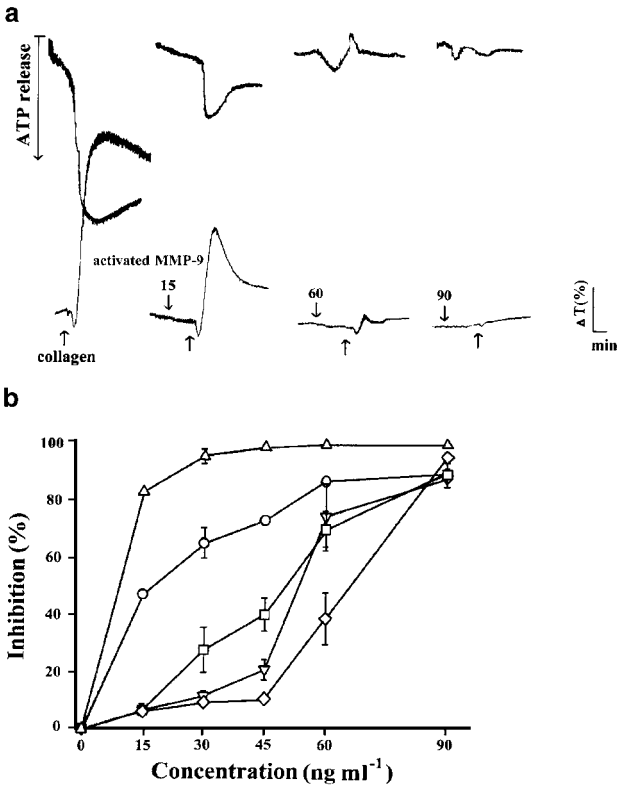

Figure 3 (a) Tracing curves of activated MMP-9 on collagen ($1\,\mu g\,ml^{-1}$)-induced aggregation and (b) concentration-inhibition curves of activated MMP-9 on collagen ($1\,\mu g\,ml^{-1}$, ○)-, thrombin ($0.01\,U\,ml^{-1}$, ▽)-, U46619 ($1\,\mu M$, □), ADP ($20\,\mu M$, ◇), and arachidonic acid ($60\,\mu M$, △)-induced platelet aggregation in washed human platelets. Platelets were preincubated with activated MMP-9 ($15–90\,ng\,ml^{-1}$); agonists were then added to trigger aggregation (lower tracings) and ATP release (upper tracings) (a). Data are presented as a percentage of the control (means ± s.e.m., $n=4$).

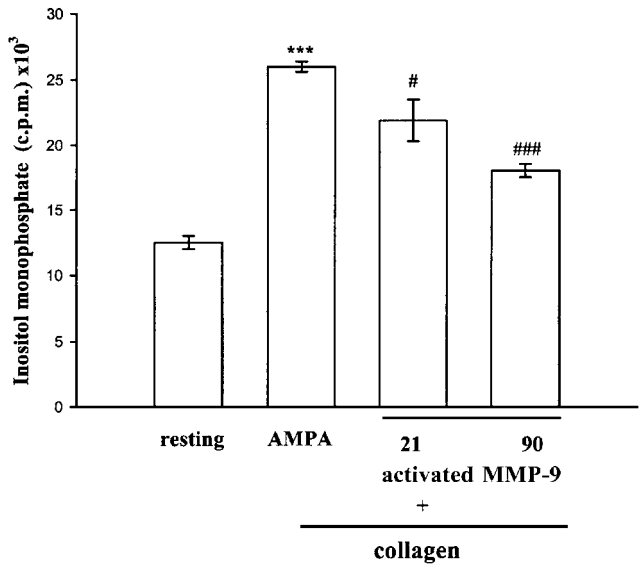

Figure 4 Effect of activated MMP-9 on collagen-induced inositol monophosphate formation in human platelets. [^{3}H]Inositol-labeled platelets were preincubated with *p*-aminophenylmercuric acetate ($10\,\mu M$) (AMPA, solvent control) or activated MMP-9 (21 and $90\,ng\,ml^{-1}$), followed by the addition of collagen ($1\,\mu g\,ml^{-1}$) to trigger platelet aggregation. Data are presented as the means ± s.e.m. ($n=4$). $^{***}P<0.001$ as compared with the resting group; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ as compared with the AMPA group.

