Wound healing activity of the human antimicrobial peptide LL37

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A R T I C L E   I N F O

Article history:
Received 24 May 2011
Received in revised form 1 June 2011
Accepted 3 June 2011
Available online 13 June 2011

Keywords:
Antimicrobial peptide LL37
Angiogenesis
Wound healing

A B S T R A C T

Antimicrobial peptides (AMPs) are part of the innate immune system and are generally defined as cationic, amphipathic peptides, with less than 50 amino acids, including multiple arginine and lysine residues. The human cathelicidin antimicrobial peptide LL37 can be found at different concentrations in many different cells, tissues and body fluids and has a broad spectrum of antimicrobial and immunomodulatory activities. The healing of wound is a complex process that involves different steps: hemostasis, inflammation, remodeling/granulation tissue formation and re-epithelialization. Inflammation and angiogenesis are two fundamental physiological conditions implicated in this process. We have recently developed a new method for the expression and purification of recombinant LL37. In this work, we show that the recombinant peptide P-LL37 with a N-terminus proline preserves its immunophysiological properties in vitro and in vivo. P-LL37 neutralized the activation of macrophages by lipopolysaccharide (LPS). Besides, the peptide induced proliferation, migration and tubule-like structures formation by endothelial cells. Wound healing experiments were performed in dexamethasone-treated mice to study the effect of LL37 on angiogenesis and wound regeneration. The topical application of synthetic and recombinant LL37 increased vascularization and re-epithelialization. Taken together, these results clearly demonstrate that LL37 may have a key role in wound regeneration through vascularization.

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1. Introduction

Cationic antimicrobial peptides (AMPs) represent the first line of defense against many invading pathogens. These small amphipathic peptides are part of the innate immune system and have a broad spectrum activity against bacteria, fungi [1] and viruses [17].

However, direct antimicrobial action is not the only, and probably not even the most important task of mammalian AMPs. In fact, these peptides present low antimicrobial activities under serum and tissue conditions [16,17]. Nevertheless, they have affinity for lipopolysaccharide (LPS) and can prevent from lethal endotoxemia by suppressing cytokine production [15]. Moreover, these AMPs appear to be involved in the orchestration of many aspects of innate immunity and the inflammatory response [17].

In mammals, at least two distinct groups of AMPs are found. Defensins are the more representatives and cathelicidins form the second group of mammalian AMPs. Cathelicidins share a highly conserved N-terminal cathelin domain, flanked by a rather variable antimicrobial peptide on the C-terminus [32]. The hCAP-18/LL37 is the only known human cathelicidin. Upon stimulation, hCAP-18 is cleaved extracellularly by proteinase 3 to generate the peptide LL37 [36]. The antimicrobial peptide is referred to as LL37, since it has a 37 amino acids sequence starting with two leucines. It is a 4.5 kDa, cationic (+6), amphipathic α-helical peptide, with a broad spectrum of antimicrobial activity. LL37 and its precursor hCAP18, were first described in leucocytes and testis, but were soon found in a large variety of cells, tissues and body fluids, as summarized by Durr et al. [11]. Upon injury or infection, there is a strong upregulation of hCAP18/LL37, indicating that LL37 assists the immune system. The downregulation of LL37 has been associated with several diseases: a deficiency of LL37 in the skin of patients with atopic dermatitis [29] or chronic ulcers [18] may account for the increased risk for skin infections. Nizet et al. [28] showed that mice with disrupted Cnlp, the gene coding for cathelin-related antimicrobial peptide (CRAMP), showed increased susceptibility to skin infections.

Besides its antimicrobial properties LL37 plays a central role in innate immune responses and inflammation. It has been identified as a potent chemoattractant for mast cells [27], monocytes, T lymphocytes and neutrophils [42] through formyl peptide receptor-like 1 (FPRL1). The fact that LL37 can attract leukocytes contributes to host defense against infections. LL37 also promotes
wound healing [18], angiogenesis and arteriogenesis [20] and acts as immune adjuvant [21].

