Outside-In Signals Delivered by Matrix Metalloproteinase-1 Regulate Platelet Function

Spencer W. Galt, Stephan Lindemann, Loren Allen, Donald J. Medd, Jeanne M. Falk, Thomas M. McIntyre, Stephen M. Prescott, Larry W. Kraiss, Guy A. Zimmerman, Andrew S. Weyrich

Abstract—Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade extracellular matrix proteins. These enzymes are implicated in a variety of physiological and pathological events characterized by extracellular matrix remodeling. Recent studies suggest that MMPs may have a signaling capacity, but direct evidence supporting this concept is lacking. In the present study, we demonstrate that outside-in signals delivered by exogenous MMP-1 (interstitial collagenase) markedly increase the number of tyrosine-phosphorylated proteins in platelets. Active MMP-1 also targets \( \beta_3 \) integrins to areas of cell contact and primes platelets for aggregation. Examination of the endogenous enzyme demonstrated that activated platelets process latent MMP-1 into its active form. Neutralization of MMP-1 activity with MMP inhibitors or specific blocking antibodies markedly attenuates agonist-induced phosphorylation of intracellular proteins, movement of \( \beta_3 \) integrins to cell contact points, and intercellular aggregation. The finding that MMP-1 is rapidly activated in platelets and controls functional responses identifies a new role for this metalloproteinase as a signaling molecule that regulates thrombotic events. (Circ Res. 2002;90:1093-1099.)

Key Words: matrix metalloproteinases | platelets | signaling | thrombosis

Dysregulated turnover of extracellular matrix (ECM) is a prominent feature of atherosclerosis and abdominal aortic aneurysm formation and of a variety of other conditions, including rheumatoid arthritis, tumor invasion and metastasis, and periodontal disease.1-3 Experimental evidence suggests that overproduction and/or unimpeded activity of matrix metalloproteinases (MMPs) contributes to these disease processes by mediating excessive ECM destruction, thereby disrupting the balance between protein synthesis and degradation necessary for normal matrix assembly, remodeling, and repair. Pro forms of MMPs are constitutively expressed or synthesized by a number of cell types. These proenzymes are then processed to active forms. This often occurs at the cell surface, thereby generating proteolytic events at contact points between the cell and the ECM.2 Degradation of the ECM by MMPs links these enzymes to the complex integrated events that underlie cell migration.4

Although the ECM is a primary target, MMPs also alter cell surface proteins, receptors, and transmembrane ECM proteins and by this mechanism regulate intercellular adhesion and signaling.2 The identities of specific MMPs that mediate these events have been elusive, but the observations are consistent with recent evidence indicating that other metalloproteinase family members can regulate intracellular signal transduction.2,5,6 In the present study, we demonstrate that endogenous and exogenous MMP-1 activates pathways that signal the phosphorylation of intracellular proteins, distributes \( \beta_3 \) integrins to cell contact points, and primes platelets for aggregation. These experiments provide new evidence that MMP-1 can regulate outside-in signaling events that control cellular function and phenotype.

Materials and Methods

Cell Isolation

Washed platelets were isolated according to protocols previously described by our laboratory.3 The platelet pellet was resuspended at \( 1 \times 10^9 \) cells/mL in serum-free M199 culture media for the experiments described subsequently, unless otherwise indicated.

Type I Collagenase Activity Assay

A type I collagenase/MMP-1 activity assay kit was purchased from Chemicon. In brief, latent FITC-labeled collagen was incubated with \( p \)-aminophenylmercuric acetate (APMA)-free active MMP-1 in the presence of specific inhibitors. The assays were conducted in buffer solution containing 0.1 mol/L Tris-HCl, pH 7.5, containing NaCl, CaCl\(_2\), and Na\(_2\)PO\(_4\). After 1 hour, the enzymatic reaction was halted, and the fluorescence intensity of the supernatant was measured. The collagenase activity is reported in units per milliliter, where one unit is defined as the amount of enzyme degrading 1 \( \mu \)g of collagen per minute. In some studies, active MMP-1 was replaced with equal...
volumes of intracellular extracts or supernatants collected from resting and thrombin-activated platelets.