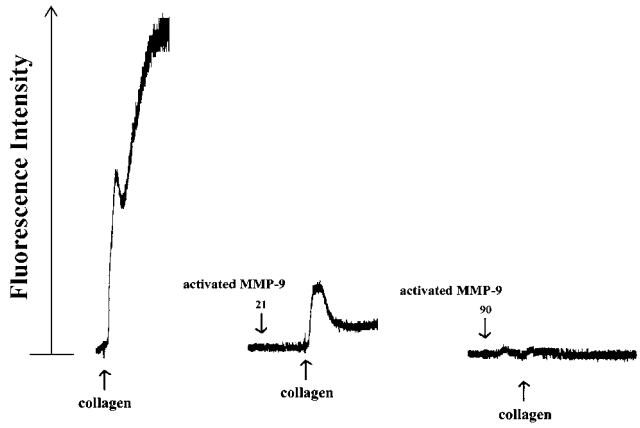

Figure 5 Effect of activated MMP-9 on collagen-induced intracellular Ca^{2+} mobilization in human platelets. Platelets were preincubated with Fura 2-AM ($5\,\mu M$) followed by the addition of collagen ($1\,\mu g\,ml^{-1}$) in the absence or presence of activated MMP-9 (21 and $90\,ng\,ml^{-1}$), which was added 3 min prior to the addition of collagen. Data are presented as the means ± s.e.m. ($n=4$). Profiles are representative examples of four similar experiments.

C, which then phosphorylates proteins of Mr 40,000–47,000 in addition to other proteins (Siess & Lapetina, 1989). In this study, phosphorylation experiments were performed to examine the role of activated MMP-9 in the activation of protein kinase C in human platelets. When PDBu (60 nM) was added to human platelets prelabeled with $^{32}PO_4$, a protein with an apparent Mr of 47,000 (P47) was predominately phosphorylated as compared with resting platelets (Figure 6). Activated MMP-9 (21 and $90\,ng\,ml^{-1}$) markedly inhibited the phosphorylation of P47 stimulated by PDBu. In this study, the extent of radioactivity in P47 was expressed as a relative detection density ($PSL\,mm^{-2}$; PSL, photostimulated luminescence) of the radioactive bands. Moreover, activated MMP-9 (21 and $90\,ng\,ml^{-1}$) also significantly inhibited collagen ($1\,\mu g\,ml^{-1}$)-induced phosphorylation of P47 in human platelets (data not shown).

Effect of activated MMP-9 on thrombus formation in microvessels of fluorescein sodium-pretreated mice

When $15\,\mu g\,kg^{-1}$ of fluorescein sodium was given, the occlusion time required was 4.7 ± 0.5 min (Figure 7). When activated MMP-9 was administered at $0.5\,\mu g\,g^{-1}$ in mice pretreated with fluorescein sodium ($15\,\mu g\,kg^{-1}$), the occlusion time was not significantly prolonged (5.3 ± 0.4 min). However with $1\,\mu g\,g^{-1}$ of activated MMP-9, the occlusion time was markedly prolonged (7.4 ± 0.7 min) (Figure 7). On the other hand, aspirin also exhibited antithrombotic activity in this experiment. When $250\,\mu g\,g^{-1}$ of aspirin was administered, the occlusion time was prolonged (Figure 7). Activated MMP-9 was over about 1×10^4-fold more potent than aspirin at prolongation of occlusion time in microvessels on a molar basis.

