Functional overlap between two classes of matrix-degrading proteases in wound healing

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Retarded wound healing was found in mice deficient in the serine protease precursor plasminogen, as well as in wild-type mice treated with the metalloprotease inhibitor galardin, but in both cases wound closure was ultimately completed in all mice within 60 days. The expression of several matrix metalloproteases in keratinocytes migrating to cover the wound was strongly enhanced by galardin treatment. However, when plasminogen-deficient mice were treated with galardin, healing was completely arrested and wound closure was not seen during an observation period of 100 days, demonstrating that protease activity is essential for skin wound healing. The requirement for both plasminogen deficiency and metalloprotease inhibition for complete inhibition of the healing process indicates that there is a functional overlap between the two classes of matrix-degrading proteases, probably in the dissection of the fibrin-rich provisional matrix by migrating keratinocytes. Each class alone is capable of maintaining sufficient keratinocyte migration to regenerate the epidermal surface, although this function would normally be performed by both classes acting in parallel. Since there are strong similarities between the proteolytic mechanisms in wound healing and cancer invasion, these results predict that complete arrest of this latter process in therapeutic settings will require the use of inhibitors of both classes of proteases.
Keywords: keratinocytes/metalloprotease/plasminogen/ serine protease/wound healing

Introduction

Wound healing is a tissue remodeling process in which the injured tissue is removed and substituted with normal tissue. Proteolytic degradation of the extracellular matrix is considered to play a crucial role in this process (Grøndahl-Hansen *et al*., 1988; Werb, 1997) as well as in a variety of other physiological and pathological processes involving tissue remodeling and cell migration. These include tropho-blast invasion (Sappino *et al*., 1989; Rinkenberger *et al*., 1997; Teesalu *et al*., 1998), post-lactational mammary gland involution (Talhouk *et al*., 1992; Lund *et al*., 1996), angiogenesis (Pepper *et al*., 1987; Mignatti *et al*., 1996), inflammation (Werb, 1997) and cancer invasion (Danø *et al*., 1985, 1994; Liotta *et al*., 1991; Blasi, 1993). Several proteolytic enzymes such as the serine protease plasmin and a number of matrix metalloproteases (MMPs) are thought to be involved in the degradation of extracellular matrix (Sternlicht and Werb, 1999). This assumption is mostly based on correlative observations and *in vitro* studies. Recently, however, a role for plasminogen (Plg), the precursor of plasmin, in a tissue remodeling process was demonstrated conclusively by the finding of impaired skin wound healing in mice with a disrupted Plg gene (Rømer *et al*., 1996). In these mice, there was a decreased rate of migration of keratinocytes from the wound edge during the re-epithelialization process, which we proposed was due to a diminished ability of these cells to dissect their way proteolytically through the fibrin-rich extracellular matrix beneath the wound crust. Strong support for this interpretation comes from the observation that virtually normal skin wound healing time is restored in mice deficient in both Plg and fibrin (Bugge *et al*., 1996).

Although wound healing in Plg-deficient mice is impaired, wound closure eventually is achieved. In our previous studies, complete healing of all wounds occurred within 13 days in wild-type mice, while 60% of the wounds in the Plg-deficient mice were healed at the termination of the experiment at day 50 (Rømer *et al*., 1996). In subsequent experiments (see below), all wounds in Plg-deficient mice were healed by around day 60. During healing of skin wounds, the migrating keratinocytes express key mediators and regulators of plasminogen activation, including urokinase Plg activator (uPA), uPA receptor (uPAR) and type 1 PA inhibitor (PAI-1) (Rømer *et al*., 1991, 1994). In addition, they express several MMPs (Hewitt and Danø, 1996; see also below). We therefore hypothesized that the MMPs may also play a role in matrix degradation during wound healing, that there is a functional overlap between plasmin and the MMPs, and that the residual wound healing capacity observed in the Plg-deficient mice reflects the ability of MMPs to perform alone the functions which they would perform together with plasmin in wild-type mice. In order to test this hypothesis, we have now studied the effect of galardin, a hydroxamate inhibitor of a broad spectrum of MMPs (Grobelny *et al*., 1992; Levy *et al*., 1998), on skin wound healing in wild-type and Plg-deficient mice.

Results

Several MMPs are expressed during skin wound healing

To examine the expression of MMPs during skin repair, full thickness incisional wounds were made in wild-type mice ($n = 12$), and sections were analyzed microscopically at time points ranging from 12 h to 7 days after wounding. mRNAs encoding the respective proteases were detected by *in situ* hybridization with probes specific for seven different MMPs, including gelatinase A (MMP-2), gelatinase B (MMP-9), collagenase-3 (MMP-13), stromelysin-1 (MMP-3), stromelysin-3 (MMP-11), MT-1 MMP (MMP-14) and macrophage metalloelastase (MMP-12). In normal skin, there was no detectable mRNA for any of these MMPs, while they were all expressed in the skin wounds.

Gelatinase B mRNA was detected already 12 h after the wounding in the keratinocytes at the wound edge, which had just begun to flatten at that time and to move into and under the wound crust. All these gelatinase B-expressing cells were located basally just at the front of the moving epidermal layer. At all subsequent time points until 7 days after wounding, gelatinase B mRNA was found in keratinocytes at the same location (Figure 1a and b). Collagenase-3 mRNA was expressed in a pattern similar to that of gelatinase B, the only difference being that collagenase-3 mRNA was also detected in many basal keratinocytes located further behind the leading edge (Figure 1e and f). Expression of gelatinase B mRNA was not detected in the granulation tissue or in any cell types other than the keratinocytes, while collagenase-3 mRNA was found occasionally in macrophage-like cells in the granulation tissue. When the wounds were completely covered with a newly formed epidermal layer, expression of mRNA for the two MMPs was no longer detectable in any cells in the skin. mRNA for gelatinase A, stromelysin-3 and MT-1 MMP were all detected in fibroblasts located in the lower dermis at the edge of the wound site from 12 h after wounding. At later time points, strong expression of mRNA from these three genes was also seen in the granulation tissue, located in fibroblast-like cells which were migrating under the wound site. Comparison of adjacent sections showed that gelatinase A and MT-1 MMP mRNA were expressed in the very same fibroblasts (not shown). In the last phase of the healing processes leading to closure of the wound site with epidermis, expression of these three MMPs was seen in fibroblast-like cells located very close to the newly formed epidermal basement membrane. After the closure of the wound site with a new epidermal layer, weak signals for gelatinase A, stromelysin-3 and MT-1 MMP mRNA were still found in some fibroblast-like cells. No expression of mRNAs for any of these three molecules was detected in keratinocytes or in any cells other than fibroblast-like cells in the wounds.

Stromelysin-1 and macrophage metalloelastase each had a unique expression pattern. Stromelysin-1 mRNA was found in both keratinocytes and fibroblasts. The expression was strongest in the basal keratinocytes behind the migrating front in the area corresponding to the initial wound edge, and in fibroblasts located below and adjacent to these keratinocytes, but stromelysin-1 mRNA was also

detected in the leading edge keratinocytes (Figure 1c and d). Macrophage metalloelastase mRNA was expressed in a subset of macrophages located below and adjacent to the wound crust (not shown). Expression of stromelysin-1 and macrophage metalloelastase mRNA was not detected in any other cells in the wounds.