Quantification of Metalloproteinases
To quantify MMP-1, MMP-2, MMP-3, and MMP-9 in platelets, ELISAs for these four metalloproteinases were purchased from Amersham and performed according to the manufacturer’s instructions. Each ELISA demonstrated negligible cross-reactivity with other MMPs. ELISAs for MMP-1 and MMP-3 recognized both the pro and active forms of the enzymes. MMP-2 and MMP-9 ELISAs were specific for the pro forms of the enzymes. For analysis, unactivated or activated platelets were pelleted, and the cell-free supernatant was collected. The cellular pellets were immediately lysed with Triton X-100 for 30 minutes on ice and centrifuged, and the soluble fractions were retained. Cell-associated and secreted metalloproteinases were subsequently measured by using the ELISAs described.

Western Blot Analysis of Intracellular Proteins
We also examined tyrosine phosphorylation of intracellular proteins in the presence or absence of MMP inhibitor-1, a synthetic tetrapeptide hydroxamic acid (Calbiochem) that preferentially inactivates MMP-1 compared with other MMPs. In these experiments, the platelets were pretreated with MMP inhibitor-1 for 1 minute and subsequently stimulated with purified catalytically active MMP-1, the C-terminal domain of MMP-1, or 0.1 U/mL thrombin. At the end of each time point, the cell suspensions (500 µL) were immediately lysed with 167 µL of 4× SDS-PAGE reducing buffer to prevent further activation. The samples were prepared for Western blot analysis as previously described by our laboratory.

In separate studies, pro and active MMP-1 was also measured by Western blot analysis with the use of an antibody that detects both forms of the enzyme (clone III12b, Neomarkers). An antibody that recognizes only the active form of MMP-1 (clone 195-1H6, Calbiochem) was used in selected studies.

Immunolocalization of MMP-1 and β1 Integrins
Immunolocalization studies for β1 integrins, MMP-1, or MMP-9 were conducted by using methods that we have previously described in detail. In brief, platelets were left quiescent or were stimulated with active MMP-1 or thrombin in the presence or absence of MMP inhibitor-1 for 1 minute. We ensured that purified active MMP-1 did not contain APMA, which is used to activate the enzyme, because we found that APMA has direct effects on platelet activation and aggregation responses that are MMP independent (data not shown). The cells were subsequently fixed with 4% paraformaldehyde and blocked with 10% normal goat serum for 1 hour at room temperature. In each study, we omitted the permeabilization step so that the primary antibodies would label plasma membrane antigens but not intracellular antigens. The MMP-1 and MMP-9 antibodies (Calbiochem) used for immunocytochemistry were clones 41-1E5 and 6-6B, respectively. The anti-β1 (clone PM6/13) antibody used for immunocytochemical analysis was purchased from Serotec Ltd. Secondary mouse antibodies, tagged to either Alexa Fluor 488 or 594, were purchased from Molecular Probes. Immunolabeled platelets were viewed by using a laser scanning confocal microscope (Bio-Rad MRC 1024).

Analysis of αIIbβ3 Surface Expression
Flow cytometry for plasma membrane αIIbβ3 and surface-bound fibrinogen was conducted as previously described. Platelets were pretreated with glycy1-L-prolyl-L-arginyl-L-proline and subsequently activated with thrombin in the presence or absence of MMP inhibitor-1. To examine αIIbβ3, an FITC-conjugated antibody that recognized the activated form was used (PA-C-1, 1 µg/mL, Becton-Dickinson). A FITC-conjugated anti-fibrinogen antibody was used to study endogenous surface-bound fibrinogen (Biodesign International).