Acute wounds normally heal in a very orderly and efficient manner characterized by four distinct, but overlapping phases: hemostasis, inflammation, proliferation and remodeling. All these phases are strictly connected and play a key role for a complete and proper restoration of the injured tissue [10]. The inflammatory phase is initiated immediately upon injury. Several inflammatory cells migrate to the wound site to fight infection and remove debris. The proliferative phase is characterized by the formation of granulation tissue, and angiogenesis. Then, the new collagen matrix becomes cross-linked and organized during the final remodeling phase. Numerous cell-signaling events are required to generate this efficient and highly controlled repair process.

In this work, we demonstrate that P-LL37 produced by recombinant techniques in our laboratory [33] preserves its immunophysiological properties and promotes wound healing through re-epithelialization and vascularization in dexamethasone-treated mice.

2. Materials and methods

2.1. Animals

Animal experiments were conducted according to accepted standards of humane animal care (Declaration of Helsinki, European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals).

2.2. Reagents

All chemicals, media and reagents were purchased from Sigma–Aldrich except stated otherwise. Synthetic LL37 (LLGDF-RFRKSKEGKFKRIVQRJKDFRLNLVPRTES) was purchased from Bachem, Switzerland. Recombinant P-LL37 (PLLGDFRKRKSKEGKFKRIVQRJKDFRLNLVPRTES) was expressed and purified as described previously [33]. Briefly, the DNA encoding the fusion protein LK-CBM3-LL37 was successfully cloned in pET21a and expressed in Escherichia coli BL21 DE3 (Novagen) in Luria-Brøth (LB) medium. The fusion protein was purified on cellulose CF11 and formic acid was applied to cleave LL37 from the fusion carrier (LK-CBM3). The resulting recombinant P-LL37 was finally purified by reverse-phase HPLC.

2.3. Culture of murine bone marrow-derived macrophages (BMDM); suppression of TNF production by LPS-activated macrophages

Macrophages were obtained from mouse bone marrow as follows: mice were sacrificed and femurs and tibias removed under aseptic conditions. Bones were flushed with Hanks’ balanced salt solution. The resulting cell suspension was centrifuged at 500 × g and resuspended in RPMI 1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS), 60 µg/ml penicillin/streptomycin, 0.005% β-mercaptoethanol (complete RPMI [cRPMI]), and 10% L929 cell conditioned medium. To remove fibroblasts or differentiated macrophages, cells were cultured on cell culture dishes (Sarstedt, Canada), overnight at 37 °C in a 5% CO₂ atmosphere. Then, nonadherent cells were collected with warm cRPMI, centrifuged at 500 × g, distributed in 96-well plates (Sarstedt, Canada) at a density of 1 × 10⁵ cells/well, and incubated at 37 °C in a 5% CO₂ atmosphere. Four days after seeding, 10% of L929 cell conditioned medium was added, and the medium was renewed on the seventh day. After 10 days in culture, cells were completely differentiated into macrophages. This method allows for the differentiation of a homogenous primary culture of macrophages that retain the morphological, physiological and surface markers characteristics of these phagocytic cells [26,39,43]. LPS was then incubated with the macrophages culture. At the same time, synthetic LL37 and P-LL37 were added at different concentrations. After 24 h, the supernatants were removed and tested for TNF-α production by ELISA (eBioscience).

2.4. Cell culture experiments

Human microvascular endothelial cells (HMECs) and human umbilical vein endothelial cells (HUVECs) were used in the experiments. HMECs were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 4.76 g/l of HEPES, 1 ml/l of epidermal growth factor (EGF) and 1 mg/l of hydrocortisone. HUVECs were cultured in medium 199 (M199) supplemented with 0.1 mg/ml of heparin, 20% FBS, 1% penicillin/streptomycin and 2 µl/ml endothelial cell growth supplement (ECGS). Rat bone-marrow mesenchymal stem cells (MSCs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells lines were maintained at 37 °C in a humidified 5% CO₂ atmosphere.