Delay of wound healing by treatment with an MMP inhibitor

The expression of several MMPs in leading edge keratinocytes during wound healing is remarkably similar to the expression we have found previously for the key components of the plasminogen activation system, uPA and uPAR (Rømer *et al.*, 1991, 1994, 1996), which could suggest that some of the MMPs, like plasmin, are involved in matrix proteolysis in the healing process. To test this hypothesis, we made standardized 20 mm long, full-thickness incisional wounds in the skin of mice, and then treated the mice with daily intraperitoneal injections of increasing doses of the metalloprotease inhibitor galardin. The wounds were examined visually and the wound length measured the day after surgery and at 2–3 day intervals thereafter. Wounds were scored as fully healed when there was a loss of the wound crust and a macroscopic closure of the incision interface with restoration of the epidermal covering. All wounds were spindle-shaped immediately after incision, with well-separated incision edges and exposure of the underlying muscle fascia. By the second day, all wounds were covered with a dry wound scab. In control mice mock treated with carboxymethylcellulose (CMC), the vehicle used for suspension of galardin, there was a gradual loss of the scab and decrease in the wound length, until all wounds were healed at day 15 (Figure 2). In the galardin-treated mice, there was a delay in the healing, which became more pronounced with higher doses of the inhibitor and was statistically highly significant with doses of 50 mg/kg and above ($P < 0.0001$ in all cases). Thus at the termination of the experiment at day 25, only 30% of the wounds were healed in mice treated daily with 100 mg/kg of galardin. The remaining wounds were covered with a dry scab and had thickened edges. As described in further detail below (see the section Keratinocyte migration and Figure 4), a microscopic analysis of the wounds revealed that the rate of migration of keratinocytes to cover the wound field was significantly inhibited by galardin treatment, probably causing the observed delay in wound closure.

Wound healing is arrested completely in Plg-deficient mice treated with MMP inhibitor

The effect of galardin on wound healing in wild-type mice is thus very similar to that of Plg deficiency caused by targeted gene inactivation, consistent with the hypothesis of similar and overlapping functions of plasmin and one or more MMPs in this process. A prediction from this hypothesis would be that an additive and perhaps synergistic effect could be produced if Plg deficiency is combined with MMP inhibition.

This prediction pre-supposes that the Plg deficiency does not in itself lead to a substantial up-regulation of MMP expression. In order to evaluate this possibility, we examined the expression of mRNAs for the seven MMPs discussed above by *in situ* hybridization of sections from
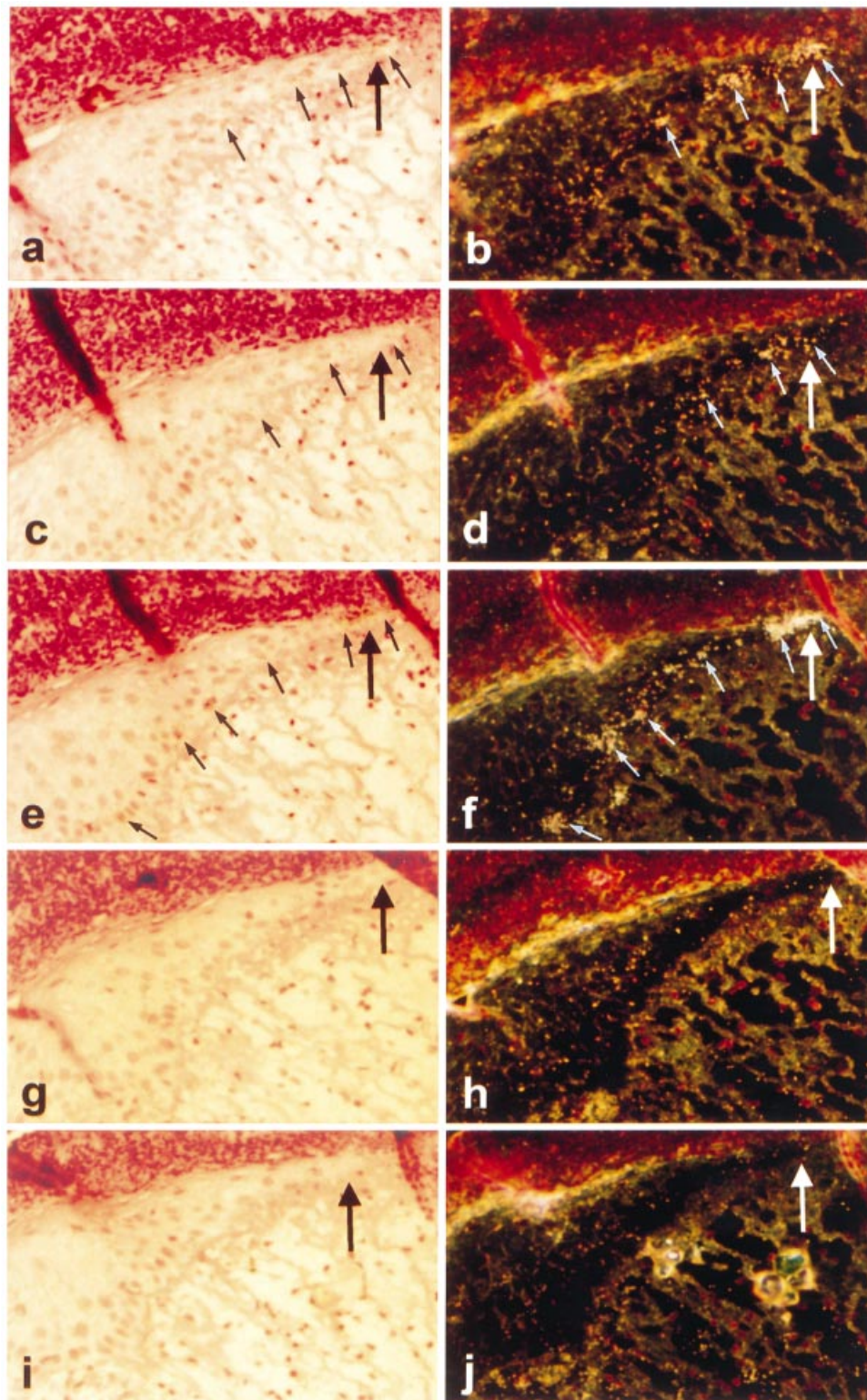
Fig. 1. Metalloprotease expression during skin wound healing. Sections of wounded skin removed 7 days after surgery of wild-type mice were analyzed by *in situ* hybridization for expression of mRNA for gelatinase B (**a** and **b**), stromelysin-1 (**c** and **d**), collagenase-3 (**e** and **f**), gelatinase A (**g** and **h**) and stromelysin-3 (**i** and **j**). The large straight arrows indicate the tip of the epidermal tongue. The small straight arrows indicate expression of gelatinase B, stromelysin-1 and collagenase-3 mRNA in the migrating keratinocytes. Note that mRNAs for gelatinase B, stromelysin-1 and collagenase-3 are expressed in the leading edge keratinocytes, whereas gelatinase A and stromelysin-3 mRNAs could not be detected in the keratinocytes. (a), (c), (e), (g) and (i) are bright-field and (b), (d), (f), (h) and (j) are dark-field micrographs. Magnification, ×160.

