Profibrinolytic Effects of Metalloproteinases during Skin Wound Healing in the Absence of Plasminogen

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Genetic ablation of plasminogen (Plg) and pharmacological inhibition of metalloproteinase activity by galardin delay skin wound healing in mice, whereas the combined inhibition of these two enzyme systems completely prevents healing. In this study, the impact of plasmin and metalloproteinases as profibrinolytic enzymes has been investigated by comparing skin wound healing in the absence and presence of fibrin. Plg deficiency impairs skin wound healing kinetics, but this delay is only partially restored in the absence of fibrin. This suggests that plasmin-mediated fibrinolysis is the primary, but not the exclusive, requirement for healing of wounds in these mice. In addition, we observe that lack of fibrin reduces Plg activation significantly during wound healing. The profibrinolytic role of metalloproteinases is revealed by the finding that lack of fibrin partially restores the otherwise arrested healing of Plg-deficient wounds after metalloproteinase inhibition. In conclusion, the residual impairment of skin wound healing in the absence of fibrin suggests the existence of a fibrin-independent substrate(s) for plasmin and metalloproteinases. Furthermore, these *in vivo* data reveal that galardin-sensitive metalloproteinases mediate compensatory fibrinolysis to facilitate wound healing in the absence of plasmin.

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INTRODUCTION

Proteolytic degradation of the extracellular matrix by the serine protease plasmin and members of the matrix metalloproteinase (MMP) family plays a key role in a variety of physiological and pathological processes that involve tissue remodeling and cell migration (Danø *et al.*, 1999; Coussens *et al.*, 2000; Egeblad and Werb, 2002; Page-McCaw *et al.*, 2007). Plasmin is formed from plasminogen (Plg) by proteolytic cleavage catalyzed by specific Plg activators. Hepatically produced Plg is present in high concentrations in plasma and interstitial fluids, and represents a large reservoir of proteolytic potential available for local activation throughout the body by its activators. Three physiologically active mammalian Plg activators are known, the urokinase type (uPA), tissue type (tPA) (Danø *et al.*, 1985; Collen and Lijnen, 2005), and a newly identified Plg activator, plasma kallikrein, demonstrated to activate Plg during adipocyte differentiation (Selvarajan *et al.*, 2001) and recently during wound healing (Lund *et al.*, 2006). It is well established that Plg deficiency has serious physiological consequences in both humans and mice (Bugge *et al.*, 1995; Mingers *et al.*, 1997; Schuster *et al.*, 1997; Drew *et al.*, 1998). A role for plasmin in tissue remodeling processes has been conclusively demonstrated by studies of skin wound healing (Rømer *et al.*, 1996), cancer metastasis (Bugge *et al.*, 1998), post-lactational mammary gland involution (Lund *et al.*, 2000), placental development (Solberg *et al.*, 2003), and neointima formation after vascular injury (Lijnen *et al.*, 1998) in mice with a disrupted Plg gene.

In Plg-deficient mice, there is a pronounced delay in healing of skin wounds, characterized by a decreased rate of keratinocyte re-epithelialization from the wound edges and an accumulation of fibrin in front of and beneath the leading edge keratinocytes, suggesting that the delay is due to a diminished ability of these cells to proteolytically dissect their way through the fibrin-rich provisional wound matrix (Rømer *et al.*, 1996). During the invasive phase of wound healing, the migrating leading edge keratinocytes express uPA and its cell surface receptor uPAR. tPA is more scarcely expressed, but has been detected in a few keratinocytes late in the re-epithelialization of human wounds and also accumulates in wound fields due to its high affinity for fibrin (Grøndahl-Hansen *et al.*, 1988; Rømer *et al.*, 1991, 1994; Drew et al., 2001). Furthermore, we have previously demonstrated that

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Abbreviations: α_2AP, α_2-antiplasmin; Ab, antibody; Fib, fibrinogen; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; PBS, phosphate-buffered saline; Plg, plasminogen; TBS, Tris-buffered saline; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor

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lack of fibrin(ogen) in the wound field could rescue the requirement for Plg to achieve timely healing (Bugge *et al.*, 1996b).

In addition to the expression of Plg activation components, several members of the MMP family, including MMP-3, MMP-9, and MMP-13, are expressed in the leading edge keratinocytes in mice (Madlener *et al.*, 1998; Lund *et al.*, 1999). The MMP family consists of more than 20 Zn-dependent endoproteases that are important for a number of tissue remodeling processes (Egeblad and Werb, 2002; Puente *et al.*, 2003). Treatment of wild-type mice with the broad-spectrum metalloproteinase inhibitor galardin, which inhibits a number of metalloproteinases (Saghatelian *et al.*, 2004), including MMP-3, MMP-9, and MMP-13 (Grobelny *et al.*, 1992; Levy *et al.*, 1998), causes a delay in wound healing (Lund *et al.*, 1999). Although delayed, complete wound healing is eventually achieved in all galardin-treated wild-type mice and all untreated Plg-deficient mice. However, when Plg-deficient mice are treated with galardin, healing is completely arrested, demonstrating that protease activity is essential for wound healing and that there is a functional overlap between the two different classes of matrix-degrading proteases in this process (Lund *et al.*, 1999).

Here, we have investigated the contribution of impaired fibrin degradation to the wound healing kinetics by comparing wound healing in Plg-deficient mice, galardin-treated wild-type mice, and galardin-treated Plg-deficient mice with the wound healing after genetically superimposing fibrinogen (Fib) deficiency. We show that reduced fibrinolysis is the primary impediment to the healing of large incisional skin wounds in Plg-deficient mice regardless of whether these are galardin-treated or not. Nonetheless, the phenotypes of Plg-deficient mice are not completely reversed by simultaneous Fib deficiency, suggesting a fibrin-independent substrate for plasmin during wound healing. Importantly, we find that Plg activation in the wounds is dependent on the presence of a fibrin-rich provisional matrix. We further demonstrate that although reduced fibrinolysis is not the primary impediment to wound healing in galardin-treated wild-type mice, galardin-sensitive MMPs are able to provide compensatory fibrinolysis that allows wound healing to proceed in the absence of Plg.

