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Plasminogen is a key pro-inflammatory regulator that accelerates the healing of acute and diabetic wounds

Short Title: Plasminogen heals diabetic wounds

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Abstract

Despite decades of research on wound healing, effective biologic agents for the treatment of chronic wounds, especially diabetic wounds, are still lacking. In this study, we report that the inert plasma protein plasminogen (plg) acts as a key regulatory molecule that potentiates wound healing in mice. Early in the healing process, plg bound to inflammatory cells is transported to the wound area, where the level of plg is locally increased. This leads to induction of cytokines and intracellular signaling events and to a potentiation of the early inflammatory response. Systemic administration of additional plg not only accelerates the healing of acute burn wounds in wild-type mice but also improves the healing of chronic diabetic wounds in a mouse model of diabetes. Our results suggest that administration of plg may be a novel therapeutic strategy to treat many different types of wounds, especially those that are chronic, such as diabetic wounds.
Introduction

Wound healing is a dynamic biological process that restores damaged cellular structures and tissue layers. This tissue-interactive process is very complex and consists of three phases: inflammation, tissue formation, and tissue remodeling. Acute wounds that do not have an underlying pathological defect normally heal readily. However, in wounds with preexisting pathophysiological problems, such as chronic wounds and diabetic wounds in particular, the healing processes are impaired. Chronic wounds not only impair the quality of life for millions of patients but also inflict an enormous burden on the healthcare system both in terms of cost and the intensity of care required. In the last few decades, large research efforts have been undertaken to dissect the molecular mechanisms behind wound healing processes, with the aim of developing new approaches to treat chronic wounds. However, thus far, very few biological substances have reached clinical practice, and no effective biologic substances are widely used for the treatment of chronic wounds.

The plasminogen activator (PA) system is a general proteolytic system, where the active protease, plasmin, is generated from the conversion of the precursor, plasminogen (plg), by either of two physiological PAs: tissue-type PA (tPA) or urokinase-type PA (uPA). The PA system is widely employed for the generation of extracellular proteolytic activity. It is well established that the PA system plays a pivotal role in fibrinolysis and in many tissue-remodeling processes, including wound healing. The PA system is also involved in the activation of intracellular signaling events and in the generation of inflammatory responses. With respect to the role of the PA system in wound healing, this system has mainly been studied and discussed in terms of fibrin degradation, generation of the extracellular proteolytic activity, and the activation of intracellular signaling pathways.
activity required for cell migration, and matrix degradation and tissue remodeling\textsuperscript{5}. However, whether plg plays a regulatory role in the initiation of the inflammatory phase of wound healing remains to be determined.

During the wound healing process, tPA and uPA are expressed by migrating keratinocytes\textsuperscript{10}. Studies in plg-deficient (plg\textsuperscript{-/-}) mice have shown that wound healing in skin is largely delayed and healing of tympanic membrane (TM) perforations is completely arrested in plg\textsuperscript{-/-} mice, indicating that plasmin plays an important role in wound healing processes\textsuperscript{5,11}. However, the concentration of the plasmin precursor plg in body fluids is as high as 2 \(\mu\text{M} \textsuperscript{12}\); it has, therefore, been assumed that plg levels are at saturation and that this molecule does not play any major regulatory role. Rather, it has been assumed that the formation of plasmin is mainly regulated by the accessibility and activity of the PAs\textsuperscript{4,12}.