2.5. MTS toxicity assay

HMECs (2 × 10⁵ cells/ml) were grown until 70–90% confluence and incubated with different concentrations of synthetic and recombinant LL37 for 24 h in 96-well plates. Toxicity was confirmed using 10% DMSO. Cells were washed twice with PBS and their viability was assessed using Cell Titer 96®Aqueous ONE Solution Reagent (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium] colorimetric assay (Promega, Madison, USA), according to the instructions provided by the manufacturer. Optical density was measured at 492 nm. Results are expressed as percentage of control, which was considered to be 100.

2.6. BrdU proliferation assay

HMECs and HUVECs (6 × 10⁴ cells/ml) were cultured in 24-well plates to adhere overnight. The cells were then incubated for 48 h with synthetic and recombinant LL37, in the presence of 5-bromodeoxyuridine (BrdU) solution at a final concentration of 0.01 mM. Proliferation was quantified using In-Situ Detection Kit (BD Biosciences Pharmigen, USA), according to manufacturer’s instructions. The results are given as mean ± SEM and are expressed as percentage of control, which was considered to be 100.

2.7. Migration analysis

Migration assays were performed in transwell BD-matrigel basement membrane matrix inserts (BD-Biosciences, Belgium). The chemotactic capacity of LL37 was evaluated by counting the cells that migrated through the membranes. Transwell inserts containing an 8 µm pore-size PET membrane coated with a uniform layer of matrigel basement membrane were used. HUVECs (5 × 10⁵ cells/ml) were seeded on inserts in serum-free medium, and placed on wells containing medium complemented with FBS 2% and recombinant or synthetic LL37 at a concentration of 5 µg/ml. For MSCs (5 × 10⁵ cells/ml), the inserts were placed on wells containing DMEM with BSA 0.1% and recombinant LL37 at a concentration of 5 µg/ml. After incubation for 24 h membranes were removed from inserts, stained with DAPI–methyl for 5 min and visualized under a fluorescence microscope. The invading cells
of each membrane were counted and the results are expressed as percentage of control, which was considered to be 100%.

2.8. Angiogenesis

The ability of LL37 to promote the formation of capillary-like structures was examined in Matrigel assay. HMECs and HUVECs (3 × 10⁴ cells/well) were incubated with 5 µg/ml of recombinant and synthetic LL37 for 24 h on matrigel-coated 96-well plates. The capillary-like structures in each well were then counted and results are expressed as percentage of control, which was considered to be 100%.

2.9. Wound healing assay

C57BL/6 mice (purchased at Charles River, Wilmington, MA) with 8–12 weeks old were used in this study. After general anesthesia, hair on the dorsal side of the animals was shaved and the skin was cleaned with 70% ethanol. A 5 mm skin biopsy punch was used to create full thickness cutaneous wounds under aseptic conditions. Two wounds were created on the dorsal surface with one on each side of the midline. LL37 (recombinant or synthetic) and sterile water were applied topically twice a day (2 × 10 µg in 50 µl). Wounds were left open and not covered by any type of dressing. To impair healing, mice were treated daily with dexamethasone (0.25 mg/kg body weight, intramuscularly). Mice were examined daily for wound-healing progression. After 7 days, the animals were euthanized and wound tissue was collected for histological and immunohistochemistry studies. Skin wound tissue specimens were fixed in 10% neutral-buffered formalin solution and paraffin-embedded. Histological analyzes were performed in 5-µm tissue sections.

2.10. Immunohistochemistry

Microvesseldensity (MVD) was evaluated in each formalin-fixed paraffin-embedded wounded tissue section by immunohistochemistry. Tissue slides were incubated overnight 8 h with an anti-CD31 antibody. Briefly, six areas with the highest visible blood vessel density (marked by the vessel marker) per section were selected, and the number of blood vessels was counted per high-power field.