RESULTS

Partial compensatory effect of Fib deficiency on the healing of wounds in Plg-deficient mice

To perform a meticulous comparison of the profibrinolytic contributions of Plg and MMPs during wound healing *in vivo*, we studied the kinetics of this process in mice with selected combinations of gene deficiencies. Central to these studies is the high reproducibility of our model system in which standardized incisional, 20-mm-long full-thickness wounds were generated in wild-type mice ($n=16$), Fib-deficient mice ($n=16$), Plg-deficient mice ($n=12$), and mice double-deficient for Fib and Plg ($n=12$). The lesions were examined by visual inspection, and the wound lengths were measured every second day. Immediately after surgery, the wounds in mice of all genotypes were spindle shaped, with well-separated incision edges and underlying muscle fascia being exposed. By the second day after wounding, the wounds in wild-type and Plg-deficient mice were all covered by a dehydrated wound crust, whereas the wounds of both Fib-deficient and Fib/Plg double-deficient mice remained open and leaking, with swollen and edematous wound edges. The crust in wild-type and Plg-deficient mice was gradually lost as healing progressed, whereas the wounds of both Fib-deficient and Fib/Plg double-deficient mice remained leaky and increasingly circular without a regular wound crust up to 14 days after wounding (Figure 1a). The lesions were judged by gross inspection, and scored as healed when there was a complete loss of the wound crust and closure of the incision interface with restoration of the epidermal covering. Macroscopic evaluation of the wound geometries revealed a slightly impaired contraction in the Fib and Fib/Plg-deficient mice in the very early processes of healing (0–5 days after wounding) as evident from the relatively larger wound widths observed in these genotypes (Figure 1b, bottom). However, this initial difference between the genotypes is lost after day 5 and only the Plg single-deficient mice exhibited a delayed wound healing in the later phases, where the re-epithelialization normally occurs by a prominent keratinocyte migration (see later). The mean time to complete healing for wild-type mice was 16.4 days. In Fib-deficient mice, the mean healing time was only slightly increased to 18.5 days ($P=0.04$), whereas the mean healing time of wounds in Plg-deficient mice was increased to 39.2 days ($P=0.0015$) (Figure 1b). Wounds in Fib/Plg double-deficient mice healed much faster than the wounds in Plg single-deficient mice (22.2 vs 39.2 days; $P=0.009$), indicating that fibrin accumulation is the major impediment to wound healing in Plg-deficient mice and that fibrin is the principal substrate for plasmin during wound healing. However, the double-deficient mice showed a mean healing time that was significantly longer when compared with wild-type mice (22.2 vs 16.4 days; $P<0.0001$) and mice with a single deficiency in Fib (22.2 vs 18.5 days; $P=0.0035$) (Figure 1b). This emphasizes that in this reproducible model, loss of Fib does not completely rescue the impaired wound healing in Plg-deficient mice inflicted with large 20 mm long wounds. The same conclusion was reached in an additional study of 15 mm long wounds (data not shown).

Aberrant re-epithelialization in Fib-deficient and Fib/Plg double-deficient mice

We next examined 20 mm long wounds from wild-type, Fib-deficient, Plg-deficient, and Fib/Plg double-deficient mice at 2, 7, and 10 days after wounding by histological methods. In both wild-type and Plg-deficient mice, the wounded area is covered by a wound crust and provisional matrix at 2 days after wounding and there is no apparent migration of keratinocytes at this time point (Figure 2a and g). Keratinocyte migration is first evident at 7 and 10 days after wounding (Figure 2b, c, h, and j). After 10 days, the keratinocytes have covered or almost covered the wound in wild-type mice, and the provisional matrix is replaced by a well-organized granulation tissue (Figure 2c). The re-epithelialization is severely retarded in Plg-deficient mice 10 days