Quantification of Surface-Bound Fibrinogen
For fibrinogen binding studies, platelets were placed in a Lumi-aggregometer (Payton) in the presence of [125I]-fibrinogen. The platelets were stimulated with thrombin in the presence or absence of MMP inhibitor-1 for 1 minute. After this time point, the cells were removed and layered onto Apiezon A oil (Apiezon). The samples were centrifuged at 12 000g for 10 minutes to separate the bound and unbound fibrinogen. Surface-bound fibrinogen was subsequently counted by using a Beckman γ-counter.

Platelet Aggregation
Washed platelets were placed in the Lumi-aggregometer, and aggregation was performed by using various agonists (ADP, arachidonic acid, collagen, MMP-1, and thrombin; Sigma Chemical Co), as previously described. In selected studies, MMP activity was inhibited by pretreating the platelets for 1 minute with MMP inhibitor-1, MMP-3 inhibitor (Calbiochem), which inhibits MMP-3, or specific azide-free anti–MMP-1 antibodies (Neomarkers). The antibody that is directed against MMP-1 and inhibits its activity is COMY 4A2 (Neomarkers), and the nonblocking MMP-1 antibody was clone III12b (Neomarkers). If any of the reagents contained sodium azide, it was removed by dialysis. This precautionary step was taken because azide concentrations used for reagent storage inhibit platelet aggregation, even when the reagents are diluted in the cell media (data not shown).

Results
Platelet Activation Increases MMP-1 Activity
Collagenolytic and elastolytic activities associated with platelets were first described in the early 1970s, but the specific enzymes and their regulation have not been fully characterized. We found that resting platelets contained small amounts of type 1 collagenase activity, which was significantly elevated when the cells were stimulated with thrombin (Figure 1A). Collagenase activity was primarily confined to cell lysates, although some activity was detected in the extracellular milieu. These results suggested that platelets constitutively express MMP-1, one of the primary interstitial collagenases. Using an ELISA that detects total MMP-1 (i.e., both the pro and active form), we found that resting platelets contain 16.5±7.2 ng (mean±SD) of MMP-1 per 1×109 cells (n=6). We also detected MMP-2, consistent with the findings of Sawicki et al., and MMP-3 in resting platelets (2.0±0.5 and 2.6±0.8 ng/109 platelets for pro-MMP-2 and total MMP-3, respectively [n=6]). Western blot analysis using an antibody that recognizes pro and active MMP-1 demonstrated that quiescent platelets primarily express pro-MMP-1 (Figure 1B). Thrombin induced a rapid increase in the amount of active MMP-1 present in platelets (Figure 1B). Similar results were found by using an antibody that is specific for the active form of MMP-1 (Figure 1C).

MMP-1 Induces Phosphorylation of Multiple Platelet Proteins
Because we detected MMP-1 in platelet lysates, we tested whether MMP-1 alters the phosphorylation status of intracellular proteins, an immediate response mechanism that regulates platelet activation and aggregation. First, we characterized the concentration-response relationship of purified active MMP-1 in cleaving type I collagen (Figure 2A). Using these concentrations, we then found that increasing amounts of MMP-1 enhanced the number of tyrosine-phosphorylated proteins in platelets (Figure 2B). MMP-3, which is present in
Platelet Aggregation