In the current study, we have used a standard burn injury mouse model to explore the healing potential of plg and to reveal the molecular mechanism underlying its effect in acute and diabetic wounds. Our data show that plg is a key regulatory molecule with a pronounced pro-inflammatory effect that potentiates the early inflammatory response during wound healing. Administration of additional plg not only accelerates the healing of acute wounds but also initiates and improves the healing of chronic diabetic wounds. Taken together, our results suggest a novel therapeutic strategy for the treatment of different types of wounds, particularly diabetic wounds.
Methods

Animals

Plg-heterozygous (plg\(^{+/}\)) mice \(^{13}\) on a C57BL/6 background were used to generate wild-type (WT), plg\(^{+/}\) and plg-deficient (plg\(^{-/}\)) mice. The mice were genotyped by a rapid chromogenic assay, as described previously, with confirmation by PCR \(^{14}\). 8 to 10-week-old mice were used for the experiments. Genetically diabetic mice (C57BLKS \(db/db\)) and control heterozygous littermates (C57BLKS \(db/+\)) were obtained from Taconic Europe A/S (Ry, Denmark). Among the \(db/db\) mice, only those that were at least 10 weeks old and with a minimal blood glucose level of 15 mmol/l were used in the experiments \(^{15}\). The control littermates (\(db/+\)) were of the same age and had a maximum blood glucose level of 7.8 mmol/l. The animals were kept under standard laboratory conditions. The Regional Ethics Committee of Umeå University approved all the experimental protocols.

Burn Wound Model

The mice were anesthetized by intravenous injection of 100 μl mixture containing 5% Ketaminol® vet. (Intervet AB, Sollentuna, Sweden) and 20% Dormitor® vet. (Orion Pharma AB, Espoo, Finland). A metal rod (25 g, 1 cm diameter) was heated to 95-100°C by submersion into boiling water. The rod was immediately positioned vertically for 6 seconds without additional pressure on the back skin of mice that had also been depilated 3 days prior to wounding. After wounding, the mice were individually caged, and the wounds were not dressed.
Analysis of wound healing

The mice received standardized wounds, as described above, and then 2 mg of human plg (Omnio AB, Umeå, Sweden) was administered daily by intravenous injection. In the control group, 2 mg BSA (Sigma-Aldrich, Steinheim, Germany) was administered daily by intravenous injection as a placebo. In WT mice and db/db mice the daily treatments were continued for 16 and 24 days respectively. Digital photographs of the dorsal wounds were taken on the specified days until the end of the experiment. The photographs of the wounded areas were analyzed by tracing the wound margins and calculating the pixel areas using the computer program Image J (National Institute of Mental Health, Bethesda, MD). The remaining wound area was calculated as a percentage area of the original wound area.

Morphological staining

6 μm thick sections were taken perpendicularly to the wounded skin from paraffin-fixed tissue samples and processed for hematoxylin/eosin staining. Images were taken with a Leica DC300F digital camera attached to a Leica DMLB microscope (Leica, Wetzlar, Germany).

Analysis of the plg levels in wounds

Wounded back skin and unwounded abdominal skin were sampled from each mouse. The samples were homogenized and lysed with lysis buffer (50 mM Tris-HCl buffer pH 8.0 with 120 mM NaCl, 20 mM NaF, 20 mM β-glycerophosphate, 1 mM EDTA, 6 mM EGTA, 1% NP-40 and 1 mM DTT). The skin lysates were then kept at -20°C until use. The total protein concentrations in the lysates were quantified using the Bio-Rad protein assay, according to the
user’s manual (Bio-Rad Laboratories, Hercules, CA). To measure the level of plg in the tissue samples, an equal amount of total protein was used for a plg-specific chromogenic assay. The plg level in the unwounded abdominal skin was used as the plg basal level. The plg level in the wounded skin or unwounded matched skin was calculated as the fold increase from the basal level.

Spatial analysis of plg levels in the wound

The mice received a standardized wound as described above. One day after the injury, tissue samples were collected from the wounded and unwounded skin. The samples were taken 0.5 cm (Sample 1, S1) and 1.5 cm (Sample 2, S2) away from the edge of the wound (Figure 2B). The plg levels in different tissue samples were analyzed as described above.

Fibrinogen depletion

The mice were defibrinogenated 3 days before the experiment, as described previously. On the experimental day, a burn was induced, as described above, and 2 mg human plg was intravenously injected 5 min post-burn. Samples were collected 24 hours after injection. Plasma fibrinogen levels were semi-quantified by densitometric analysis of fibrinogen by Western blotting, using rabbit anti-mouse fibrinogen serum as the primary antibody (1:1000; Nordic Immunological Laboratories, Tilburg, The Netherlands).