Table 1 Effect of activated MMP-9 on thromboxane B_2, nitrate and cyclic GMP formation in washed human platelets

	Concentration	*Thromboxane B_2* (ng ml^{-1}) $n=5$	*Nitrate* (μM) $n=6$	*Cyclic GMP* (pmol ml^{-1})
Resting		193.0±11.5	1.2±0.2	5.7±0.2
Collagen (μg ml^{-1})	1	619.0±23.8***	4.6±0.4***	—
+Activated MMP-9 (ng ml^{-1})	21	392.8±16.1$^{###}$	—	—
	90	325.3±17.8$^{###}$	—	—
Nitroglycerin (10 μM)	10	—	—	12.6±1.6**
Activated MMP-9 (ng ml^{-1})	21	—	5.8±0.8***	9.5±0.9**
	90	—	6.6±0.5***	11.7±0.6***

Platelet suspensions were preincubated with activated MMP-9 (21 and 90 ng ml^{-1}) for 3 min at 37°C, and then collagen (1 μg ml^{-1}) was added to trigger thromboxane B_2 formation. Addition of nitroglycerin into the platelet suspensions served as positive control of cyclic GMP. Data are presented as the means±s.e.m. (n=number). ** $P<0.01$ and *** $P<0.001$ as compared with the resting groups; $^{###}P<0.001$ as compared with the collagen group.

Discussion

The results obtained from this study demonstrate for the first time the presence of MMP-9 both in resting and activated human platelets by quantitative and qualitative assays. CZE analysis detected the presence of pro-MMP-9 in the homogenate of resting platelets. Using electron microscopy, the presence of MMP-9 (pro-MMP-9 and/or activated MMP-9) immunoreactivity was labeled in the cytoplasma, OCS, and α-granules both in resting and activated platelets. These results clearly indicate that pro-MMP-9/activated MMP-9 is present in human platelets. In addition, the principal objective of this study was to describe the detailed inhibitory mechanisms of activated MMP-9 in platelet activation. This inhibitory effect of activated MMP-9 was demonstrable with the use of various agonists: collagen, thrombin, ADP, U46619, and arachidonic acid. The inhibition was directly proportional to the pharmacological concentrations of activated MMP-9 used. Nakamura *et al*. (1998) suggested that human plasma MMP-9 concentrations ranged from 34.2±16.6 to 52.4±26.6 ng ml^{-1}. In this study, activated MMP-9 was employed at concentrations of about 15–90 ng ml^{-1}, which inhibited platelet aggregation induced by agonists. This result indicates that the pharmacological concentrations of activated MMP-9 employed to inhibit platelet aggregation *in vitro* are reasonably close to those of physiological concentrations obtained for plasma MMP-9.

In this study, platelet aggregation induced by these agonists (i.e. collagen) appeared to be affected in the presence of activated MMP-9. Therefore, this partly infers that activated MMP-9 may affect Ca^{2+} release from intracellular Ca^{2+}-storage sites (i.e. dense tubular systems or dense bodies), and this is in accord with the concept that intracellular Ca^{2+} release is responsible for platelet aggregation (Charo *et al*., 1976). Moreover, this inhibitory effect of activated MMP-9 was dependent on the catalytic activity of this enzyme since the zymogen, pro-MMP-9, did not significantly inhibit platelet aggregation stimulated by agonists. These data also indicate that MMP-9 generated in an autocrine or paracrine way regulates platelet aggregation.

Although the action mechanisms of various platelet aggregation agonists, such as collagen, thrombin, ADP, U46619, and arachidonic acid, differ, activated MMP-9 significantly inhibited platelet aggregation stimulated by all of them. This implies that activated MMP-9 may block a common step shared by these inducers. These results also indicate that the site of action of activated MMP-9 is not at the receptor level of individual agonists. Stimulation of platelets by agonists (i.e., collagen) results in phospholipase C-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Kirk *et al*., 1981). There is strong evidence that IP_3 induces the release of Ca^{2+} from intracellular stores (Berridge, 1983). Diacylglycerol activates protein kinase C, inducing protein phosphorylation and a release reaction. In this study, phosphoinositide breakdown of collagen-activated platelets was inhibited by activated MMP-9, suggesting that inhibition of platelet aggregation by activated MMP-9 is related to inhibition of phospholipase C activation. Moreover, TxA_2 is an important mediator of the release reaction and aggregation of platelets (Hornby, 1982). Collagen-induced TxB_2 formation, a stable metabolite of TxA_2, was markedly inhibited by activated MMP-9. It has been demonstrated that phosphoinositide breakdown can induce TxA_2 formation *via* free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A_2 from membrane phospholipids (McKean *et al*., 1981). Thus, it seems likely that TxB_2 formation plays a role in mediating the inhibitory effect of activated MMP-9 on human platelets.