3. Results

3.1. Recombinant P-LL37 neutralizes the activation of macrophages by LPS

Murine macrophages are known to induce several cytokines, including tumor necrosis factor-α (TNF-α), upon LPS activation. LPS was added to macrophage cultures to a concentration of 10 ng/ml, either alone or in the presence of 20 µg/ml of recombinant P-LL37 or synthetic LL37. Cultures were then incubated for 24 h. The absence of LPS in the recombinant protein was confirmed using the limulus amoebocyte lysate test (data not shown). As expected, the recombinant P-LL37, as well as the synthetic peptide, neutralized the LPS-mediated activation of macrophages (Fig. 1).

3.2. Toxicity

In order to evaluate the cytotoxicity of recombinant P-LL37, HMECs were cultured for 24 h in the presence of the peptide and the cell viability was evaluated by MTS. DMSO 10% was used as cytotoxic agent, a positive control. As shown in Fig. 2, P-LL37 did not affect viability of HMECs at any of the concentrations used. Actually, with 50 and 500 ng/ml of P-LL37, a slight increase of viability was observed, indicating that the recombinant peptide P-LL37 might have promoted proliferation, as previously shown by Koczulla et al. [20].

3.3. P-LL37 promotes proliferation, migration and tubule-like structures formation by endothelial cells

Proliferation of HMECs and HUVECs was evaluated using the BrdU incorporation assay. Synthetic LL37 (5 µg/ml) was used as a positive control and 100 µM of deoxycholic acid (DCA), an antagonist of FPRL-1, as negative control. DCA attenuates the activation of FPRL-1, by binding to the cell membrane and interfering with the access of the receptor to its agonists [23]. Fig. 3 demonstrates that P-LL37 promoted proliferation with concentrations as low as 50 ng/ml in HMECs (a) and the effect at 5 µg/ml was very similar to the one obtained using the synthetic LL37. The proliferation of HUVECs (b) was inferior but once again there were no significant differences between synthetic and recombinant peptides.

Next, the chemotactic and angiogenic activities of P-LL37 were evaluated. Fig. 4 illustrates the invasion assays performed with HUVECs (a) and MSCs (b). Again, P-LL37 was able to increase cell invasiveness capacity both in HUVECs and in MSCs. The capacity of LL37 to promote the formation of capillary-like structures was assessed with HUVECs and HMECs using matrigel as three-dimensional scaffold (Fig. 5). The use of LL37 had a strong effect in angiogenesis. In fact, it almost doubled the number of capillaries formed by HUVECs and HMECs.

3.4. Wound healing assays

To further examine the effects and biological activities of synthetic and recombinant LL37, wound healing experiments in dexamethasone-treated mice were performed. The peptides or vehicle were topically applied daily for 7 days in the 5 mm skin wounds. Dexamethasone was used to impair healing. Fig. 6 illus-
Fig. 3. Effects of synthetic and recombinant LL37 in HMECs (a) and HUVECs (b) proliferation assessed by BrdU assay. P-LL37 and synthetic LL37 promoted similar proliferation of endothelial cells. Three independent experiments were performed in duplicate. *P ≤ 0.05 and **P ≤ 0.01 vs. control.

Fig. 4. Invading cells relative to the initial amount of HUVECs (a) and MSCs (b) cultured using a double-chamber assay. Results are means ± SEM of three independent experiments performed in duplicate and expressed relatively to control. *P ≤ 0.05 and **P ≤ 0.01 vs. control.