Neutralization of Endogenous MMP-1 Attenuates Platelet Aggregation

Because exogenous active MMP-1 redistributed β3 integrins to discrete areas on the cell periphery and primed the platelets for aggregation, we next tested whether endogenous MMP-1 regulates aggregatory responses. We first determined whether a portion of MMP-1 was expressed on the surface of resting cells by immunolocalizing the MMP in nonpermeabilized fixed platelets (see Materials and Methods). Under these conditions, we detected MMP-1 (Figure 4A) on platelets, whereas no staining was present when an isotype-matched antibody that recognizes MMP-9 was substituted (Figure 4B). Instead, it primed platelets for aggregation in response to submaximal stimulation with thrombin (Figure 3B, left), a response that was abolished by the blocking antibody directed against MMP-1 (Figure 3B; right) or MMP inhibitor-1 (data not shown). When we examined the cells by microscopy, we also found that MMP-1 did not induce platelet aggregation (Figure 3C, middle). However, active MMP-1 did redistribute β integrins to discrete areas on the cell periphery (Figure 3C, middle), a response that was blocked when MMP-1 activity was neutralized (Figure 3C, right). Although active MMP-1 clustered integrins to these areas, it did not significantly alter the conformational state of αIIbβ3 integrins, as tested by the binding of monoclonal antibody PAC-1 to MMP-1–stimulated platelets (see online Figure 1, which can be accessed in the data supplement available at http://www.circresaha.org). In addition, active MMP-1 did not increase the binding of radiolabeled fibrinogen to platelets, and neutralization of the endogenous enzyme had no effect on the increased binding of fibrinogen to thrombin-stimulated platelets (see online Figure 2). Similarly, MMP-1 did not regulate the binding of endogenous fibrinogen to platelet membranes, as measured by flow cytometry (data not shown).

MMP-1 Primes Platelet Aggregatory Responses

Numerous investigators have demonstrated that tyrosine phosphorylation of intracellular proteins is a necessary prerequisite for platelet aggregation. Therefore, we questioned whether exogenous MMP-1 regulates platelet aggregation by acting as a direct agonist. Pro-MMP-1 did not degrade collagen fibrils nor did it activate or block platelet aggregation (data not shown). APMA-free active MMP-1 readily cleaved type I collagen, a response that was completely abolished by MMP inhibitor-1 or a blocking MMP-1 antibody (Figure 3A). Catalytically active MMP-1 did not induce platelet aggregation when added alone, nor did it have appreciable effects on platelet shape change, intracellular calcium fluxes, translocation of P-selectin to the plasma membrane, or thromboxane synthesis (data not shown). Instead, it primed platelets for aggregation in response to submaximal stimulation with thrombin (Figure 3B, left), a response that was abolished by the blocking antibody directed against MMP-1 (Figure 3B; right) or MMP inhibitor-1 (data not shown). When we examined the cells by microscopy, we also found that MMP-1 did not induce platelet aggregation (Figure 3C, middle). However, active MMP-1 did redistribute β integrins to discrete areas on the cell periphery (Figure 3C, middle), a response that was blocked when MMP-1 activity was neutralized (Figure 3C, right). Although active MMP-1 clustered integrins to these areas, it did not significantly alter the conformational state of αIIbβ3 integrins, as tested by the binding of monoclonal antibody PAC-1 to MMP-1–stimulated platelets (see online Figure 1, which can be accessed in the data supplement available at http://www.circresaha.org). In addition, active MMP-1 did not increase the binding of radiolabeled fibrinogen to platelets, and neutralization of the endogenous enzyme had no effect on the increased binding of fibrinogen to thrombin-stimulated platelets (see online Figure 2). Similarly, MMP-1 did not regulate the binding of endogenous fibrinogen to platelet membranes, as measured by flow cytometry (data not shown).

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MMP-1 and β3 integrins were localized to cell contact points in aggregated cells (Figures 4E and 4F). These data indicate that a portion of MMP-1 had colocalized with β3 integrins in cell contact sites after activation and aggregation.

We then determined whether neutralization of endogenous MMP-1 activity altered agonist-induced platelet aggregation. Platelets were pretreated for 1 minute with MMP inhibitor-1, which preferentially neutralizes MMP-1 activity,8 a selective MMP-3 inhibitor, 16 or with blocking or nonblocking antibodies directed against MMP-1. Signal-dependent increases in type I collagenase activity induced by thrombin were attenuated in the presence of MMP inhibitor-1 or blocking MMP-1 antibodies (Figure 5A). Inhibition approximated 50%, regardless of the thrombin concentration used to induce collagenase activity (data not shown). The MMP-3 inhibitor or nonblocking antibodies did not reduce collagenase activity (Figure 5A). MMP inhibitor-1, but not the MMP-3 inhibitor, decreased thrombin-induced platelet aggregation (Figure 5B). Inhibition was more robust when aggregation was induced with smaller concentrations of thrombin. Treatment of the cells with the blocking antibody directed against MMP-1 had similar effects on platelet aggregation (Figure 5C). The nonblocking MMP-1 antibody (Figure 5C) did not decrease aggregation. Similar patterns were observed when collagen, ADP, and arachidonic acid were used as agonists, indicating that neutralization of endogenous MMP-1 activity is not restricted to responses induced via the thrombin receptor (data not shown).

