Maggot therapy´s modes of action : effect of maggot secretions on microbiological, haematological and immunological processes

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CHAPTER FOUR

Maggot secretions enhance plasminogen activator-induced fibrinolysis by cleavage of plasminogen

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Abstract

Maggots of the blowfly *Lucilia sericata* are used for the treatment of chronic wounds. As hemostatic processes play an important role in wound healing, this study focused on the effects of maggot secretions on coagulation and fibrinolysis.
The results showed maggot secretions to enhance the plasminogen activator (tPA and uPA)-induced formation of plasmin and subsequent fibrinolysis without affecting coagulation. Secretions themselves did not induce plasmin formation. Furthermore, we found secretions to interact with plasminogen, but not with plasminogen activators, in a dose- and time-dependent manner. Using acid-urea gels and selective protease inhibitors we found that a serine protease within the secretions was responsible for cleavage of plasminogen, leading to a derivative which is activated more easily by plasminogen activators. We conclude that a serine protease within maggot secretions enhances plasminogen activator-induced fibrinolysis by cleavage of plasminogen.

Introduction

Maggots of the green bottle blowfly *Lucilia sericata* are used for the treatment of many types of wounds including venous ulcers[1], traumatic and post-surgical wounds[2], osteomyelitis[3] and burns[4]. Although maggots are known primarily for debridement - removal of necrotic tissue and fibrin slough - of chronic wounds, they exert many additional effects. Earlier we reported maggot excretions/secretions to breakdown bacterial biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*[5]. Furthermore, maggots ingest and subsequently kill bacteria in their digestive tract[6]. In addition to antibacterial effects, we showed secretions to inhibit the pro-inflammatory responses of human neutrophils[7] and monocytes[8] without affecting the antimicrobial activities of these phagocytes. Moreover, maggot secretions skew the monocyte-macrophage differentiation away from a pro-inflammatory to a pro-angiogenic type (submitted for publication). Others reported accelerated fibroblast migration induced by maggot excretions/secretions[9,10]. However, there is an overall lack of information on the effect of maggots on coagulation and fibrinolysis.

Coagulation refers to the formation of insoluble fibrin, which stops hemorrhage and provides a provisional matrix essential for cell migration thereby aiding in the repair of damaged vessels and tissues[11,12]. In a balanced wound healing process these fibrin clots are broken down (during remodelling of the tissue) in the fibrinolytic phase; plasminogen is converted by plasminogen activators (uPA or tPA) to plasmin, which subsequently cuts the fibrin mesh by proteolytic degradation[12]. In chronic wounds, fibrin clots may be partially degraded by proteolytic enzymes derived from immune cells, like neutrophils and macrophages. These clots no longer support re-epithelialisation and granulation tissue formation and therefore have to be removed[11,13]. However, this cannot be accomplished by the wound components itself as, for instance, fibrinolysis may be impaired in chronic wounds due to enhanced levels of the fibrinolysis inhibitor PAI. These processes contribute to the formation of necrotic tissue and fibrin slough which contain trapped leucocytes and are a rich source of nutrients for bacteria. If necrotic tissue and/or fibrin slough are left unattended, it is very difficult to keep the wound free of infection, to prevent excessive inflammatory responses and to ensure closure of the wound. Therefore, debridement is essential for healing of these wounds and, as mentioned above, this can be exerted by maggots. It has been reported that after debridement has been accomplished by maggots minor bleeding may occur[14]. On the other hand, it has been reported that the excretory substances from the larvae have a potent hemostatic effect in haemorrhage[15]. Based upon the above considerations and clinical observations, the aim of this study was to investigate the effects of maggot secretions on coagulation and fibrinolysis.

Materials and methods

Preparation of maggot secretions

Sterile second- and third-instar larvae of *L. sericata* were a kind gift from BioMonde GmbH (Barsbüttel, Germany). Maggot secretions were collected as described[8]. Prior to use, sterile preparations of secretions were pooled and centrifuged at 1,300xg for 5 min at 4°C to remove particulate material. Subsequently, the protein concentration of these pools was determined using the Pierce BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instructions. In each assay, at least 3 different pools were used.

