Loss of matrix metalloproteinase 2 in platelets reduces arterial thrombosis in vivo

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Platelet activation at a site of vascular injury is essential for the arrest of bleeding; however, excessive platelet activation at a site of arterial damage can result in the unwarranted formation of arterial thrombi, precipitating acute myocardial infarction, or ischemic stroke. Activation of platelets beyond the purpose of hemostasis may occur when substances facilitating thrombus growth and stability accumulate. Human platelets contain matrix metalloproteinase 2 (MMP-2) and release it upon activation. Active MMP-2 amplifies the platelet aggregation response to several agonists by potentiating phosphatidylinositol 3-kinase activation. Using several in vivo thrombosis models, we show that the inactivation of the MMP-2 gene prevented thrombosis induced by weak, but not strong, stimuli in mice but produced only a moderate prolongation of the bleeding time. Moreover, using cross-transfusion experiments and *wild-type/MMP-2*$^{-/-}$ chimeric mice, we show that it is platelet-derived MMP-2 that facilitates thrombus formation. Finally, we show that platelets activated by a mild vascular damage induce thrombus formation at a downstream arterial injury site by releasing MMP-2. Thus, platelet-derived MMP-2 plays a crucial role in thrombus formation by amplifying the response of platelets to weak activating stimuli. These findings open new possibilities for the prevention of thrombosis by the development of MMP-2 inhibitors.

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Abbreviations used: MMP2, matrix metalloproteinase 2; mRNA, messenger RNA; PI3K, phosphatidylinositol 3-kinase; PRP, platelet-rich plasma.

Ischemic cardiovascular disorders, the leading cause of death and disability worldwide (Mathers and Loncar, 2006), are generated by occlusive arterial thrombi that are principally formed by activated platelets (Davì and Patrono, 2007). Enhanced platelet reactivity, which is associated with several pathological states such as dyslipidemia, diabetes, obesity, hypertension, or smoke, plays an important role in the pathophysiology of occlusive arterial thrombi (Davì et al., 1997; Kabbani et al., 2001; Davì and Patrono, 2007). In fact, the activation of platelets by the exposed subendothelial surface may proceed beyond the purpose of hemostasis and lead to occlusive thrombus formation when an excessive accumulation of substances facilitating thrombus growth and stability takes place (Davì et al., 1997; Kabbani et al., 2001; Weyrich et al., 2007; Brass et al., 2008). Several molecules that facilitate the activation of platelets by primary stimuli, potentially transforming a normal hemostatic response in the formation of an occlusive thrombus, have been described recently (Podrez et al., 2007; Gresele et al., 2008a).

For most of these substances, a priming activity on platelets has been characterized only in vitro (Brass et al., 2008; Gresele et al., 2008b) and little evidence is available so far for a role of this class of molecules in regulating platelet function in vivo (Gresele et al., 2008a). Moreover, a pathogenic role of platelet hyperresponsiveness in thrombus formation in vivo has so far only been hypothesized and not demonstrated.

Matrix metalloproteinase 2 (MMP-2), the most abundant MMP which is constitutively expressed in cells of mesenchymal origin (Galis and Khatri, 2002), is contained in platelets and released upon aggregation and displays a potentiating effect on platelet activation in vitro (Sawicki et al., 1997; Kazes et al., 2000; Falcinelli

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et al., 2005). MMP-2 is randomly distributed in the cytosol of resting human platelets and is then translocated to the extracellular space during aggregation (Sawicki et al., 1998). Active MMP-2, but not its proenzyme, amplifies the platelet aggregation response to weak concentrations of a range of agonists acting on different receptors, such as U46619 (TxA_2/PGH_2 receptor agonist), ADP, or thrombin (Sawicki et al., 1997; Kazes et al., 2000; Falcinelli et al., 2005) by functionally potentiating phosphatidylinositol 3-kinase (PI3K) activation (Falcinelli et al., 2005). Enhanced activation of PI3K, in turn, magnifies the triggering of several other signaling events, like protein kinase C activation, calcium transients, and, ultimately, integrin $\alpha_{IIb}\beta_3$ activation, ending in the enhanced expression of fibrinogen-binding sites (Falcinelli et al., 2005; Choi et al., 2008; Santos-Martínez et al., 2008). The concentrations of MMP-2 that exert this priming activity are in the range of those secreted by activated platelets in vitro (Falcinelli et al., 2005). Moreover, the release of MMP-2 by activated platelets, in amounts similar to those found to potentiate platelet activation, occurs in vivo in healthy humans during primary hemostasis (Falcinelli et al., 2007) or in the coronary bed of patients with acute coronary syndromes during myocardial ischemia (Falcinelli, E., M. Leone, G. Cimmino, T. Corazzi, P. Golino, and P. Gresele. 2005. Congress of the International Society on Thrombosis and Haemostasis. Abstr. OR370).

Altogether, these observations suggest that MMP-2 may play a role in the regulation of platelet activation in vivo by amplifying the response to weak stimuli and, thus, leading to unrestrained thrombus formation (Gresele et al., 2008b). In this paper, using *MMP-2*$^{-/-}$ and chimeric mice, we provide the first genetic evidence that platelet-released MMP-2 plays a role in promoting arterial thrombosis in vivo and that this represents one of the mechanisms producing a prothrombotic phenotype and transforming a normal hemostatic response to a vessel injury in the unwarranted formation of an occlusive thrombus. These findings identify the effect of MMP-2 on blood platelets as a novel potential therapeutic target for the prevention of thrombosis.

RESULTS

Phenotype and genotype data

Zymography of plasma, platelet lysates, and lung extracts confirmed that MMP-2 is not expressed in MMP-2 knockout mice. On the contrary, in WT mice MMP-2 was detectable by zymography in all three types of biological samples (unpublished data). Pro–MMP-2 was present in platelet lysates (43.9 ± 2.1 ng/10^8 platelets) from WT mice, and thrombin, collagen, or ADP induced a rapid increase in the amount of active MMP-2 in the supernatant to a maximal value of 36.4 ± 2.5 ng/10^8 platelets. As expected, active MMP-2 was not detected in the supernatant of platelets from *MMP-2*$^{-/-}$ mice (unpublished data).

RT-PCR of spleen extracts confirmed that WT mice, but not *MMP-2*$^{-/-}$ mice, contain MMP-2 messenger RNA (mRNA). MMP-2 mRNA was reduced by 51% in spleen extracts from *MMP-2*$^{+/-}$ mice, as compared with WT mice. The expression of mRNA for MMP-9, TIMP-1, and TIMP-2 was also evaluated in mouse spleens, and it was found that although mRNA for MMP-9 was not significantly different between *MMP-2*$^{-/-}$, *MMP-2*$^{+/-}$, and WT mice, mRNA for TIMP-1 and TIMP-2 was reduced both in heterozygous (30% reduction for both) and in homozygous *MMP-2*$^{-/-}$ mice (36 and 33% reduction, respectively).

MMP-2$^{-/-}$ mice have hyporeactive platelets

MMP-2$^{-/-}$ mice had a normal platelet count (949.000 ± 68.000 vs. 1,047.000 ± 22.630 platelets/μl of WT mice; $n = 10$; P = NS) and platelets appeared ultrastructurally normal at electron microscopy (Fig. S1). Surface expression of the major platelet glycoproteins (GPIIb/IIIa, GPIbα, GPVI, and CD9) did not differ between *MMP-2*$^{-/-}$ and WT mice. Platelet ATP content was similar in WT and *MMP-2*$^{-/-}$ mice (17.5 ± 1.7 and 18.8 ± 0.75 nmol/10^8 platelets, respectively; $n = 5$). On the contrary, although platelet lysates of WT mice contained MMP-2 (43.9 ± 2.1 ng/10^8 platelets; $n = 5$), platelets from *MMP-2*$^{-/-}$ were completely devoid of it (<0.1 ng/10^8 platelets; $n = 5$). 0.1 U/ml thrombin induced a rapid release of active MMP-2 in the supernatant of WT platelets (36.4 ± 2.47 ng/10^8 platelets; $n = 3$) but not of platelets of *MMP-2*$^{-/-}$ mice. A time course of the appearance of active MMP-2 on the surface of platelets upon activation was performed with gel-filtered WT mouse platelets by flow cytometry, using an antibody that specifically recognizes active MMP-2. Resting platelets did not express active MMP-2 on their surface. The expression of active MMP-2 on mouse platelets was evident already at 30 s (baseline = 2.5 ± 0.3%, 30 s = 9.3 ± 0.2% of positive platelets) after thrombin stimulation, reached a maximum at 2 min (21.4 ± 1.04% of positive platelets), and remained expressed for up to 15 min ($n = 3$).

The ability of 0.5 ng/ml of human active MMP-2 to potentiate mouse platelet aggregation and the time course of this effect was evaluated with washed platelets from WT mice. With all the agonists used (0.8 μg/ml collagen, 3 μM U46619, and 0.05 U/ml of purified human α-thrombin), the potentiation of platelet aggregation was maximal within 2 min and decreased thereafter to disappear in 30 min (Table S1). Platelets of *MMP-2*$^{-/-}$ mice revealed a significant functional defect. In fact, although platelets from WT mice, as well as from *MMP-2*$^{+/-}$ mice, aggregated dose dependently in response to ADP (0.2, 0.4, 2, and 10 μM), collagen (2, 5, and 10 μg/ml), arachidonic acid (0.1, 0.4, and 0.6 mM), epinephrine (10 and 100 μM), U46619 (3 and 4 μM), and purified human α-thrombin (0.05 and 0.1 U/ml), platelets from *MMP-2*$^{-/-}$ mice failed to aggregate in response to the lowest concentrations of each of the agonists tested, and only the highest concentrations induced aggregation (Fig. 1 A; Table S2).

Secretion of dense granules in response to ADP or arachidonic acid (evaluated by the release of ATP) was also impaired in platelets from *MMP-2*$^{-/-}$ mice (Fig. 1 B; Table S2). Similarly, the expression on the platelet surface of P-selectin, an α-granule protein, in response to collagen, thrombin, or ADP was reduced in platelets from *MMP-2*$^{-/-}$ mice (Fig. 1 C; Table S2).