Discussion

MMPs are members of the family of MB metallopeptidases. Collectively, these enzymes are able to degrade all components of the ECM. MMPs have protean involvement in health and disease, including physiological actions in growth, development, and tissue remodeling. In contrast, they are likewise implicated in pathological processes of tissue destruction in fibrotic diseases and tumor metastasis. They may be of particular importance in cardiovascular diseases, including atherosclerotic plaque rupture and abdominal aortic aneurysm formation, in which the vessel wall matrix is altered. The properties of MMPs, including mechanisms of catalytic activation, substrate targeting, and strategies to inhibit their enzymatic activity, have been extensively investigated, and it is clear that they regulate biological responses via enzymatic cleavage of protein substrates.3

Less is known regarding other actions of MMPs, but emerging evidence suggests that these enzymes also regulate cellular signaling events.5 In the present study, we show that platelet activation triggers cleavage of endogenous pro-MMP-1, a portion of which is on the cell surface, generating its active form. Active MMP-1, from either endogenous or exogenous sources, induces tyrosine phosphorylation of intracellular proteins, clusters β3 integrins to the cell periphery, and primes platelets for aggregation. Despite modulating these events, active MMP-1 did not have appreciable effects on platelet shape change, intracellular calcium fluxes, trans-
demonstrate that MMP-1 colocalizes with thrombospondin in platelets, as described in Materials and Methods. Panels A through C are representative of 3 independent experiments.

location of P-selectin to the cell surface, or thromboxane synthesis (data not shown), indicating that MMP-1 regulates distinct platelet responses.

The complete mechanism by which MMP-1 delivers outside-in signals to intracellular tyrosine kinase pathways in platelets is not known. Agonists that induce similar responses, such as ADP, collagen, platelet-activating factor, thromboxane A₂ and thrombin, deliver signals through cell surface receptors.

It is possible the MMP-1 elicits signals through a specific plasma membrane receptor that has not yet been identified. In other cell types, pro-MMP-1 directly binds α₃β₁ integrins and regulates cell migration.

MMP-2 binds α₅β₃ and ligation to α₅β₃ integrins facilitates MMP-2 activation.

We found that MMP-1 colocalizes with β₃ integrins in activated platelets at points of intercellular contact (see Figure 4). We have also coimmunoprecipitated MMP-1 with β₃ integrins in preliminary studies (data not shown). However, numerous proteins sediment with β₃ integrins in coimmunoprecipitation studies, and we do not yet know whether a specific protein-protein interaction occurs between MMP-1 and α₅β₃ (authors’ unpublished data, 2001). Although this remains to be determined, our data demonstrate that MMP-1 colocalizes with α₅β₃ and regulates the spatial distribution of this integrin in activated platelets.

We found that proteolytically active MMP-1 is required for signaling. Deletion of the catalytic site or neutralization of exogenous or endogenous MMP activity, prevents MMP-1 from inducing functional responses in platelets. Other investigators have demonstrated that ECM proteinases release cadherins and syndecans from the cytoskeleton and, consequently, alter signaling events.

MMP-1 may be acting in a similar fashion by influencing the association of β₃ integrins with the complex cytoskeleton that underlies platelet membranes.