Coagulation assays

Clot formation was measured after incubating 10 µL of secretions (final concentration 50 µg/mL) or H_2O as a control with 90 µL of citrated plasma for 10 and 30 min at room temperature (RT). To initiate the intrinsic pathway of coagulation, 100 µL of APTT reagent (Kordia Life Sciences, Leiden, The Netherlands) and 100 µL of 25 mM Ca^{2+} were added (APTT-test). The extrinsic pathway was started by adding 200 µL of Thromborel S (Dade Behring BV, Leusden, The Netherlands) to the secretions-plasma mixture (PT test) while the Thrombin time (TT-test) was measured after adding 25 µL of Thrombin (100 U/mL; Enzyme Research Laboratories Inc, South Bend, IN, USA). The time needed for clot formation was measured at 37°C.

Clot lysis assay

Clot lysis was measured by a turbidimetric method using 96-wells microlon plates (Greiner Bio-One, Alphen aan de Rijn, The Netherlands). Mixtures of 80 µL were made containing 75% citrated plasma, 7.5 U/mL of tissue-type plasminogen activator (tPA; kindly provided by TNO, Leiden, The Netherlands) and secretions (range 1.25-5 µg) or, as a control, H_2O and transferred to the wells. Subsequently, 20 µL of a second mixture was added consisting of a 100 fold dilution of Innovin (Dade Behring) in TEA-buffer (containing 25 mM triethanolamine, 0.05% Tween and 50 mM NaCl) supplemented with 100 mM $CaCl_2$. Next, the plate was shaken for 30 s after which the absorbance (405 nm) was measured 60 times with an interval of 10 min at 31°C using a Tecan reader. The time needed to obtain 50% lysis of the clot (X50) was calculated. Results, being the average X50 of samples measured in duplicate, were normalized by dividing them by the X50 obtained in the absence of secretions (Ratio).

Plasminogen activation

The effect of secretions on the kinetics of fibrinolysis was investigated in a system of purified proteins (tPA and plasminogen) using the chromogenic substrate for plasmin S2403.

Mixtures were made containing tPA (25-600 U/mL), secretions (0.78-100 µg/mL) or, as a control, H_2O, Glu-plasminogen (Plg; 0.25-2 U/mL = 0.33-2.68 µM) and 0.375 mM S2403 (Chromogenix, Milano, Italy) and transferred to 96-wells microlon plates (Greiner Bio-One). Next, the plates were shaken for 30 s after which the absorbance (405 nm) was measured 30 times with an interval of 20 s at 31°C using an ELISA reader. Subsequently, absorbance values were corrected for the absorbance in the absence of Plg and tPA (due to enzymatic activity present in secretions[16]) at each time interval. The resulting values were plotted against the time square and the slope of this line, which reflects the rate of plasmin production, was calculated for each sample. Next, the Δabsorbance/sec^2 was converted into rates of plasmin production (nM/sec) using purified human plasmin as a standard (Enzyme Research Laboratories, South Bend, IN, USA).

Acid-urea gel electrophoreses

Acid-urea gels (AU-page; 10%) were prepared as described[17]. Plg in TEA buffer was incubated with tPA and/or secretions for the indicated time intervals at 31°C and mixed with sample buffer (9.5 M urea in 5% acetic acid) in a 1:1 ratio. Next, samples were transferred to the slots and gels were run in 5% acetic acid for 90-120 min at 150V using reversed polarity. Thereafter, gels were stained with Coomassie Blue.

