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INVESTIGATIVE REPORT

Stability of the Cathelicidin Peptide LL-37 in a Non-healing Wound Environment

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The endogenous cathelicidin peptide LL-37 is strongly expressed at the wound edge early in the process of acute wound healing, but only weakly expressed in chronic wounds. Excessive proteolysis may limit the therapeutic usefulness of exogenous LL-37, especially in ulcers colonized with *Pseudomonas aeruginosa* that produce elastase, which degrades LL-37. This study investigated the stability of synthetic LL-37 against two types of proteinases in the presence or absence of wound fluid samples (diluted to 10–20%) from nine non-healing venous leg ulcers. Incubation of LL-37 (10 µg/ml) at 37°C for 6 h resulted in complete degradation by the serine proteinase trypsin (≥10 ng/ml), while no degradation was observed with matrix metalloproteinase-9. LL-37 susceptibility to trypsin was diminished considerably in the presence of wound fluid, and there was no apparent cleavage of exogenous LL-37 incubated in wound fluid for up to 24 h at 37°C even when using fluids from ulcers with resident *P. aeruginosa* ($n=2$). In conclusion, LL-37 was degraded by trypsin, but not by matrix metalloproteinase-9, and was fairly resistant to proteolytic cleavage *ex vivo* by incubation with wound fluid from non-healing venous leg ulcers. Thus, the proteolytic environment of chronic wounds does not seem to prevent the therapeutic use of topical LL-37. *Key words: cathelicidin; hCAP-18; LL-37; MMP-9; proteinase; trypsin; neutrophil elastase; venous leg ulcer; wound healing.*

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LL-37 is an endogenous human peptide that has attracted much attention during recent years because of its pleiotropic biological activities. Originally described as an antimicrobial peptide, LL-37 has subsequently been shown to mediate a multitude of activities on eukaryotic cells, implicating a role in innate immunity, immunomodulation, cell growth, cell survival, angiogenesis and wound healing (1, 2).

LL-37 is generated by extracellular proteolytic processing of the antimicrobial cathelicidin hCAP-18. Proteinase-3 is the predominating proteinase operating on neutrophil-derived cathelicidin, generating LL-37 (3), while in epididymis gastricin at low pH gives rise to an alternative fragment, ALL-38 (4). Additional enzymes from the kallikrein family have also been implicated in the natural processing and degradation of LL-37 in epithelia (5).

LL-37 is a potential wound-healing stimulator (6, 7) and is highly upregulated during physiological wound healing. Although chronic wound tissue and fluids contain hCAP-18, as judged from immunoblot analysis (6, 8, 9), the amount of LL-37 in chronic wounds is low or undetectable (6, 10). One possibility is that LL-37 is rapidly lost via proteolytic degradation by endogenous or bacterially-derived proteinases present in chronic wound fluid (9, 11). Under such circumstances, this may attenuate the biological effects of locally applied peptide in a chronic wound. Studies on recombinant polypeptide growth factors indicate accelerated proteolysis by chronic wound fluids (12–15). LL-37 incubated with supernatants and proteolytic enzymes from common pathogens showed loss of bioactivity (bactericidal activity) already after 30 min, and addition of selected proteinase inhibitors attenuated the proteolytic degradation (9). Although only a limited number of chronic wound fluids were investigated, in the presence of wound fluid, LL-37 was shown to be stable for 4 h unless the wound fluid was sampled from ulcers with *P. aeruginosa* (9).

We have undertaken a study in which the stability of exogenous LL-37 in wound fluids from nine non-healing venous leg ulcers was assessed *ex vivo*. In addition, the influence of prototypic serine and metalloproteinases on LL-37 degradation was studied. The results show that LL-37 is readily degraded by the serine proteinase trypsin (16), but not by matrix metalloproteinase-9 (MMP-9), and that LL-37 is surprisingly stable in an *ex vivo* chronic wound environment.

