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Antimicrobial peptide-gold nanoscale therapeutic formulation with high skin regenerative potential

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ABSTRACT

Chronic skin wounds affect ≈ 3% of persons aged > 60 years (Davies et al., 2007) [1]. These wounds are typically difficult to heal by conventional therapies and in many cases they get infected making even harder the regeneration process. The antimicrobial peptide (AMP) LL37 combines antimicrobial with pro-regenerative properties and thus represents a promising topical therapy to address both problems. Here, we investigated the wound healing potential of soluble and immobilized LL37 (LL37-conjugated gold nanoparticles, LL37-Au NPs), both *in vitro* (migration of keratinocytes) and *in vivo* (skin wound healing). Our results show that LL37-Au NPs, but not LL37 peptide, have the capacity to prolong the phosphorylation of EGFR and ERK1/2 and enhance the migratory properties of keratinocytes in a large *in vitro* wound model. We further report that both LL37 and LL37-Au NPs promote keratinocyte migration by the transactivation of EGFR, a process that seems to be initiated at the P2X7 receptor, as confirmed by chemical and genetic inhibition studies. Finally, we show *in vivo* that LL37-Au NPs have higher wound healing activity than LL37 peptide in a splinted mouse full thickness excisional model. Animal wounds treated by LL37-Au NPs have higher expression of collagen, IL6 and VEGF than the ones treated with LL37 peptide or NPs without LL37. Altogether, the conjugation of AMPs to NPs offers a promising platform to enhance their pro-regenerative properties.

1. Introduction

Topical therapies that combine antimicrobial and pro-regenerative effects are of great potential in the context of skin wound healing [1]. Antimicrobial peptides (AMPs) act as a first line of defense in the human body against bacteria, virus and fungi [2,3], and some of them modulate regeneration processes [4]. In the last years, these small peptides (typically below 40 aminoacids) have been tested as a potential anti-infective therapy, at least for some indications, and thus an alternative to conventional antibiotics [5]. Currently, there are 10 AMP compounds in clinical trials, mostly for skin applications [5]. Two AMPs are in phase 3 clinical trials (to treat *C. difficile* infections and diabetic foot ulcers). Some of these AMPs combine antimicrobial properties (targeting microorganisms) with immunomodulatory, pro-angiogenic and tissue regenerative properties (both targeting human cells). For example, LL37 (one of the AMPs currently in clinical trials [6]) is an AMP predominantly found in human skin that acts at different levels of skin homeostasis [7]. LL37 is a chemoattractant of mast cells, monocytes, T lymphocytes and neutrophils, and regulates inflammation, angiogenesis and wound healing [4,8]. The wound healing properties of LL37 peptide (either in a soluble formulation [4,8,9] or in a formulation consisting in polymeric nanoparticles releasing LL37 [10]) has been demonstrated in wound animal models, as well as in a recent phase I/II clinical trial [6].

One of the approaches being tested to deliver AMPs *in vivo* and prevent their potential toxicity while increasing their stability against protease degradation and serum inactivation is through the chemical immobilization of AMPs in nanoparticles (NPs) [11–20]. We and others have recently demonstrated that AMP-conjugated NPs may offer higher stability, lower toxicity, enhanced antimicrobial properties (due to an

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increase in the local density of positive charges and peptide mass) and improved targeting compared with free AMP [11–13,18,19]. However, to the best of our knowledge, AMP-conjugated NPs have not been tested either *in vitro* or *in vivo* in the context of their regenerative potential relatively to soluble AMPs. It is unclear whether multivalent AMP-conjugated NPs may modulate cellular signaling and bioactivity. Although it has been demonstrated that in some cases multivalent ligand-containing NPs have superior bioactivity than soluble ligands [21], such activity profile are likely dependent in the type of cellular target (*e.g.* receptor, protein, ionic channel) and type of cell.

Here, we investigated the wound healing potential of LL37-conjugated NPs both *in vitro* (migration of keratinocytes) and *in vivo* (skin wound healing). These NPs have a gold (Au) core and a hydrophilic cationic LL37 peptide shell. We have selected Au NPs because it is relatively easy the modification of their properties (*e.g.* size), the immobilization of high concentrations of AMPs per surface area [13], and they have a biomedical track [22]. LL37-conjugated NPs were prepared by a one step procedure [13]. Initially, the physico-chemical properties of LL37-Au NPs were characterized. To show the unique properties of LL37-Au NPs we have evaluated their pro-migratory properties against keratinocytes, an important player in the context of skin healing, and evaluated their mechanism relatively to LL37 peptide. Finally, we have evaluated *in vivo* the regenerative potential of LL37 and LL37-Au NPs in a splinted mouse full thickness excisional model. Overall our results indicate that LL37-conjugated NPs have enhanced wound-healing properties than LL37 peptide because they prolong in time the biological activity of the peptide.

2. Materials and methods

Detailed materials and methods section is provided in Supplementary information.

2.1. Materials

$HAuCl_4{\cdot}3H_2O$, $Na_3C_6H_5O_7$ and HEPES, all acquired to Sigma-Aldrich, were used as received. Lyophilized LL37 peptide modified with a C-terminal cysteine (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPR TESC) was purchased from Caslo Laboratory, Denmark. The peptide was synthesized by conventional solid-phase synthesis, purified by high performance liquid chromatography, and characterized by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy. The purity of the peptide was 96%. Rhodamine B isothiocyanate and HEPES were purchased from Sigma.