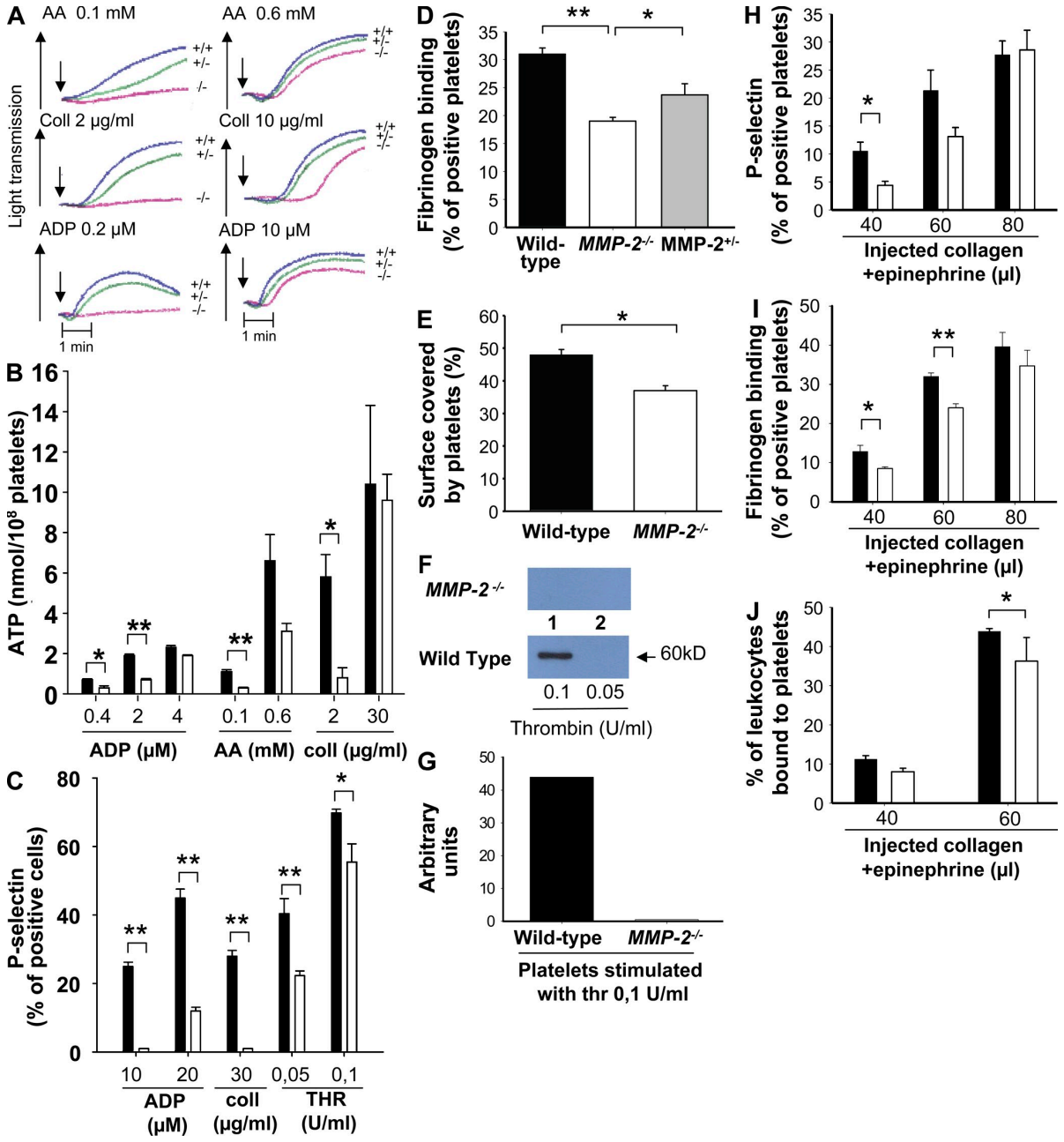

Figure 1. Impaired platelet function in *MMP-2*$^{-/-}$ mice. (A) Aggregation of platelets from WT mice (blue, $^{+/+}$) and from heterozygous (green, $^{+/-}$) or homozygous (red, $^{-/-}$) MMP-2–deficient mice. PRP from four to six animals was pooled and stimulated with different agonists. Platelets from *MMP-2*$^{-/-}$ mice did not aggregate in response to low concentrations of arachidonic acid, collagen, or ADP, which was different from platelets of WT mice, whereas higher concentrations of the stimuli induced irreversible aggregation. Platelets from *MMP-2*$^{+/-}$ mice aggregated almost normally in response to the agonists used. Arrows: addition of the agonist. Platelet aggregation tracings are representative of four independent experiments. (B and C) Secretion of dense granules in response to ADP, collagen (coll), or arachidonic acid (AA), assessed by the release of ATP (B), as well as secretion of α-granules, evaluated by platelet P-selectin expression by flow cytometry (C), were defective with *MMP-2*$^{-/-}$ platelets. (D) Fibrinogen binding to platelets activated by 10 µM ADP and assessed by flow cytometry was also impaired with platelets from *MMP-2*$^{-/-}$ mice as compared with WT platelets. (E) The adhesion of *MMP-2*$^{-/-}$ platelets to a collagen-coated surface, under flow conditions (3000 s^{-1}), was reduced as compared with WT platelets ($n = 6$). (F) Western blotting of phospho–Akt Ser^{473}. Akt phosphorylation, induced by stimulation with thrombin, was almost absent with MMP-2$^{-/-}$ platelets, whereas it was evident with WT platelets with the highest dose of thrombin. (G) Akt phosphorylation. Data from lane 1 in F were quantified by densitometry and expressed as arbitrary units. (H–J) Expression of P-selectin (H) or fibrinogen (I) on platelets, and circulating platelet-leukocyte aggregates (J), in blood collected 2 min after the i.v. injection of increasing volumes of a mixture of collagen plus epinephrine in WT and *MMP-2*$^{-/-}$ mice. *MMP-2*$^{-/-}$ mice show defective in vivo platelet activation and platelet-leukocyte aggregate formation as compared with WT animals, especially when low doses of the platelet-activating challenge are used. Results in B–D, H, J, and K are obtained from three independent experiments. Error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$.

Table I. Platelet activation in vivo in collagen plus epinephrine-challenged WT and *MMP-2*$^{-/-}$ mice

Parameter	Collagen plus epinephrine	WT mice	*MMP-2*$^{-/-}$ mice	Reduction in *MMP-2*$^{-/-}$ versus WT mice	P-value
	μl	%	%	%	
P-selectin	80	27.7 ± 2.5 (*n* = 5)	28.6 ± 3.5 (*n* = 5)	0	NS
	60	21.3 ± 3.7 (*n* = 5)	13.1 ± 1.6 (*n* = 5)	38.5	NS
	40	10.5 ± 1.6 (*n* = 8)	4.4 ± 0.7 (*n* = 8)	58	0.01315
Platelet/leukocyte aggregates	60	43.84 ± 0.79 (*n* = 6)	36.2 ± 6.05 (*n* = 4)	17	NS
	40	11.2 ± 0.9 (*n* = 10)	8 ± 0.9 (*n* = 10)	27.3	0.015
Fibrinogen binding	80	39.6 ± 3.67 (*n* = 5)	34.7 ± 4.01 (*n* = 5)	12.4	NS
	60	32 ± 0.91 (*n* = 5)	24 ± 1.06 (*n* = 5)	25	0.00128
	40	12.8 ± 1.62 (*n* = 5)	8.5 ± 0.44 (*n* = 5)	33.6	0.018

P-selectin (expressed as percentage of positive platelets): basal value, 4.9 ± 0.8% of positive cells; Platelet/leukocyte aggregates (expressed as percentage of leukocytes bound to platelets): basal value, 6.3 ± 0.6% of leukocytes bound to platelets; Fibrinogen binding (expressed as percentage of positive platelets): basal value, 3.3 ± 0.14% of positive cells.

Serum thromboxane B_2 levels did not differ between WT and *MMP-2*$^{-/-}$ mice (WT, 95 ± 13.6 ng/ml; *MMP-2*$^{-/-}$, 92 ± 10.1 ng/ml; $n = 5$; P = NS), excluding defective arachidonic acid metabolism as a cause of impaired secretion. Fibrinogen binding to platelets stimulated in vitro with 10 μM ADP was significantly reduced in *MMP-2*$^{-/-}$ as compared with WT mice, whereas platelets from *MMP-2*$^{+/-}$ mice showed an intermediate value (Fig. 1 D). Platelet adhesion to collagen, under flow conditions at high shear rate, was significantly lower in *MMP-2*$^{-/-}$ mice as compared with WT mice (Fig. 1 E). The phosphorylation of Akt, the principal downstream effector of the PI3K signaling pathway, induced by stimulation with thrombin was almost absent in platelets from *MMP-2*$^{-/-}$ mice as compared with platelets from WT mice (Fig. 1, F and G).

The expression of P-selectin on circulating platelets was enhanced dose dependently by the i.v. injection of a mixture of collagen plus epinephrine, significantly more so in WT mice than in *MMP-2*$^{-/-}$ mice (Fig. 1 H; Table I). Similarly, fibrinogen binding to platelets induced by the i.v. injection of collagen plus epinephrine was also lower in *MMP2*$^{-/-}$ as compared with WT mice (Fig. 1 I; Table I). Finally, platelet/leukocyte aggregates circulating in whole blood were enhanced by the i.v. injection of collagen plus epinephrine significantly more in WT mice than in *MMP-2*$^{-/-}$ mice (Fig. 1 J; Table I).

MMP-2$^{-/-}$ mice have a defective thrombotic response and mildly impaired hemostasis

The i.v. injection of collagen plus epinephrine in WT mice induced platelet pulmonary thromboembolism, with a dose-dependent increase of mortality of the number of lung vessels occluded by platelet thromboemboli and a marked drop of the number of circulating platelets. *MMP-2*$^{-/-}$ mice were partially protected against platelet pulmonary thromboembolism. This was more evident when low doses of the thrombogenic challenge were used, whereas the thromboembolic response was close to normal when a strong stimulus was used (Fig. 2 A). Histological analysis of slices of lungs obtained 2 min after the i.v. injection of collagen plus epinephrine showed that a significantly higher number of lung vessels were totally or partially occluded by platelet thrombi in WT animals than in *MMP-2*$^{-/-}$ mice when low doses of the thrombotic challenge were used (Fig. 2, B and C). The injection of collagen plus epinephrine dose-dependently induced a drop of the platelet count that was stronger in WT mice as compared with *MMP-2*$^{-/-}$ mice, indicating that in vivo activation and sequestration was more sustained in WT than in knockout mice (Fig. 2 D).