Macrophage depletion

One day before the experiment, the mice received an intraperitoneal injection of 300 µl
clodronate liposomes, which led to a depletion of macrophages of at least 80% over the course of the experiment. Empty liposomes were used in the no depletion group. On the day of the experiment, a burn was induced as described above, followed by an intravenous injection of 2 mg human plg 5 min post-burn. Samples were collected 24 hours after injection.

**Neutrophil depletion**

For the 2 days prior to the experiment, the mice received daily 0.5 ml intraperitoneal injections of saline-diluted (1:3) rabbit anti-mouse neutrophil polyclonal antibody (Accurate Chemical & Scientific, Westbury, NY, USA), which resulted in a depletion of circulating neutrophils of at least 70% on the day of burn induction. Normal rabbit serum (Accurate Chemical & Scientific, Westbury, NY) was used in the no depletion group. Burns were induced as described above, and intravenous injections of 2 mg human plg (Omnio AB) followed 5 min post-burn. Samples were collected 24 hours after injection.

**Western blot analysis**

24 hours after wounding and injection, skin lysates were prepared and quantified, as described above. Anti-STAT3 and anti-phosphorylated-STAT3 (Tyr705) antibodies were obtained from Cell Signaling Technology (Boston, MA). Anti-β-actin antibody was obtained from Sigma-Aldrich Sweden AB (Stockholm, Sweden). Western blotting was performed according to the manufacturer’s instructions and visualized using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Uppsala, Sweden). The integrated density of the bands was quantified using ImageJ 1.41o analysis software (National Institutes of Health,
Bethesda, MD)¹⁹.

**ELISA**

24 hours after wounding and injection, skin lysates were prepared, and total protein was quantified, as described above. IL-6 levels were measured using an IL-6 ELISA kit (eBioscience, San Diego, CA). BSA levels were measured using a BSA ELISA kit (Acris Antibodies GmbH, Herford, Germany).

**Statistics analysis**

The results are expressed as the mean ± SD. Comparisons between two groups were analyzed by 2-tailed t-tests. Comparisons between multiple groups were analyzed by one-way ANOVA tests. $P < 0.05$ was considered to be significant.
Results

Plg accelerates the healing of burn wounds in wild-type mice

Previous studies from our group have shown that the healing of TM perforations is completely arrested in plg−/− mice but that healing can be restored following supplementation with plg 11. To explore if increased levels of circulating plg have any effect on the rate of wound healing, full thickness standardized burn wounds (1 cm in diameter) were induced in wild-type (WT) mice. Immediately after the injury, each mouse in the plg-treated group was given 2 mg of plg by intravenous injection, whereas the mice in the control group received 2 mg of Bovine Serum Albumin (BSA) by intravenous injection. This treatment was continued daily for 16 days. Quantification of the wound area at different time points showed that from day 6 post-injury, healing in the plg-treated group was significantly faster compared to that in the control group (Figure 1A). As shown in Figure 1B, the time to healing (scab falling off) in the plg-treated group was also approximately 2 days earlier than in the control group. A morphological analysis at 11 days post-injury revealed that the epithelium layer in the control group remained open and was covered by a large scab (Figure 1C), whereas in the plg-treated group, the epithelium layer had fused to reepithelialize the wound completely, and only a small scab remained lightly attached above the wound (Figure 1D). These data indicate that an increased level of plg in the circulation accelerates the rate of healing in WT mice.