2.2. NP preparation

LL37 (0.5 mM) were dissolved initially in DMF (100 μL) followed by addition of HEPES (900 μL, 100 mM, pH 5). $HAuCl_4{\cdot}3H_2O$ (10^{-2} M, 50 μL) was added to a peptide solution (0.25 mM, 950 mL; therefore the final concentration of $HAuCl_4$ was 0.5 mM) and the NP synthesis was carried out at 25 °C. LL37-Au NPs were also synthesized using $HAuCl_4.3H_2O$ (final concentration 0.25 mM, 0.5 mM and 1 mM), and HEPES (100 mM, pH 6 and pH 7.5) by the same procedure at 25 °C. The synthesized Au NPs were centrifuged at 14,000 rpm for 20 min at 4 °C followed by washing with Milli-Q water to remove unreacted peptides and HEPES, frozen and freeze-dried at 223 K using a Snijders Scientific freeze-dryer. Spherical Au NPs were also synthesized *via* citrate reduction method [23]. An aqueous $HAuCl_4$ solution (0.5 mM, 100 mL of water) was boiled in a 250 mL round bottom flask while being stirred after which an aqueous sodium citrate solution (2%, w/v, in water) was added. The reaction was allowed to run until the solution reached a wine red color, indicating the reaction was completed. Fluorescent Au NPs and LL37-Au NPs were prepared by addition of DMSO (0.5 mM) solution of rhodamine to achieve a final concentration of 25 μM for flow cytometry and confocal microscopy studies. Free rhodamine molecules in the colloidal gold solution were removed by centrifugation at 12,000 rpm for 15 min at 4 °C followed by one washings with Milli-Q water. The pellet obtained after centrifugation was redispersed in Milli-Q water and then dialysed. The methods for NP characterization are presented in the Supplementary information.

2.3. Cell culture

Human keratinocyte cell line (HaCaT cell line, CLS, Eppelheim, Germany; passage 35–40) and human primary keratinocytes (NHEK, PromoCell, Heidelberg, Germany; passage 3) were cultured as recommended by the vendor. Briefly, HaCaT cells were cultivated using DMEM supplemented with 1% (v/v) penicillin and streptomycin (Invitrogen) and 10% (v/v) fetal bovine serum (FBS, Invitrogen) until 90% of confluence. Human primary keratinocytes were cultured using KGM-2 media (PromoCell, Heidelberg, Germany). For cell passage, keratinocytes were initially trypsinized and then scraped. The cells were sub-cultured at a ratio of 1:3 until achieving the number of cells required for the experiment. The methods to evaluate the cytotoxicity, internalization and intracellular trafficking of LL37-Au NPs and LL37 peptide are described in detail in the Supplementary information.

2.4. NP bioactivity

2.4.1. Scratch assay

Keratinocytes were seeded at a density of 2×10^4 cells/well in fibronectin-coated 96-well plate in DMEM supplemented with 1% (v/v) penicillin and streptomycin and 10% (v/v) FBS. After 48 h, cells were initially starved for 15 h in DMEM with 0.5% FBS, inactivated with mitomycin (5 μg/mL) for 2 h, and then incubated with LL37 (1 μg/mL), LL37-Au NPs or Au NPs (both at 200 μg/mL) for 5 h at 37 °C and 5% CO_2. In case of chemical inhibition, the chemical inhibitors for FPRL1 (WRW4, Calbiochem, 10 μM), EGFR (Erlotinib HCL, OSI-744, Selleck Chemicals, 2 nM), ADAM17 (Marimastat, Sigma, 10 μM), P2X (PPADS, Sigma, 100 μM), for P2X7 (A-740003, Sigma, at a concentration of 500 nM), were added to the cells 1 h before the incubation with LL37 peptide and LL37-Au NPs and then maintained during the assay. We tested all inhibitors used in our study, and none of them showed any significant toxicity to cells under our experimental conditions (data not shown). After the 5 h treatment, cells were washed twice with PBS to remove non-internalized NPs and a scratch was created with a sterile pipette tip. The detached cells were washed twice with PBS and then plates were re-coated with fibronectin (10 μg/mL in starvation medium) for 1 h at 37 °C. Cells were washed and maintained in starvation medium up to 72 h. Cell migration was monitored overtime by a In Cell Microscope 2000 (GE Healthcare) (objective 2 ×). The cells treated with LL37 peptide were cultured with starvation medium containing LL37 for the entire duration of the experiment. Scratch areas were quantified using the AxioVision software (Carl Zeiss). Wound areas were normalized by the initial area ($n = 6$ images). In case of siRNA knock-down studies, on-target plus human ADAM17 siRNA or P2X7R siRNA (both from Dharmacon) were used to silence ADAM17 or P2X7 before performing the scratch assay. Keratinocytes were transfected with 50 nM siRNA using 0.25 μL of Lipofectamine RNAiMAX (Life Technologies) for 24 h in antibiotic-free complete medium before starting the scratch assay.

To test the prolonged effect of LL37-Au NPs on keratinocyte migration, cells were plated in fibronectin-coated 24-well plate (1×10^5 cells/well), after 48 h cells were starved for 15 h in DMEM with 0.5% FBS and then the scratch was made with a sterile pipette tip. Only after this step, the cells were incubated with LL37 (5 μg/mL), LL37-Au NPs and Au NPs (15 μg/mL) in starvation medium up to 96 h. During this time, cell migration was monitored by scratch area quantification. To test the effect of LL37-Au NPs in primary keratinocytes, cells were plated in a 24 well plate (pre-warmed for 1 h with 1 mL medium; cells added dropwise) (4×10^4 cells/well) and allowed to