skin wounds derived from our previous study of wound healing in mice homozygous for a disrupted Plg gene (Plg$^{-/-}$ mice) (Rømer *et al.*, 1996). Littermates that were

homozygous for the wild-type Plg gene (Plg$^{+/+}$ mice) were included as controls. All seven mRNAs were expressed in the Plg$^{-/-}$ mice in a pattern and to a degree similar to
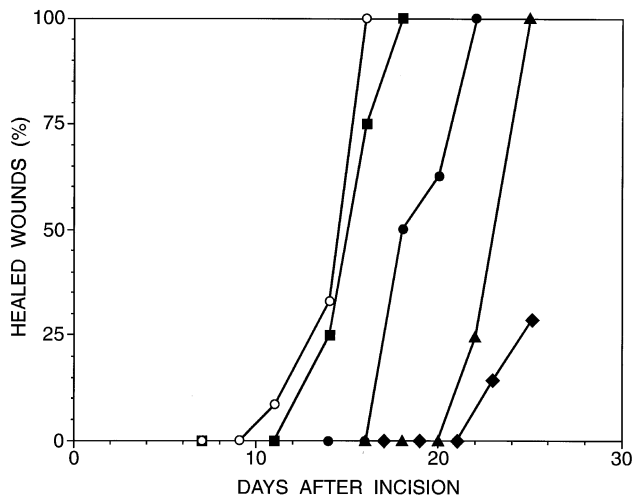
Fig. 2. Effect of the MMP inhibitor galardin on skin wound healing in wild-type mice. The percentage fractions of mice with healing defined as loss of the wound scab and complete covering of the epidermis are plotted versus time after the incision, for mice treated i.p. with galardin in daily doses of 25 mg/kg (■), 50 mg/kg (●), 75 mg/kg (▲) and 100 mg/kg (◆) or mock-treated with 4% CMC in 0.9% saline (○), the vehicle used for suspension of galardin.

that described above for wild-type mice. This pattern of expression was also observed in the Plg$^{+/+}$ littermate control mice, taking into account that the wound healing is strongly delayed in the Plg-deficient mice, i.e. at the same stage of healing the MMP mRNA expression observed in mice of the two genotypes was similar with respect to both location and intensity of the respective hybridization signals. We thus observed no sign of transcriptional up-regulation of MMP genes in the Plg-deficient mice.

We then treated wounded Plg$^{-/-}$ mice with galardin (Figure 3). As controls, we included littermate Plg$^{+/+}$ mice and groups of mice of each genotype that were given mock treatment with the vehicle CMC alone. In this experiment, we used mice backcrossed into NIHS mice. Wild-type mice of this genetic background had a somewhat slower wound healing than the C57BL/6J mice used in the experiment shown in Figure 2, and the effect of galardin on this process was less pronounced; 100 mg/kg only causing a moderate and statistically insignificant ($P = 0.06$) delay. In the mock-treated Plg-deficient mice, there was a stronger and statistically highly significant ($P < 0.0001$) delay in healing, similar to what we found in our previous study. The macroscopic appearance of the wounds was similar in galardin-treated wild-type mice and mock-treated Plg-deficient mice. These wounds were characterized by a long period with a gaping and red wound field, which had a hard and scaly surface, lacked epidermal covering, and often granulation tissue protruded between the wound edges. However, in both groups, all wounds were healed at approximately day 60 (Figure 3b). The healed skin in these two groups differed from that in the mock-treated wild-type mice in that it was palpably thicker than the surrounding skin, indicating an abnormal healing process.

In the group of mice which were both Plg-deficient and treated with galardin, all wounds remained open within
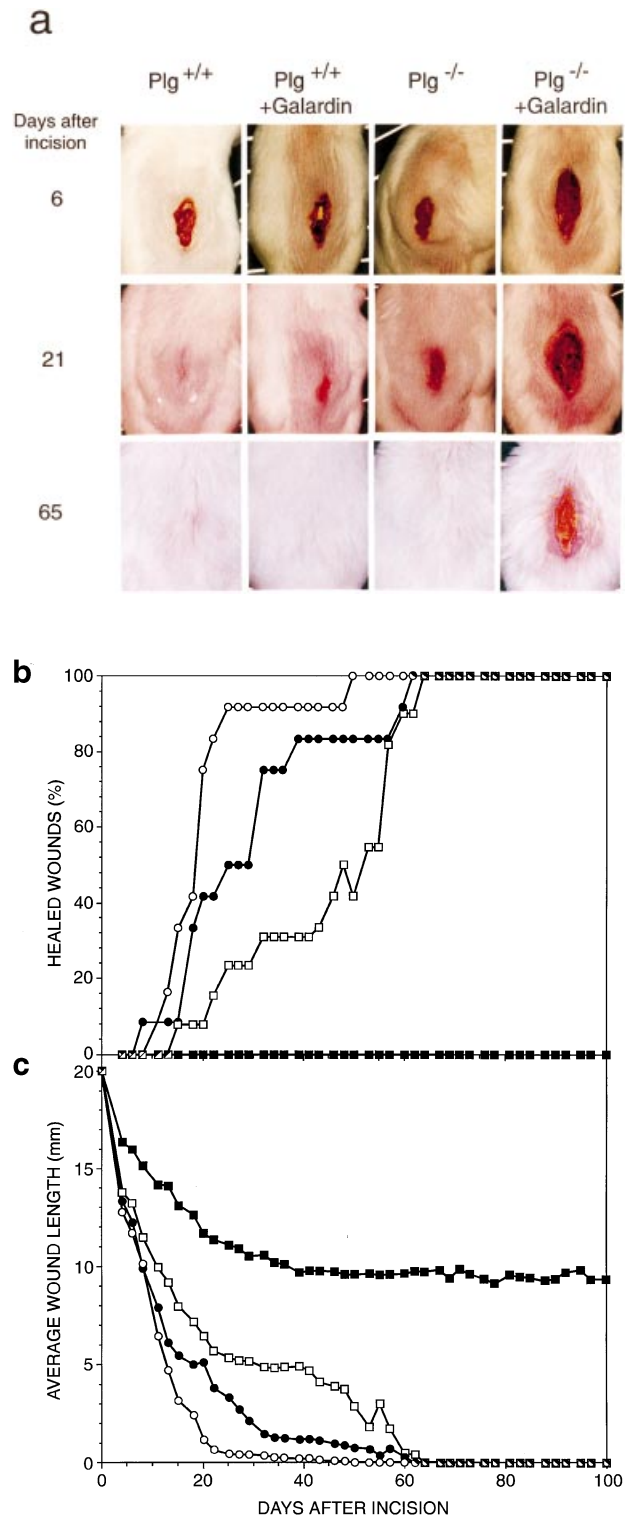

Fig. 3. Combined effect of Plg deficiency and MMP inhibition on skin wound healing. (**a**) Examples of the progress of repair processes in wild-type and Plg-deficient mice with and without galardin treatment. (**b**) The percentage fractions of mice with healing are plotted versus time after the incision for wild-type mice that were mock-treated with CMC (○) or treated daily i.p. with 100 mg/kg of galardin (●) and Plg-deficient mice that were mock-treated (□) or treated daily i.p. with 100 mg/kg of galardin (■). (**c**) The average lengths of the healing incisional wounds are plotted versus time for the same groups of mice. The mice in this experiment were obtained by backcrossing of Plg gene-targeted mice to NIHS mice for six generations.

the observation period of 100 days (Figure 3b), in stark contrast to the complete wound healing eventually observed in all of the mice which were either deprived of Plg or treated with the MMP inhibitor. Combination of Plg deficiency and MMP inhibition thus had a synergistic effect on the overall wound healing process. The lack of complete healing in the galardin-treated Plg-deficient mice was also reflected in the length of the wounds, which initially decreased, but then from approximately day 30 after incision remained stable at around half of the initial length (Figure 3c), the wounds being broad with a hard, dry and scaly surface. We did not observe any effect of Plg deficiency, galardin treatment or both on other parameters that potentially could influence the healing process, such as initial dryness of the wound bed, skin contraction or infection.