Photochemically induced thrombosis of the femoral artery was correlated with the dose of rose Bengal infused. An occluding thrombus occurred after 13 ± 2.4 min ($n = 5$) and 7.6 ± 0.5 min ($n = 5$) from the beginning of the irradiation with 20 or 50 mg/kg of rose Bengal, respectively, in WT mice. In *MMP-2*$^{-/-}$ mice, instead, the infusion of the lowest dose of rose Bengal (20 mg/kg) did not lead to the development of an occluding thrombus (>30 min; $n = 7$; P < 0.01 vs. WT), whereas blood flow stopped in 8.6 ± 1.6 min ($n = 5$; P = NS vs. WT) when the strongest thrombotic stimulus was used (50 mg/kg of rose Bengal; Fig. 2 E). Thrombus size was 0.33 ± 0.06 mg with 20 mg/kg of rose Bengal and 0.86 ± 0.17 mg with 50 mg/kg of rose Bengal (+165%; $n = 5$) in WT mice. When the weaker thrombogenic stimulus was used (20 mg/kg of rose Bengal), thrombus weight was particularly reduced in *MMP-2*$^{-/-}$ mice, to 78% smaller on average than in WT mice, whereas it was only 28% smaller with the strongest stimulus (Fig. 2 F). Bleeding time was 175 ± 14 s (median 155 s; 95% confidence intervals [CIs] 143.3–205.7 s) in WT mice, 306.5 ± 8.6 s (median 197.5 s; 95% CIs 124.2–488.8 s) in heterozygous mice (*MMP-2*$^{+/-}$; P < 0.01 vs. WT), and 555.5 ± 97.7 s (median 452.5 s; 95% CIs 334.5–776.5 s) in *MMP-2*$^{-/-}$ (P < 0.0001 vs. WT and P < 0.001 vs. heterozygous; Fig. 2 G).

Human pro–MMP-2 restores platelet function of MMP-2$^{-/-}$ mice

To confirm that pro–MMP-2 is rapidly transformed into active MMP-2 at the surface of activated mouse platelets, as was previously shown for human platelets (Kazes et al., 2000), gel-filtered platelets from *MMP-2*$^{-/-}$ mice were preincubated with 100 ng/ml of human pro–MMP-2 and stimulated

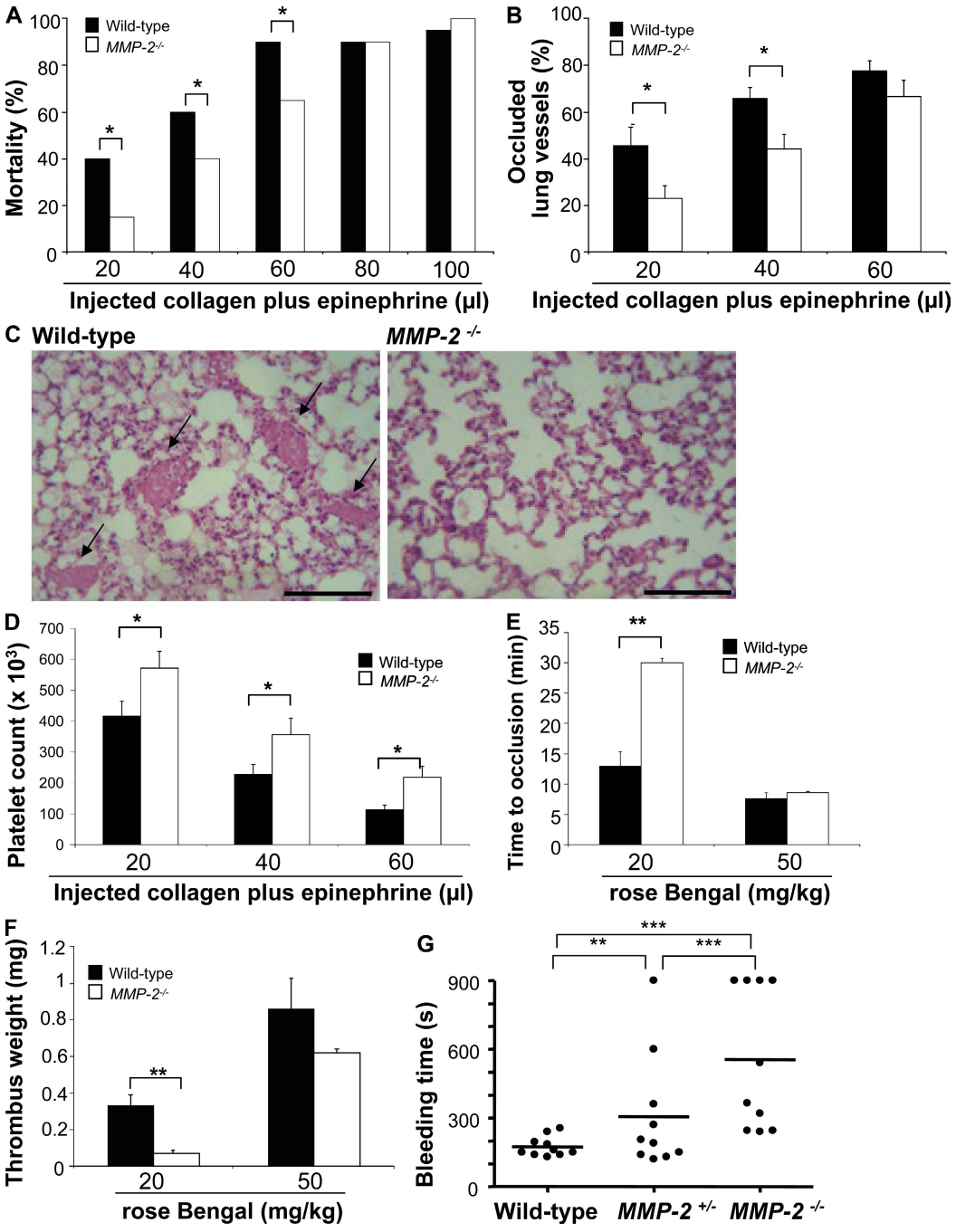

Figure 2. ***MMP-2*$^{-/-}$ mice have a defective thrombotic response.** (A) Pulmonary thromboembolism-associated mortality induced by the i.v. injection of increasing volumes of collagen plus epinephrine in WT and *MMP-2*$^{-/-}$ mice ($n = 20$ per group; *, $P < 0.05$ vs. WT). Mortality was lower in *MMP-2*$^{-/-}$ mice with the lower, but not with the higher, doses of the thrombotic challenge. Error bars represent SEM. (B) Number of lung vessels occluded by platelet emboli in WT and in *MMP-2*$^{-/-}$ mice 2 min after the injection of collagen plus epinephrine ($n = 5$ mice per group). Numbers represents means ± SEM of the percentage of occluded vessels out of the total number of lung vessels counted in 10 microscopic fields for each lung section. *, $P < 0.05$ versus WT. (C) Light microscopy (hematoxylin and eosin staining) of lungs after injection of 40 µl collagen plus epinephrine reveals platelet-rich thrombi (arrows) occluding lung vessels in WT mice but not in *MMP-2*$^{-/-}$ mice. Bars, 100 µm. (D) Platelet count in WT and *MMP-2*$^{-/-}$ mice 2 min after the i.v. injection of different doses of collagen plus epinephrine ($n = 5$ mice per group; $P < 0.05$ vs. WT). (E and F) Photochemically induced femoral artery thrombosis, as assessed by time-to-occlusion measured by laser Doppler (E) or as weight of the thrombus (F), was correlated to the amount of rose Bengal infused. *MMP-2*$^{-/-}$ mice showed markedly impaired thrombosis with the weaker stimulus (20 mg/kg of rose Bengal) but not with the strongest stimulus (50 mg/kg). Data are means ± SEM ($n = 5$ mice per group). (G) Tail-bleeding time in WT ($n = 10$), *MMP-2*$^{+/-}$ ($n = 10$), and *MMP-2*$^{-/-}$ mice ($n = 10$). Results in A are obtained from four experiments; results in B and D–G are obtained from three experiments; and results in C are representative of three experiments. Horizontal bars indicate the mean value. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