MATERIALS AND METHODS

Patients

Wound fluids were isolated from patients who had ankle/brachial index > 0.8, and one or two venous leg ulcers older than 3 months, larger than 3 cm^2 and with lack of healing in the past 3 months, and had given their written consent. Exclusion criteria were: diabetes, systemic corticoid treatment within the last 3 months, chemotherapy within the last 3 months, cellulitis, osteomyelitis or other infections in the vicinity of the ulcer, necrotic ulcer, intermittent claudication, pregnancy or breastfeeding.

The samples were obtained at the Copenhagen Wound Healing Center at Bispebjerg Hospital in Copenhagen, Denmark,

which is a multidisciplinary expert national referral wound healing centre (17). The local ethics committee approved the study (H-KF-311969).

Wound fluid sampling

The ulcers were cleansed with sterile saline, and a charcoal cotton swab was taken from the central ulcer surface for bacteriological examination, as described earlier (18). Hydrophobic polyurethane foam was applied to the ulcer and covered with an occlusive polyurethane adhesive dressing (Tegaderm®, 3M, St Paul, MN, USA) and compression bandage (19). After 24 h, the accumulated wound fluid was sterile-filtered (0.20 µm) and stored at –80°C until analysed for MMP-9 and neutrophil elastase activities, or used in the LL-37 stability tests.

Protein concentrations of wound fluids (18–67 mg/ml) were determined according to a modified Bradford assay (20).

In vitro *stability assay*

LL-37 (> 95%, 4492 Da) was produced by PolyPeptide Laboratories (Limhamn, Sweden). LL-37 (10 µg/ml, final concentration) in phosphate-buffered saline (PBS) was incubated alone, or in the presence of 10 µl (10%, v/v) or 20 µl (20%) chronic wound fluid in a total volume of 100 µl in Nunc-Immuno™ Minisorp polyethylene tubes (466982; Nunc, Roskilde, Denmark). In some conditions, samples were supplemented with trypsin from porcine pancreas (93614; Sigma-Aldrich, St Louis, MO, USA) at 1, 10 or 100 ng/ml or with APMA-activated recombinant mouse MMP-9 (21) at 0.05, 0.5, 5 and 50 nM. Trypsin (100 ng/ml) was also added to subsets of tubes containing synthetic LL-37 and 10% or 20% chronic wound fluid. After incubation for 0 (baseline), 3 and 6 h at 37°C, a proteinase inhibitor cocktail (Complete® EDTA-free, 11836170; Roche Applied Science, Mannheim, Germany) was added to the samples, which were then immediately frozen at –80°C. One additional set of tubes with three chronic wound fluids (numbers 5, 8 and 9) was incubated for 24 h at 37°C without or with the proteinase inhibitor cocktail supplemented with the metalloproteinase inhibitor 1,10-phenanthroline (2 mM) from the start.

Western blot analysis

The samples were electrophoresed in 16.5% Tris-Tricine gels (Bio-Rad Laboratories, Hercules, CA, USA), and then electrically transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories) according to standard protocols. The membranes were blocked with 4% non-fat milk proteins and incubated overnight with primary affinity-purified rabbit anti-hCAP-18 antibodies (Agrisera, Umeå, Sweden) at 1:1,000 dilution. Horseradish peroxidase-conjugated secondary goat anti-rabbit antibodies (Cell Signaling, Stockholm, Sweden) were used at 1:2,000 dilution and incubated with membrane for 2 h at room temperature. Bound antibodies were detected by enhanced chemoluminescence (ECL) (ECL Plus; GE Healthcare, Uppsala, Sweden). A charge-coupled device (CCD) camera captured ECL signals, and the intensity of the bands was determined with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Gelatin zymography

Latent and active forms of MMP-9 were determined using 0.5 mg/ml gelatin as a substrate in a 7.5% sodium dodecyl sulphate-polyacrylamide gel (19). Five µl of samples, appropriately diluted in saline, were mixed with 5 µl of 2 × sample buffer (Invitrogen, Carlsbad, CA, USA) and applied to each lane. Samples were electrophoresed at constant 125 V using the XCell SureLock™ Mini-Cell (Invitrogen) for 90 min. Gels were renatured for 30 min at ambient temperature and then incubated for 18 h at 37°C in developing buffer (Invitrogen). Gels were stained with EZBlue™, destained and assembled using the DryEase® Mini-Gel Drying System (Invitrogen).