Partial characterization of the active component in secretions

To obtain information about the nature of the active component(s), secretions were incubated with 0.1-1% of SDS, 6 M urea, 50 mM DTT or, as a control, H_2O for 1 h at RT followed by overnight dialysis using a 12-14 kD tubing membrane (Visking, Medicell International Ltd, London, UK) against ultrapure H_2O at 4°C. In addition, secretions were boiled for 2 min or 2 h. Subsequently, the effects of treated secretions on fibrinolysis were tested in the clot lysis assay. Furthermore, secretions were incubated with Serine Protease Inhibitor Cocktail Set I (SPIC-1; Calbiochem, EMD Biosciences, Inc, La Jolla, Ca, USA) or 10 mM of PMSF at various time intervals at 31°C before testing in the AU-page.

Statistical analysis

Statistical analyses were performed using Graphpad Prism version 4.02. Differences between values obtained with samples incubated with secretions and control samples were analyzed with a paired t-test. $P<0.05$ was considered significant.

Results

Effect of secretions on coagulation

To investigate whether maggot secretions interfered with the formation of blood clots, their effect on the intrinsic pathway (APTT-test), the extrinsic pathway (PT-test) and fibrin formation (TT-test) were assessed. The results showed no effect of secretions (50 µg/mL) on coagulation (Table 1).

Table 1 Effect of 50 µg of secretions/mL on coagulation

Test	10 min		30 min	
	Control	Secretions	Control	Secretions
APTT	41.0 ± 1.4	44.3 ± 1.8	41.6 ± 2.3	41.3 ± 1.6
PT	25.3 ± 0.2	22.4 ± 0.3	24.0 ± 0.3	22.2 ± 0.2
TT	19.8 ± 0.2	20.5 ± 0.6	20.5 ± 0.4	20.2 ± 0.6

The results, expressed in seconds, are means ± SEM of 6 experiments.
PT, Prothrombin Time; TT, Thrombin Time.

Effect of secretions on tPA-induced fibrinolysis

To investigate whether maggot secretions affect breakdown of plasma clots, a turbidimetric method was used. The results showed that secretions dose-dependently decreased the lysis time (Table 2; Figure 1). Secretions added to the wells 15 min after the fibrin clot was formed also reduced the lysis time -although less efficient- indicating that secretions are effective against preformed clots as well (Table 3). Similar results were obtained when using uPA (30 U/mL) instead of tPA (data not shown). Importantly, the addition of a plasminogen activator was essential as secretions themselves did not induce clot lysis. When the experiments were repeated with plasma deficient in α2-antiplasmin or TAFI, similar effects were obtained as in normal plasma indicating that the accelerating effect of secretions on fibrinolysis was not due to inhibition/inactivation of these fibrinolysis inhibitors. Two min of boiling was sufficient to abrogate the profibrinolytic effect of secretions, indicating that the active component is likely a protein (n = 3). Treatment of secretions with SDS or urea abrogated their activity (n = 3) indicating that the tertiary structure of the protein(s) is essential for its biological activity. As DTT had no effect on the activity of secretions (n = 3) it seems unlikely that the active component contains disulfide bonds.

Table 2 Effect of secretions on the lysis time of plasma clots

Secretions (µg/mL)	X50 (min)	Ratio
0	207 ± 2	1.00
12.5	195 ± 5	0.94 ± 0.03
25	181 ± 3	0.87 ± 0.02**
50	165 ± 3	0.79 ± 0.02**

Results, expressed as the time needed to obtain 50% lysis of the clot (X50) and as Ratio (X50-secretions/X50-control), are means ± SEM of 7-8 experiments. **Values are significantly ($p<0.005$) different compared to the control.