Furthermore, activated MMP-9 significantly inhibited PDBu-induced activation of protein kinase C. PDBu is known to intercalate with membrane phospholipids and form a complex with protein kinase C translocated to the membrane (Kraft & Anderson, 1983). Moreover, increased cyclic GMP can negatively affect agonist-induced protein kinase C activation (Murohara *et al*., 1995). Signaling by cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca^{2+}-mobilizing second messengers (McDonald & Murad, 1996). Activated MMP-9 increases the level of cyclic GMP in human platelets; therefore, the inhibitory effect of activated MMP-9 in PDBu-induced activation of protein kinase C may be due, at least partly, to mediating the increase in cyclic GMP. In this study, we found that activated MMP-9 induced NO formation in human platelets. This result is in accord with the result of a cyclic GMP study, because most cellular actions of NO occur via stimulation of intracellular guanylate cyclase, leading to an increase in cyclic GMP (McDonald & Murad, 1996).

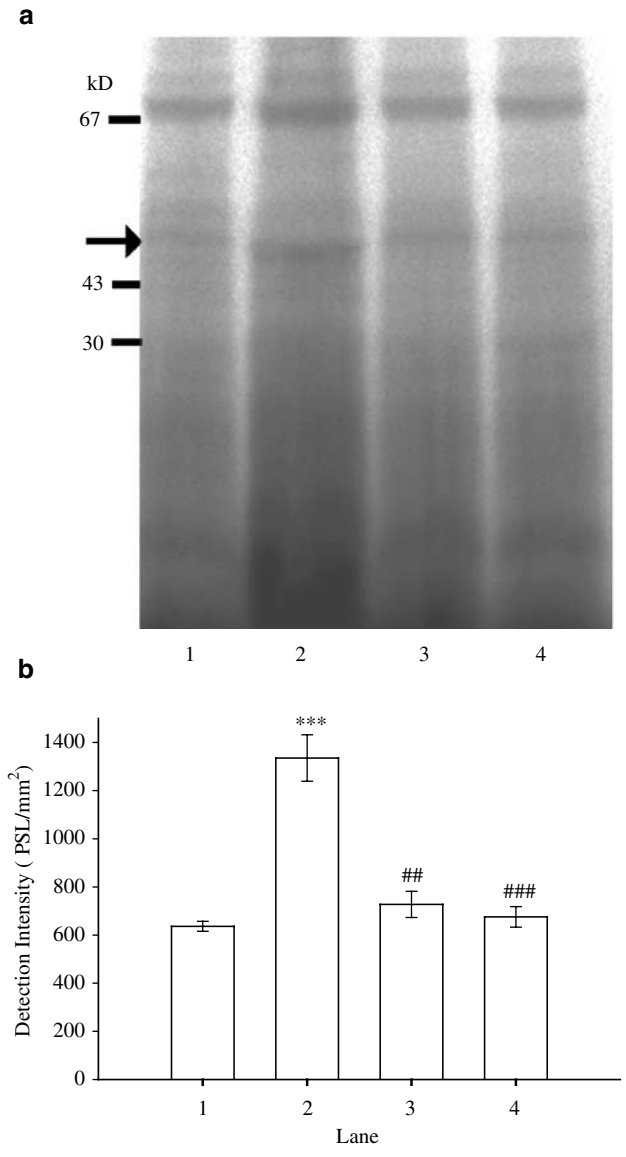

Figure 6 Effect of activated MMP-9 on phosphorylation of a protein of Mr 47,000 (P47) in human platelets challenged with phorbol-12, 13-dibutyrate (PDBu). Platelets were preincubated with activated MMP-9 (21 and 90 ng ml^{-1}) before challenge with PDBu (60 nM). Lane 1, platelets with Tyrode's solution; lane 2, platelets with PDBu (60 nM); lane 3, platelets with activated MMP-9 (21 ng ml^{-1}); and lane 4, with activated MMP-9 (90 ng ml^{-1}) followed by the addition of PDBu. (a) The arrow indicates a protein of Mr 47,000 (P47). (b) The relative detection densities of the radioactive bands are expressed as PSL mm^{-2} (PSL, photostimulated luminescence). Data are presented as the means ± s.e.m. ($n=4$). ***$P<0.001$ as compared with the resting group; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ as compared with the PDBu group.

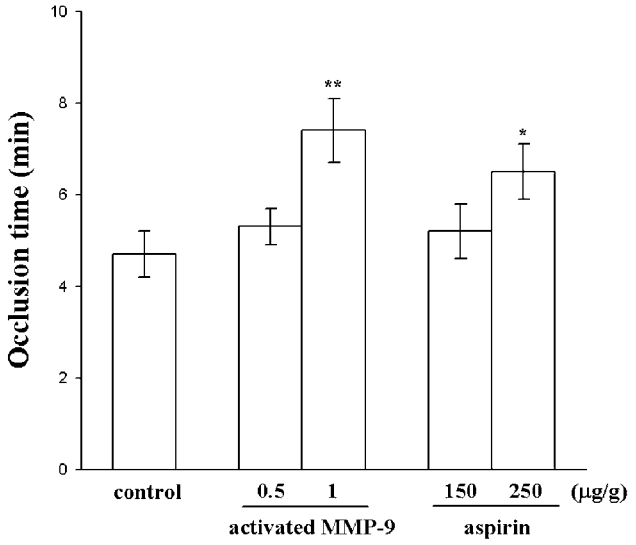

Figure 7 Effect of activated MMP-9 (0.5 and 1 μg g^{-1}) and aspirin (150 and 250 μg g^{-1}) on occlusion times for inducing thrombus formation upon light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (15 μg kg^{-1}). Data are presented as the means ± s.e.m. of occlusion time (min) of platelet plug formation ($n=8$). *$P<0.05$ and **$P<0.01$ as compared with the control group.