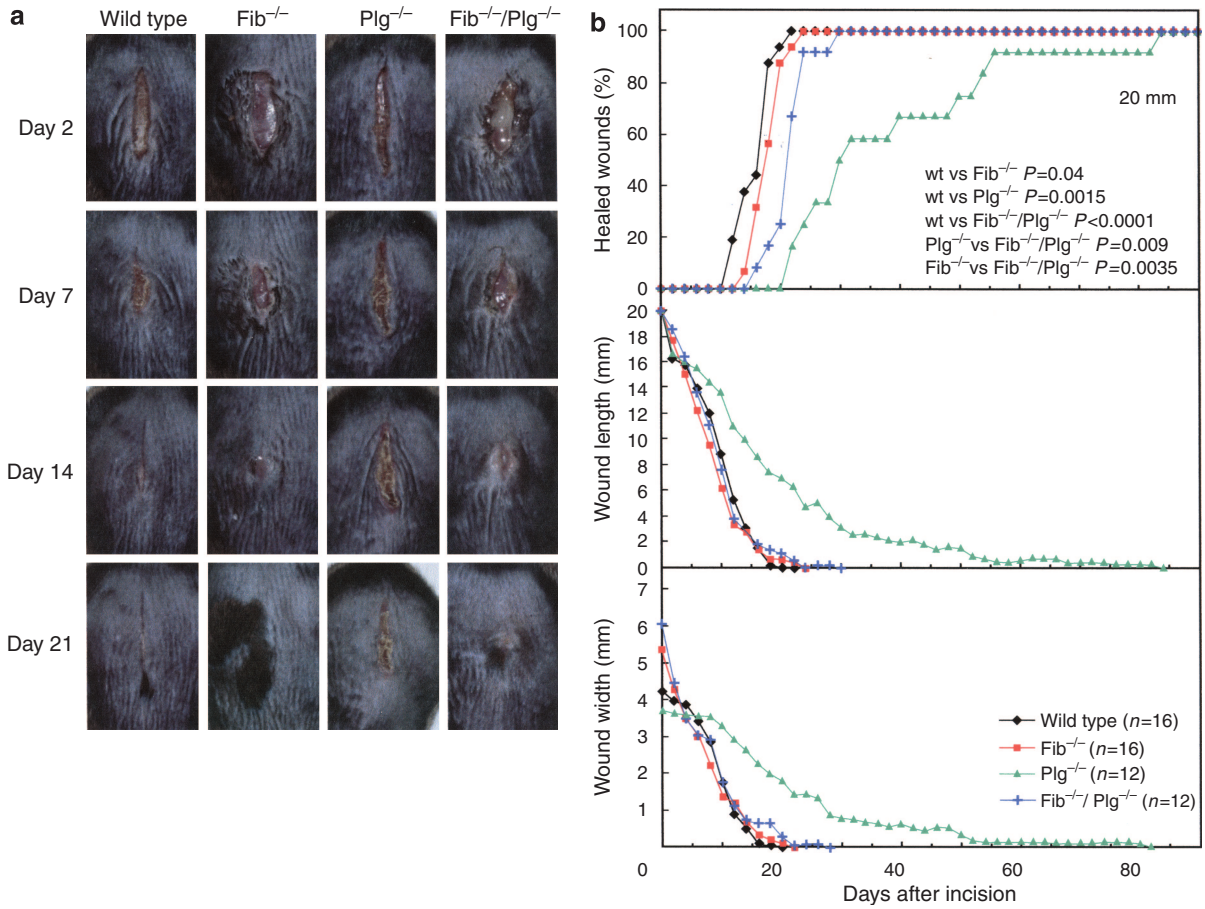

Figure 1. Time course of skin wound healing in wild-type, Fib-deficient, Plg-deficient, and Fib/Plg double-deficient mice. (**a**) Representative examples of the progress of wound repair processes in wild-type, Fib-deficient, Plg-deficient, and Fib/Plg double-deficient mice. (**b**) The percentage fraction of mice with complete re-epithelialization is plotted vs time after incision of 20 mm long wounds. The severely impaired wound healing in Plg-deficient mice is to a large extent rescued in Fib/Plg double-deficient mice. Wound healing in Fib/Plg double-deficient mice is still significantly delayed compared to wild-type mice, whereas mice with a single deficiency in Fib show only a modest delay in healing time. Wound geometries (length and width) were measured every second day for the individual single- and double-deficient genotypes and are plotted as a function of time in the middle and lower panel, respectively.

after wounding (Figure 2j). In contrast, there is no apparent provisional matrix or wound crust in either Fib-deficient or Fib/Plg double-deficient wounds at any of the time points analyzed (Figure 2d–f and k–m). Instead of using the provisional matrix as a substrate for migration, the keratinocytes move along the surfaces of the tissue resulting in a clearly aberrant re-epithelialization pattern (Figure 2f and m).

We quantified the impact of Fib deficiency, Plg deficiency, and Fib/Plg double deficiency on re-epithelialization by morphometric analysis. This was done by calculating the relative migration distance of the keratinocytes inward from the wound edges. We found the relative migration distances of the keratinocytes at day 10 after wounding to be significantly different between the wild-type and Plg-deficient wounds, as reported previously (Rømer *et al.*, 1996; Lund *et al.*, 2006). The aberrant pattern of re-epithelialization of Fib-deficient and Fib/Plg double-deficient mice complicates the interpretation of the migration distance measurements in these genotypes and excludes a direct comparison of this parameter between Fib-deficient and Fib-sufficient mice. Whereas Fib-sufficient mice exhibited a significant reduction ($P=0.003$) of the migration distance in the absence of Plg (Figure 2n), such differences in the relative migration distances were not observed in Fib-deficient mice with or without Plg (Figure 2o).

Fibrin accumulates in front of leading edge keratinocytes in Plg-deficient mice

We next used double immunofluorescence staining of tissue sections for fibrin(ogen) and cytokeratin to visualize fibrin (ogen) and keratinocytes in the wounds (Figure 3). This analysis demonstrated an accumulation of fibrin underneath and in some cases in front of the migrating keratinocytes in Plg-deficient wounds (Figure 3a), whereas no fibrin was detectable beneath the adjacent non-injured skin (data not shown). Compared with the sporadically fenestrated fibrin staining seen in wild-type mice, the wounds in Plg-deficient mice showed an almost confluent fibrin staining in the same areas (Figure 3a and b), suggestive of an additional fibrin accumulation in the absence of Plg. In the Fib-deficient and