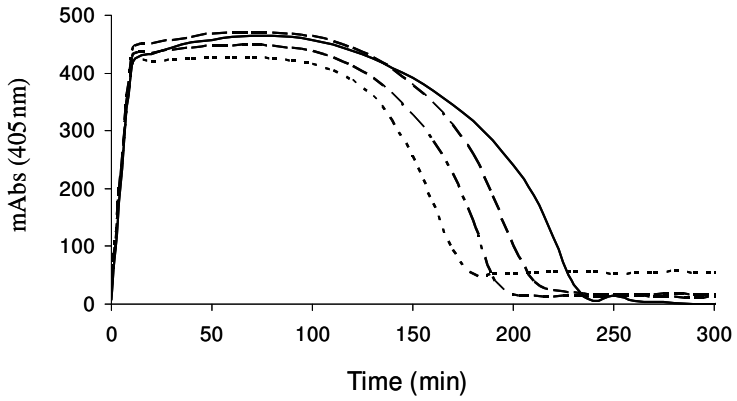

Figure 1 Representative example of the effect of maggot secretions on the tPA-induced lysis of plasma clots. No secretions —; 12.5 µg of secretions/mL — —; 25 µg of secretions/mL – —; 50 µg of secretions/mL – – –.

Table 3 Effect of secretions on the lysis time of established plasma clots

Secretions (µg/mL)	Ratio
0	1.00
25	0.96 ± 0.04
50	0.92 ± 0.03*
100	0.86 ± 0.06*
200	0.81 ± 0.04*
250	0.73 ± 0.02*

Results, expressed as Ratio (X50-secretions/X50-control), are means ± SEM of 6-8 experiments. *Values are significantly ($p<0.05$) different compared to the control.

Stimulation of tPA-induced plasmin formation by secretions

As the presence of a plasminogen activator was essential for secretions-stimulated fibrinolysis, we further studied the effect of secretions on the tPA-induced plasminogen activation. The results showed secretions to enhance the rate of plasmin formation in the presence of 0.33 µM of Plg and 50 U/mL of tPA but not in the absence of tPA (Figure 2A). Maximum stimulation was observed for 12.5 µg of secretions/mL; this concentration was independent of the tPA concentration used (data not shown). In contrast, maximum stimulation was obtained at higher secretions concentrations when the Plg concentration was increased (Figure 2B). These data indicate that secretions interact with Plg but not with tPA.

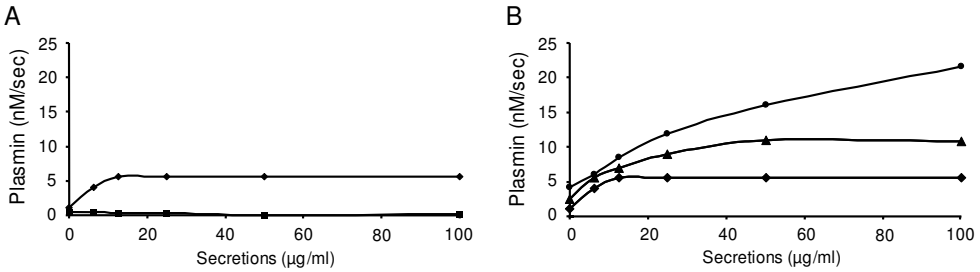

Figure 2 Effect of secretions on the tPA induced conversion of Glu-plasminogen.
A) The effect of secretions (1.56-100 µg/mL) on plasmin formation from 0.33 µM of Plg and 50 U/mL of tPA (diamonds) or no tPA (squares). B) The effect of secretions (1.56-100 µg/mL) on plasmin formation from 0.33 µM (diamonds), 0.67 µM (triangles) and 1.34 µM (circles) of Plg/mL in the presence of 50 U/mL of tPA. The shown results are of representative examples out of 3-6 experiments.

To investigate this interaction in more detail, secretions and plasminogen (0.33 µM) were incubated for various time intervals before addition of tPA (50 U/mL) and the plasmin substrate. The results showed that plasmin formation was dependent on the pre-incubation time (Figure 3A), indicating that enzymes in the secretions are responsible for the observed stimulatory effect. Plg incubated with secretions (12.5-100 µg/mL) for 24 h was no longer activated by tPA although lower concentrations of secretions were still able to stimulate Plg activation. Incubating tPA with secretions before addition of Plg and the plasmin substrate had virtually no effect on plasmin formation (Figure 3B).