Effect of the wound fluid collection material on neutrophil elastase activity

The effect of the polyurethane material on human neutrophil elastase activity was studied *in vitro*. The foam (1.5 cm^2) was saturated with 200 µl of a solution containing purified human neutrophil elastase (30 mU/ml) (Enzo Life Sciences, Plymouth Meeting, PA, USA) in assay buffer (100 mM Hepes, pH 7.25, 500 mM NaCl and 0.05% Tween-20) supplemented with 0.5% bovine serum albumin and incubated for 1 h at 37°C. In parallel, the neutrophil elastase solution was incubated in the absence of the foam.

Neutrophil elastase activity assay

Neutrophil elastase activity was determined in 10 µl neutrophil elastase solution samples, 1 µl wound fluid samples, and purified human neutrophil elastase (0.22 mU) using the chromogenic substrate methoxysuccinyl-ala-ala-pro-val p-nitroanilide (100 µM) in a total reaction volume of 100 µl, essentially as described by Rao et al. (22). $OD_{405\ nm}$ was read at 30 s intervals for 5 min at 37°C by a microplate reader (Benchmark; Bio-Rad Laboratories) and the reaction velocity in mU/min determined by the software Microplate Manager® 4.0 (Bio-Rad Laboratories).

RESULTS

Proteinase susceptibility of LL-37

Because serine proteinase activity is present in chronic wounds (13, 16, 22), we wanted to test the susceptibility of LL-37 to the serine proteinase trypsin in buffer solution. Addition of trypsin to LL-37 caused a dose-dependent decrease in the amount of intact LL-37 as detected by Western blot analysis. Incubation of LL-37 at 37°C for 3 h with 10 ng/ml and 100 ng/ml trypsin resulted in almost complete disappearance, whereas with 1 ng/ml trypsin approximately 50% of the initial amount of LL-37 remained after 3 h of incubation (Fig. 1A). Prolonged incubation with 1 ng/ml trypsin fragmented LL-37 almost entirely and only traces of LL-37 remained after 6 h. The presence of the proteinase inhibitor cocktail blocked LL-37 degradation (Fig. 1B).

MMP-9 is a zinc-dependant proteinase that is present in wound tissue and fluid (19, 23, 24). The MMP-9 level in wound fluid from non-healing venous leg ulcers has been estimated to be 50 nM (24). Therefore, we also tested the effect of MMP-9 on LL-37 integrity. The amount of LL-37 was unaffected by the addition of 50 nM MMP-9 (Fig. 1C).

In conclusion, LL-37 is susceptible to degradation by trypsin, but proteolytically resistant to MMP-9.

Characterization of samples of chronic wound fluids used for the study on LL-37 stability

We next wanted to investigate the influence of proteinases and natural proteinase inhibitors on the degradation of LL-37 in a chronic wound environment. This was

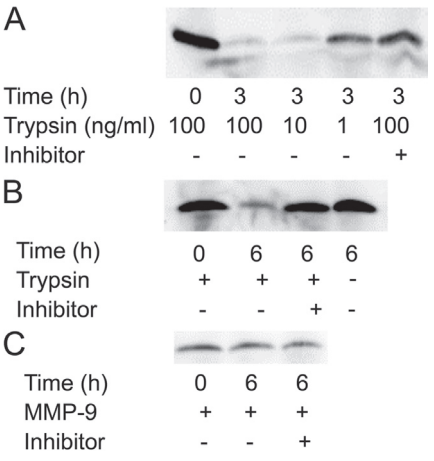

Fig. 1. LL-37 is degraded by trypsin but not by matrix metalloproteinase-9 (MMP-9). LL-37 (10 µg/ml) was incubated at 37°C in the presence of 1, 10 or 100 ng/ml trypsin for 3 h (A), with 1 ng/ml trypsin for 6 h (B), or with 50 nM MMP-9 for 6 h (C) in the absence or presence of a proteinase inhibitor cocktail. Incubations were terminated by addition of the proteinase inhibitor cocktail and freezing at –80°C. Sample volumes corresponding to 26 ng (A) and 12.5 ng (B and C) of added LL-37 were analysed by gel electrophoresis and immunoblotting with a polyclonal anti-LL-37 antibody.