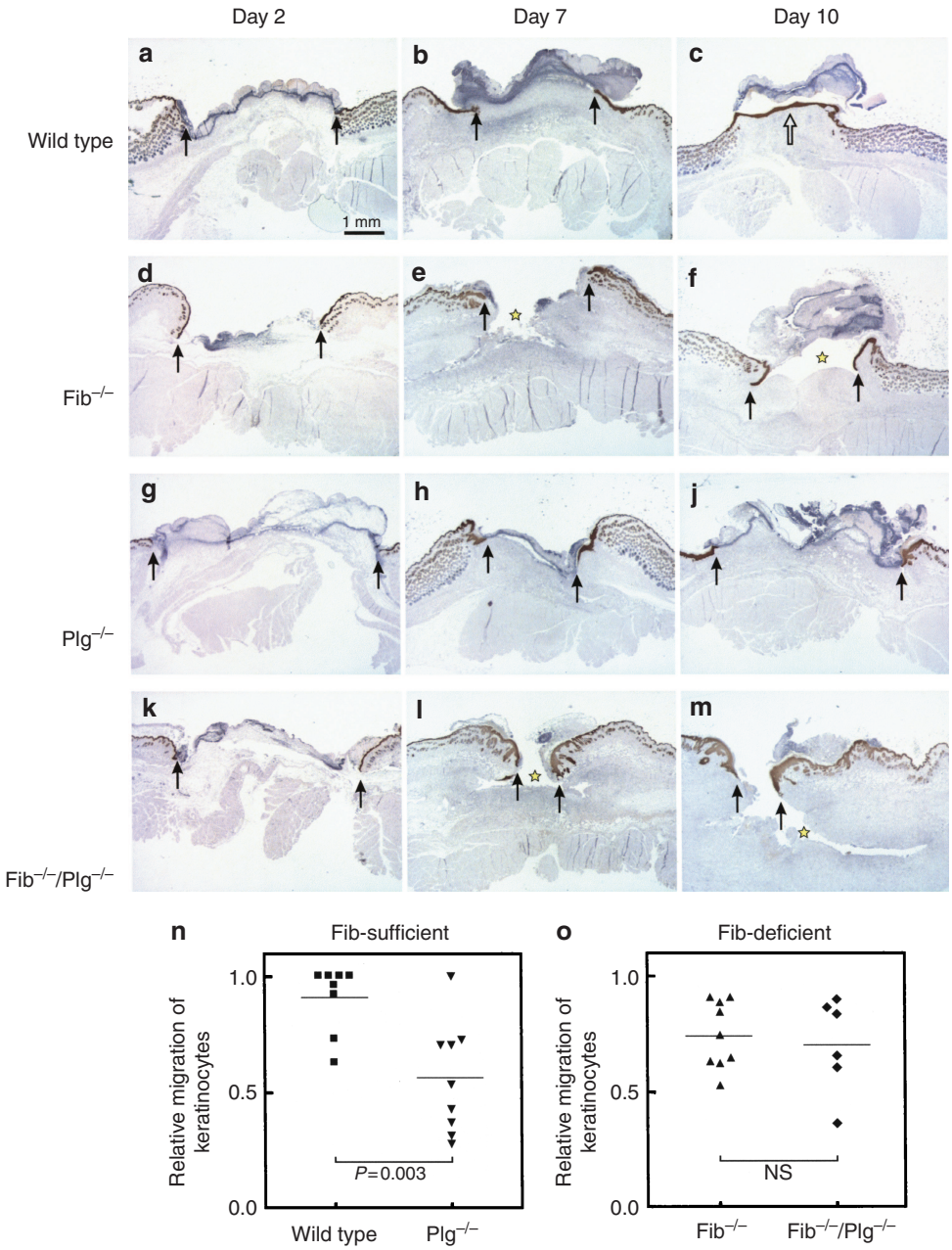

Figure 2. Kinetics of re-epithelialization in skin wounds from wild type, Fib-deficient, Plg-deficient and Fib/Plg double-deficient mice. Re-epithelialization of incisional wounds is visualized by immunostaining of keratinocytes with anti-mouse keratins 10 and 14 in wild type (**a**–**c**), Fib-deficient (**d**–**f**), Plg-deficient (**g**–**j**), and Fib/Plg-deficient (**k**–**m**) mice. The leading edge keratinocytes are indicated by solid arrows, whereas the open arrow highlights the complete restoration of the epidermal surface in a wild-type wound. The star indicates the lack of a provisional matrix in the absence of fibrin (**e**, **f**, **l**, and **m**). Scale bar = 1 mm. (**n** and **o**) Quantitative evaluation of the relative migration distance of keratinocytes measured at day 10. With this method, complete re-epithelialization is scored as 1.0 and each data point refers to an individual wound. The mean values are depicted as horizontal lines. The migration of keratinocytes in Plg-deficient wounds is significantly decreased compared with wild-type wounds (see panel n, $P = 0.003$). In contrast, there is no difference between the keratinocyte migration distances in Fib-deficient and Fib/Plg double-deficient wounds (see panel o).

Fib/Plg double-deficient wounds used as controls, no specific immunofluorescence was detected (Figure 3c and d). We next tested whether lack of fibrin or the observed differences in fibrin accumulation between Plg-deficient and Plg-sufficient mice affected the leading edge keratinocyte expression of the laminin-5 (laminin-332) γ2 chain, which is a marker for migrating keratinocytes (Larjava *et al.*, 1993; Pyke *et al.*, 1994). Immunohistochemical staining for the laminin-5 γ2 chain revealed no differences in staining intensity or localization in or beneath the migrating basal keratinocytes between any of the four genotypes analyzed (data not shown).

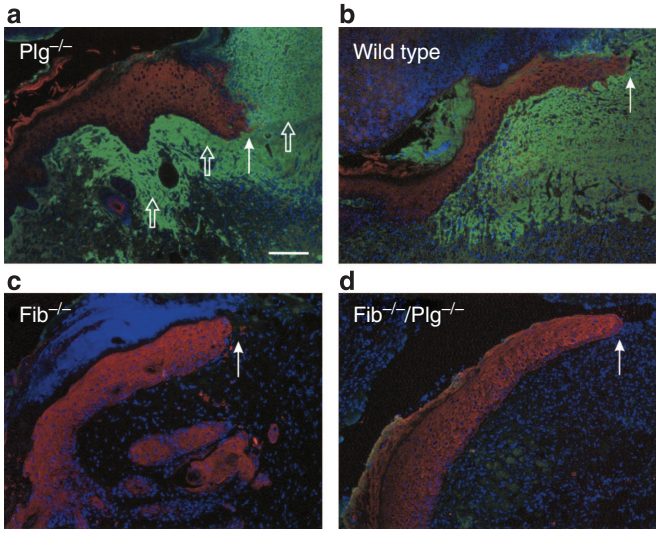

Figure 3. Fibrin accumulation in the provisional matrix of skin wounds as revealed by immunofluorescence. Tissue sections of 10-day-old wounds from Plg-deficient (**a**), wild-type (**b**), Fib-deficient (**c**), and Fib/Plg double-deficient (**d**) mice were stained by immunofluorescence with antibodies to cytokeratin 8 (red) and fibrin(ogen) (green) accompanied by nuclear staining with Hoechst (blue). The tip of the leading edge keratinocytes is shown for each genotype (closed arrows). In the wild-type wound, the keratinocytes migrate through the fibrin matrix, whereas in the Plg-deficient wound, the migrating tip is blunt-ended and a dense accumulation of fibrin is observed in front of and under the migrating keratinocytes (open arrows). As expected, no signal for fibrin is detectable in the Fib-deficient and Fib/Plg double-deficient wounds.

Activation of Plg in the wound field is dependent on the presence of a fibrin-containing provisional matrix

As fibrin is the principal substrate for plasmin, we next examined the Plg activation status in wound extracts pooled from three mice of each of the relevant genotypes. The excised wounds were lysed by ultrasonication in the presence of 10 μg ml^{-1} aprotinin, and Plg derivatives were affinity-purified on a lysine-Sepharose microcolumn (Lund *et al.*, 2006). The eluates were analyzed by SDS-PAGE followed by either silver staining or western blot analysis using a polyclonal anti-human Plg antibody (Ab) that crossreacts with murine Plg (Figure 4a and b). In the reduced and alkylated samples, the A-chain of plasmin, but not the B-chain, was detected in wounds from wild-type mice, whereas neither A- nor B-chains were detected in non-injured skin or plasma by silver staining or western blotting (Figure 4a). Surprisingly, the A-chain was also undetectable in wound extracts from Fib-deficient mice. Parallel studies performed under non-reducing conditions revealed the presence of a stable complex between plasmin and α_2-antiplasmin (α_2AP) in the wound extracts from wild-type mice, whereas this complex was undetectable in Fib-deficient mice, non-injured wild-type skin, and plasma (Figure 4b). In addition to this covalent serpin–plasmin complex, we also observed a number of fragments derived from the Plg A-chain (that is, K1–K5 and K1–K3). Peptide mass mapping by matrix assisted laser desorption ionization mass