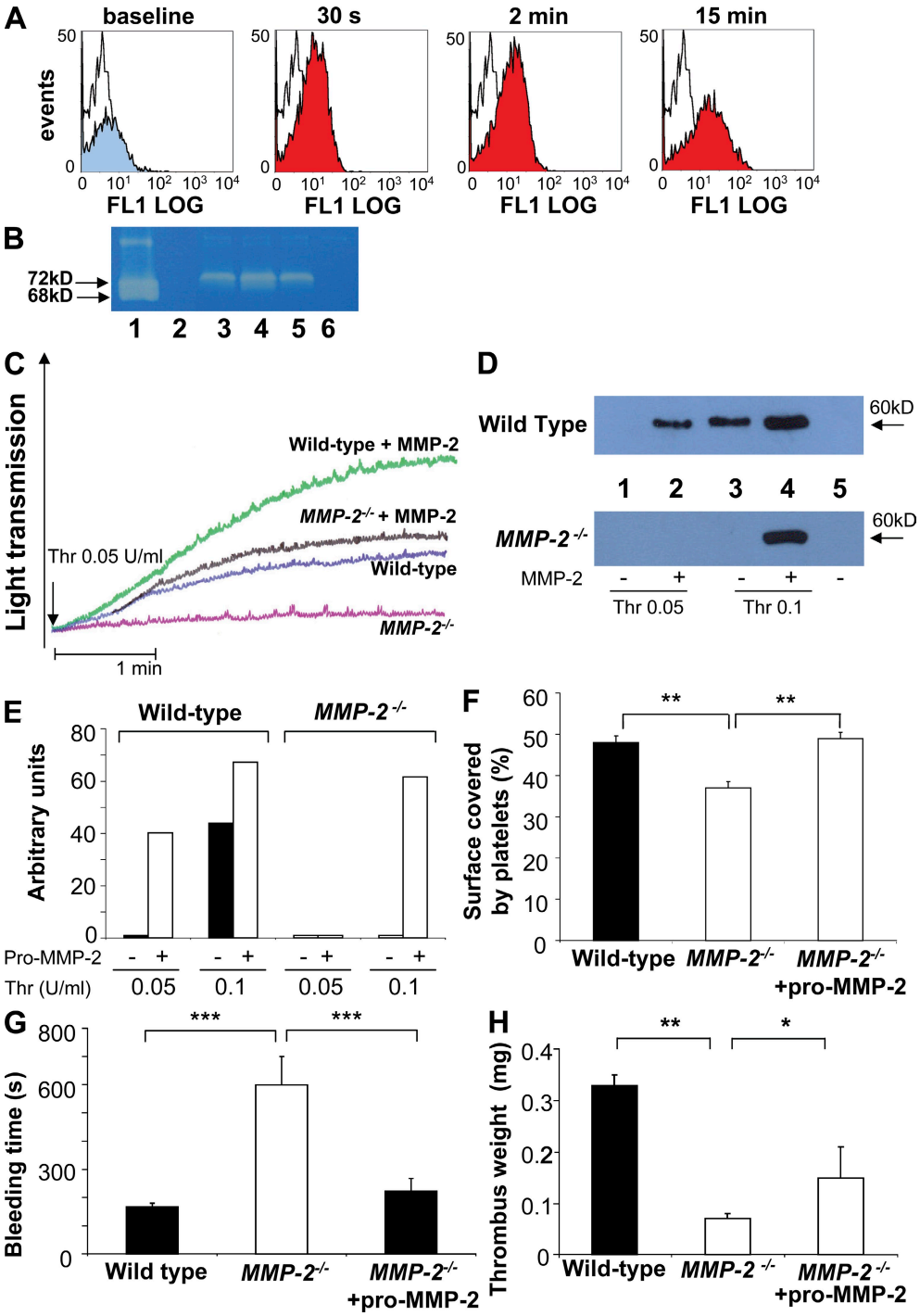

Figure 3. Human pro–MMP-2 restores platelet function of MMP-2 mice. Expression of active MMP-2 by mouse platelets. (A) The addition of 100 ng/ml of human pro–MMP-2 to gel-filtered *MMP-2*$^{-/-}$ platelets, followed by stimulation with human α-thrombin, led to the expression of active MMP-2 at the platelet surface at only 30 s after stimulation, reached a maximum at 2 min, and remained expressed at up to 15 min. Representative examples of flow cytometry plots of platelets stained with a specific rabbit anti–mouse active MMP-2 antibody: active MMP-2 expression at baseline, 30 s, and 2 and 15 min after stimulation. White, aspecific IgG antibody; blue, gel-filtered *MMP-2*$^{-/-}$ platelet baseline; red, expression of active MMP-2 on the surface of α-thrombin–activated gel-filtered *MMP-2*$^{-/-}$ platelets ($n = 3$). (B) 100 ng/ml of human pro–MMP-2 was added to gel-filtered platelets of *MMP-2*$^{-/-}$ mice. Active MMP-2 was released in the supernatant of 0.1 U/ml of α-thrombin–activated gel-filtered platelets 2 min after stimulation (lane 1, mouse MMP-2 standard; lane 2, sample buffer; lane 3, platelet supernatant 15 min after simulation; lane 4, platelet supernatant 2 min after simulation; lane 5, supernatant of resting platelets plus pro–MMP-2; lane 6, supernatant of resting platelets from *MMP-2*$^{-/-}$ mice). (C)The addition of 100 ng/ml of human pro–MMP-2 to PRP of *MMP-2*$^{-/-}$ mice (red), 2 min before stimulation with 0.05 U/ml thrombin, restores platelet aggregation (black) to a level similar to

with human α-thrombin. Active MMP-2, which is not present on resting platelets, was already detectable at their surface 30 s after stimulation, reached a maximum at 2 min, and remained expressed at up to 15 min, as revealed by flow cytometry (Fig. 3 A). Gelatinolytic activity at 68 kD (active MMP-2) was visualized by zymography in the supernatant of gel-filtered platelets from *MMP-2*$^{-/-}$ mice incubated with 100 ng/ml of human pro–MMP-2 and stimulated with α-thrombin 2 min after stimulation (Fig. 3 B).

The addition of 100 ng/ml of human pro–MMP-2 to mouse platelet-rich plasma (PRP), 2 min before challenge with thrombin, potentiated platelet aggregation in both WT and *MMP-2*$^{-/-}$ mice (Fig. 3 C). Preincubation of mouse-washed platelets with human 100 ng/ml of pro–MMP-2, 2 min before 0.1 U/ml thrombin, enhanced Akt phosphorylation in platelets from both WT (+53%) and *MMP-2*$^{-/-}$ (+61.6%) mice (Fig. 3, D and E). The plasmatic half-life of human recombinant pro–MMP-2, assessed after the i.v. injection of 500 ng of the proenzyme in *MMP-2*$^{-/-}$ mice, was 19 min. Accordingly, in the subsequent experiments assessing the in vivo effects of human pro–MMP-2, all tests were performed between 2 and 15 min from its i.v. injection. The injection of 100 ng/mouse of human pro–MMP-2 into *MMP-2*$^{-/-}$ mice, 2 min before blood sampling, normalized ex vivo platelet adhesion (from 37 ± 1.5 to 48.7 ± 1.5% of surface covered; $n = 5$; $P < 0.01$; Fig. 3 F). The i.v. injection of 100 ng/mouse of human pro–MMP-2, 15 min earlier, shortened the bleeding time in *MMP-2*$^{-/-}$ mice (from 599.2 ± 74.1 to 222 ± 47 s; $n = 10$; Fig. 3 G). The thrombotic defect of *MMP-2*$^{-/-}$ mice upon photochemically induced femoral artery injury was partially reversed by the i.v. injection of 100 ng/mouse of purified pro–MMP-2 ($n = 5$; Fig. 3 H). In WT mice that were infused with a low dose of Rose Bengal (20 mg/kg), the injection of 100 ng/mouse of human pro–MMP-2 significantly shortened the time to occlusion (from 13 ± 2.4 to 6.9 ± 0.6 min; $n = 5$; $P < 0.01$), whereas it did not exert any appreciable effect in WT mice infused with 50 mg/kg of rose Bengal (from 7.6 ± 0.5 to 6.4 ± 0.8 min; $n = 5$; P = NS).

Role of platelet-derived MMP-2 in hemostasis and thrombosis

To establish the contribution of MMP-2 derived from platelets in the hemostatic and thrombotic defect observed in *MMP-2*$^{-/-}$ mice, cross-transfusion experiments were performed (Pitchford et al., 2004). WT mice that were made deeply thrombocytopenic (platelet count <5% of basal) by the i.v. injection of a rabbit anti–mouse platelet antiserum had a very prolonged bleeding time (≥900 s). The reconstitution of an almost normal platelet count by the transfusion of platelets from WT mice shortened the bleeding time by 47.5% (473 ± 89 s; $P < 0.01$; $n = 11$), whereas the reconstitution of the platelet count by the transfusion of platelets from *MMP-2*$^{-/-}$ mice shortened the bleeding time by only 12% (785.5 ± 61 s; $n = 6$; Fig. 4 A).

To confirm that platelet-derived MMP-2 was the cause of the hemostatic effect of transfused WT platelets and to exclude that other factors, such as plasma components, were involved, we created chimeric mice with *MMP-2*$^{-/-}$ blood cells and WT vessels. The tail bleeding time of WT mice engrafted with bone marrow from WT animals (controls) was 296 ± 71 s ($n = 10$), whereas *MMP-2*$^{-/-}$ bone marrow chimeras had a prolonged bleeding time, which was similar to that of *MMP-2*$^{-/-}$ mice. The bleeding time of *MMP-2*$^{-/-}$ mice engrafted with bone marrow from WT mice was normal (Fig. 4 B).

The bleeding time of *MMP-2*$^{-/-}$ mice transplanted with bone marrow from the same genotype (880 ± 144.6 s) was somewhat longer than that of *MMP-2*$^{-/-}$ mice (555.5 ± 97.7 s). Considering that in cross-transplantation experiments the reconstitution of the platelet count reached ∼70% of the normal, we performed experiments to assess the effect of a mild reduction of the platelet count on the bleeding time in *MMP-2*$^{-/-}$ mice. The reduction of the number of circulating platelets to ∼70% in *MMP-2*$^{-/-}$ mice by the administration of a low dose of a rabbit anti–mouse platelet antiserum induced a prolongation of the bleeding time to a level similar to that seen in *MMP-2*$^{-/-}$/*MMP-2*$^{-/-}$ cross-transplanted animals (755 ± 57 s; $n = 5$).

Femoral artery thrombosis was photochemically induced in chimeric mice upon infusion of a low dose of rose Bengal (20 mg/kg). Blood flow stopped after 10.6 ± 2.6 min ($n = 5$) from the beginning of irradiation in chimeric mice with *MMP-2*$^{+/+}$ blood cells. In contrast, in chimeric mice with *MMP-2*$^{-/-}$ blood cells, blood flow stopped after 27.8 ± 2.2 min ($n = 5$; $P < 0.001$ vs. chimeric mice with normal bone marrow; Fig. 4 C). The reduced thrombotic phenotype observed in *MMP-2*$^{-/-}$ bone marrow chimeras was associated with defective platelet function: ex vivo platelet adhesion to a collagen-coated surface under high shear was lower in *MMP-2*$^{-/-}$ bone marrow chimeras than in normal bone marrow mice (Fig. 4 D);

that of WT platelets (blue). Arrow denotes addition of the agonist. As expected, MMP-2 potentiated platelet aggregation of WT mice (green). (D) Preincubation of washed platelets from *MMP-2*$^{-/-}$ mice with 100 ng/ml of active human MMP-2 enhanced Akt phosphorylation only when the highest dose of thrombin (0.1 U/ml, lanes 3 and 4) was used as a stimulus, and preincubation with pro–MMP-2 of washed platelets from WT mice also enhanced Akt phosphorylation when the lowest dose of thrombin (0.05 U/ml) was used. In the absence of thrombin, MMP-2 has no effect on Akt phosphorylation (lane 5). Results in A–D are representative of three independent experiments. (E) Data from lanes 1–4 in D were quantified by densitometry and expressed as arbitrary units (white bars, mouse platelets added with pro–MMP-2; black bars, mouse platelets without the addition of pro–MMP-2). Results in A–E are representative of three independent experiments. (F) The i.v. injection of 100 ng/mouse of human pro–MMP-2 into *MMP-2*$^{-/-}$ mice, 2 min before sampling, restores ex vivo–impaired adhesion to a collagen-coated surface almost to the level observed in WT mice ($n = 5$). (G) The i.v. injection of 100 ng/mouse of human pro–MMP-2 into *MMP-2*$^{-/-}$ mice shortens the prolonged tail tip bleeding time ($n = 10$). (H) The i.v. injection of 100 ng/mouse of human pro–MMP-2 into *MMP-2*$^{-/-}$ mice significantly increases photochemically induced femoral artery thrombosis, as measured by thrombus weight ($n = 5$). Results in E–H are the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

platelet P-selectin expression after the i.v. injection of 60 µl of collagen plus epinephrine was significantly lower in *MMP-2*$^{-/-}$ bone marrow chimeras (Fig. 4 E); and similar findings were observed for platelet/leukocyte aggregates (Fig. 4 F).