mimicked by wound fluids collected from non-healing venous leg ulcers ($n = 9$) in eight patients (4 women and 4 men, aged 67–88 years (mean ± SEM, 81 ± 2 years)). Five ulcers were located on the medial right leg, three on the lateral left and one on the anterior right leg. The ulcers were 5–636 months old (94 ± 78 months), with a surface area of 7–104 cm^2 (35 ± 11 cm^2). Five ulcers increased and four decreased in size with no overall ulcer area change over the 4-week period. Four of the ulcers (numbers 1, 4, 5 and 8) were colonized with *P. aeruginosa* at the first sampling. Two (numbers 1 and 8) of these four remained positive for *P. aeruginosa* at termination week 4. There were no apparent differences in the percentage change of ulcer areas over 4 weeks between *P. aeruginosa*-positive and *P. aeruginosa*-negative venous ulcers.

The wound fluids contained 913 ± 130 nM hCAP-18, as determined by an enzyme-linked immunosorbent assay (ELISA) (Hycult Biotechnology, Uden, The Netherlands). When analysed by immunoblotting, there was no detectable staining of LL-37 (Fig. 2).

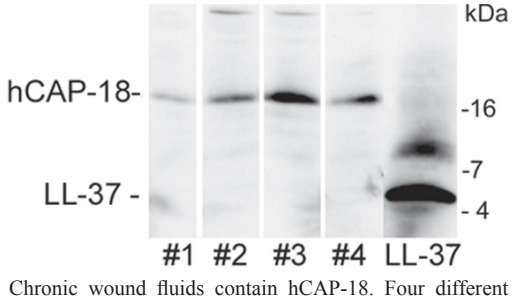

Fig. 2. Chronic wound fluids contain hCAP-18. Four different wound fluids (0.26 µl) (numbers 1–4) were analysed by gel electrophoresis and immunoblotting using a polyclonal anti-LL-37 antibody. LL-37 (75 ng) and molecular weight markers were used as reference.

Proteolytic activity of the wound fluids was assessed by gelatin zymography (Fig. 3). The activity of the wound fluids showed large inter-patient variations, but was elevated compared with serum. Serum is representative of the factors released locally at the initiation of wound healing and serves as baseline. Five times more of the serum than the wound fluid samples on a total protein basis was loaded, to enable detection of MMP-9 in serum (S in Fig. 3A). We could also demonstrate the anticipated activity of the recombinant MMP-9 used above (rM-9 in Fig. 3A). The gelatinolytic bands were abolished when incubated in the presence of metalloproteinase inhibitor, except for one ulcer (cf. 6^0 in Fig. 3A and Fig. 3B). The origin of this non-metal dependent proteinase is unknown. It has been shown previously that LL-37 is susceptible to degradation by the endogenous serine proteinase human neutrophil elastase (25). Neutrophil elastase activity was detected in nine and undetectable in three (numbers 2 and 3) of the 12 analysed wound fluids, and was 18.7 ± 5.4 mU/mg ($n = 12$). In comparison, Rao et al. (22) determined the neutrophil elastase activity of eight wound fluids obtained from venous leg ulcers to 4.5 ± 0.8 mU/mg.

Stability of LL-37 in chronic wound fluids with or without added trypsin

LL-37 (10 µg/ml, final concentration) was incubated for 3–24 h at 37°C in 10–20% (v/v) wound fluid. Western blot data for nine wound fluids incubated for 6 h are shown in Fig. 4A and image analysis of the Western blot bands for all 12 fluids revealed no significant ($p = 0.36$, paired *t*-test) difference in the relative amount of LL-37 after 6 h (Fig. 4B). Prolonged incubation of three wound fluids for 24 h, with or without proteinase inhibitors present from start of incubation, indicated no appreciable further degradation of exogenous LL-37 (Fig. 4C). One concern was that the material used to collect wound fluid sequestered endogenous neutrophil elastase (26, 27). This is unlikely, because negligible loss of purified neutrophil elastase activity (30 mU/ml) was observed when incubated for 1 h at 37°C in the presence of the polyurethane foam material (27.2 mU/ml) compared with the absence of foam (28.9 mU/ml).

