Accelerated Skin Wound Healing in Plasminogen Activator Inhibitor-1-Deficient Mice

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Components of the fibrinolytic system have been implicated in cell migratory events associated with tissue remodeling. Studies in plasminogen-deficient mice ($PG^{-/-}$) indicated that skin wound healing is impaired, but is resolved with an additional fibrinogen deficiency. Plasminogen activator inhibitor-1 (PAI-1) expression by keratinocytes has been identified shortly after wound injury. PAI-1 expression could affect wound healing by regulating the fibrinolytic environment of the wounded area, as well as influencing events associated with cell attachment and detachment through interactions with matrix proteins. The present study directly assesses PAI-1 involvement in skin wound healing through analyses of a dermal biopsy punch model in PAI-1-deficient ($PAI-1^{-/-}$) mice. While the cellular events associated with the healing process are similar between wild-type (WT) and $PAI-1^{-/-}$ mice, the rate of wound closure is significantly accelerated in $PAI-1^{-/-}$ mice. (*Am J Pathol* 2001, 159:1681–1688)

The fibrinolytic system consists of the zymogen, plasminogen (Pg), its serine protease activated form, plasmin (Pm); plasminogen activators, viz., urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), which are also serine proteases; receptors for these proteins viz., the uPA receptor (uPAR); serine-type inhibitors, viz., plasminogen activator inhibitor-1 (PAI-1); and fibrinogen/fibrin. While the major physiological function of this system is to maintain vascular patency through fibrin surveillance, a number of *in vitro* and *in vivo* studies have implicated Pm in playing an important role in facilitating cell migration through direct proteolysis of extracellular matrix protein or indirectly through activation of other matrix degrading pathways, e.g., metalloproteases. These events are pivotal in wound healing and tissue remodeling processes. Alternatively, inhibitors of this proteolytic pathway serve to regulate the extent of tissue degradative processes and thus balance destructive and repair events. Indeed, previous studies have indicated that uPA and PAI-1 are regulated in their

expression, both spatially and temporally, during the migration of keratinocytes and connective tissue cells during reepithelialization, and tissue remodeling associated with wound healing.¹ Additional studies of wound healing using the keratinocyte cell line, HaCaT, transfected with an antisense PAI-1 vector, indicated that the rate and extent of wound closure was impaired.² Direct studies in plasminogen-deficient ($PG^{-/-}$) mice have indicated that keratinocyte migration during skin wound healing is attenuated, a phenomenon which appears to be the result of unresolved provisional fibrin matrix.^{3,4} However, vascular injury models have shown that PAI-1 plays an inhibitory role during wound healing and arterial neointima formation following injury.^{5–7} Therefore, the location and cell types affected play important roles in determining whether alterations in PAI-1 expression impact the wound healing responses.

Using a skin wound healing model and $PAI-1^{-/-}$ mice, we have investigated the effects of alterations in the expression of PAI-1 on skin wound healing processes. The results of this investigation are reported herein.

Materials and Methods

Animals

The generation of mice homozygous for a total PAI-1 deficiency ($PAI-1^{-/-}$) has been described.⁸ $PAI-1^{-/-}$ mice were back-crossed to at least the F7 generation in strain C57Bl/6J (~99% C57Bl/6J background). The animals were housed in micro-isolation cages on a constant 12 hours light/dark cycle with controlled temperature and humidity and given access to food and water *ad libitum*. C57Bl/6J animals (Jackson Laboratories, Bar Harbor, ME) were used as wild-type (WT) controls. All mice used in these studies were between 8 and 12 weeks of age and of mixed gender. All animal experiments were per-

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formed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Induction of the Skin Wound

Mice were anesthetized by intraperitoneal injection of rodent cocktail (0.015 mg xylazine/0.075 mg ketamine/0.0025 mg acepromazine per gram body weight). The backs of the mice were shaved and sterilized with alcohol, followed by 1% iodine solution. A full thickness wound, approximately 8 mm in diameter, was made using a dermal biopsy punch, down, but not through, the muscle fascia. Mice were singly caged, without bedding, for the first several days until a provisional matrix had formed. Wound areas (width $\times$ length) were measured every other day. Mice were sacrificed at various time points during healing or at the time when the wound appeared closed (endpoint). At least three mice were sacrificed for each time point. The wounded tissues and the surrounding skin were carefully excised, pinned to a corkboard, and fixed flat in 10% neutral buffered formalin (NBF) for 3 hours before alcoholic dehydration and paraffinization. Wounds were bisected and embedded in paraffin. Microtomy was performed at 4 μ m.