Platelet aggregation plays a pathophysiological role in a variety of thromboembolic disorders. Therefore, inhibition of platelet aggregation by drugs may represent an increased therapeutic possibility for such diseases. We have demonstrated that endothelial cell injury induces platelet aggregation and adhesion to the vessel wall *in vivo* (Sheu *et al*., 1999b). In this study, activated MMP-9 significantly prolonged the occlusion times in mice mainly through its inhibition of platelet aggregation.

In conclusion, the most important findings of this study suggest that activated MMP-9 can markedly inhibit agonist-induced platelet aggregation. This inhibitory effect may possible involve the following two mechanisms. (1) Activated MMP-9 may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane A_2 formation, thereby leading to inhibition of the activation of protein kinase C and intracellular Ca^{2+} mobilization. (2) Moreover, activated MMP-9 increases NO/cyclic GMP formation and subsequently inhibits phosphoinositide breakdown and protein kinase C activity, ultimately resulting in inhibition of both the phosphorylation of P47 and intracellular Ca^{2+} mobilization. The important findings for vascular thrombosis suggest that MMP-9 appears to represent a novel platelet-regulatory system based on its antiaggregatory effect. Therefore, it is possible that an imbalance in the generation and/or release of MMP-9 contributes to pathological vascular diseases associated with platelet aggregation such as stroke, atherosclerotic plaque rupture, myocardial infarction, and thrombosis. We therefore believe that our data represent a physiologically relevant function for MMP-9 as a negative feedback regulator during platelet activation. When MMP-9 accumulates in the microenvironment of a generating thrombus, the recruitment and activation of nearly platelets is then prevented. However, the physiological relevance of a direct antiaggregatory effect of MMP-9 still remains to be further studied.

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