Plg accumulates in the wounded area during wound healing
The above data suggest that the level of plg in circulation affects the rate of the wound healing process. Therefore, we further investigated how the level of plg is regulated at and around wound sites during the wound healing process. A standardized burn wound was induced on the back skin of WT and plg-heterozygous (plg\(^+/\)) mice. Plg\(^+/\) mice were included, as they have approximately half the amount of plg in their blood \(^{13}\) and tissue compared with WT mice (data not shown). At different time points after the injury, wounded back skin and unwounded skin from the same mouse were collected for the measurement of plg levels in wounded and unwounded skin, respectively. As shown in Figure 2A, 1 day after the injury, the level of plg increased approximately 6-fold in the wound sites in the WT mice and 4-fold in the plg\(^+/\) mice, as compared with the basal level of plg in the control unwounded skin. In both WT and plg\(^+/\) mice, the change in the plg levels in the wound sites coincided with inflammation activity in the wound sites. The plg levels were highest during the early inflammatory phase, became lower during the tissue formation phase, and nearly returned to basal levels during the late tissue remodeling phase.

We then studied the spatial distribution of plg at and around the wound sites on day 1 post-injury. Tissue samples were taken from and around the wound sites as indicated in Figure 2B, and unwounded skin from the same mouse was collected for the measurement of basal plg levels. A spatial analysis showed that the plg levels only increased in the vicinity of the wound and were at basal levels outside of the wounded area (Figure 2C). These results indicate that plg specifically accumulates at inflamed areas during the early inflammation phase of the wound-healing process. As shown in Figure 2A, the plg\(^+/\) mice had a similar pattern of plg distribution but the increase was smaller than in the WT mice, suggesting that the increase of
plg in the wound sites appears to correlate with the concentration of plg in circulation. To examine if this is also the case when the plg concentration in circulation is higher than normal, the WT mice were intravenously injected with 2 mg of plg 5 min post-wounding. We then analyzed the level of plg in the wound sites 24 hours after plg injection. As shown in Figure 2D, there was a 22-fold increase of plg in the wound sites of the mice that had been intravenously injected with 2 mg of plg. This increase was dramatically higher than the increase seen in the normal wound group, where there was a 6.5-fold increase (Figure 2C). Moreover, a spatial analysis of the WT mice supplemented with plg showed that the dramatic increase of plg was restricted to the wound area (Figure 2D). These findings were further confirmed by using measurements of plg antigen levels by ELISA and Western blot analysis (Supplemental Figure S1).

Taken together, our data suggest that the healing effect of plg might be related to its wound specific accumulation and that the extent of the accumulation effect can be further enhanced by supplementation with exogenous plg.

Plg is mainly transported to the wounded area by inflammatory cells

To elucidate the underlying mechanisms behind the healing potential of plg, we performed a set of experiments to investigate the mechanisms by which plg is accumulated in the wound area following systemic supplementation.

Fibrin formation followed by fibrinolysis performed by plasmin is one of the early hemostatic events after a wound injury. As plg binds to fibrin surfaces with high affinity,
we investigated if the binding of plg to fibrin is responsible for the accumulation of plg in the wound. WT mice were first treated with the defibrinogenation reagent ancrod (3 U/day) for 3 days, resulting in an 80% reduction of plasma fibrinogen levels (data not shown)\textsuperscript{16}. After this treatment, a standardized burn wound was induced on the back of the defibrinogenated mice, and 5 min later, each mouse was intravenously given 2 mg plg. Tissue samples were collected 24 hours after the injection. As shown in Figure 3A, there was no significant reduction in plg accumulation in the wounds of fibrinogen-depleted mice, as compared to the control group. This indicates that the binding of plg to fibrin is not a major mechanism for the local accumulation of plg in the wound.