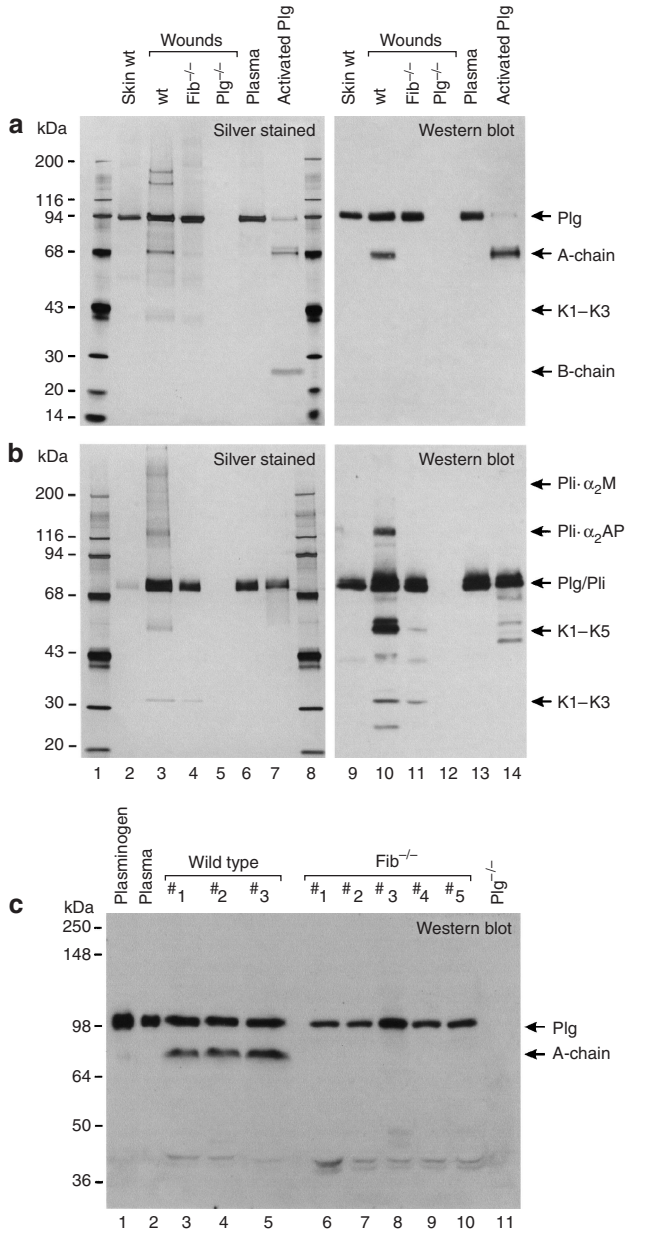

Figure 4. Attenuated plasmin generation in skin wounds from Fib-deficient mice. Plasminogen-derived fragments and complexes were purified from murine tissue extracts (pools of three mice) and plasma by lysine-Sepharose affinity chromatography. (**a**) An analysis of these purified components by SDS-PAGE after reduction and alkylation followed by either silver-staining (left) or western blotting using a polyclonal anti-plasminogen (Plg) antibody (right). (**b**) The corresponding analysis of unreduced samples. The skin extracts analyzed are from non-injured wild-type skin (lanes 2 and 9), wild-type wounds (lanes 3 and 10), Fib-deficient wounds (lanes 4 and11), and Plg-deficient wounds (lanes 5 and 12). Purified Plg from wild-type plasma served as control before (lanes 6 and 13) and after uPA-mediated *in vitro* conversion to plasmin in the presence of 10 μg ml^{-1} aprotinin (lanes 7 and 14). (**c**) Crude extracts of 7-day-old incisional wounds from individual mice were analyzed for Plg conversion by western blotting after reduction and alkylation (16 μl extract applied in each case). Extracts prepared from three wild-type mice are analyzed (lanes 3–5) and those from 5 Fib-deficient mice (lanes 6–10). To control for nonspecific binding, 16 μl of extract from a Plg-deficient wound were analyzed in parallel (lane 11) and 2 μl of plasma from a wild-type mouse served as a positive control (lane 2).