An arterial damage facilitates downstream thrombus formation by inducing the release of platelet MMP-2

When only a mild damage to the femoral artery was induced by the infusion of a low dose of rose Bengal (10 mg/kg; Fig. 5 A), no occluding thrombus occurred in WT mice (time to occlusion, >30 min; $n = 5$; Fig. 5 B). However, when a second arterial damage of identical intensity was induced downstream of the first insult (Fig. 5 A) a complete occlusion occurred (time to occlusion, 19.4 ± 2.3 min; $n = 5$; Fig. 5 B). When the sequence of the two arterial damages was inverted, first downstream and then upstream of the first insult, no occlusive thrombus occurred. These data suggest that the first arterial damage induces the release of mediators, possibly platelet derived, flowing downstream and potentiating the thrombotic response to a second stimulus. Indeed, a

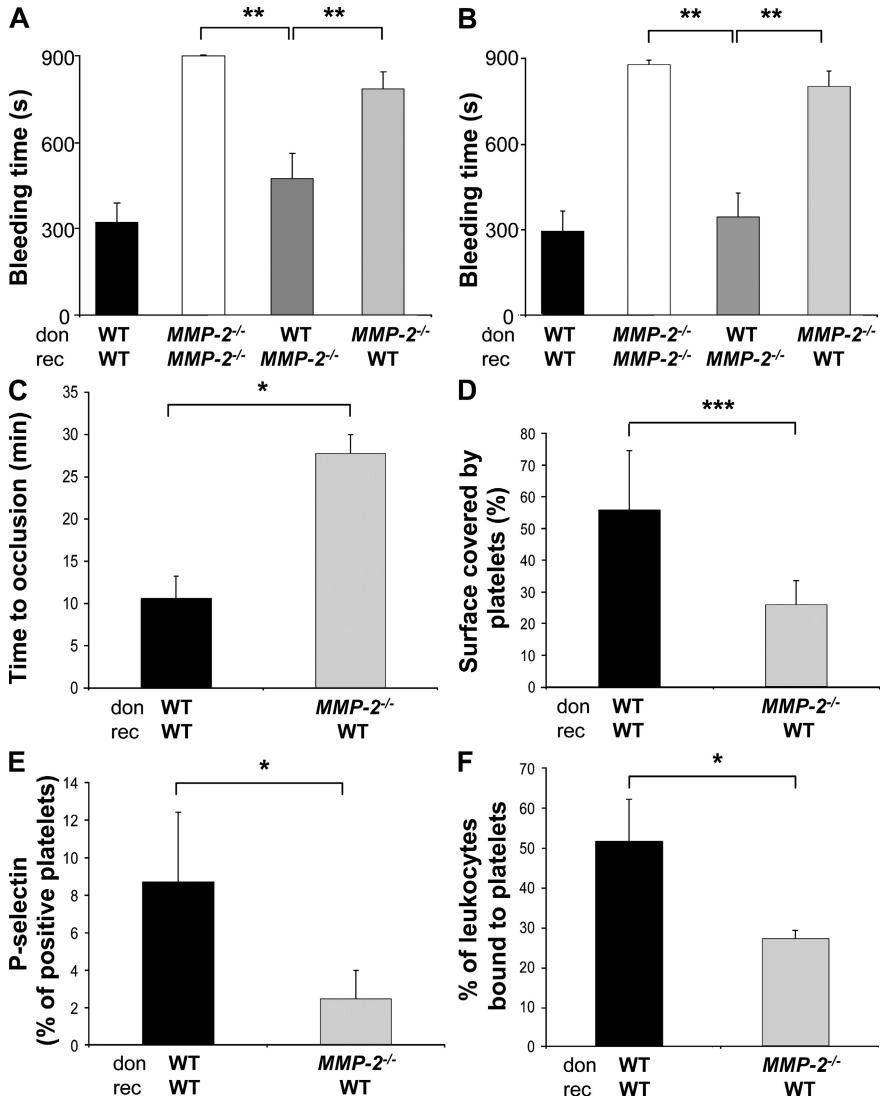

Figure 4. Role of platelet-derived MMP-2 in hemostasis and thrombosis. (A) Mice made thrombocytopenic by the i.v. injection of a rabbit anti-mouse platelet antiserum (rec, recipient) were transfused with platelets from either WT or *MMP-2*$^{-/-}$ donor (don) mice. The tail tip bleeding time was significantly shortened by the transfusion of WT platelets but not of *MMP-2*$^{-/-}$ platelets. (B) Chimeric mice were generated by the transplantation of bone marrow from donor WT or *MMP-2*$^{-/-}$ mice into lethally irradiated recipient mice. Chimeras with WT bone marrow had a short bleeding time, whereas chimeras with *MMP-2*$^{-/-}$ bone marrow had a prolonged bleeding time. (C–F) Photochemically induced femoral artery thrombosis was significantly impaired in chimeras with *MMP-2*$^{-/-}$ bone marrow (C). Ex vivo platelet function in chimeras with bone marrow from *MMP-2*$^{-/-}$ mice was significantly reduced as compared with chimeras with bone marrow from WT mice, as assessed by platelet adhesion to a collagen-coated surface (D), expression of P-selectin (E), or platelet-leukocyte aggregates (F) after the i.v. injection of a 60-µl mixture of collagen plus epinephrine. Results in A–F represent the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

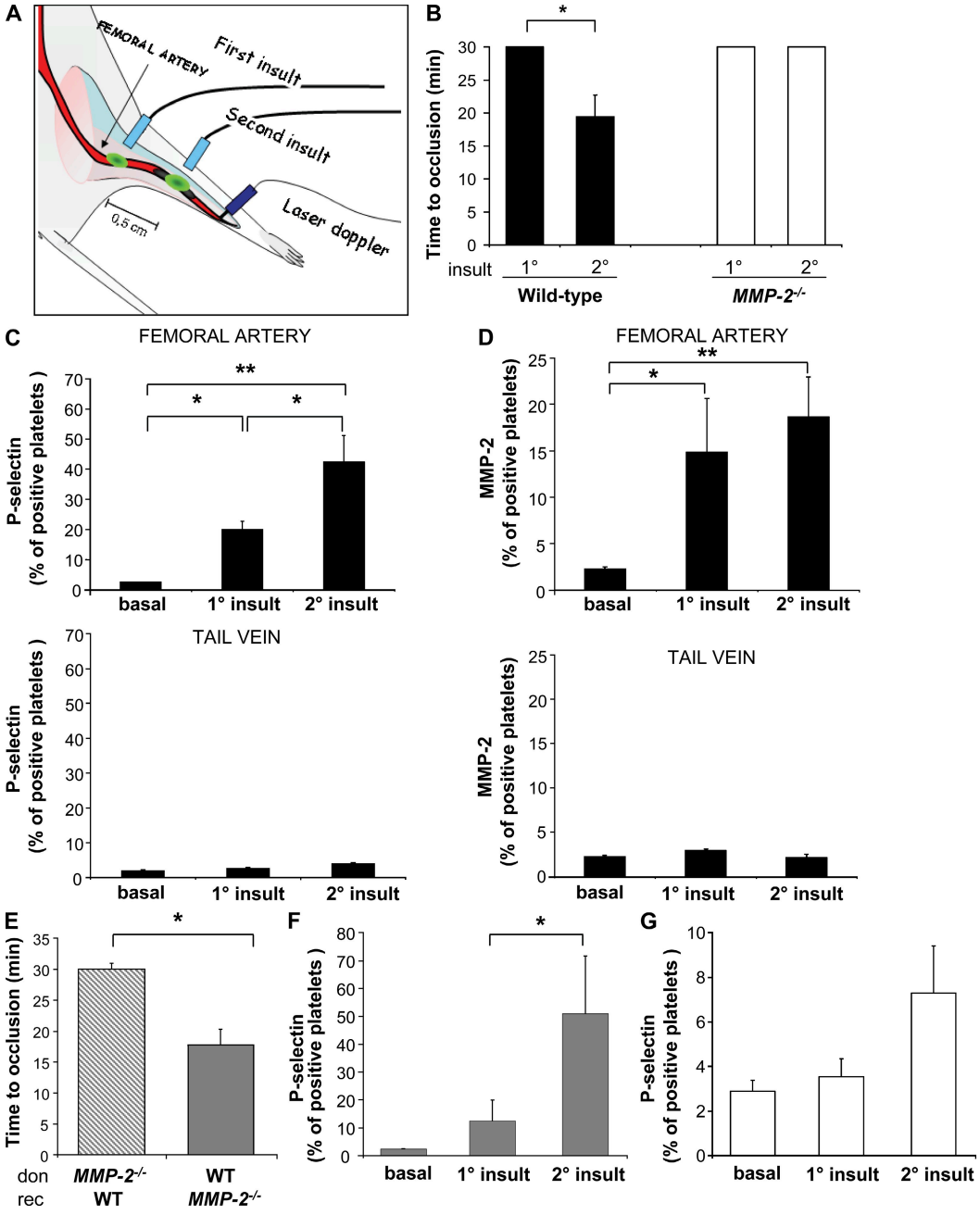

Figure 5. A mild arterial damage facilitates downstream thrombus formation. (A) Cartoon illustrating the model. A first thrombogenic stimulus was induced by irradiation with green light during infusion of 10 mg/kg of rose Bengal. After 30 min, a second arterial damage was produced with the same modalities 0.5 cm downstream. The formation of an occluding thrombus was assessed by the measurement of blood flow with a laser Doppler probe. (B) Time to occlusion of the femoral artery, as assessed by laser Doppler, after repeated photochemical damage generated upon the infusion of a low dose of rose Bengal (10 mg/kg). In WT mice, the first photochemical insult does not induce thrombus formation, whereas an occlusion is observed after the second insult ($n = 5$). In *MMP-2*$^{-/-}$ mice no thrombus forms after either insult ($n = 5$). (C and D) Expression of P-selectin (C) or MMP-2 (D) on platelets in blood collected downstream to the femoral insult (femoral artery) or in the systemic circulation (tail vein) in WT mice ($n = 5$). Platelet activation is observed in the femoral artery after the first arterial injury and, even more, after the second arterial injury, whereas no changes are detected in the systemic circulation. Results in B–D represent the mean ± SEM of five independent experiments. (E) The cross-transfusion of *MMP-2*$^{-/-}$ platelets in platelet-depleted WT mice ($n = 4$) abolished thrombus formation after the second arterial insult, whereas thrombus formation was completely restored by the cross-transfusion of WT platelets into platelet-depleted *MMP-2*$^{-/-}$ mice ($n = 7$). (F) Platelet-depleted *MMP-2*$^{-/-}$ mice cross-transfused with platelets from WT mice showed platelet activation, as shown by platelet P-selectin increase after the first femoral artery insult and, even more, after the second femoral artery insult ($n = 7$). (G) The expression of P-selectin on platelets in blood collected downstream of the femoral insult was strikingly reduced in *MMP-2*$^{-/-}$ mice ($n = 4$) as compared with WT mice and with platelet-depleted *MMP-2*$^{-/-}$ mice cross-transfused with platelets from WT mice. Results in E–G represent the mean ± SEM of four independent experiments. *, $P < 0.05$; **, $P < 0.01$.