As shown in Figure 2A, the accumulation of plg in wounds is greatest during the early inflammatory phase. Immunostaining of human plg in the wound after plg treatment showed that human plg was clearly localized at the edge of the wound where the inflammatory cells infiltrate (Supplemental Figure S2). It is well known that inflammatory cells are recruited to a wounded area after injury, and several \textit{in vitro} studies have shown that plg binds to inflammatory cells through different plg receptors \textsuperscript{22-24}. Consistently, after systemic supplementation of fluorescent plg into WT mice, exogenous fluorescent plg was bound to peripheral inflammatory cells (Supplemental Figure S3). However, the \textit{in vivo} significance of such binding in wound healing has not been explored. We therefore studied the contribution of two primary inflammatory cells, macrophages and neutrophils, to plg accumulation. In 2 sets of experiments, \textit{in vivo} cell depletion techniques were used to deplete neutrophils and macrophages before a burn wound was induced. Macrophages or neutrophils were depleted as described in the methods section, and burn wounds were induced. At 5 min after the burn
induction, each mouse was intravenously given 2 mg plg, and tissue samples were collected 24 hours after the injection. As shown in Figure 3B, the level of plg in the wound after macrophage depletion was reduced by 52% compared with the no depletion control group. Similarly, the plg accumulation after neutrophil depletion was reduced by 23% (Figure 3C). These data suggest that the wound-specific accumulation of plg after systemic supplementation is mainly due to the transportation of plg by macrophages and neutrophils.

Burn injuries cause vasodilation, increased blood flow, and an increased vessel permeability, which results in the increased transport of plasma proteins to the wound. Albumin is commonly used to measure vessel permeability. To investigate if part of the wound-specific accumulation of plg is caused by these mechanisms, we used albumin to measure changes in vessel permeability during burn injury in our model. After burn induction, WT mice either received an intravenous injection of 2 mg of BSA or plg. As demonstrated in Figure 3D, the level of plg increased approximately 22-fold in the wound, whereas the level of BSA only increased approximately 5-fold.

Taken together, our results suggest that the bulk of plg is transported to the wound site bound to inflammatory cells, and only a minor part is due to vessel leakage. However, the binding of plg to fibrin appears to play no or only a minor role.

**Plg enhances the expression of IL-6 and augments the activation of STAT3 in wounded skin**

A proper inflammation response after injury is considered to be a prerequisite for wound
healing, and pro-inflammatory cytokines are known to be involved in this process. IL-6 is an important mediator of host responses to tissue injury that can be induced through both TNF-α-dependent and other pathways. Therefore, we measured IL-6 levels in plg−/− and WT mice that were treated with BSA (control) or plg. As shown in Figure 4A, IL-6 levels in unwounded skin were low irrespective of genotype and treatment. However, the level of IL-6 in the wounded skin of the WT mice was significantly higher than that in the wounded skin of the plg−/− mice, suggesting that the inflammation response after the injury was compromised in the plg−/− mice. After plg treatment, the level of IL-6 in the wounds was enhanced both in the WT and plg−/− mice. Moreover, the level of IL-6 in the wounded skin in the plg−/− mice was almost restored to normal WT levels. These data indicate that plg enhances the expression of IL-6 in wounds.

STAT3 is a key intracellular molecule that is involved in signaling during acute inflammatory responses mediated by several inflammatory cytokines, including IL-6. To study how plg treatment may affect STAT3 activation, we measured the levels of phosphorylated STAT3 (pSTAT3) in wounds 24 hours after plg treatment. As shown in Figure 4B, the level of pSTAT3 is higher in wounded skin than in unwounded skin, and the pSTAT3 level was further increased following plg treatment, suggesting that plg treatment could enhance the activation of STAT3 in the wound. These data indicate that during wound healing, plg treatment enhances pro-inflammatory cytokines and activates intracellular signaling events.

Plg improves the healing of burn wounds in a mouse model of diabetes (db/db mice)
An imbalanced inflammatory response is one of the reasons for wound healing defects in diabetic patients. To explore the healing potential of plg in diabetic wounds, we utilized \(db/db\) mice that suffer from a severe wound healing impairment. A standardized burn wound was first induced on the backs of \(db/db\) mice. Immediately after the injury, the mice in the plg-treated group were intravenously administered 2 mg plg, whereas every mouse in the control group received 2 mg BSA. These injections were repeated daily for 24 days. As shown in Figure 5A, the healing rate in the plg-treated group was significantly faster than that of the control group. The time to healing (scab falling off) in the plg-treated group was also significantly earlier (approximately 3 days earlier than the control group) (Figure 5B). A morphological analysis at 18 days after the injury revealed that the front of the epithelium layer in the control group had barely fused and was covered by a scab. In addition, the tissue underneath the scab was inflamed (Figure 5C). In contrast, in the plg-treated group, the injured epithelium layer and the underlying tissue had healed (Figure 5D).