Fig. 5. Recombinant and synthetic LL37 promote angiogenesis in vitro. (Left) Capillary-like structures formation of HMECs, after treatment with 5 μg/ml of LL37, visualized under a phase-contrast microscope. Figures are representative of the whole cultures. (Right) Quantification of capillary-like structures assembly. Results are means ± SEM of three independent experiments performed in triplicate and expressed relatively to control. *P ≤ 0.05 and **P ≤ 0.01 vs. control.
The human antimicrobial peptide LL37 was initially recognized for its antimicrobial properties. In fact, it has a broad spectrum of antimicrobial activity against bacteria, fungi, and viral pathogens, as summarized by Dunn et al. [11]. Several studies reported that AMPs lose their antimicrobial activity in elevated salt concentrations and under serum conditions [2,13,24]. This inhibition is less pronounced in the case of LL37, probably due to its α-helical structure [30]; however, the peptide becomes inactive against certain organisms—susceptible under low salt conditions—in media with 100 mM NaCl [38]. Furthermore, LL37 had no killing activity against Staphylococcus aureus or Salmonella typhimurium in the presence of tissue-culture medium [5]. Therefore, the relevance of antimicrobial activity in vivo is not clear. However, AMPs are imperative in innate immunity, since animals lacking gene expression of these peptides were more susceptible to infections [28]. Besides its antimicrobial properties, many other activities have been reported for the human peptide LL37. As already stated, LL37 is involved in many aspects of innate immunity, like chemotaxis, angiogenesis or wound healing. Although not completely understood, these activities have been associated to some cells receptors like FPRL-1, P2X7 and epidermal growth factor receptor (EGFR) [12,19,37]. Among these, LL37 only binds directly to FPRL-1 [42], inducing cellular signaling and Ca²⁺ flux [20]. So, we have tested the effects of P-LL37 in proliferation, migration and angiogenesis of endothelial cells to ascertain whether the recombinant peptide binds the receptor FPRL-1. The results confirmed that P-LL37 promotes proliferation, migration and tube-like structures formation by endothelial cells. The effect of P-LL37 in the migration of HUVECs and MSCs is significant, with an increase of almost 100% invading cells being observed in both cases. In the experiments with HUVECs, synthetic LL37 promoted a very strong increase in invading capacity, much superior than the one obtained with P-LL37. This result was surprising, since no significant differences were found in the proliferation assays. P-LL37 is obtained by recombinant techniques and the batch used in this experiment could not be as pure as expected, misleading the concentration quantification. A smaller concentration of the recombinant peptide might have been used, which would explain this result. In fact, as illustrated in Fig. 5, the formation of capillary structures was very similar using both peptides. The invasion assay with MSCs was only performed with control and recombinant LL37. Coffelt et al. [8] reported that LL37 was able to chemoattract MSCs through the activation of FPRL-1. Considering all the results obtained, we clearly demonstrate that the recombinant LL37 produced in our laboratory is fully active in vitro. The N-terminus proline, result of the formic acid hydrolysis, does not interfere with the binding of LL37–LPS and to the receptor FPRL-1. Glucocorticoids affect almost every phase of wound healing because of their inhibitory effect on gene expression in various cells [40]. Beer et al. [3] showed that dexamethasone inhibits recruitment of various inflammatory cells and gene expression of many key wound healing cytokines and growth factors. During the proliferative and remodeling phases, dexamethasone can inhibit the synthesis of several dermal extracellular matrix (ECM) proteins, and delay re-epithelialization and fibroplasia. The healing of a skin wound is a complex process requiring the collaborative efforts of many different tissues and cell lineages and involves the formation of new extracellular matrix, cell infiltration, and tissue remodeling. Inflammation and angiogenesis are two fundamental physiological conditions implicated in this process. In

4. Discussion

A variety of endotoxins released by the outer membrane of Gram-negative bacteria, namely the lipopolysaccharide (LPS)–protein complex, play an important role in pathogenesis of many exogenous respiratory diseases. The massive release of LPS can lead in extreme cases to endotoxic shock and therefore, death [14]. Many antibiotics release endotoxin by disrupting the cell wall [9,31], and patients cured of bacterial infections can suffer from endotoxic shock. The activation of macrophages is started when LPS binds the LPS-binding protein (LBP). In turn, LBP binds the macrophage surface protein CD14, the primary receptor of LPS, and is 100-fold more potent than LPS alone [34]. The antimicrobial activity of the peptide LL37 results from the disruption of the bacteria cell wall; however, LL37 can also neutralize LPS activity by forming high affinity complexes [22,35].