We show that a portion of MMP-1, although typically regarded as a secreted protein, is located at the plasma membrane of platelets, ideally positioning itself for proteolytic modification of membrane proteins, such as integrin α₅β₃ or its binding partners. In other cell types, enzymatic modification by ECM proteinases alters integrin anchorage, focal adhesions, cytoskeletal architecture, and a number of signaling molecules. Recent evidence demonstrates that overexpression of MT1-MMP in nucleated cells processes the α subunit of α₅β₃. Cleavage of this subunit enhances tyrosine phosphorylation of focal adhesion kinase. It is possible that processing of latent MMP-1 to its active form in platelets triggers the proteolytic modification of transmembrane receptors and/or adhesion molecules that are directly linked to intracellular tyrosine kinase pathways (Figure 2).

Numerous studies have demonstrated that tyrosine kinase inhibitors block agonist-induced platelet aggregation. This suggests that activation of intracellular tyrosine kinase pathways may be a mechanism by which MMP-1 primes platelets for aggregation. Using a mutated integrin, Hato et al demonstrated that clustering of α₅β₃ integrins induces intracellular tyrosine phosphorylation events that enhance ligand binding and cell adhesion. We found that exogenous MMP-1 clusters β₃ integrins on the cell surface and primes platelets for aggregation without inducing detectable conformational changes in α₅β₃ (see online Figure 1). Although MMP-1 did not alter the conformation state of α₅β₃, there is evidence that MMP-2 regulates interactions between fibrinogen and its receptor as well as glycoprotein Ib and von Willebrand factor, suggesting that MMPs may directly modify cell surface integrins. The targeting of β₃ integrins to cell contact areas by MMP-1 was associated with increased intracellular tyrosine phosphorylation. From our studies, it is not clear whether MMP-1 clusters surface integrins and then elicits tyrosine kinase cascades or whether the converse holds true.

Figure 3. MMP-1 primes platelets for enhanced aggregation. A, Degradation of collagen fibrils by purified APMA-free active MMP-1 (75 ng/mL) in the presence of Inh 1, MMP-3 inhibitor, and blocking (B) or nonblocking (NB) antibodies directed against MMP-1 (see Materials and Methods). B, Left, Platelets were pretreated for 1 minute with active MMP-1 (75 ng/mL) or buffer alone and, subsequently, were stimulated with a submaximal concentration of Thr (0.01 U/mL). Platelet aggregation was measured as described in Materials and Methods. B, Right, Platelets were pretreated for 1 minute with active MMP-1 (75 ng/mL), which was coincubated with either B antibody or NB antibody directed against MMP-1. The cells were subsequently stimulated with a submaximal concentration of Thr (0.01 U/mL). Platelet aggregation was measured as described in Materials and Methods. C, Platelets were pretreated with either Inh 1 or buffer alone. The cells were left quiescent (baseline) or were stimulated with active MMP-1 (75 ng/mL) for 1 minute, and β₃ integrins were identified on the plasma membrane of platelets, as described in Materials and Methods. Panels A through C are representative of 3 independent experiments.
Nevertheless, integrin clustering enhances and stabilizes adhesive interactions mediated by a conformational change in $\alpha_{IIb}\beta_3$, a likely mechanism underlying the priming effect of exogenous MMP-1 on platelet aggregation (Figure 3). The proaggregatory characteristics of MMP-1 are strikingly similar to those of phosphatidylinositol 3-kinase (PI3K), which also associates with focal adhesions during platelet aggregation. Like MMP-1, PI3K does not directly activate $\alpha_{IIb}\beta_3$, nor does it increase fibrinogen binding. Rather, it regulates the clustering of surface integrins. PI3K controls clustering through cytoskeletal changes that regulate the translocation and topography of key platelet molecules and organelles within the cell. In additional experiments, we found that neutralization of endogenous MMP-1 activity in thrombin-stimulated platelets prevents $\beta_3$ integrins from associating with the actin-rich cytoskeleton (data not shown). These results suggest that MMP-1 may target $\beta_3$ integrins to areas of cell contact by modifying cytoskeletal responses within the cell.