To further dissect the molecular mechanisms underlying the improved healing mediated by plg in \(db/db\) mice, we determined the plg level in wounded and unwounded skin in \(db/db\) and non-diabetic control \(db/+\) mice. As shown in Figure 6A, the plg levels in the unwounded skin were low, irrespective of genotype or treatment. After burn induction, the plg levels in the wounds of the \(db/db\) mice were only slightly enhanced compared with the control \(db/+\) mice, where the plg level after wounding increased approximately 5-fold. However, after receiving systemic plg treatment, the plg levels in the wounds of the \(db/db\) mice reached approximately the same level as in the control \(db/+\) mice. Studies of the IL-6 levels 24 hours after injury in the
control and diabetic wounds showed that IL-6 levels were significantly lower in the wounds of the \textit{db/db} mice than in the control \textit{db/+} mice (Figure 6B). However, after plg treatment, the IL-6 levels in the wounds of the \textit{db/db} mice were clearly increased (Figure 6B). We also measured the levels of pSTAT3 in skin from unwounded, wounded \textit{db/db} mice, and wounded \textit{db/db} mice treated with plg. As shown in Figure 6C, the levels of pSTAT3 in the wound sites increased after wounding and further increased after plg treatment.

Taken together, these data indicate that the impaired wound healing in diabetic \textit{db/db} mice may in part be due to the dysregulation of plg. Plg supplementation appears to rebalance the early inflammatory response in these mice, which leads to improved wound healing. Plg treatment may, therefore, become a novel therapeutic strategy for the treatment of diabetic wounds.
Discussion

The present study demonstrates for the first time that plg acts as a key regulatory molecule that plays a pivotal role in the control of wound healing. Plg potentiates the early inflammatory response, and systemic administration of additional plg to mice not only accelerates the healing of acute wounds in healthy WT mice but also improves the healing of chronic diabetic wounds in db/db mice. Our results suggest that the administration of plg may be a novel therapeutic strategy to treat different types of wounds, especially diabetic wounds for which there are no effective, widely used treatments.

It is well established from studies of gene deficient mice that the PA system and formed plasmin play an important role in wound healing. Based on these studies, it has been suggested that the activation of plg mainly plays a role in fibrin degradation, cell migration, and the degradation of the extracellular matrix during tissue remodeling events. However, our studies on the healing of TM perforation in plg−/− mice have revealed that plg/plasmin appears to play a profoundly different role in the healing process than what had been previously assumed based on studies of skin wounds. In particular, our studies have indicated that plasmin may not just be a protease involved in fibrin degradation and matrix remodeling but may also act as an initiator of wound healing.

In the present study, we demonstrated that plg supplementation improves and speeds up skin wound healing in healthy WT mice. These mice do not have impaired fibrinolysis or a defect in extracellular matrix degradation. This suggests that plg may play further roles in wound healing in addition to acting as a protease that dissolves fibrin and tissue barriers to pave the way for cells to migrate. We also demonstrated that plg levels specifically increase at the
inflammation site and in a limited area around the wound. This accumulation of plg occurs during the early inflammation phase of wound healing and can be further enhanced following plg supplementation. Systemic plg supplementation also leads to an accelerated wound-healing process. This indicates that plg acts as a key pro-inflammatory factor that regulates the wound-healing process by activating the early inflammatory reaction.