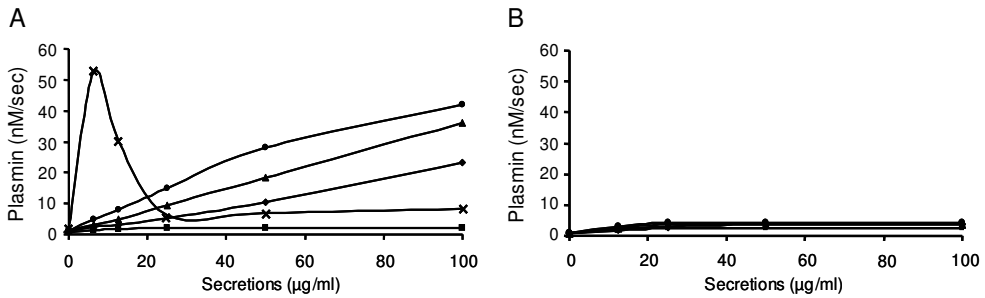

Figure 3 Effect of pre-incubation of Glu-plasminogen or tPA with secretions on plasmin formation. 0.33 µM Glu-Plg (A) or 50 U/mL of tPA (B) were pre-incubated with secretions for various time intervals before measuring plasmin formation. Pre-incubation time: 0 min = squares; 20 min = diamonds; 40 min = triangles; 60 min = circles; 24 h = cross. The results are of representative examples out of 3-6 experiments.

Effect of secretions on plasminogen

As secretions enhance tPA-induced plasmin formation by interacting with Plg, we considered the possibility that secretions promote the proteolytic conversion of Plg into (a) derivative(s) with a higher affinity for plasminogen activators. The results showed secretions to cleave plasminogen in a dose- (Figure 4A) and time-dependent (Figure 4B) manner eventually leading to complete fragmentation. Incubating secretions with 10 mM of PMSF or SPIC-1 abrogated the observed cleavage of Plg (Figure 4C) indicating that a serine protease within secretions was responsible for these effects. However, formation of Lys-plasminogen, a known derivative of Glu-plasminogen[18], and/or plasmin was not observed when incubating Plg with secretions whereas incubation of Plg with tPA resulted in the formation of Lys-plasminogen and/or plasmin (Figure 4D).