The TNF-α production was very low in the presence of either the recombinant or the synthetic peptides, indicating that recombinant P-LL37 has affinity for LPS. The recombinant peptide only differs from the synthetic in one amino acid, a neutral proline resulting from the formic acid hydrolysis [33]. Therefore, the global net charge (+5) remains unaltered. Since electrostatic and hydrophobic interactions are the predominant forces driving the binding between antimicrobial peptides and LPS, these results were expected. However, Rosenfeld et al. [34] described that a strong binding of AMPs–LPS might not be sufficient to neutralize LPS-induced macrophage activation. In fact, those authors showed that the AMP magainin, although binding LPS with high affinity, did not reduce the production of TNF-α. Some AMPs, including LL37, can compete with LPS on its binding site within the CD14 receptor, modulating the TLR signaling pathway involving NFκB [25]. Therefore, the inhibition of TNF-α production by LL37 is not only caused by the interaction with LPS. Overall, these results demonstrate that the recombinant peptide P-LL37 is biologically active.
Control

s-LL37

P-LL37

Fig. 7. Hematoxylin and eosin stained micrographs of wound tissue sections treated with control, synthetic and recombinant LL37. The rectangle identifies an area further investigated in a higher magnification picture. At day seven, re-epithelialization is almost complete in wound treated with LL37 and a high number of blood vessels are present (pointed by arrows).

In fact, the formation of new blood vessels is a prerequisite of tissue repair and wound healing [6]. As already stated and confirmed in this work, LL37 promotes angiogenesis. Moreover, LL37 appears to have a very important role in wound healing. In fact, Heilborn et al. [18] showed that the use of LL37 antibody inhibited re-epithelialization in a concentration-dependent manner in an organ cultured full-thickness ex vivo wound model. Moreover, Carretero et al. [7] transferred an adenoviral vector containing LL37 via intradermal injection to excisional wounds in ob/ob mice, improving re-epithelialization and granulation tissue formation. Considering all these results, we decided to study the effects of the topical application of LL37 on wounds. Synthetic and recombinant LL37 strongly accelerated the healing process with a re-epithelialization almost complete after 7 days. Moreover, a significant number of new blood vessels were present in the wounds treated with the peptide.

The importance of LL37 in wound healing has already been presented, but the topical application of LL37 on skin lesions with impaired healing has not been studied so far. In this work, we demonstrate that LL37 can be used topically to support wound healing. The topical application of the peptide allows a better regulation of the process. The applied concentrations can be easily controlled and the administration can be stopped at any moment. Moreover, delivery systems can be used to obtain a regulated administration. The tight regulation of AMPs is very important since elevated amounts may lead to a chronic inflammatory process, as it has been demonstrated for psoriasis and rosacea [29,41]. In order to better understand the role of LL37 in wound healing, further experiments will be performed, namely with diabetic mice. Diabetic mice have impaired healing and it is well known that the healing treatments of diabetic ulcers did not deliver satisfactory results. In this type of lesions, angiogenesis seems to be the most compromised
phase with impairment in VEGF synthesis [4]. Regarding the positive results obtained in this work, we believe that the treatment of diabetic ulcers with LL37 will improve wound healing.

Conflict of interest

All authors disclose that there is no conflict of interest including any financial, personal or other relationships with other people or organizations. All authors have materially participated in the research and/or article preparation. All authors have approved the final article.

Acknowledgements

This work was supported by the individual Grant SFRH/BD/27404/2006 from Fundação para a Ciência e a Tecnologia (Portugal). We are grateful to Joana Almeida, at the Department of Biochemistry, University of Porto, for the help with the histological analyzes.

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