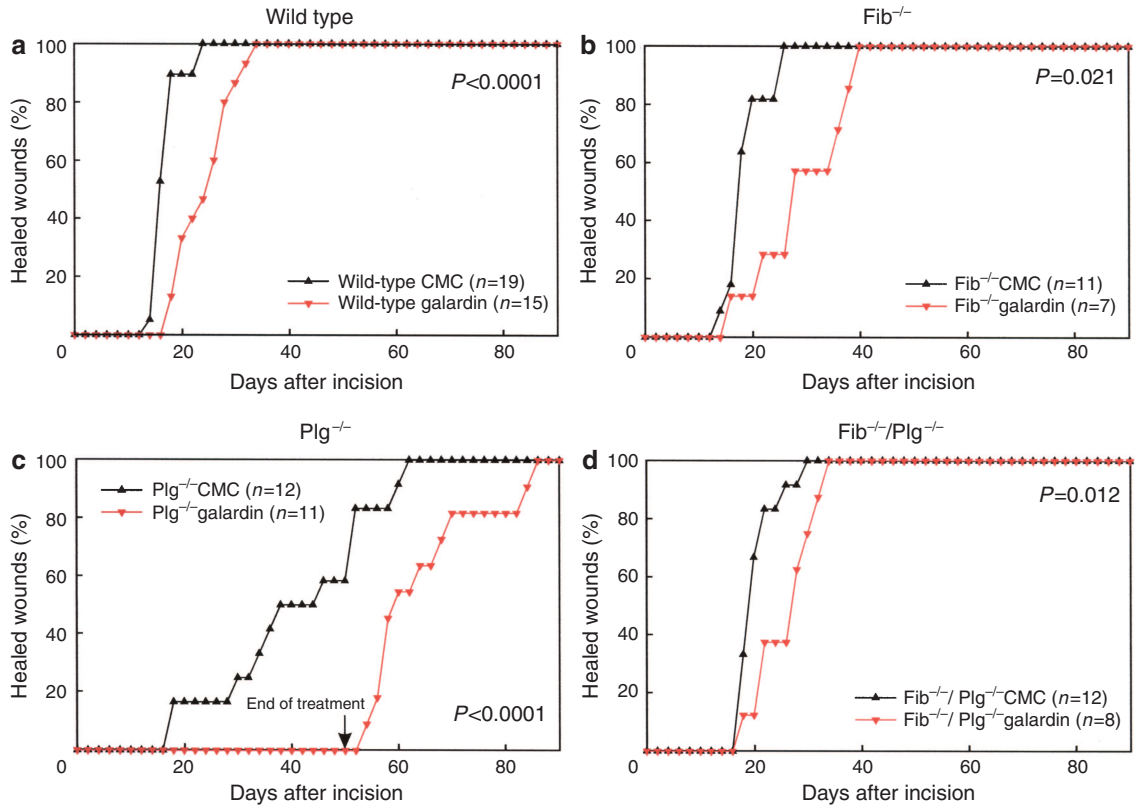

Figure 5. Effect of pharmacological inhibition of metalloproteinase activity on the healing kinetics of skin wounds in mice genetically deficient in Fib and Plg. The percentage of mice with complete healing is plotted vs time after incision of the skin. Wild-type (**a**), Fib-deficient (**b**), and Fib/Plg-deficient (**d**) mice treated with a metalloproteinase inhibitor (galardin, in red) exhibited a significant delay in healing compared with vehicle-treated mice (in black). In contrast, inhibitor treatment of Plg-deficient mice (**c**) completely blocked the repair process. This blockage of wound healing was reversible, as cessation of galardin treatment at 50 days after wounding (arrow in **c**) reinstated the healing process. Within 36 days after galardin withdrawing, all the wounds of these mice were completely covered with a new epidermal layer.

spectrometry (MALDI-MS/MS) sequencing after in-gel digestion of the excised bands confirmed the identity of the plasmin·α_2AP complex as well as the A-chain fragments shown in Figure 4a and b (data not shown). The identity of the plasmin·α_2AP complex was further substantiated by a comparison of the electrophoretic mobility of complexes formed *in vitro* and by its reactivity with anti-Plg (Figure 4b, right panel) and anti-α_2AP Abs in western blotting (data not shown). Identification of the plasmin·α_2-macroglobulin complex was based on a comparison of electrophoretic mobility with complexes formed *in vitro* (data not shown). Despite the lack of detectable plasmin formation in the absence of fibrin, the phenotypic difference observed between the healing times in Fib-deficient and Fib/Plg double-deficient mice (Figure 1b) may indicate that minor amounts of plasmin are actually formed in the Fib-deficient mice. Nonetheless, these biochemical data demonstrate a significant role *in vivo* of local fibrin deposition for Plg activation during wound healing as evident from the specific accumulation of inhibited plasmin complexes in the wild-type wounds only. This genotype-specific Plg activation was not influenced by the lysine-Sepharose chromatography, as a similar activation status was observed by western blotting using crude wound extracts from individual mice (Figure 4c).

Lack of fibrin decreases wound healing times in galardin-treated Plg-deficient mice, but not in galardin-treated wild-type mice

We previously demonstrated that the broad-spectrum metalloproteinase inhibitor galardin delays wound healing in wild-type mice and that wound healing in galardin-treated Plg-deficient mice is completely blocked (Lund *et al.*, 1999). To explore the impact of fibrin(ogen) under these conditions, we next examined the contribution of impaired fibrin degradation to wound healing kinetics in galardin-treated wild-type mice and in galardin-treated Plg-deficient mice. Galardin treatment increased the mean wound healing time in wild-type mice ($n=15$) to 24.9 days compared with 17.5 days in mock-treated wild-type mice ($n=19$) ($P<0.0001$) (Figure 5a). Similarly, the mean wound healing time of galardin-treated Fib-deficient mice ($n=7$) was increased to 29.7 days compared with 19.3 days in mock-treated Fib-deficient mice ($n=11$) ($P=0.021$) (Figure 5b). There was no significant difference in the healing times between galardin-treated wild-type mice and galardin-treated Fib-deficient mice (24.9 vs 29.7 days; $P=0.12$) (compare Figure 5a and b). This indicates that reduced fibrinolysis is not the major cause of the delayed wound healing in galardin-treated Plg-sufficient mice. No appreciable wound healing takes place in galardin-treated Plg-deficient mice ($n=11$) (Figure 5c).

Importantly, when Fib deficiency is superimposed on this phenotype, the resulting galardin-treated Fib/Plg-deficient mice ($n=8$) displays only a modest delay in wound healing compared with mock-treated Fib/Plg-deficient mice ($n=12$) (26.8 vs 21.0 days; $P=0.012$) (Figure 5d). This shows that galardin-sensitive MMPs are profibrinolytic in the absence of Plg and that the complete loss of healing capacity in galardin-treated Plg-deficient mice is largely attributable to the inability to proteolytically remove accumulated fibrin from the wound field.

DISCUSSION

In this study, we have shown that galardin-sensitive metalloproteinases are endowed with profibrinolytic activity *in vivo*, as they, to some extent, can substitute for Plg in the removal of fibrin during skin wound healing in a reproducible mouse model. Another important conclusion from these studies is that although the primary impediment to the healing of wounds in Plg-deficient mice is compromised fibrin dissolution, it is not the only defect. This indicates that plasmin has other as yet unidentified substrates during skin wound healing. Intriguingly, these experiments also highlight the importance of a Fib/fibrin matrix in the biochemical activation of Plg *in vivo*.

Using an identical genetic approach, it was previously reported that removal of fibrin(ogen) from the wound field negates the requirement for Plg for timely healing (Bugge *et al.*, 1996b), suggesting that plasmin-mediated fibrinolysis is the only rate-limiting factor in the dissolution of the provisional matrix in the wound field. Nevertheless, in this study, we consistently find a small, but significant, residual impairment in the healing kinetics of wounds in Fib/Plg double-deficient mice compared to littermate wild-type controls. The reason for the discrepancy between the two studies is at present unknown, but it may have a bearing on the differences in genetic background of the mice, wound size and geometry, and animal housing facilities. These variations in the experimental conditions may have caused shorter healing times (11–13 days) in the previous study (Bugge *et al.*, 1996b) compared with a mean healing time of 16.4 days in this study, thus providing a larger dynamic range in the latter study enabling the identification of minor differences between genotypes. Therefore, our study provides clear evidence that plasmin cleavage of substrates different from fibrin(ogen) is ultimately rate-limiting for incisional skin wound healing, as also reported previously for the removal of necrotic hepatic debris after carbon tetrachloride intoxication of Plg-deficient mice (Bezerra *et al.*, 1999). Extracellular proteases have pleiotropic functions in tissue repair (Page-McCaw *et al.*, 2007). Besides the removal of provisional extracellular matrix, these include the liberation of latent growth factor precursors from the cell surface and the extracellular matrix, the activation of protease activated receptors, the shedding of growth factor and cytokine receptors, and the generation of chemotactic degradation products of extracellular matrix. Identification of the relevant non-fibrin(ogen) substrates cleaved by plasmin during skin wound healing, therefore represents, a challenging enterprise.