In a separate experiment with C57Bl/6J mice and a 42 day treatment and observation period, similar results were obtained. At day 42, no wounds were healed in Plg-deficient C57Bl/6J mice treated daily with 100 mg/kg of galardin, 60% were healed in mock-treated Plg-deficient mice and 56% in galardin-treated, littermate, wild-type mice, while all wounds in mock-treated, littermate, wild-type mice were healed at day 18.

Keratinocyte migration

In a separate experiment, we analyzed the impact of galardin treatment on keratinocyte migration in wild-type and littermate Plg-deficient mice. Groups of the two genotypes were either treated with 100 mg/kg daily of galardin for 7 days after skin wounding or mock treated. Keratin-stained sections of wound tissue from each group of mice were then analyzed microscopically, and the length of the epidermal tongue along its base from the wound edge to the tip of the wedge (see Figure 4) was measured blindly in three or four sections per wound by computer-assisted morphometry. This distance included both the actual migration distance covered by the keratinocytes and the epidermal area just behind, consisting of proliferating keratinocytes. In the mock-treated wild-type mice ($n = 5$), the length of the epidermal tongue was 1227 ± 114 µm (mean value $\pm$ SEM), compared with 908 ± 119 µm in the galardin-treated wild-type mice ($n = 5$), 950 ± 217 µm in the mock-treated Plg-deficient mice ($n = 4$) and 514 ± 74 µm in the galardin-treated Plg-deficient mice mice ($n = 5$). Evaluated by a nested analysis of variance (ANOVA), the decrease in the length of the epidermal tongue caused by galardin treatment of wild-type mice was statistically significant and this was also the case for the decrease caused by Plg deficiency alone ($P < 0.01$ in both cases). In the galardin-treated Plg-deficient mice, the decrease was significant in comparison with the mock-treated wild-type mice, the galardin-treated wild-type mice and the mock-treated Plg-deficient mice ($P < 0.01$ in all cases). Thus, MMP inhibition and Plg deprivation each reduced the length of the epidermal tongue, and in combination had at least an additive effect on this parameter.

In the galardin-treated Plg-deficient mice, the tip of the epidermal tongue was located very close to the initial wound edge, identified as the area just beneath the edge of the wound crust. The measured length of the epidermal tongue in these mice therefore represented almost entirely the zone of proliferation behind the leading edge keratinocytes, reflecting that the keratinocyte migration was virtually blocked. Staining of the wound sections with Masson-trichrome to reveal the collagen distribution showed that in both the mock-treated and galardin-treated wild-type mice, the epidermal outgrowth was wedge shaped and surrounded by a loose network of the provisional extracellular matrix (Figure 4a–d). Occasionally, the epidermal tongue in the galardin-treated wild-type mice was surrounded by a dense collagenous matrix (not shown). In the mock- and galardin-treated Plg-deficient mice, the epidermal tongues were blunted and surrounded by a dense layer of fibrillar non-collagenous material (curved arrows in Figure 4e–h). This layer was most pronounced in the galardin-treated Plg-deficient mice.

Immunohistochemical staining for fibrin(ogen) revealed that increased amounts of fibrin were present below and in front of the epidermal outgrowths in all of the galardin-treated wild-type mice (Figure 5c), the mock-treated Plg-deficient mice (Figure 5b) and the galardin-treated Plg-deficient mice (Figure 5d), compared with the mock-treated wild-type (Figure 5a) mice ($n = 5$ in each group). It is particularly noteworthy that the compact and band-shaped fibrin immunoreactivity was found below and in front of the keratinocytes in all five wild-type mice treated with galardin, whereas such a pattern of fibrin immunoreactivity was absent in four out of five mock-treated wild-type mice (Figure 5a shows a representative example). These findings indicate that galardin treatment alone, like Plg deficiency alone, may impair keratinocyte migration by hampering the degradation of fibrin immediately in front of the leading edge keratinocytes. We also immunohistochemically investigated the localization of fibronectin and laminin in the wounds, but found no obvious differences in the immunostaining patterns of these proteins between mock- and galardin-treated wild-type and Plg-deficient mice.

In the galardin-treated wild-type mice, and both the mock- and galardin-treated Plg-deficient mice, there was abundant formation of granulation tissue with inflammatory cells, endothelial cells and fibroblasts (Figure 4). We did not observe any apparent signs of reduced angiogenesis in the wounds from any of these three groups of mice.