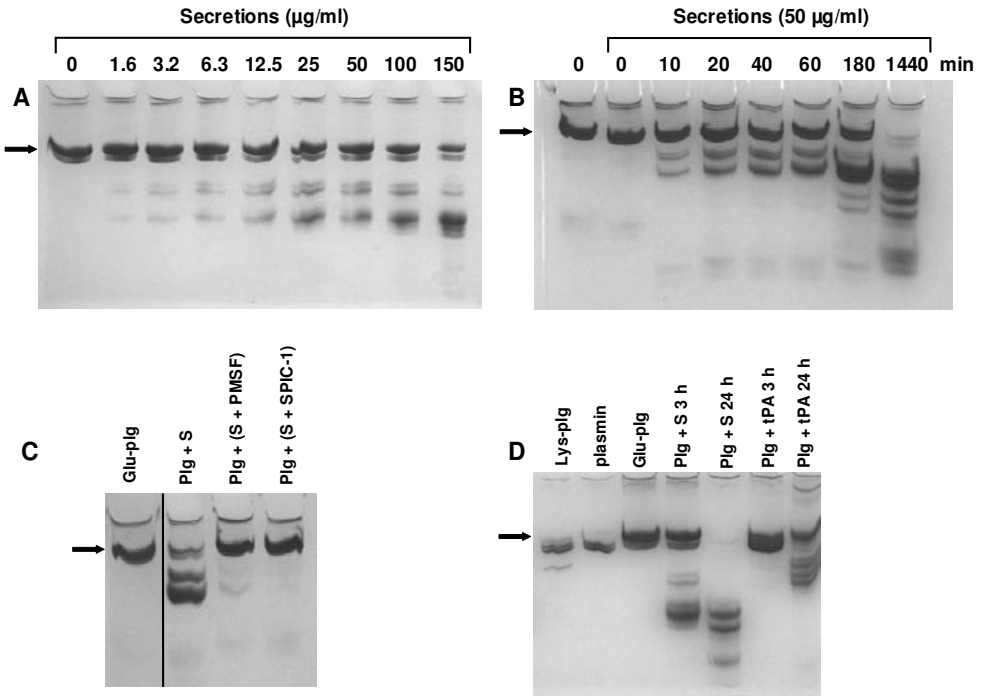

Figure 4 Effect of secretions (S) on cleavage of Glu-plasminogen visualized with AU-page. Plg was incubated with A) various amounts of secretions for 1 h, or B) with 50 µg/mL of secretions for various time intervals. C) Plg was incubated for 1 h with a mixture of 50 µg of secretions/mL pre-incubated for 24 h with the serine protease inhibitors PMSF or SPIC-1. D) The formation of Lys-plg/plasmin by secretions or tPA for 3 h and 24 h. Arrow: Glu-plasminogen.

Discussion

The main conclusion from the present study is that a serine protease present in the secretions of maggots enhances plasminogen activator-induced fibrinolysis by cleavage of plasminogen. This conclusion is based on the following observations. First, the tPA (and uPA) induced lysis of fibrin clots was enhanced by secretions when added either before or after the formation of clots from plasma; secretions could not induce lysis in the absence of these activators. Secretions exerted similar effects on clots formed with TAFI or α2-antiplasmin deficient plasma indicating that the observed enhanced fibrinolysis was not the result of inactivation of these inhibitors. Second, using a system of purified proteins, we observed secretions to enhance the tPA-induced plasminogen activation in a dose-dependent manner. Pre-incubating Plg with secretions further enhanced this process whereas pre-incubation of tPA with secretions did not lead to increased plasmin formation. Third, secretions were inactive after treatment with SDS and urea as well as after boiling

indicating that the active component is a protein with a tertiary structure essential for its biological activity. We hypothesized that protease activity in secretions[19] was responsible for cleavage of plasminogen into a derivative that is activated more easily by plasminogen activators. Using AU-page we found a serine protease within secretions to cleave Glu-plg in a dose- and time-dependent manner, although no formation of the Lys-plg[18] and/or plasmin was observed. Moreover, using the plasmin substrate assay we found secretions to enhance the tPA-induced plasmin formation from purified Lys-plg as well (data not shown). Therefore, digestion of Plg by secretions probably leads to formation of a small derivative like miniplasminogen (kringle 5 attached to the protease part)[20], which still can be activated to plasmin by plasminogen activators. In summary, a serine protease within secretions enhances the plasminogen activator-induced fibrinolysis by cleavage of Plg though the cleavage site itself is unknown and should be further investigated.

The second conclusion from this study is that maggot secretions have no effect on coagulation, at concentrations that clearly affect fibrinolysis. Therefore, the observations by Weil *et al* that the excretory substances from the larvae have a potent hemostatic effect in haemorrhage[15] may be due to other, yet unknown, causes. However, no other reports mention effects of maggots on haemorrhage as observed by Weil. In contrast, it has been reported that bleeding may occur after debridement of wounds by maggots. A possible explanation could be that the clot is broken down before the underlying tissue has healed. However, in none of the patients treated with the contained form of maggot therapy (biobags) has bleeding been observed[21]. It is unclear whether bleeding is the result of crawling of maggots[22] or of the amount of lysing components as it is likely that a large part of the maggot products stick to the biobags leading to less active molecules in the wounds.