It is well established that intravascular plasmin generation is strictly confined to the dissolving thrombus, as explained by the very potent stimulation of tPA activity by fibrin, and by the protection of fibrin-bound plasmin from neutralization by its endogenous inhibitors (Collen and Lijnen, 2005). An unanticipated finding in this study was that plasmin generation is also strongly dependent on fibrin(ogen) in the extravascular environment of a wound field. This is particularly unexpected, as we have previously shown that uPA, which has no known fibrin-stimulated Plg activator activity, has the major impact on plasmin generation during skin wound healing compared to tPA (Lund *et al.*, 2006). Intriguingly, this suggests that fibrin(ogen) is also a potent promoter of the reciprocal pro-uPA/Plg zymogen activation *in vivo* and that this effect may be well governed by the resistance of fibrin-bound plasmin toward inhibition by α_2AP and α_2-macroglobulin. Nevertheless, studies on a combined deficiency of both uPA and tPA demonstrate an important functional overlap of these enzymes in skin wound healing and plasmin generation (Bugge *et al.*, 1996a; Lund *et al.*, 2006). Detailed studies of plasmin generation in tPA-deficient mice with and without a genetic superposition of fibrin(ogen) deficiency would be excellently suited to further investigate this interesting phenomenon. Our findings also raise the rather curious possibility that the minor, but significant, delay in wound healing observed here in Fib-deficient mice may be caused by the loss of fibrin(ogen)-dependent plasmin formation and the subsequent absence of plasmin cleavage of non-fibrin(ogen) substrates. Nonetheless, it should be emphasized that this observation could equally be well rationalized by the absence of specific fibrin-derived bioactive peptides in the Fib-deficient mice, which normally would provoke a pro-inflammatory response in the provisional matrix by recruitment of neutrophils and monocytes, and the absence of these stimuli would cause the observed delay in wound healing (Richardson *et al.*, 1976; Gross *et al.*, 1997; Laurens *et al.*, 2007). An analogous pro-inflammatory role of transforming growth factorβ has been proposed to influence wound healing (Wahl *et al.*, 1987).

We have previously demonstrated a functional overlap between Plg and galardin-sensitive metalloproteinases during skin wound healing (Lund *et al.*, 1999, 2006). We now provide previously unidentified mechanistic insights into this phenomenon by showing that fibrin(ogen) is an important mutual substrate for the two extracellular proteolytic systems. Furthermore, we show that metalloproteinase-mediated fibrin dissolution becomes functionally important only when plasmin generation is absent, but fibrin(ogen)-independent metalloproteinase activity is quantitatively important for wound healing both in the presence and absence of Plg. Our data are concordant with previous studies showing that some MMPs are endowed with significant fibrinolytic capacity in cell-based *ex vivo* models of cell migration (Hiraoka *et al.*, 1998; Hotary *et al.*, 2002). We previously reported that most cell types critical to skin wound healing, including endothelial cells, fibroblasts, and inflammatory cells, accumulate at normal rates in the Plg-deficient wound

field and that the subsequent granulation tissue formation involving these cells is seemingly unimpaired by the absence of Plg (Rømer *et al.*, 1996). This strongly suggests that the compensatory fibrinolysis mediated by galardin-sensitive metalloproteinases during healing of Plg-deficient wounds is directly related to the dissolution of fibrin required for the migration of granulation tissue cells and keratinocytes into the fibrin-rich provisional matrix during tissue repair. This hypothesis is in excellent agreement with our previous demonstration of a colocalization of Plg activator (uPA) and several galardin-sensitive metalloproteinases (MMP-3, MMP-9, MMP-13) in leading edge wound keratinocytes (Lund *et al.*, 1999), which may explain the complete arrest of keratinocyte migration at the wound edge of galardin-treated Plg-deficient mice.

In summary, our studies have revealed that galardin-sensitive metalloproteinases are equipped with profibrinolytic activity during skin wound healing, the existence of fibrin(ogen)-independent substrates for both Plg and galardin-sensitive metalloproteinases that are quantitatively relevant to skin wound healing, and uncovered a strict requirement for fibrin(ogen) in the generation of plasmin within the extravascular environment of a healing wound.

MATERIALS AND METHODS

Animals and animal treatment

Fib gene-targeted mice on a C57BL/6J background (Ploplis *et al.*, 2000) were backcrossed into an FVB/n background (Panum Institute, Copenhagen, Denmark) for eight generations, as less than 5% Fib-deficient mice born on a C57BL/6J background survived to weaning. Heterozygous mice were crossed with heterozygous Plg mice backcrossed to C57BL/6J for 22 generations to obtain an F1 generation of double heterozygous $Fib^{+/-}/Plg^{+/-}$ siblings. These double-deficient heterozygous mice were crossed to obtain an F2 generation of siblings, which revealed the expected Mendelian distribution. Chromosomal DNA from an approximately 2 mm piece of tail tip was isolated as described (Lund *et al.*, 1999). The Plg genotype was determined as described (Bugge *et al.*, 1996b). The Fib genotype was determined by multiplex PCR using the following three primers. mfib(g)-1p (GTGCCACAGCTGCTTGTCATTAG) hybridizing to position 10 138–10 160 in the mouse Fib(γ) gene (GenBank: CAAA01040047); mfib(g)-2m (ATGCTGGAGATG GGTTCCTGTC) hybridizing to position 10 449–10 470 generate a 333 bp product specific for the endogenous allele; and mfib(g)-1p and Neo-3p (TCAGCAGCCTCTGTTCCACATAC) hybridizing within the neomycin cassette generate a 499 bp product specific for the targeted allele.

All mice used for experiments were between 6 and 8 weeks old at the start of the experiment. Investigators unaware of the mouse genotype performed all experimental evaluations, tissue isolations, and microscopic analyses. The MMP inhibitor *N*-[(2R)-2-(hydroxamido-carbonylmethyl)-4-methylpentanoyl]-L-tryptophanmethylamide (Galardin) was synthesized as described (Grobelny *et al.*, 1992). Galardin was clinically formulated as a 20 mg ml^{-1} slurry in 4% carboxymethylcellulose in phosphate-buffered saline (PBS) and was administered daily intraperitoneally at 100 mg per kg body weight. Mock treatment included 4% carboxymethylcellulose in PBS.