Increased MMP expression after treatment with MMP inhibitor

Seven days after surgery, the expression of gelatinase B, collagenase-3, stromelysin-1, stromelysin-3 and gelatinase A was examined by *in situ* hybridization of wound tissue from mock-treated Plg-deficient and wild-type mice, and from mice of both genotypes that had been treated with daily doses of 100 mg/kg of galardin. In the mock-treated mice, the expression of these MMPs was as described above for untreated animals (compare Figure 6a and e). However, in both wild-type and Plg-deficient mice treated with galardin, the expression of gelatinase B (Figure 6a, e, f and j), stromelysin-1 (Figure 6b and g) and collagenase-3 (Figure 6c and h) mRNA was dramatically up-regulated in the leading edge keratinocytes compared with the mock-treated mice of the same genotype. Expression of gelatinase B, stromelysin-1, collagenase-3 and gelatinase A was also up-regulated in both groups of galardin-treated mice in the granulation
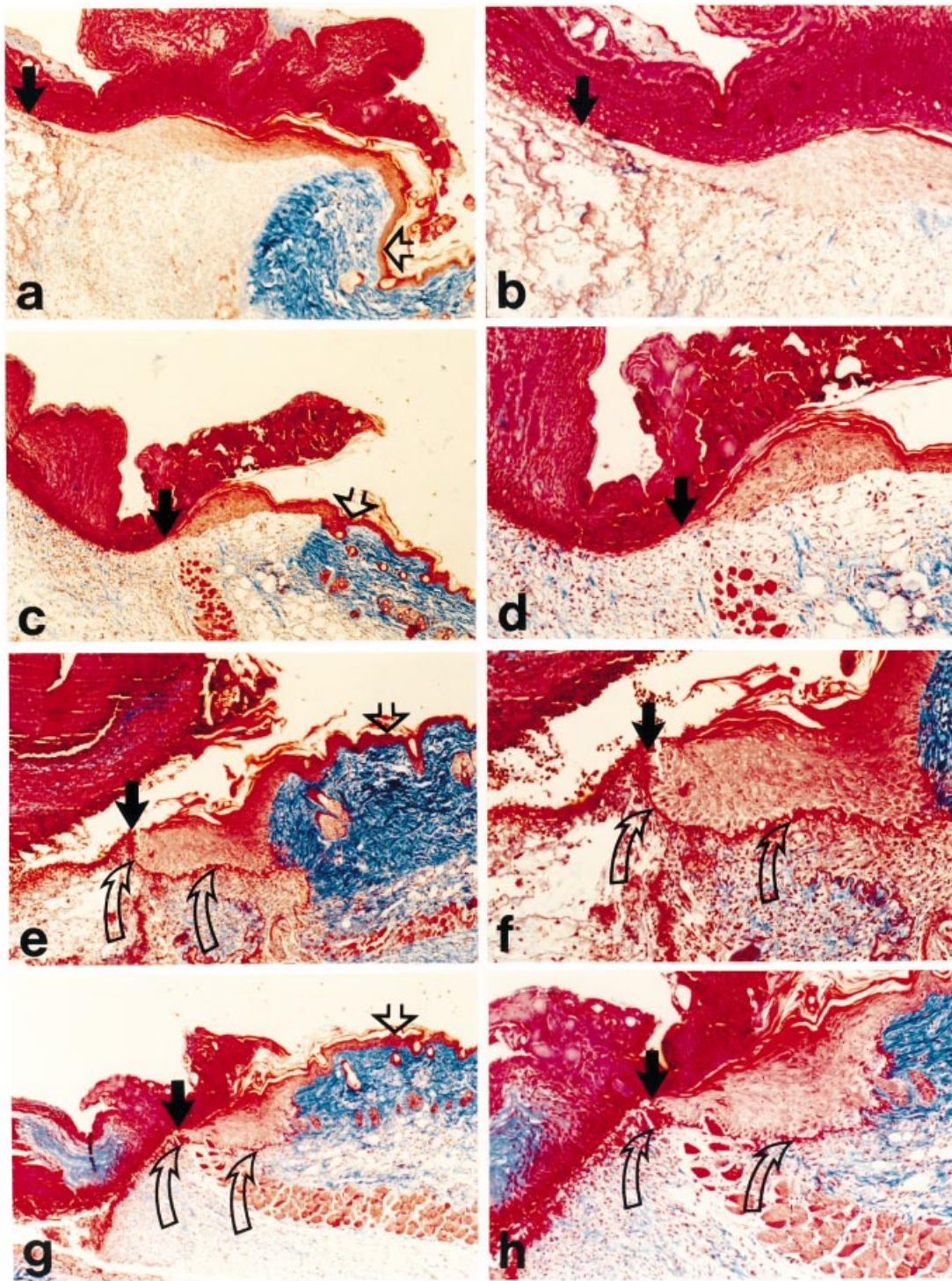
Fig. 4. Microscopic appearance of skin wounds in mock- and galardin-treated Plg-deficient and wild-type mice. Sections of skin wounds 7 days after surgery were stained with Masson-trichrome. In mock-treated (**a** and **b**) and galardin-treated (**c** and **d**) wild-type mice, the epidermal tongue is wedge shaped and surrounded by a loose network of extracellular matrix. In mock-treated (**e** and **f**) and galardin-treated (**g** and **h**) Plg-deficient mice, the epidermal ends are blunted and surrounded by a dense layer of fibrillar material. The fibrillar material is shown by curved arrows in (e–h) and is most pronounced in the galardin-treated Plg-deficient mice (h). In (a), (c), (e) and (g), the straight closed arrows indicate the tip of the epidermal tongue and the straight open arrows indicate the point at the start of the zone of proliferating keratinocytes used for measurements of the length of the epidermal tongue. (b), (d), (f) and (h) are larger magnifications of (a), (c), (e) and (g), respectively. Magnification: (a), (c), (e) and (g), ×45; (b), (d), (f) and (h), ×90.

tissue located just beneath the leading edge keratinocytes (curved arrows in Figure 6b–d and f–i). Stromelysin-1 and gelatinase A were expressed in fibroblast-like cells in the galardin-treated mice, as in the mock-treated mice, while gelatinase B and collagenase-3 were expressed in macrophage-like cells in the galardin-treated mice. Furthermore, expression of both gelatinase A and stromelysin-3 mRNA was up-regulated in fibroblast-like
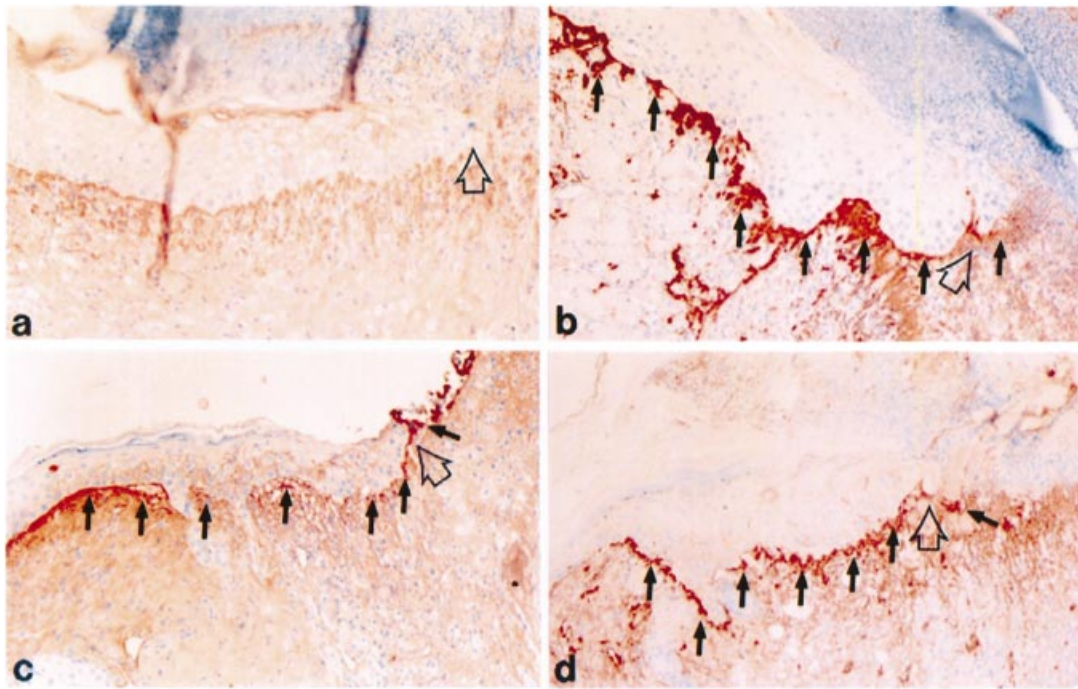
Fig. 5. Immunohistochemical staining for fibrin in wounds from Plg$^{+/+}$ and Plg$^{-/-}$ mice treated with galardin or vehicle 7 days after surgery. Sections from mock-treated wild-type (**a**) and plasminogen-deficient (**b**) mice, and from galardin-treated wild-type (**c**) and plasminogen-deficient (**d**) mice were stained immunohistochemically for fibrin(ogen). Both in the wild-type mice treated with galardin and in the Plg-deficient mice treated with either vehicle or galardin, fibrillar deposits are seen containing fibrin in front of and below the epidermal outgrowth (small straight arrows). There is no apparent difference in the intensity of the fibrin staining between the wild-type mice treated with galardin and the Plg-deficient mice treated with either vehicle or galardin. The open arrows in (a–d) show the ends of the epidermal outgrowth. Magnification, ×170.

cells in the deep part of the granulation tissue bordering the normal subcutis in the galardin-treated wild-type and Plg-deficient mice (not shown). Gelatinase A mRNA expression in these animals was also detectable in the leading edge keratinocytes, in contrast to the lack of detectable gelatinase A expression in keratinocytes in the mock-treated mice (Figure 6d and i).