What could be the clinical relevance of our findings? In a balanced wound healing process proteases are involved in autolytic/enzymatic degradation of the clot/provisional matrix which is essential for remodelling and repair of the tissue[23]. However, proteases, such as elastase and matrix metalloproteases (MMPs), in chronic wounds not only partially degrade clots and extracellular matrix but, due to excessive proteolytic enzyme production by immune cells[11,13,24], also damage surrounding healthy tissue. The clots no longer support re-epithelialisation and granulation tissue formation and therefore have to be removed. However, elevated levels of pro-inflammatory mediators, like TNF-α and C5a, in chronic wounds may lead to enhanced production of the fibrinolysis inhibitor PAI-1[25,26] as is reported for obese and diabetic patients[27,28]. PAI-1 binds to and inactivates uPA and tPA which results in impaired lysis of clots and fibrin cuffs[27,29]. Additionally, enhanced levels of methylglyoxal found in diabetic patients result in decreased activation of plasminogen[30]. Clearly, failure in the removal of impaired clots may promote formation of ulcers and/or (more) necrotic tissue and/or fibrin slough, which facilitates bacterial colonization and infection and consequently pro-inflammatory responses. In contrast to many ‘conventional’ therapies, maggots actively induce healing of chronic wounds. Maggots not only inhibit the

production of pro-inflammatory mediators by leucocytes[7,8] but also debride the wounds of necrotic tissue and fibrin slough. Debridement is often explained to result from enzymatic activity of the excretions/secretions of the maggots involving serine proteases, peptidases, and lipases[31-33]. However, our results showed that secretions do not degrade plasma clots in the absence of plasminogen activators. This is in contrast with the results of Chambers *et al*[16] who reported that maggot ES induced lysis of a fibrin matrix and various clot/matrix components suspended in Tris-HCL (pH 8), such as fibronectin, laminin and acid-solubilized collagen types I and III. This discrepancy was not the result of differences in collecting the maggot products (data not shown) but likely the result of differences in the composition of the clots. Chambers *et al* formed 'clots' containing fibrin only, whereas in our studies clots were formed using plasma which contains a large variety of other proteins that can be incorporated in the clot. Hence, the composition of the clot may be an important factor for the activity of the enzymes within secretions. In agreement, clots/matrices in chronic wounds obtain an altered composition and structure compared to those in acute wounds as some of their components have been degraded by proteases[13,34,35]. This altered structure/ composition may result in direct debridement of wounds by enzymes in secretions due to easier accessibility to the different clot/matrix components.

It should be noted that the relation between chronic wounds and the levels of tPA, uPA and PAI-1 are unclear. Classically, uPA is associated with cell-mediated activation of plasminogen in tissue whereas tPA is related to lysis of fibrin clots in the circulation[36]. However, both uPA and tPA are present in chronic ulcers. Elevated levels/activity of uPA, tPA, and PAI-1 have been reported, as well as no differences in and/or decreased activity[24,37,38]. Clearly, the localisation, activity and subsequent role of tPA, uPA and PAI-1 in chronic ulcers remains unclear and should be investigated in more detail. However, the observation that serine protease inhibitors, like PAI-1, prevent fibroblast migration into fibrin clots[39], as is seen in chronic wounds, suggests the absence of active molecules involved in plasmin formation. In agreement, impaired wound healing observed in uPA/tPA double-deficient and plasminogen-deficient mice results from the diminished ability of wound edge cells to migrate through the provisional and/or extracellular matrix[40,41].

In summary, a serine protease within maggot secretions enhances the plasminogen activator-induced fibrinolysis by cleavage of plasminogen making suboptimal levels of plasminogen activators likely sufficient to obtain lysis of clots as well as fibrin cuffs below the wound surface. These effects of secretions may explain their ability to effectively debride wounds which may be further enhanced by direct enzymatic activity of the secretions. In addition, maggot secretions enhance cAMP levels in leucocytes[7,8] which leads to reduced production of pro-inflammatory cytokines and PAI-1 further enhancing fibrinolysis. Taken together, these actions of secretions may prevent ongoing inflammation and tissue destruction in chronic wounds and hence contribute to the beneficial effects of maggot treatment.

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