We also added trypsin (100 ng/ml) to two wound fluids. Trypsin was ineffective under these experimental conditions, in sharp contrast to the digestive effect of trypsin in the absence of wound fluids (Fig. 5). Although *P. aeruginosa* can produce an elastase that degrades LL-37 (9), wound fluids obtained from the *P. aeruginosa*-positive ulcer (number 8) did not deviate from the general pattern and produced no significant proteolysis of LL-37. In contrast, the endogenous hCAP-18 band decreased slightly after 6 h in chronic wound fluid with trypsin added. The presence of the proteinase inhibitor cocktail blocked this decrease in hCAP-18.

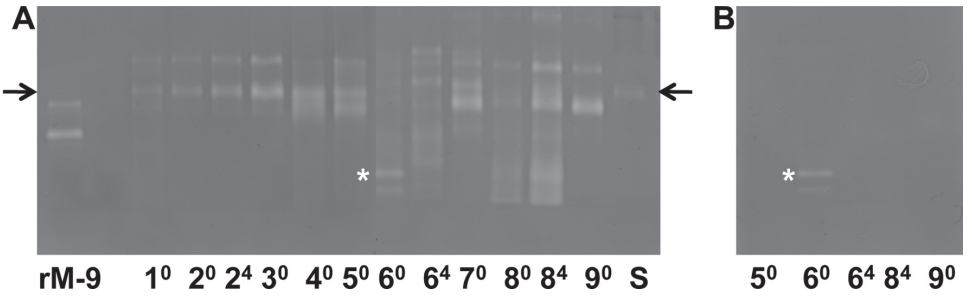

Fig. 3. Enzymatic activity assessed by gelatin zymography of the 12 wound fluids from the nine venous ulcers (1–9) used to test LL-37 stability. The amount of wound fluids loaded was normalized to 250 ng and serum (S) to 1250 ng total proteins. The superscript 0 refers to the start week 0 and the superscript 4 to the last wound fluid sampling week 4. A. rM-9, recombinant and activated matrix metalloproteinase-9 (MMP-9) (250 fmol) loaded in the same proportions as used in the stability assay. Arrows indicate the position of the latent form of MMP-9. B. Gel incubated in the presence of a zinc chelator (2 mM 1,10-phenanthroline). Asterisk in (A) and (B) indicates gelatinolytic band that was not blocked by the zinc chelator.

Taken together, exogenous synthetic LL-37 remained detectable and stable after 24 h of incubation at 37°C in 20% chronic wound fluid irrespective of *P. aeruginosa* colonization.

These data suggest that LL-37 is relatively resistant to degradation in chronic wound fluid, possibly because of endogenous proteinase inhibitors that prevent the action of endogenous as well as added proteinases.

DISCUSSION

This study investigated the fate of synthetic LL-37 peptide in wound fluid collected from non-healing chronic venous leg ulcers. The study showed that most of the 1 μg added peptide remained intact after 24 h of incubation in 20% wound fluid. LL-37 was readily degraded in buffer alone by trypsin, but not by MMP-9. The presence of wound fluid inhibited the effect of trypsin. These results clearly show that the wound fluid environment is not rapidly destroying LL-37, and even if wound fluid contains proteolytic enzymes it also contains proteinase inhibitors that apparently antagonize excessive peptide degradation. We also demonstrated that the wound fluids used possessed MMP-9 and neutrophil elastase of expected activities (19, 22–24).