significant but localized platelet activation occurred after the first insult. In fact, the expression of P-selectin on the platelet surface rose from 2.4 ± 0.15% (baseline) to 19.9 ± 2.7% in blood collected immediately downstream of arterial damage (Fig. 5 C and Fig. S2), whereas it did not change on platelets from systemic blood tail vein (Fig. 5 C). One of the platelet activation–potentiating mediators may be MMP-2. Interestingly, the expression of MMP-2 on platelets from blood withdrawn from the femoral artery was clearly enhanced after the first arterial injury (Fig. 5 D). Moreover, when an identical experiment was performed in *MMP-2*$^{-/-}$ mice, no occlusive thrombus formed either after the first or after the second downstream arterial insult (time to occlusion, >30 min; $n = 4$; Fig. 5 B). However, the cross-transfusion of WT platelets into platelet-depleted *MMP-2*$^{-/-}$ mice restored the ability to form an occluding thrombus after the second downstream arterial insult (time to occlusion, 17.7 ± 2.65 min; $n = 7$; $P < 0.05$ vs. *MMP-2*$^{-/-}$; Fig. 5 E). On the contrary, the cross-transfusion of *MMP-2*$^{-/-}$ platelets into platelet-depleted WT mice also abolished thrombus formation after the second downstream damage to the femoral artery (time to occlusion, >30 min; $n = 4$). The expression of P-selectin and of MMP-2 (MMP-2 expression rose from 2.5 ± 0.5 to 18.2 ± 4.6% of positive cells; $n = 7$) on platelets from blood collected immediately downstream of the arterial insult was significantly enhanced in *MMP-2*$^{-/-}$ mice cross-transfused with WT platelets after the photochemically induced arterial damage (Fig. 5 F) as compared with *MMP-2*$^{-/-}$ mice (Fig. 5 G).

To confirm the role of platelet-released MMP-2 in the thrombogenic response downstream to a first arterial damage, we infused systemically WT mice i.v. with 1 μg/mouse of the selective inhibitor of MMP-2, TIMP-2. Immediately, downstream of the first arterial insult, thrombus formation was abolished. Moreover, P-sel and MMP-2 expression at the surface of circulating platelets collected downstream of the second arterial insult were not different from baseline in TIMP-2–treated mice. In contrast, when the first weak, normally nonthrombogenic, arterial damage was performed in WT animals systemically infused with 100 ng/mouse of pro–MMP-2, a thrombus had already developed after the first arterial insult (time to occlusion, 15 ± 1.08 min).

All these data are consistent with a model in which localized platelet activation induced by a first arterial damage generates the release of platelet-stored pro–MMP-2, which gets rapidly activated to active MMP-2 and acts on the neighboring platelets, making them hyperresponsive to a second stimulus like that generated by an arterial damage downstream to the first, to produce the formation of an occluding thrombus. The fact that this priming effect is essentially limited to the local circulatory bed derives from the transient nature of the priming effect of MMP-2 on platelets and from the dilution and/or inactivation in the systemic circulation of thrombogenic substances generated by localized platelet activation. This was confirmed by the subsequent experiments. The potentiating activity of human active MMP-2 on mouse platelet aggregation was maximal after a 2-min preincubation and it almost disappeared after 10 min. The infusion of thrombin-activated platelets directly into the femoral artery led to the appearance of P-selectin–positive platelets in the systemic circulation within the first 2 min after the injection (baseline, 3 ± 0.1%; 2 min, 7.7 ± 1.3% of positive platelets) and, thereafter, the expression of P-selectin on platelets in systemic blood disappeared.

DISCUSSION

We show in this paper that *MMP-2*$^{-/-}$ mice are strongly protected against thrombosis induced by mild thrombogenic stimuli and have only a moderate impairment of hemostasis, that these alterations are the consequence of an abnormal platelet function, and that platelet-derived MMP-2 plays a crucial role in platelet activation and thrombus formation in vivo. Mice in which the MMP-2 gene has been disrupted do not suffer spontaneous bleeding and have an only moderately prolonged tail tip bleeding time as compared with WT mice, which is at variance from mice in which mechanisms of essential importance for the hemostatic function of platelets have been disrupted, such as the GPIIb/IIIa-von Willebrand factor interaction (Hodivala-Dilke et al., 1999; Marx et al., 2008). In contrast, *MMP-2*$^{-/-}$ mice are resistant to platelet-dependent thrombosis, like pulmonary platelet thromboembolism (Gresele et al., 1990; Momi et al., 2005) or photochemically induced thrombosis of the femoral artery (Kikuchi et al., 1998; Momi et al., 2005). Several data suggest that the hemostatic defect and impaired thrombus formation of *MMP*$^{-/-}$ mice are the result of defective platelet activation.

Indeed, although the structure and number of platelets from *MMP-2*$^{-/-}$ mice are normal, they show defective aggregation in response to a variety of stimuli in vitro, reduced adhesion to collagen, an impaired expression of platelet P-selectin, and diminished formation of platelet/leukocyte aggregates upon activation in vivo. Interestingly, defective platelet activation in vitro and in vivo is especially evident when weak stimuli are used, whereas it is much less apparent with strong stimuli, which is in agreement with an autocrine- or paracrine-potentiating action of platelet-released MMP-2, as had been previously observed in vitro with human platelets (Sawicki et al., 1997; Falcinelli et al., 2005; Santos-Martínez et al., 2008). Defective platelet function of *MMP-2*$^{-/-}$ mice was restored by the in vitro addition of human pro-MMP-2; moreover, the i.v. injection of human pro–MMP-2 into *MMP-2*$^{-/-}$ mice restored platelet function in terms of adhesion, aggregation, and P-selectin expression, indicating a pivotal role of MMP-2 for platelet activation. The human pro–MMP-2 injected into *MMP-2*$^{-/-}$ mice was rapidly activated at the surface of platelets, and the concentrations of active MMP-2 measured in plasma 2 min after the i.v. injection of pro–MMP-2 were in the range of those potentiating platelet activation in vitro (Falcinelli et al., 2005) and, thus, sufficient to reverse the deficit of platelet function of knockout mice.

The hemostatic defect and defective thrombus formation observed in mice in which the gene for MMP-2 has been disrupted is mainly the result of the absence of MMP-2 from platelets, as shown by the observation that the cross-transfusion

of *MMP-2*$^{-/-}$ platelets into thrombocytopenic WT mice does not correct the prolonged bleeding time. Similarly, chimeric mice in which the hematopoietic cell line derives from bone marrow cells of *MMP-2*$^{-/-}$ mice have moderately impaired hemostasis and defective photochemical injury-induced arterial thrombosis, which is similar to what observed in *MMP-2*$^{-/-}$ mice, whereas *MMP-2*$^{-/-}$ mice with bone marrow derived from WT mice, and, therefore, with circulating platelets containing normal amounts of MMP-2 but with blood vessels devoid of MMP-2, showed a normal thrombotic response upon arterial injury.

Altogether, our results are compatible with a model in which MMP-2 contained in platelets is released upon activation and acts in an autocrine and paracrine way on the neighboring platelets, facilitating their activation response to physiological or pathological stimuli (Gresele et al., 2008b). This may become crucial in transforming a normal hemostatic platelet activation response to mild vessel wall damage into an uncontrolled thrombotic response. This is confirmed by the observations that when two mild arterial insults are induced sequentially, a thrombus forms only at the level of the second arterial damage downstream, that this phenomenon does not take place when circulating platelets are devoid of MMP-2, and that a selective MMP-2 inhibitor blocks downstream thrombus formation. These observations might explain why in the coronary bed of patients dying from acute myocardial infarction, where arterial plaques are widely disseminated and more than one may be fissured or eroded, only a single acute occluding thrombus forms and is ultimately responsible for the acute ischemic event (Casscells et al., 2003). In contrast, pathological conditions in which circulating levels of MMP-2 are enhanced as a result of the release of MMP-2 from tissue, like ruptured inflammed atherosclerotic plaques (Galis et al., 1994; Galis and Khatri, 2002), may be associated with platelet hyperreactivity and with an enhanced tendency to thrombosis. Indeed, the release of MMP-2 in the coronary circulation of patients with acute coronary syndromes has been previously shown (Hojo et al., 2002; Falcinelli, E., M. Leone, G. Cimmino, T. Corazzi, P. Golino, and P. Gresele. 2005. Congress of the International Society on Thrombosis and Haemostasis. Abstr. OR370). Our data, showing that an increase of circulating MMP-2, which is obtained by the infusion of pro–MMP-2 in the systemic circulation of normal mice, transforms a mild nonthrombogenic stimulus into a strong thrombotic insult, are in favor of this hypothesis.

The important role played by platelet-released MMP-2 in arterial thrombosis identifies a new potential target for novel antithrombotic therapies. This is especially relevant if one considers that aspirin intake does not prevent the in vivo release of MMP-2 from activated platelets (Falcinelli et al., 2007). The modulation of platelet-released MMP-2 by the development of specific monoclonal antibodies, of selective small molecule inhibitors, or by the application of gene silencing techniques can be one of the strategies to overcome the narrow gap between insufficient efficacy and excessive bleeding typical of current antiplatelet drugs (Bhatt, 2007; Gresele et al., 2008b).