Inflammatory cell depletion experiments revealed that the majority of the plg that accumulates in wound sites is connected to neutrophils and macrophages. Most likely plg is transported to the inflammation site bound to the surface receptors present on these cells. This is consistent with previous studies showing that the capacity of leukocytes to bind plg is increased after cell activation. Pro-inflammatory cytokines play important roles in the activation of inflammatory responses. In vitro studies on monocytes have shown that active plasmin bound to the cell surface of monocytes through lysine binding sites can activate several intracellular signaling cascades that lead to activation of pro-inflammatory genes. Consistent with this, we found that the level of IL-6 is enhanced and STAT3 is activated in the wounded area. Moreover, supplementation with additional plg not only caused additional IL-6 enhancement and a further activation of STAT3 but also significantly improved the healing process.

Based on the above studies and previous results, we propose the following working model for the role of plg in wound healing. In this model, we propose that plg, in addition to its well known role as a protease involved in fibrinolysis, cell migration and tissue remodeling, may also play an even more profound role as a key regulatory molecule that potentiates and perhaps even initiates the healing process. Under normal physiological conditions, plg binding to the
surface of inflammatory cells is low. However, upon injury, inflammatory cytokines are released, thereby providing an “injury signal” to inflammatory cells, which results in an increased plg binding capacity, and a locally increased vessel permeability. Plg bound to inflammatory cells is then transported to the wounded area where it is activated to plasmin, which somehow mediates proteolytic cell activation, possibly through the proteolytic cleavage of a yet unidentified receptor. Plasmin then activates several signaling cascades and elicits a pro-inflammatory reaction that potentiates the early inflammatory response during wound healing. Our data showing that plg is a key pro-inflammatory regulator that potentiates wound healing, together with its previously known roles in fibrin degradation, cell migration and extracellular matrix degradation and tissue remodeling, give plg a more central role during wound healing than previously appreciated.

Diabetic wounds are among the most severe types of chronic wounds. More than 20% of diabetic wounds result in amputations of the affected foot or leg. However, much of the available information on the biology of wound healing relates to acute and experimental wounds and may, therefore, not be directly relevant to diabetic wound healing. Based on our findings in acute wounds in healthy wild-type mice, we investigated whether plg was dysregulated in diabetic wound healing and if plg supplementation had any stimulatory effect on the healing of diabetic wounds. As demonstrated in Figure 5 and 6, there was no wound-specific accumulation of plg in the wounds of diabetic db/db mice (Figure 6A), and the healing rate was also slower in these mice (Figure 5A). It has been shown that impaired healing in diabetic wounds is accompanied by decreased early infiltration of inflammatory cells. Our binding studies indicate that the binding of plg to the peripheral inflammatory
cells in WT mice and db/db mice were similar (unpublished data). The impaired wound-specific accumulation of plg is therefore most likely due to a decreased infiltration of inflammatory cells. Following plg administration, the level of plg in the wounds of the diabetic mice increased to the same level as that found in non-diabetic control mice. This also resulted in the activation of intracellular signaling events and a significantly increased healing rate. Our data, therefore, indicate that the dysregulation of plg may be part of the pathogenesis of diabetic wound healing. Our data also show that supplementation with plg results in significantly improved diabetic wound healing.

In conclusion, our results show for the first time that plg, in addition to its role in extracellular matrix degradation, plays a pivotal role as a key pro-inflammatory factor that potentiates the early inflammatory response during wound healing. Based on our findings, we propose a novel therapeutic strategy for the treatment of different types of wounds, including chronic diabetic wounds. This treatment would consist of exogenous plg administrated locally or systemically.
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Authorship

Contributions: Y.S., T.N. and J.L. conceived and designed the experiments. Y.S., Y.G., P.M. and R.S. performed the experiments. Y.S., Y.G., M.W., T.N. and J.L. analyzed the data. Y.S., T.N. and J.L. wrote and prepared the manuscript.

Conflict-of-interest disclosure: T.N. and J.L. have patented the use of plasminogen for the treatment of wound healing. They are also stock holders in a start-up company which owns the right to develop plasminogen for therapeutic purposes.