The present study extends a previous report investigating LL-37 stability in wound fluid. Schmidtchen et al. (9) showed that 1 μg LL-37 was stable for 4 h in acute wound fluid (7% concentration), but was completely degraded in chronic wound fluid positive for elastase-producing *P. aeruginosa* (9). We tested 12 wound fluids from nine venous leg ulcers and found that no, or only negligible, degradation of synthetic LL-37 occurred after incubation for 24 h with up to 20% wound fluid, whether or not the ulcers were colonized with *P. aeruginosa*. However, neither the degree of colonization nor the quantity of *P. aeruginosa* elastase was measured. It has been reported that different strains of *P. aueruginosa* isolated from leg ulcers produce highly variable levels of elastase (28) detected by gelatin zymography, with the major band corresponding to 150

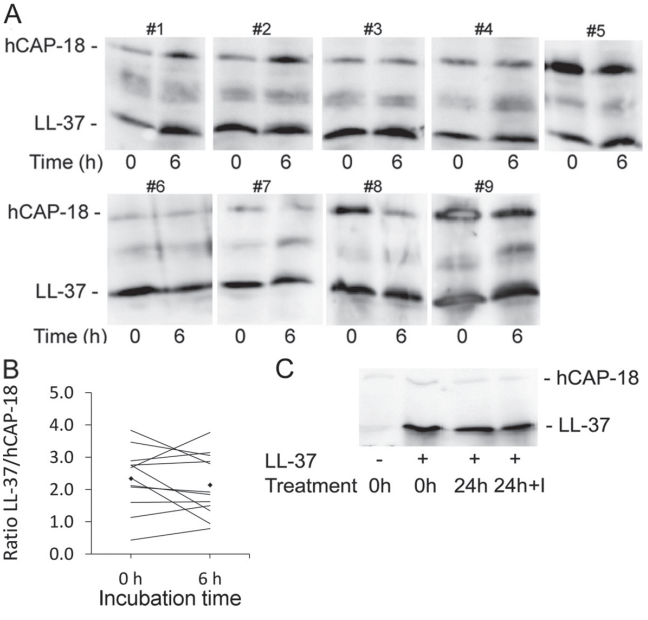

Fig. 4. LL-37 is resistant to degradation in the presence of wound fluid as visualized by Western blotting using a polyclonal anti-LL-37 antibody. (A) Western blot data for LL-37 (10 μg/ml) incubated in 20% (v/v) wound fluid from nine ulcers for 6 h. (B) Western blot image analysis data for 12 wound fluids expressed as a ratio between LL-37 and hCAP-18. Diamonds indicate mean values. (C) LL-37 (10 μg/ml) incubated in 20% wound fluid from one ulcer with resident *P. aeruginosa* for 24 h in the presence and absence of proteinase inhibitors (I).

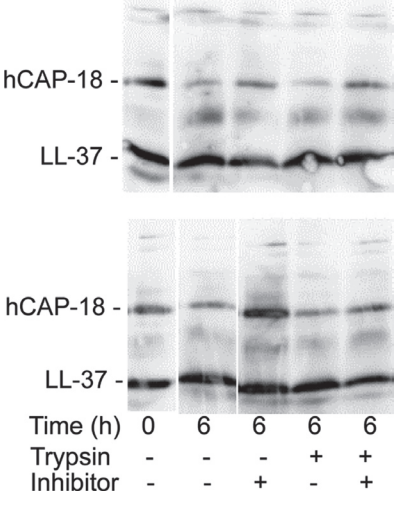

Fig. 5. LL-37 is resistant to degradation by trypsin in the presence of wound fluid as visualized by Western blotting using a polyclonal anti-LL-37 antibody. LL-37 (10 μg/ml) incubated in 10% (v/v) wound fluid from one *P. aeruginosa*-positive ulcer (*upper panel*) or one *P. aeruginosa*-negative ulcer (*lower panel*) for 6 h with trypsin (100 ng/ml) and proteinase inhibitors added as indicated before the start of incubation.

kDa (9, 28). This band presumably represents multimers of *P. aeruginosa* elastase (28). On the other hand, locally applied LL-37 was bioactive in experimental wounds inoculated with *P. aeruginosa* (29).

In conclusion, our results indicate that LL-37 is relatively stable in a hostile chronic wound environment for several hours *ex vivo*. Furthermore, these findings imply that LL-37 deficiency in venous leg ulcers is due to decreased synthesis of hCAP-18/LL-37 rather than increased proteolysis.

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