Discussion

The findings in the present study demonstrate that treatment of mice with a metalloprotease inhibitor, galardin, impairs skin wound healing in mice. We have reported previously that Plg deficiency achieved by targeted gene disruption results in a similar delay in wound repair. In both cases, the repair process does, however, eventually proceed, leading to complete healing in all mice. In contrast, the wound healing process is effectively brought to a standstill and complete wound healing is not observed in any Plg-deficient mice treated with galardin, thus demonstrating that protease activity is essential for this tissue remodeling process, and that there is a synergistic effect of Plg deficiency and galardin treatment.

In the case of both Plg deficiency and galardin treatment, there is an impairment of the migration of keratinocytes from the wound edge, while we did not observe any decrease in the formation of granulation tissue or in neovascularization in any of the cases. With respect to Plg deficiency, a reversal to virtually normal skin wound healing time in mice made deficient in both Plg and fibrinogen (Bugge *et al.*, 1996) indicates that the main reason for the impairment is a decreased ability of the Plg-deprived keratinocytes to dissect their way proteolytically

through the fibrin-rich extracellular matrix beneath the wound crust. Our present finding of excessive amounts of fibrin below and in front of the migrating keratinocytes in galardin-treated wild-type mice makes it likely that the mechanism of action of the galardin-induced impairment of wound healing is a similar inhibition of degradation of fibrin and possibly other matrix components by the migrating keratinocytes. In favor of this proposal is the finding that these keratinocytes express not only the key regulators of Plg activation, uPA and uPAR, but also the three MMPs gelatinase B, collagenase-3 and stromelysin-1. Moreover, at least two MMPs, stromelysin-1 (Bini *et al.*, 1996) and MT-1 MMP (Hiraoka *et al.*, 1998), have been shown to degrade fibrin, and in the latter case the data strongly argue that in some tissue remodeling processes MMPs may even be more important than plasmin in providing the fibrinolytic activity required for cell migration (Hiraoka *et al.*, 1998).

Galardin is a peptide-based zinc-chelating hydroxamate that inhibits the proteolytic activity of several MMPs, including gelatinase A (0.5 nM), gelatinase B (0.2 nM), interstitial collagenase (0.4 nM), neutrophil collagenase (0.1 nM) and stromelysin 1 (30 nM) with the K_is indicated (Grobelny *et al.*, 1992; Levy *et al.*, 1998). In at least four studies of systemic galardin treatment in animal models, specific effects were observed which, as in the present study, most probably were due to inhibition of MMP-mediated degradation of the extracellular matrix, while there were no observations of general toxicity (Galardy *et al.*, 1994; Gijbels, 1994; Strauss *et al.*, 1996; Witte *et al.*, 1998). However, it cannot be excluded at present that the inhibition of metalloprotease-mediated activation of latent growth factors by galardin may also
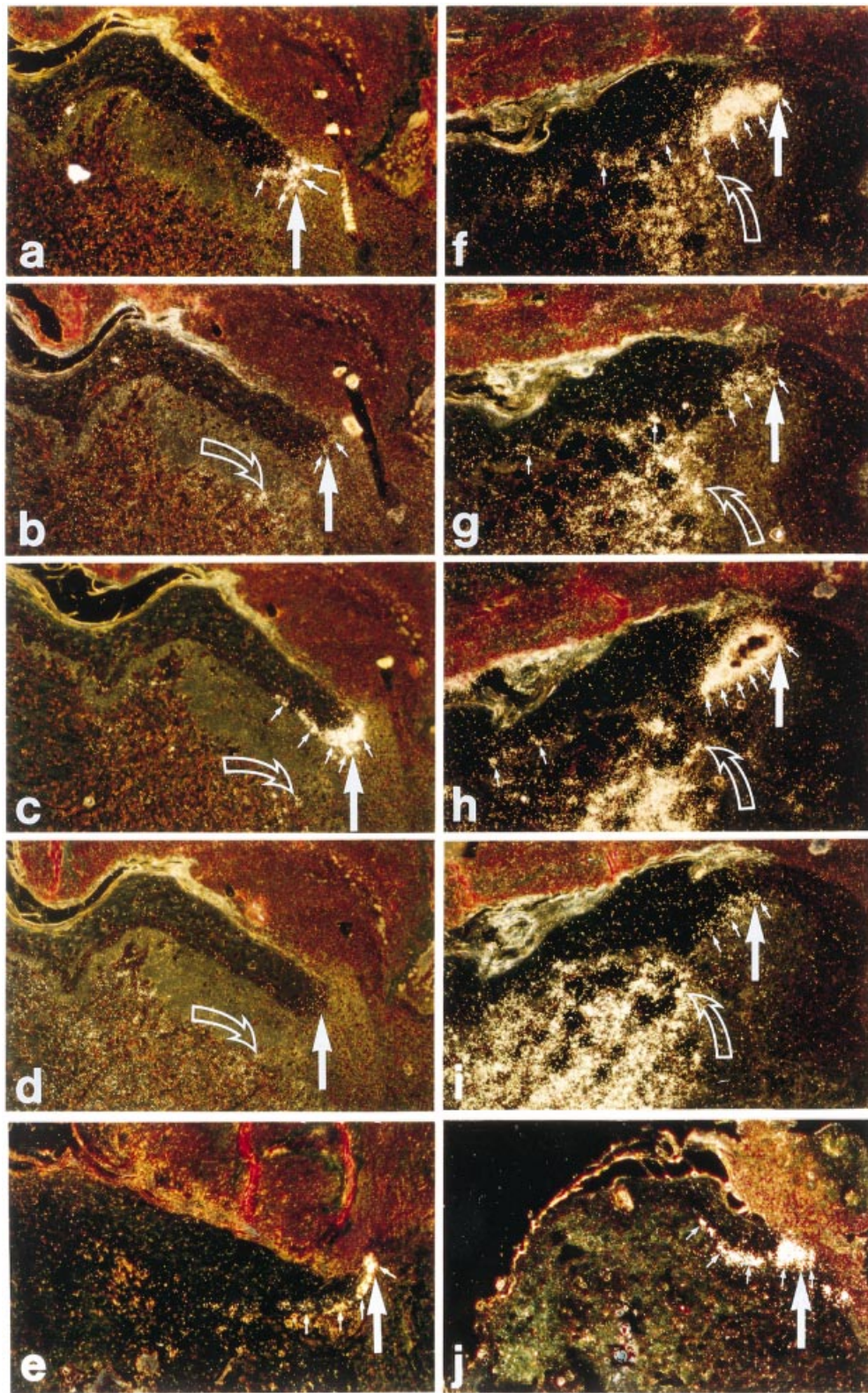
Fig. 6. Increased metalloprotease expression after treatment with galardin. The expression of metalloproteases in skin wounds from mock-treated (a–e) or galardin-treated (f–j) Plg-deficient (a–d and f–i) or wild-type (e and j) mice were analyzed by *in situ* hybridization 7 days after surgery. The expression of mRNA for gelatinase B (**a**, **e**, **f** and **j**), stromelysin-1 (**b** and **g**), collagenase-3 (**c** and **h**) and gelatinase A (**d** and **i**) is increased in all the galardin-treated animals. The large straight arrows indicate the tip of the epidermal tongue. The small straight arrows show expression of gelatinase B (a, e, f and j), stromelysin-1 (b and g) and collagenase-3 (c and h) in the leading edge keratinocytes of both the mock- and galardin-treated Plg-deficient or wild-type mice, and of gelatinase A (i) in the tip of the leading edge keratinocytes in the galardin-treated animals. The curved arrows indicate expression at the front of the granulation tissue of gelatinase B (f) in galardin-treated Plg-deficient mice, and of stromelysin-1 (b and g), collagenase-3 (c and h) and gelatinase A (d and i) in both mock- and galardin-treated Plg-deficient animals. Note that the expression of all four MMPs is up-regulated both in the leading edge keratinocytes and in the granulation tissue cells in the galardin-treated animals. All micrographs are dark-field images. Magnification, ×90.