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Figure Legends

Figure 1. Effect of plg treatment on burn wound healing in wild-type (WT) mice. (A)
Quantification of the remaining wound area at different time points post-wounding in the
BSA-treated control group (n = 6) and the plg-treated group (n = 7). (B) Comparison of the
healing times (scab falling off) in the days post-wounding. * p < 0.05; ** p < 0.01. (C-D)
Representative pictures of the control group and the plg-treated group on day 11 post-injury.
The leading edges of the epithelium layer are indicated by arrows, and the scab is indicated by
an asterisk. The magnification is ×50.

Figure 2. Temporal and spatial regulation of plg during wound healing. (A) Kinetics of
the plg levels in the wounds for 18 days after burn wounding in WT (n ≥ 5) and plg+/- mice (n ≥
3). (B) The experimental design used to determine the spatial regulation of plg after wounding.
(C) Comparison of the plg levels at different distances from the wound 24 hours
post-wounding in WT mice (n = 5). (D) Comparison of the plg levels at different distances
from the wound 24 hours after the intravenous injection of plg in WT mice (n = 5).

Figure 3. Mechanisms regulating plg accumulation in wounds after burn wounding. (A)
Comparison of the plg levels in the wound sites in the control and fibrinogen-depleted mice 24
hours after the intravenous injection of plg (n = 5). (B) Comparison of the plg levels in the
wound sites in the control and macrophage-depleted mice 24 hours after the intravenous
injection of plg (n ≥ 4). (C) Comparison of the plg levels in the wound sites in the control and
neutrophil-depleted mice 24 hours after the intravenous injection of plg (n ≥ 6). (D) Comparison of the plg or BSA accumulation in the wounded areas in the plg-injected (n = 12) and BSA-injected mice (n = 6). The level of plg or BSA in the abdominal skin was used as basal level. The level of plg or BSA in the wound was determined and displayed as the fold increase from the basal level. ns = not significant, * p < 0.05; ** p < 0.01.

**Figure 4. Effect of plg on IL-6 expression and STAT3 activation in the wound.** (A) Comparison of the IL-6 levels between the WT (n ≥ 9) and plg-/- mice (n ≥ 9) treated with BSA (control) or plg. (B) Western blot analysis of the phosphorylated STAT3 (pSTAT3) levels in skin lysates from the unwounded WT mice, the wounded WT mice and the wounded WT mice treated with plg (n ≥ 5). β-actin served as loading control. The level of pSTAT3 was quantified using ImageJ. Values are expressed as the intensity of pSTAT3 divided by the intensity of total STAT3. ns = not significant, * p < 0.05; ** p < 0.01.

**Figure 5. Effect of plg treatment on wound healing in db/db mice.** (A) Quantification of the remaining wound area at different time points post-wounding in the BSA-treated control group (n = 6) and in the plg-treated group (n = 6). (B) Comparison of the healing times (scab falling off) in days post-wounding. (C-D) Representative pictures of the control group and the plg-treated group on day 18 post-injury. The inflamed tissue is indicated by arrows, and the scab is indicated by an asterisk. The magnification is ×50.

**Figure 6. Effect of plg treatment on IL-6 expression and STAT3 activation in db/db mice.**
(A) Comparison of plg levels in the unwounded and wounded areas in the db/db mice (n = 8), the db/db mice treated with plg (n = 4) and the db/+ mice (n = 4). (B) Comparison of the IL-6 levels between the db/db mice (n = 8), the db/db mice treated with plg (n = 8) and the db/+ mice (n = 10). (C) Western blot analysis of the pSTAT3 levels in the skin lysates from the unwounded db/db mice, the wounded db/db mice and the wounded db/db mice treated with plg (n = 4). β-actin served as loading control. The level of pSTAT3 was quantified using ImageJ. Values are expressed as the intensity of pSTAT3 divided by the intensity of total STAT3. ns = not significant, * p < 0.05; ** p < 0.01.
Figure 1.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Plasminogen is a key pro-inflammatory regulator that accelerates the healing of acute and diabetic wounds

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