Tissue preparation

Six- to eight-week old mice were anesthetized by subcutaneous administration of 0.03 ml per 10 g of a 1:1 mixture of Dormicum (Midazolam 5 mg ml^{-1}) and Hypnorm (Fluanison 5 and Fentanyl 0.1 mg ml^{-1}) before surgery. Incisional skin wounds of 15- or 20-mm-long full-thickness were made middorsally with a scalpel. The wounds were neither dressed nor sutured. The mice were caged individually until killing, at the time they were anesthetized as described above, followed by perfusion fixation with PBS and 4% paraformaldehyde. The wound areas were removed, bisected in the midtransversal plane, fixed in 4% paraformaldehyde overnight and then paraffin-embedded as described previously (Lund *et al.*, 1999). The sections were cut perpendicular to the longitudinal direction of the wounds. For histological analysis, a total of 90 mice were wounded, from which tissue was isolated for each genotype (wild type, Fib-deficient, Plg-deficient, and Fib/Plg double-deficient) at day 2 after wounding from 5, 5, 4, and 3 mice of the respective genotypes; at day 7 from 4, 4, 5, and 5 mice; at day 10 from 8, 9, 10, and 6 mice; and at the time of healing from 7, 5, 3, and 7 mice. Animal care at The Department of Experimental Medicine, University of Copenhagen and Rigshospitalet, Copenhagen, Denmark, was in accordance with institutional and national guidelines.

Computer-assisted morphometry

Keratinocyte migration was measured microscopically on tissue sections stained immunohistochemically with anti-mouse keratins 10 and 14 IgG (see below). The length of the epidermal tongue was measured as the linear distance between the tip of the leading edge keratinocytes and the edge of the wound, defined as the point in the zone of proliferation where a shift from two to three layers of keratinocytes could be identified. The relative migration was determined as the sum of the lengths of both epidermal tongues divided by the width of the wound, defined as the linear distance between the wound edges. The sections used for these measurements were carefully selected from the midline of the wound field. Identification of this point in the proliferation zone and the tip of the epidermal wedge by image analysis were performed on two sections per wound by an observer unaware of the genotype of the mice.

Statistical analysis

The StatView software package (version 5.0; SAS Institute, Cary, NC) was used for statistical analysis. The distribution of time to macroscopic wound closure and the migration distance of keratinocytes in histological sections were found to be normal. Pre-specified tests of hypothesis comparing experimental groups were carried out using two sample *t*-tests. The assumption of variance homogeneity was tested by the folded F-test, and the two sample *t*-tests assuming unequal variances was used if the hypothesis of variance homogeneity was rejected. The level of significance was set at 5%.

Immunohistochemistry

Tissue sections were immunohistochemically stained by the peroxidase anti-peroxidase method with rabbit polyclonal Abs directed against mouse keratins 10 and 14 (Cappel, Organon Teknika, PA). Each experiment included controls with omission of the primary Ab and substitution of the primary Ab with non-immune rabbit IgG. These were all negative.

Double immunofluorescence

Tissue sections were de-paraffinized in xylene and hydrated through graded ethanol/water dilutions. Antigen retrieval was by incubation with proteinase K for 15 minutes at 37 °C. Sections were then washed in running tap water for 5 minutes and Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.6) for 5 minutes. The slides were then mounted with TBS containing 0.5% Triton X-100 in immunostaining chambers (Thermo Shandon, cat. no. 7211013, Pittsburgh, PA) for subsequent incubations. Slides were incubated overnight together with rabbit anti-mouse fibrin(ogen) Ab (1:2,000; Bugge *et al.*, 1995) and rat anti-mouse cytokeratin-8 Ab (clone Troma-1, 1:100; Kemler *et al.*, 1981) in TBS containing 0.25% BSA at 4 °C. All Ab incubations were followed by washes in TBS. Rabbit anti-fibrin(ogen) Ab was detected with Alexa Fluor 488-linked donkey anti-rabbit Ab (1:200, cat. no. A21206; Molecular Probes, Carlsbad, CA), and rat anti-cytokeratin-8 Ab was detected with Alexa Fluor 594-linked goat anti-rat Ab (1:200, cat. no. A11007; Molecular Probes) in TBS containing 0.25% BSA for 45 minutes at ambient temperature. The slides were counterstained with Hoechst 33342 (1:5,000, cat. no. H3570; Molecular Probes) in TBS for 10 minutes at ambient temperature and mounted in fluorescent mounting medium (cat. no. S3023; DakoCytomation, Glostrup, Denmark). Negative controls without anti-fibrin(ogen) primary Ab were negative for unspecific staining.

Western blot analysis

Frozen tissue powder containing both the wound rim and granulation tissue (pool of three mice per genotype) was resuspended in 5 µl extraction buffer (0.1 M Tris/HCl (pH 7.4) containing 10 µg ml^{-1} aprotinin) per milligram wet weight tissue and treated for 2 × 8 minutes in an ultrasonication bath. The resulting supernatants were collected after centrifugation at 12,000 *g* for 30 minutes at 4 °C. After SDS-PAGE, the samples were transferred to polyvinylidene difluoride membranes by electroblotting, and additional binding was blocked by incubation for 1 hour at room temperature with either 5% non-fat-dried milk (crude lysates) or 2% BSA (purified Plg preparations) in PBS containing 0.05% Tween-20 (PBS-T). Primary Ab was incubated overnight at 4 °C with 0.1 or 0.5 µg ml^{-1} polyclonal rabbit anti-human Plg Ab (cat. no. A0081; DakoCytomation). After washing in PBS-T (5 × 5 minutes), membranes were incubated for 1.5 hours at room temperature with horseradish peroxidase-linked secondary Ab (cat. no. P0217, 1:5,000; DakoCytomation) in blocking buffer. After washing in PBS-T and PBS, horseradish peroxidase activity was detected using enhanced chemiluminescence reagents (Amersham Biosciences, Hillerød, Denmark). Mouse Plg purified from plasma served as controls as did plasmin generated *in vitro* from purified mouse Plg by uPA activation in the presence of 10 µg ml^{-1} aprotinin. A negative control without primary Ab included was carried out in parallel.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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