contribute to the observed impairment of keratinocyte migration. Indeed, a recent report provides evidence that some functional inhibition of tumor necrosis factor-α (TNF-α)-converting enzyme (TACE)—a metalloprotease disintegrin (Wolfberg *et al*., 1995; Moss *et al*., 1997)—may be achieved *in vivo* by systemic galardin treatment of mice (Solorzano *et al*., 1997). It should be noted, however, that deficiency in one of the principle substrates of TACE, transforming growth factor-α (Peschon *et al*., 1998), did not affect keratinocyte migration or other components of the healing process in a skin wound healing model similar to the one applied in this study (Guo *et al*., 1996). Furthermore, to our knowledge, effects on skin wound healing of deficiencies in other substrates of TACE, such as the TNF-α–TNF-α receptor complex and L-selectin, have not been reported.

It is noteworthy that we found a strong increase in the expression of several MMPs in the keratinocytes after treatment with galardin, indicating that the galardin was bioavailable at the wound site, and could exert an effect there. However, this up-regulation also suggests that there are compensatory regulatory systems, whereby the persistence of specific matrix components increases MMP gene expression. The mechanism of the up-regulation is yet to be clarified, but recent reports on collagen V-induced up-regulation of MMP-1 expression through an interaction with the discoidin domain receptor 2 indicate that such feedback mechanisms exist (Shrivastava *et al*., 1997; Vogel *et al*., 1997). However, due to the number of MMPs that are known to be inhibited by galardin, we are unable to specify which MMPs are critically involved in wound healing. In preliminary experiments with mice deficient in either gelatinase B or stromelysin-1, we have not found any retardation of wound healing (unpublished results), and it appears likely that several of the at least 17 known MMPs need to be inactivated concomitantly in order to obtain an impairment of the healing process.

The observation that wound healing does proceed in the absence of Plg indicates that alternative mechanisms exist which can perform the function of plasmin. The complete arrest obtained with galardin treatment in Plg-deficient mice, but not in wild-type mice, shows that these alternative mechanisms involve metalloprotease activity. We therefore conclude that there are one or more functions that can be exerted by either of the two classes of proteases. The lack of detectable up-regulation of metalloprotease expression in the Plg-deficient mice makes it likely that the metalloproteases also exert this function in wild-type mice, and that there is normally a functional overlap between the two classes of proteases. A probable explanation for the functional overlap in skin wound healing demonstrated in this study is that cleavage of one or more extracellular matrix components is critical for keratinocyte migration, and that this cleavage can be achieved by either of the two protease systems. Our previous studies on Plg-deficient mice (Rømer *et al*., 1996), together with studies of mice with combined deficits in Plg and fibrinogen (Bugge *et al*., 1996), indicate that degradation of fibrin is a critical function of plasmin in skin wound repair. As noted above, our present findings of residual fibrin-rich matrix in galardin-treated mice argue that MMPs also contribute to fibrin degradation by migrating keratinocytes, so that fibrin may be a target for both plasmin and MMPs.

In view of these findings, the slow but ultimately successful repair of wounds in Plg-deficient mice is likely to be due to slow clearance of fibrin by MMPs. We did not find any detectable changes in the staining of fibronectin and laminin, which are also known to be substrates for both plasmin and MMPs *in vitro* and, probably, *in vivo* (Danø *et al*., 1985; Murphy *et al*., 1992; Chen and Strickland, 1998; Tsirka *et al*., 1998). We cannot, however, exclude that insufficient cleavage of these or other matrix proteins, such as vitronectin and proteoglycans (Whitelock *et al*., 1996; Waltz *et al*., 1997), may also contribute to the complete arrest of wound healing that we observed in galardin-treated Plg-deficient mice. The hypothesis that one or more matrix components are common substrates for plasmin and MMPs, and that this explains their functional overlap in wound healing, predicts that the healing would be restored in Plg-deficient, galardin-treated mice if the animals also lacked one or more of those matrix components. With respect to fibrin, this prediction can be tested using available fibrinogen knockout mice (Bugge *et al*., 1996).

In vitro observations suggest that plasmin plays an important role in the activation of some pro-metalloproteases (Murphy *et al*., 1992; Mazzieri *et al*., 1997). The pronounced effect of galardin treatment on wound healing in Plg-deficient mice observed in this study does, however, clearly demonstrate that at least some metalloproteases involved in wound healing are not dependent on plasmin activation in order to promote tissue remodeling. This conclusion is supported further by the finding that active gelatinase B, as identified by zymography (see Lund *et al*., 1996), is present in extracts of wound tissue from Plg-deficient mice (unpublished results). The arrest of wound healing seen in galardin-treated Plg-deficient mice, but not in galardin-treated wild-type mice, argues that Plg has more functions in tissue remodeling than activation of MMPs.

The present findings may have implications not only for our understanding of the proteolytic mechanisms involved in wound healing, but also for other normal and pathological tissue remodeling processes, including trophoblast invasion, organ involution, inflammatory diseases and in particular cancer invasion. It is becoming increasingly clear that the same repertoire of proteases is involved in degradation of the extracellular matrix in such processes (Danø *et al*., 1985; Hewitt and Danø, 1996; Edwards *et al*., 1998; Johnsen *et al*., 1998), and that there are also strong similarities in the way in which epithelial cells interact with stromal connective tissue cells in the generation and regulation of extracellular proteolysis (Hewitt and Danø, 1996; Johnsen *et al*., 1998). Particularly striking are the similarities between wound healing and cancer (Dvorak *et al*., 1986). These similarities were extended recently to findings in Plg-deficient mice in which, besides impaired wound healing (Rømer *et al*., 1996), there is also an impairment of primary growth of the transplanted Lewis lung carcinoma (Bugge *et al*., 1997) and of metastasis of mammary tumors genetically induced by overexpression of the polyoma virus middle T antigen (Bugge *et al*., 1998). Like the skin wound healing, tumor growth and metastasis do, however, eventually proceed in the absence of Plg, probably reflecting the notion that also in these processes there is a functional

overlap between plasmin and metalloproteases. The present findings predict that effective arrest of cancer progression will require the combined use of inhibitors of MMPs and inhibitors of the plasmin/Plg activation system. Several MMP inhibitors are already in clinical trials for cancer treatment (Brown, 1999). The use of inhibitors that effectively block the enzymatic activity of plasmin will probably produce severe side effects (see below), while an attractive approach for inhibition of Plg activation in cancer is blocking of the receptor binding of uPA (Danø *et al.*, 1994; Kim *et al.*, 1998), which will probably be virtually non-toxic (Bugge *et al.*, 1995b). If the massive up-regulation of MMP expression seen upon galardin treatment in this study also occurs during treatment of cancer with MMP inhibitors, this may raise problems as a mechanism for cancer cells to acquire resistance.