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Charles River. Mice deficient in MMP-2 were generated by gene targeting (Itoh et al., 1997; Wielockx et al., 2001). The mutation heterozygous mice were obtained by crossing the chimeras to C57BL/6J one to five times, and then crossing to obtain the mutation homozygous mice. These mice, and controls with matching genetic backgrounds, were bred as homozygous lines and were used at the age of 8–12 wk. Mice were maintained with a 12:12-h light/dark cycle and had free access to food and water.

Genotyping of mice was performed by PCR analysis. Littermate tail tip DNA extraction was performed with the NucleoSpin tissue kit (M-Medical). Primer sequences used for mouse MMP-2 were 5′-AACGATGGAGGCACGAGTGGCC-3′ (forward) and 5′-TGTTTTAGGGGCGACATCGCCG-3′ (reverse). Primer sequences used for NEO detection were 5′-CAACGCTATGTCCTGATAGCGGTCC-3′ and 5′-CGTGTTCCGGCTGTCAGCGCAGG-3′. In addition, the absence of MMP-2 protein in MMP-2$^{-/-}$ mice was evaluated by zymography in plasma (Fig. S3). All animal protocols were approved by the Committee on Ethics of the University of Perugia (protocol 186/2006 and 216/2007) and by the Italian Ministry of Public Health (authorization protocol 74/2007 and 76/2007-B).

Assessment of MMPs expression by RT-PCR

MMP-2, MMP-9, TIMP-1, and TIMP-2 gene expression was assessed by RT-PCR in spleens taken from anesthetized WT, *MMP-2*$^{-/-}$, and *MMP-2*$^{+/-}$ mice after perfusion with 20 ml of saline via left ventricular puncture at an infusion pressure ≤100 mm Hg, and immediately frozen in liquid nitrogen. Total RNA was extracted using an RNA purification kit (Absolutely RNA Miniprep kit; Agilent Technologies) and treated with DNase to avoid any genomic DNA contamination. Quantitative PCR was performed by monitoring in real time the increase in fluorescence of the Sybr Green Dye in a real-time PCR system (Mx 3000P; Agilent Technologies).

MMP-2, MMP-9, TIMP-1, and TIMP-2 segments of each resultant complementary DNA sample were PCR amplified in the presence of specific sense and antisense primers (200 nmol/liter each), using a power Sybr Green PCR Master Mix (Applied Biosystems) in a final reaction volume of 25 µl. Cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and extension). β2 microglobulin complementary DNA was amplified as an internal control. Each sample was tested in triplicate for each gene, and then a melting curve to ensure the specificity of amplification and the expression level of the gene was calculated by the $\Delta\Delta C_T$ method (Antonov et al., 2005).

Zymography

The gelatinolytic activity of MMP-2 was measured in plasma, in the supernatant of activated platelets, or in tissue extracts (20 µg of protein extracts) by zymography (Kleiner and Stetler-Stevenson, 1994), as previously described (Falcinelli et al., 2005). In brief, samples were mixed with SDS sample buffer without 2-mercaptoethanol and applied to 10% polyacrylamide gels copolymerized with 2 mg/ml of gelatin. After electrophoresis, gels were incubated for 1 h with 2.5% Triton X-100 to remove SDS and then overnight in buffer (50 mM Tris-HCl buffer with 20 mM NaCl, 5 mM $CaCl_2$, and 0.01% Brij35, pH 7.5) at 37°C. Gels were then stained with 0.05% Coomassie Brilliant blue G-250 in a mixture of methanol, acetic acid, and water (3:1:6, vol/vol) and destained in 30% methanol with 10% acetic acid. Gelatinolytic activities were detected as transparent bands against the background of Coomassie Brillant blue–stained gelatin and quantified by analyzing the zymograms by densitometry. Intensity of pro–MMP-2 and MMP-2 bands was quantified by densitometry using the software Quantity One (version 4.1.1; Bio-Rad Laboratories). Gelatinolytic bands were compared with a standard curve built using increasing amounts of recombinant human MMP-2 (EMD). Tissue or plasma protein content was measured by a protein assay (Bio-Rad Laboratories).

Platelet studies

Platelet count. Blood was collected by cardiac puncture from mice under ether anesthesia and anticoagulated with 1/10 vol of tripotassium EDTA.

After thorough mixing, platelets were counted optically, using the Brecher-Cronkite method (Brecher and Cronkite, 1950), by an operator unaware of the experimental groups (Momi et al., 2005).

Electron microscopy. Resting washed platelets were fixed for 4 h at 4°C, using cacodylate-HCl buffer (pH 7.4) containing 4% wt/vol of glutaraldehyde. The samples were then washed, maintained in cacodylate buffer for a further 4 h, and placed in 1% osmium tetraoxide and pelleted by centrifugation at 10,000 *g* for 30 s. Ultrathin sections of the platelet pellets were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope at 80 kv (Optic EM208; Phillips; Gresele et al., 2009).

Platelet aggregation. The effect of MMP-2 on platelet aggregation was studied by the photometric method using either washed platelets (Vezza et al., 2002) or PRP (Momi et al., 2005). Whole blood, drawn in 4% sodium citrate, was centrifuged at 150 *g* for 15 min to obtain PRP, which was then adjusted to 300,000 platelets/µl using autologous platelet-poor plasma. Washed platelets were prepared in the presence of 0.2 µM PGI_2, 50 U/ml heparin, and 0.01 mg/ml apyrase. Finally, platelets were resuspended in standard Tyrode's buffer, pH 7.4, containing 0.35% BSA and 0.1% glucose, to give a final concentration of 250,000 platelets/µl. Gel-filtered mouse platelets were prepared as previously described (Pulcinelli et al., 1998) and adjusted to 10^8/ml in Hepes-Tyrode's buffer containing 0.1% BSA, pH 7.4. Platelet aggregation in response to different doses of collagen, arachidonic acid, ADP, purified human α-thrombin (given by R. De Cristofaro, Catholic University of Rome, Rome, Italy), or U46619, a stable analogue of TxA_2 (Vezza et al., 1993), was measured using an automatic platelet aggregation analyzer (APACT 4; Alfa Wassermann).

ATP secretion. Platelet ATP release was measured using firefly luciferase (7.3 µg/ml final concentration) and luciferin (800 U/ml final concentration). Luminescence generated by platelet-secreted ATP was monitored using a lumi-aggregometer (Chrono-Log Corporation) and compared with that obtained by adding a known concentration of ATP standard (Emiliani et al., 2006).

Platelet adhesion. Platelet adhesion to a collagen-coated surface under flow conditions was studied in a parallel-plate perfusion chamber (Sixma et al., 1998), as previously described (Momi et al., 2005). In brief, 0.5 ml of citrated blood was passed through a perfusion chamber, over a plastic coverslip sprayed with ∼30 µg/cm² collagen from equine tendon (Hormon-Chemie), at a wall shear rate of 3000 s^{-1}. The chamber was then perfused with BSA-containing saline to remove all residual blood, and the coverslip was harvested, gently washed with 10 mmol/liter Hepes, and fixed with 0.25% glutaric dialdehyde in PBS. Adhering platelets were stained with May-Grünwald/Giemsa, observed under an optical microscope, and the area covered by platelets was measured using a computerized image analyzer (Scion Image; National Institutes of Health; Momi et al., 2005).

Flow cytometry. Platelet surface P-selectin, MMP-2, and fibrinogen expression and circulating platelet-leukocyte aggregates were measured in whole blood by flow cytometry (EPICS XL-MCL; Beckman Coulter) as previously described (Ciferri et al., 2000; Pitchford et al., 2003). Blood samples, collected in 4% sodium citrate and diluted 1:10 with PBS, were stimulated with a mixture of 0.5–5–20 µg/ml collagen plus 1–10–50 µM epinephrine, 30 µg/ml collagen, 0.05 or 0.1 U/ml of human α-thrombin, or 20 µM ADP for 30 min under continuous stirring. Aliquots of 5 µl were then stained with a PE-labeled anti-CD41 antibody (Leo.D2; Emfret Analytics) as a platelet identifier and with an FITC-labeled anti–P-selectin antibody (CD62P; Emfret Analytics) or with an FITC-labeled anti-fibrinogen antibody (P140-1; Emfret Analytics) for 30 min in the dark. The reaction was stopped with 1 ml of 1% PFA. P-selectin, MMP-2, and fibrinogen expression are reported as a percentage of positive cells. For platelet-leukocyte aggregates, 50 µl of undiluted whole blood were fixed with 1% PFA and incubated for 30 min with saturating concentrations of FITC-labeled anti-CD41/CD61 ($\alpha_{IIb}\beta_3$; Leo.D2; Emfret Analytics) and Peridin chlorophyl protein–conjugated anti–mouse CD45 (clone 30-F11; BD). Subsequently, red blood cells were lysed by adding 400 µl of water and, after 10 min, 1 ml of PBS.

Leukocytes were identified on the basis of their morphology and of the positivity for CD45. Platelet-leukocyte aggregates were quantified as the percentage of leukocytes positive for the platelet antigen CD41/CD61 or as the mean fluorescence intensity of the platelet antigen CD41/CD61 on the leukocyte surface. In some selected experiments, platelet P-selectin expression, fibrinogen binding, and platelet/leukocyte aggregates were assessed in blood collected from WT and *MMP-2* $^{-/-}$ mice 2 min after the i.v. injection of a mixture of 150 µg/ml collagen plus 0.25 µM epinephrine.

For platelet MMP-2 expression, 5 µl of diluted whole blood was incubated for 30 min with saturating concentrations of rabbit PE-labeled anti–mouse CD41 (Leo.F2; Emfret Analytics) and goat anti–mouse MMP-2 (R&D Systems). Subsequently, an Alexa Fluor 488 anti–goat antibody (1:500; Invitrogen) was added. At the end of incubation period the reaction was stopped with 1 ml of 1% PFA.

For the study of the time course of expression of active MMP-2 on platelet surface, gel-filtered platelets from WT or *MMP-2*$^{-/-}$ mice, incubated with 100 ng/ml of human pro–MMP-2, were activated by 0.1 U/ml of human α-thrombin, and the reaction was stopped at 30 s and 2 and 15 min after the addition of α-thrombin with 1 ml of 1% PFA. Active MMP-2 was assessed with a specific rabbit anti–mouse active MMP-2 antibody (Millipore) and a goat anti–rabbit antibody (1:500; Invitrogen) by flow cytometry.