Histology, Histochemistry, and Immunohistochemistry

Sections were stained with hematoxylin and eosin (H&E) to examine general tissue and cellular morphology, and with Masson's Trichrome for identification of collagen IV.⁹ The Ayoub-Shklar method was used for identification of keratin and prekeratin structures.¹⁰ The periodic acid-Schiff procedure (PAS) was used for identification of basement membranes.¹¹

A number of immunohistochemical stains were performed. Fibrin was identified with a polyclonal goat-anti-mouse fibrin(ogen) antibody (Accurate Chemicals, Westbury, NY). Antigen retrieval was performed under high temperature and pressure with citrate buffer (BioGenex, San Ramon, CA), followed by endogenous peroxidase blocking with Peroxoblock (Zymed, South San Francisco, CA). Subsequent to incubation, first with rabbit serum and then with the primary antibody, the slides were incubated with the secondary rabbit anti-goat IgG (Dako, Carpinteria, CA), followed by goat peroxidase anti-peroxidase (Dako). Peroxidase activity was detected with the substrate 3-amino-9-ethylcarbazole (AEC), (Biomedex, Foster City, CA). PAI-1 antigen was detected by citrate buffer antigen retrieval and a rabbit anti-rat PAI-1 IgG (American Diagnostica, Greenwich, CT). Slides were then incubated with a swine anti-rabbit biotin F(ab)₂ IgG, followed by streptavidin-HRP (BioGenex) and AEC detection. A similar procedure was used for detection of smooth muscle cell α -actin with an anti- α -actin monoclonal antibody (Sigma Chemical Co., St Louis, MO) and vascular endothelial growth factor (VEGF) with a mouse anti-human monoclonal antibody (Oncogene Research Products, Boston, MA). CD45-positive leukocytes were detected using citrate buffer antigen retrieval and a biotinylated rat anti-mouse CD45 monoclonal antibody

Figure 1. Kinetics of skin wound healing in *PAI-1^{-/-}* and *WT* mice. The percentage area of the wound (width $\times$ length) remaining as a function of time after skin incision in *PAI-1^{-/-}* (●) and *WT* (■) mice. At least 4 mice/genotype/time point are graphically represented and expressed as the average $\pm$ SE. *P* values at days 3–9 < 0.05.

(PharMingen, San Diego, CA). Following a streptavidin incubation (NEN, Boston, MA), detection was performed with diaminobenzidine (DAB) (Dako). Urokinase was identified using a rabbit anti-rodent antibody (American Diagnostica), followed by biotin-conjugated swine anti-rabbit F(ab)₂ IgG and then streptavidin conjugated HRP. AEC chromogen was used for visualization and antigen retrieval was accomplished using Tris-HCl, pH 8.0. All immunohistochemical slides were counterstained with hematoxylin (Biomedex).

Results

Kinetics of Skin Wound Healing in WT and *PAI-1^{-/-}* Mice

After dermal incision, wound areas were measured every other day for 15 days and expressed as percent wound remaining as a function of time. The rate of wound healing was significantly increased in *PAI-1^{-/-}* mice relative to *WT* mice, day 3–9 comparisons *P* < 0.05 (Figure 1). By day 7, approximately 90% of the wound was closed in *PAI-1^{-/-}* mice compared to 50% in *WT* mice. Between days 11 and 13, the wound was completely sealed in *PAI-1^{-/-}* mice while in *WT* mice wound closure was still incomplete.

Histological Analyses of Skin Wound Healing in WT and *PAI-1^{-/-}* Mice 5 Days after Incision

At day 5, it was already evident that wound healing was accelerated in *PAI-1^{-/-}* mice relative to *WT* mice. The newly formed neoepithelial layer from the marginal edges of the wound were thickened and connected underneath the provisional matrix in *PAI-1^{-/-}* mice (Figure 2a). This is in contrast to *WT* mice, at this time interval, where the neoepithelial layer was still associated with the incisional edges of the wound, marginal to the matrix (Figure 2b). Additionally, weak PAI-1 immunoreactivity was observed

Figure 2. Histological analysis of a skin lesion 5 days following administration of the wound in *PAI-1*^{-/-} and *WT* mice ($\times 40$ – 100). H&E staining of a newly connected and thickened neoepithelial layer underneath the formed matrix, which is now attached only at a small, single point (*) in *PAI-1*^{-/-} mice (**a**) ($\times 40$). Under the same magnification, the leading edges of the migrating epithelium (*) have not yet associated in *WT* mice skin wounds (**b**). The underlying dermal layer in *PAI-1*^{-/-} mice (**c**) is not yet developed although Masson's trichrome staining indicates the initiation of collagen deposition (*) in this area ($\times 40$). Some collagen is also present under the granulation tissue (arrow) of *WT* mice lesions ($\times 40$) (**d**). Ayoub-Shklar staining demonstrates that some erythrocytes (arrow) are still present in the immature and unconnected area in *PAI-1*^{-/-} mice (**e**) ($\times 100$) but is much more substantial in the *WT* mice wound (**f**) (arrows $\times 100$).