The conclusions based on observations on tissue remodeling processes in Plg-deficient mice are probably very relevant also for the human situation. Recently, the rare, but long known and well described, inherited disease ligneous conjunctivitis was linked to Plg deficiency (Mingers *et al.*, 1997; Schuster *et al.*, 1997, 1999; Schott *et al.*, 1998). This disease is characterized by pseudo-membranous 'wood-like' lesions of the conjunctiva and other mucous membranes (Bouisson *et al.*, 1847; Bateman *et al.*, 1986; Hydayat *et al.*, 1987; Schuster *et al.*, 1997), and there are many similarities with the phenotype observed in the Plg-deficient mice (Bugge *et al.*, 1995a; Ploplis *et al.*, 1995; Rømer *et al.*, 1996; Drew *et al.*, 1998), including the impaired wound healing which was recently described in two patients with inherited Plg deficiency (Mingers *et al.*, 1997; Schott *et al.*, 1998). Ligneous conjunctivitis patients often display serious symptoms, such as hydro-cephalus and blindness, indicating that long-term use of effective plasmin inhibitors for therapeutic purposes, including the treatment of cancer patients, may not be feasible due to severe side effects.

Materials and methods

Tissue preparation
Adult mice were anaesthetized by subcutaneous administration of 0.03 ml/10 g of a 1:1 mixture of Dormicum (midazolam 5 mg/ml) and Hypnorm (fluanison 5 mg/ml and fentanyl 0.1 mg/ml) before surgery. Full-thickness 20 mm long incisional skin wounds were made mid-dorsally with a scalpel. The wounds were neither dressed nor sutured. In this wound model, the formation of a dry scab between the wound edges has the effect that the epidermal edges are kept well separated, despite the initial wound contraction (Rømer *et al.*, 1996). This means that the new epidermal layer is formed entirely by keratinocytes migrating from the wound edges and not from underlying hair follicles. In this model, the significance of skin contraction and the hair growth cycle is minimized during the restoration of an intact epidermal wound cover. The mice were caged individually until sacrifice by perfusion–fixation with phosphate-buffered saline (PBS) and 4% paraformaldehyde. The wound areas were removed, bisected in the mid-transversal plane, fixed in 4% paraformaldehyde overnight (Rømer *et al.*, 1991, 1994) and then paraffin embedded. Sections were cut perpendicular to the longitudinal direction of the wounds. Animal care at The Department of Experimental Medicine, University of Copenhagen and Rigshospitalet, Copenhagen, Denmark was in accordance with the national guidelines. There were no gross or microscopic signs of infections of the wounds or mice during the course of any of the experiments.

Animals and animal treatment
Plg gene-targeted mice of a mixed 129/Black Swiss background (Bugge *et al.*, 1995) were backcrossed to C57Bl/6J mice for 2–6 generations and used throughout the study, except for the experiment with mice backcrossed into the NIHS background shown in Figure 2. For all breeding, heterozygous $Plg^{+/-}$ mice were used. In all experiments involving wild-type mice as controls, these were littermates of the Plg-deficient mice. All mice used for experiments were between 7 and 11 weeks old at the start of the experiment. All experimental evaluations were performed by an investigator unaware of animal genotype. Genotyping of the Plg alleles was performed as described (Bugge *et al.*, 1996). The MMP inhibitor *N*-[(2R)-2-(hydroxamido-carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (galardin) was synthesized as described (Grobelny *et al.*, 1992). Galardin inhibits the enzymatic activity of a number of MMPs, including gelatinase A, gelatinase B, interstitial collagenase, neutrophil collagenase and stromelysin-1 (Grobelny *et al.*, 1992; Levy *et al.*, 1998). Galardin was administered i.p. as a 20 mg/ml slurry in 4% CMC, 0.9% saline. Injections began immediately after wounding and were continued every 24 h thereafter until the end of the experiment. Statistical evaluation of differences between groups of mice in the time taken to complete wound healing was done with the log rank test.

Computer-assisted morphometry
Keratinocyte migration was measured microscopically on tissue sections stained immunohistochemically with anti-keratin IgG (see below). The length of the epidermal tongue was measured from the tip of the leading edge keratinocytes to the point in the zone of proliferation, where a shift from two to three layers of keratinocytes was identified. Indication of this point in the proliferation zone and the tip of the epidermal wedge, and the drawing of a line along the base of the tongue between these sites by image analysis (NIH Image/ppc 1.56b30 software for Macintosh) were performed by an observer unaware of the genotype and treatment of the mice. The mean distance of migration in each group was calculated as an estimated mean, and the standard error of the mean (SEM) as the standard deviation divided by the square root of the number of animals in the group.

Immunohistochemistry
Tissue sections were stained immunohistochemically by the peroxidase–anti-peroxidase method (Rømer *et al.*, 1991) with rabbit polyclonal antibodies directed against mouse keratin (Cappel, Organon Teknika, PA) or mouse fibrinogen (Bugge *et al.*, 1995a), fibronectin (Dako, Denmark) or laminin (L9393, Sigma, IL). Each experiment included controls with omission of the primary antibody and substitution of the primary antibody with non-immune rabbit IgG. These were all negative.

In situ hybridization
In situ hybridization was performed on paraffin sections essentially as described (Rømer *et al.*, 1991). ^{35}S-labeled RNA sense and antisense probes were generated by *in vitro* transcription from subclones of the following mouse cDNA fragments: gelatinase A (604–1165) in pSP64 and pSP65 or (1924–2259) in pGEM-3 (Reponen *et al.*, 1992); gelatinase B (779–1073) or (1918–2239) in pSP64 and pSP65 (Reponen *et al.*, 1994); and macrophage metalloelastase (900–1250) in T3T7-19 (Shapiro *et al.*, 1992). From stromelysin-1 cDNA (Hammani *et al.*, 1992) fragments of (2205–2918) and (3115–4051); from stromelysin-3 cDNA (Lefebvre *et al.*, 1992) a fragment of (1–996); and from collagenase-3 cDNA (Henriet *et al.*, 1992), the two *Eco*RI fragments of 485 and 811 bp were subcloned in pBluescript KS+. The mouse MT-1 MMP fragment (1122–1867, DDBJ/EMBL/GenBank accession No. X83536) was generated by PCR amplification of reverse-transcribed mRNA extracted from involuting mammary gland tissue with the primers: 5′ACATCTGTGACGGGAACTTTGACA and 5′ATGGCGTCTGAAG-AAGAAGACAGCGAG and subcloned in pBluescript KS+. Expression of mRNA for gelatinase A, gelatinase B, stromelysin-1 and collagenase-3 was detected in some of the experiments with two non-overlapping probes. In these cases, identical results were obtained. Sense probes were included in all experiments and in all cases gave negative results.

Acknowledgements

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