Half-life of infused human pro–MMP-2 in mice. 500 ng of human recombinant pro–MMP-2 (Millipore) was injected i.v. in *MMP-2*$^{-/-}$ mice (n = 15). After 2 min, and then every 30 min for a total of 120 min, whole blood was withdrawn in sodium citrate 4% (1:9 vol/vol) and quickly centrifuged to obtain platelet-poor plasma that was snap frozen for later determination of MMP-2 activity. The amount of active and total MMP-2 in mouse plasma was assed by ELISA (MMP-2 Biotrak activity assay system; GE Healthcare) and by gelatin zymography. The plasma half-life of MMP-2 was determined as the time required by human MMP-2 to be degraded to half the concentration found in plasma 2 min after MMP-2 injection.

Platelet-derived thromboxane B_2. For serum TxB_2 measurement, non-anticoagulated blood was collected in glass tubes and placed in a water bath at 37°C for 60 min and then centrifuged (Patrono et al., 1980). Immunoreactive TxB_2 was measured in unextracted highly diluted serum samples by a highly specific radioimmunoassay, as previously described (Gresele et al., 1987).

Akt phosphorylation. Akt phosphorylation was studied in washed platelets incubated for 2 min with activated human MMP-2 (Falcinelli et al., 2005) or with its vehicle before the addition of 0.05–0.1 U/ml thrombin. After 10 min, the reaction was stopped with SDS-PAGE sample buffer, platelets were lysed with 2% SDS, and samples were carefully mixed and stored on ice. Proteins (measured by the Bradford assay) were analyzed by SDS-PAGE followed by immunoblotting, using an antibody against Akt or phospho-Akt Ser^{473} (1:2,000). In each electrophoresis well, an identical amount of proteins was loaded (20 µg; Falcinelli et al., 2005). Data were quantified using the QuantiScan software (BIOSOFT) and expressed as percentage of control (i.e., phosphorylation of Akt in thrombin-stimulated platelets). Human pro–MMP-2 was activated by incubation with 1 mmol/liter APMA (para-aminophenylmercuric acetate), in a buffer containing 50 mmol L^{-1} NaCl, 5 mmol L^{-1} $CaCl_2$, and 0.01% Brij 35, pH 7.5, at 37°C for 30 min (Falcinelli et al., 2005). APMA was then removed by filtration through Amicon filters (Microcon YM-10; Millipore).

Thrombosis models

Platelet pulmonary thromboembolism. Collagen plus epinephrine–induced pulmonary thromboembolism was performed in *MMP-2*$^{-/-}$ and in WT mice at 8–12 wk of age, using a method described previously (Gresele et al., 1990; Momi et al., 2005). Mice were caged and fed a regular diet for at least 1 wk before use. Thrombotic challenge was generated by the rapid i.v. injection of a mixture of 250 µg/ml collagen 1.5 µg/ml and epinephrine

into one of the tail veins. *MMP-2*$^{-/-}$ and WT mice were challenged with increasing volumes (from 20 to 100 μl) of the collagen plus epinephrine mixture and mortality was recorded. The effect of the i.v. challenge with collagen plus epinephrine was evaluated as described; the cumulative end point was death of the animal or paralysis of the hind limbs for more than 15 min (Gresele et al., 1990; Momi et al., 2005). In each session, at least five animals per experimental group were tested. Controls were run at the beginning and at the end of each experimental session. At the end of each experimental session, surviving animals were sacrificed by an overdose of anesthesia. Mice were accustomed to handling and the injections were performed by skilled investigators with minimal disturbance to the animals. Data are presented as a percentage of animals dead per total number of animals tested.

Lung histology. The total number of lung vessels occluded by platelet thrombi was counted by light microscopy in paraffin-embedded sections stained with hematoxylin and eosin (Momi et al., 2005).

Femoral artery thrombosis. Photochemical-induced femoral artery thrombosis was induced in anesthetized mice by a method described previously (Kikuchi et al., 1998; Momi et al., 2005). In brief, mice were anaesthetized by 60 mg/kg of intraperitoneal sodium pentobarbital and placed on a heated operating table. A 25G-needle venous butterfly was inserted in one of the tail veins for the infusion of rose Bengal. The left femoral artery was carefully exposed, and a laser Doppler probe (Transonic System Inc.) was positioned onto the branch point of the deep femoral artery, distal to the inguinal ligament, for monitoring blood flow. The exposed artery was irradiated with green light (wavelength, 540 nm) of a Xenon lamp (L4887; Hamamatsu Photonics) equipped with a heat-absorbing filter via a 3-mm diameter optic fiber attached to a manipulator. Light irradiation was protracted for 20 min. The infusion of 10–50 mg/kg of rose-Bengal was started 5 min after the beginning of irradiation and lasted for 5 min. The end point was set as the cessation of blood flow for >30 s. If no occlusion occurred after 30 min, the time was recorded as 30 min (Kikuchi et al., 1998; Momi et al., 2005). 1 h after the end of the irradiation period, the femoral artery was excised and opened, and the thrombus was removed, dried at 37°C for 24 h, and then weighed. In some experiments, arterial damage was induced sequentially at two different sites of the femoral artery by two irradiation injuries separated from one another by 30 min, with the second 0.5 cm downstream of the first. For flow cytometry measurement of platelet P-selectin or platelet MMP-2 expression, blood was collected immediately after the first or the second vessel injury by arterial puncture at a femoral artery site distal to the injury or systemically from a tail vein.

To assess the detectability on the systemic circulation of localized platelet activation in the femoral artery, platelets obtained from WT mice were isolated by gel filtration and activated with 0.1 U/ml of purified human α-thrombin for 15 min. Thrombin was neutralized with equimolar hirudin (Huo et al., 2003), and then 3×10^8 activated platelets were injected directly into the femoral artery of recipient mice. Blood for the detection of platelet P-selectin expression by flow cytometry was collected after platelet injection systemically, from a tail vein, at 2, 5, 10, and 15 min.

Bleeding time. Bleeding time was measured by the tail tip transection method, as previously reported (Momi et al., 2005). In brief, mice were positioned in a special immobilization cage that keeps the tail of the animal steady and immersed in saline thermostated at 37°C. After 2 min, the tip of the tail was transected with a razor blade at 2 mm from its end. The tail was immediately reimmersed in thermostated saline and time taken to stop bleeding was measured. The end point was the arrest of bleeding lasting for more than 30 s. Bleeding was recorded for a maximum of 900 s.

Cross-transfusion experiments. In some selected experiments, platelets were isolated by centrifugation at 150 *g* for 10 min from whole blood of donor mice anticoagulated with acid citrate dextrose (1:6, vol/vol). The PRP obtained was pooled and infused in recipient mice that had been previously rendered deeply thrombocytopenic by one single i.v. injection of a saturating amount (100 μl) of an anti–mouse anti-platelet serum (APAS), prepared as previously described (Pitchford et al., 2004, 2008), or by γ irradiation (10Gy; Clinac 600/C; Varian Medical Systems, Inc.). APAS was injected 15 min before platelet transfusion. Platelet counts decreased by ≥95% within 2 min, and a gradual increase in the number of circulating platelets was seen only 48 h after administration (Pitchford et al., 2004). In γ-irradiated mice, the number of circulating platelets dropped by 96% by day 5 and platelets were reinfused at that time. Approximately 1.0×10^9 platelets were reinfused into each animal, leading to a restoration of the platelet count to ∼80% of basal (Pitchford et al., 2004). Bleeding time measurements or photochemically induced arterial damage was performed 1 h after platelet transfusion.

Bone marrow transplantation. 8–12-wk-old, male and female *MMP-2*$^{-/-}$ (C57BL/6 background) and C57BL/6 (WT) mice were used. Recipient mice were exposed to a single lethal dose of 8 Gy from an 18-mV photon beam linear accelerator (Clinac 600/C) with a focus-to-skin distance of 63 cm and a dose rate of 0.2 Gy/min. After irradiation, all mice not receiving bone marrow transplantation died within 14 d. Donor bone marrow cells were collected into PBS by flushing the shafts of the femur and tibia. The cells were suspended and washed with PBS, and then red blood cells were lysed with ammonium chloride–potassium buffer and the remaining cells were washed twice with PBS. Finally, bone marrow cells were resuspended at a final concentration of 20×10^6 cells per ml in PBS and, the day after total body irradiation, 10×10^6 cells/mouse were injected into recipient mice i.v. via the lateral tail vein (Ruggeri et al., 2002).

In some experiments *MMP-2*$^{-/-}$ mice were used as donors and WT as recipients, in others WT mice were used as donors and *MMP-2*$^{-/-}$ as recipients. Control experiments were performed by transplanting bone marrow from WT donors into WT recipients and from *MMP-2*$^{-/-}$ donors into *MMP-2*$^{-/-}$ recipients. 1 mo after bone marrow transplantation, both chimeric and control mice had almost normal platelet counts (746.000 ± 39.975 and 800.000 ± 30.551 platelets/μl, respectively). MMP-2 activity by zymography and MMP-2 expression by RT-PCR were assessed in blood cells, aorta, kidney, and lung extracts obtained from recipients 8 wk after bone marrow transplantation to ensure that full engraftment had taken place.

Statistics

Data are expressed as arithmetic means ± SEM and were analyzed by one way analysis of variance, followed by the Newman-Keuls multiple comparison tests between all groups. Differences in mortality were assessed by the Fisher's exact test. All analyses were performed by Prism 4.0 (GraphPad Software, Inc.). A p-value of <0.05 was considered to be statistically significant.

Online supplemental material

Fig. S1 shows electron microscopy of resting platelets from *MMP-2*$^{-/-}$ and WT mice. Fig. S2 shows flow cytometry plots of the expression of P-selectin and active MMP-2 on the platelet surface in WT and *MMP-2*$^{-/-}$ mice, in blood collected at baseline and immediately downstream of an arterial damage. Fig. S3 shows an example of zymography that confirms the absence of MMP-2 in *MMP-2*$^{-/-}$ mice. Table S1 reports data on the potentiation of platelet aggregation induced by active MMP-2. Table S2 summarizes platelet aggregation, ATP release, and P-selectin expression in response to various agonists in WT and *MMP-2*$^{-/-}$ mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090687/DC1.

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