in *WT* wounds at the marginal edges and no differences in uPA localization were observed in wounds from *PAI-1*^{-/-} and *WT* mice (data not shown). While the underlying dermal layer in *PAI-1*^{-/-} mice was not yet developed,

organization of collagen fiber formation was initiated at this time in *PAI-1*^{-/-} mice, with little evidence of this occurring in *WT* mice (Figure 2, c and d). A small amount of bleeding was still observed in *PAI-1*^{-/-} mice wounds

Figure 3. Immunohistochemical analysis of a skin lesion 5 days following wound placement in *PAI-1*^{-/-} and *WT* mice. Anti-fibrin(ogen) immunostaining shows diffuse fibrin deposition (brown) within the healing lesion in *PAI-1*^{-/-} mice (**a**) and between the wound and the provisional matrix (**arrow**), whereas fibrin deposition in the *WT* mice skin lesions (**b**) remains generally unresolved ($\times 100$). Similarly, CD45-positive leukocytes are still prevalent in the granulation tissue (*) under the matrix of the *WT* mice lesions, (**d**) but not as prevalent in lesions from *PAI-1*^{-/-} mice (**c**) ($\times 400$). VEGF expression was prominent in the granulation tissue (*) of skin wounds from *WT* mice (**f**) but scarce in wounds from *PAI-1*^{-/-} mice (**e**) ($\times 100$).

in unconnected areas under the matrix but was much more evident in *WT* mice wounds where there was a larger open area of injury (Figure 2, e and f). While wound-associated fibrin(ogen) was observed to be diffuse in *PAI-1*^{-/-} animals, it was much more evident in wounds from *WT* mice (Figure 3, a and b). This supports observations made in *PG*^{-/-} mice and *PG*^{-/-}/*FG*^{-/-} double deletions, where it was demonstrated that a lack of Pm activity resulted in diminished skin wound healing which was resolved with a fibrinogen deficiency.^{3,4} Inflammation appeared to be much more prominent in *WT* wounds at day 5 than in *PAI-1*^{-/-} wounds consistent with the different stages of wound healing in these mice (Figure 3, c and d). VEGF, a growth factor known to be up-regulated during skin wound healing,¹² was evident in the granulation tissue in *WT* mice wounds but very poorly expressed in *PAI-1*^{-/-} skin wounds.

Histological Analyses of Healed Skin Wounds in *PAI-1*^{-/-} Mice

Healed wounds in *PAI-1*^{-/-} mice demonstrated a thickened epidermal layer within the newly healed lesion that is not seen in uninjured skin (Figure 4, a and b). There is also evidence of hair follicle structure development that is represented by areas of invagination of the epidermis (Figure 4a). The dermal layer contains diffuse deposition of collagen fibers that are not organized as in uninjured skin (Figure 4, c and d). A thickened keratinized layer is more evident above the epidermal layer of the healed lesion relative to non-wounded skin (Figure 4, e and f). Reticular fiber structures of a developing basement membrane are unorganized and, for the most part, cellular in the healed lesion as compared to that observed in non-wounded skin (Figure 4, g and h). The finding of localized α -actin positive cells within the developing basement membrane indicates that this region is fibroblast-rich (Figure 5, a and b). Additionally, the lack of significant accumulation of fibrin(ogen) (Figure 5, c and d) or inflammatory cells (Figure 5, e and f) within the lesion is indicative of a resolved wound and is similar in appearance to nonwounded skin. These findings are similar to those observed in healed *WT* skin lesions.

Discussion

Wound healing is a multifactorial process involving 3 distinct yet interrelated phases that temporally overlap. The inflammatory phase, or exudative phase, is involved in removal of the damaged tissue and cleansing of the wound site. The proliferative phase involves formation of the granulation tissue which acts as a provisional replacement tissue. Finally, the differentiation or regeneration phase supports scar formation and reepithelialization of the wound site. Components of the fibrinolytic system have been implicated in a number of the events associated with wound healing and direct studies in *PG*^{-/-} mice have indicated a significantly attenuated rate of wound healing which is resolved in a fibrinogen deficient state. While wound healing is impaired in *PG*^{-/-} mice, it

eventually occurs, indicating the involvement of other proteolytic pathways in these events. Additional studies support a functional overlap between the fibrinolytic system and the MMP family of matrix degrading proteases in skin wound healing processes¹³ and have shown that inhibition of both pathways leads to a complete arrest of wound healing and closure. Therefore, imbalances in either proteolytic system could manifest itself in altered wound healing events.