The display of endogenous MMP-1 on the surfaces of platelets is consistent with observations indicating that many cell types, particularly cancer cells, express MMPs on their plasma membranes even though the proteinases are soluble. Previously, it has been reported that platelets contain collagenolytic activity, that 59% of platelet collagenase is confined to the plasma membrane, and that collagenolytic activity increases with platelet activation. Although the authors were unable to identify the specific enzyme at that time, our data demonstrate that a portion of the collagenolytic activity is due to MMP-1. MMP-2 and MT1-MMP have also been found in platelets. Like MMP-1, MMP-2 has been shown to regulate platelet aggregation, but the mechanisms involved are not yet defined. It is possible that multiple MMPs cooperate with one another to initiate intracellular signaling cascades that control adhesive functions of platelets.

The fact that MMPs alter platelet aggregation suggests that neutralization of their activity may have clinical efficacy, especially because the proaggregatory effect of MMP-1 is independent of fibrinogen binding to $\alpha_{IIb}\beta_3$. This indicates that MMP inhibitors regulate platelet aggregation through a
mechanism that is different from clinically available α5β1 antagonists abciximab (reopro) and eptifibatide (integrin). Moreover, neutralization of MMP-1 in activated platelets leaves many signaling pathways intact, and high concentrations of agonists can override the antiaggregatory properties of MMP inhibitors. Inhibition of selective responses without globally blocking platelet function is similar to the effects of low-dose aspirin (a drug that is commonly used in clinics to decrease thrombotic episodes) on platelets. MMP inhibitors may also be effective "anti-platelet" compounds that precisely blunt aggregation with only a slight decline in normal hemostatic function.

Acknowledgments
This study was supported by grants from the NIH (HL-66277 to Dr Weyrich and HL-44525 to Dr Zimmerman), an Atrorvastatin Research Award (Dr Weyrich), and the Department of Veteran Affairs Medical Research funds (Dr Galt). We thank Mary Madsen for help with the project, and the HMBG graphic staff (Diana Lim for preparing this manuscript. We are grateful to our colleagues in the Medical Research funds (Dr Galt). We thank Mary Madsen for help with the project, and the HMBG graphic staff (Diana Lim, London, UK) for discussing the manuscript and kindly providing neutralization of P-selectin protects feline heart and endothelium in myo-

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Circ Res. 2002;90:1093-1099; originally published online April 25, 2002; doi: 10.1161/01.RES.000019241.12929.EB

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplementary Figure Legends

Supplementary Figure 1. Active MMP-1 does not alter binding of a conformation-dependent antibody against \( \alpha_{\text{III}}\beta_3 \). Platelets were isolated as described in the Methods section and stirred in the aggregometer for each study. Stirred platelets were left unstimulated (control; top left panel), were treated with 150 ng/ml of active MMP-1 (top right panel), or were stimulated with thrombin (0.1 U/ml) for 1 minute in the presence (bottom right panel) or absence (bottom left panel) of MMP-Inhibitor 1 (10 \( \mu \)M). The platelets were subsequently incubated with PAC-1, an anti-\( \alpha_{\text{III}}\beta_3 \) antibody that only recognizes the integrin after its conformational change, and examined by flow cytometry as described in the Methods section. This supplemental figure is representative of four independent experiments.

Supplementary Figure 2. Active MMP-1 does not alter binding of exogenous fibrinogen to platelets. Platelets were isolated as described in the Methods section and stirred in the aggregometer for each study. Stirred platelets were left unstimulated (control), were treated with active MMP-1 (150 ng/ml), or were stimulated with 0.1 U/ml of thrombin for 1 minute in the presence (Thr + Inh 1) or absence of MMP-Inhibitor 1 (10 \( \mu \)M). \(^{125}\text{I}-\text{fibrinogen} \) was incubated with the platelets and surface-bound fibrinogen was quantitated as described in the Methods section. Supplemental figure 2 is the mean±SEM of three independent experiments.
Supplementary figure 1
Galt et al.
Supplementary figure 2
Galt et al.