An important physiological regulator for the generation of plasmin is PAI-1. While PAI-1 is not normally expressed by keratinocytes in the epidermis, it has been shown to be increased in expression following *in vitro* and *in vivo* wound injury^{1,14} and localized to the hyperproliferative migrating zone of epithelial cells. Clinically, it has been demonstrated that elevated PAI-1 levels occur in diabetic patients who also suffer from delayed wound healing responses resulting in skin ulcerations and other serious clinical complications.^{15,16} The current study supports a role for PAI-1 in wound healing and indicates that a loss of PAI-1 function results in accelerated wound healing. Interestingly, as observed in other models in our laboratory^{7,17}, PAI-1 expression appears to regulate VEGF expression. Further studies to characterize the mechanisms associated with this observation are ongoing.

PAI-2 is also expressed in the epidermis and could play a role in skin wound healing.¹⁸ Surprisingly, a wound healing model using *PAI-2*^{-/-} and *PAI-2*^{-/-}/*PAI-1*^{-/-} double deficient mice failed to show an effect on the healing process.¹⁹ PAI-2 is a member of the ovalbumin family of serpins and it has been demonstrated that mice produce a much larger array of these serpins relative to humans.²⁰ Perhaps a loss of PAI-2 expression is compensated for by increased expression of one or more of these other inhibitors. Indeed, the ovalbumin serpin, proteinase inhibitor 6, PI-6, is up-regulated during keratinocyte differentiation²¹ and hurpin, proteinase inhibitor 13 (PI13), is up-regulated in keratinocytes during psoriasis.²²

Additional studies using artificially wounded keratinocytes, in cell culture, have indicated that healing events are impaired after transfection with an antisense PAI-1 vector.² However, these studies were performed on human transformed cells and with a single population of cell type, keratinocytes. Normal wound healing involves a number of different cells, i.e., leukocytes, endothelial cells, and fibroblasts.

PAI-1, through its ability to alter the fibrinolytic environment, could manifest its effects during wound healing by temporally regulating the extent of the fibrin-rich provisional matrix allowing for fibroblast migration into the wound and eventual replacement of fibrin with collagen. Other studies have indicated that vitronectin receptor and plasminogen activators are increased in expression in migrating cells during wound healing.^{1,23-25} PAI-1 has been shown to inhibit cell migration by blocking the vitronectin receptor, α V β 3, interaction with vitronectin.²⁶ PAI-1 interaction with plasminogen activators, in this setting, results in a loss of affinity of PAI-1 for vitronectin, which restores cell migration. In this context, PAI-1 exerts its effects by regulating cell adhesion and detachment during cell migratory events of

Figure 4. Histological comparisons of healed skin wounds (**a,c,e,g**) versus non-wounded skin (**b,d,f,h**) in *PAI-1*^{-/-} mice (×200). H&E staining demonstrating a significantly thickened epidermal layer (*) within the healed lesion (**a**) as compared to uninjured skin (**b**). Invaginations of epidermal epithelium (arrow), which will ultimately result in the development of hair follicle structure, are observed. Masson's Trichrome staining indicates premature, diffuse deposition of collagen (blue) (**c**) as compared to the rich organization of collagen fibers (*) in non-wounded skin (**d**). Ayoub Shklar staining demonstrates a thickened keratinized layer above the epidermis (arrow) (**e**). PAS staining demonstrates that the reticular fiber structure (pink) is still cellular and does not yet demonstrate the organized basement membrane (**g**) as compared to the more non-cellular reticular layer of fibrous network (*) seen in the non-wounded skin (**h**). These findings are similar to those observed in healed *WT* skin lesions.

Figure 5. Immunohistochemical analysis of healed (**a**, **c**, **e**) and non-wounded skin (**b**, **d**, **f**) in *PAI-1*^{-/-} mice (×200). Anti- α -actin immunostaining demonstrates a reorganization of α -actin positive cells with particular staining in the deep fibroblast-rich reticular region (*) of the healed lesion (**a**) compared to a more scattered and less localized presence in the dermis of non-wounded skin (**b**). There is no longer a significant presence of extravascular fibrin(ogen) (**c**) or CD45-positive leukocytes (**e**) within the healed area although nonspecific staining of the sebaceous glands is typical for the latter stain as demonstrated in **f**. Fibrinogen in non-wounded skin is primarily associated within the vasculature (**d**).

wound healing. A role for vitronectin in wound healing was confirmed using a skin wound healing model in vitronectin deficient animals in which it was demonstrated that healing was delayed.²⁷ This would indicate that vitronectin/receptor interactions facilitate cell migration during wound healing and that blocking that interaction, through PAI-I, could alter the healing process.

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