LL-37-induced caspase-independent apoptosis is associated with plasma membrane permeabilization in human osteoblast-like cells

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ABSTRACT

The host defense peptide LL-37 is active against both gram-positive and gram-negative bacteria, but it has also been shown to reduce human host cell viability. However, the mechanisms behind LL-37-induced human host cell cytotoxicity are not yet fully understood. Here, we assess if LL-37-evoked attenuation of human osteoblast-like MG63 cell viability is associated with apoptosis, and if the underlying mechanism may involve LL-37-induced plasma membrane permeabilization. MG63 cell viability and plasma membrane permeabilization were investigated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and by measuring lactate dehydrogenase (LDH) release, respectively. Apoptosis was assessed by the terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) assay and Annexin V flow cytometry, and caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage were determined by Western blot. LL-37 (4 and 10 μM) reduced both cell number and cell viability, and these effects were associated with a pro-apoptotic effect demonstrated by positive TUNEL staining and Annexin V flow cytometry. LL-37-induced apoptosis was not coupled to either caspase-3 or PARP cleavage, suggesting that LL-37 causes caspase-independent apoptosis in MG63 cells. Both LL-37 and the well-known plasma membrane permeabilizer Triton X-100 reduced cell viability and stimulated LDH release. Triton X-100-treated cells showed positive TUNEL staining, and the detergent accumulated cells in late apoptosis/necrosis. Similar to LL-37, Triton X-100 caused no PARP cleavage. We conclude that LL-37 promotes caspase-independent apoptosis, and that this effect seems coupled to plasma membrane permeabilization in human MG63 cells.

Keywords: Antimicrobial peptides (AMP) Apoptosis Cathelicidin Cytotoxicity Innate immunity

ARTICLE INFO

1. Introduction

Host defense peptides represent an important part of innate immunity and make up the first line of defense against invading microorganisms [1]. They are mainly produced by neutrophils but also by other types of immune cells, as well as by epithelial cells aligning the mucosal areas of e.g. the airways and the gastrointestinal tract [2,3]. LL-37 is the only member of the cathelicidin family of host defense peptides in humans, and the peptide is processed from its pre-cursor protein hCAP18 via extracellular cleavage, a reaction catalyzed by either serine protease 3 or kallikrein 5 [4,5]. The functional importance of LL-37 is regarded to be associated with its α-helical cationic structure showing both hydrophilic and hydrophobic properties [6]. LL-37 exerts antimicrobial activity by permeabilization of both gram-positive and gram-negative bacteria, causing cell lysis, and through neutralization of bacterial endotoxins [2,7,8]. However, the peptide has also been shown to permeabilize plasma membranes of human host cells, assessed by inflow of Ca2+; demonstrating that LL-37-induced plasma membrane permeabilization is not restricted to the bacterial cell wall but also involves host cells [9]. Apart from its antimicrobial activity, LL-37 has been shown to act as a modulator of innate immunity by for example operating as a chemoattractant for immune cells and regulate their production of pro-inflammatory cytokines [10,11].

In cell culture experiments, high concentrations of LL-37 (>4 μM) have been shown to be cytotoxic for several types of human host cells, but the mechanisms and signaling pathways involved in LL-37-evoked attenuation of cell viability is not yet fully understood [12-15]. Notably, LL-37 has been reported to have pro-apoptotic effects in many human cell types including osteoblast-like MG63 cells, but also LL-37-induced anti-apoptotic effects have been reported [9,15-19].

ABSTRACT

The host defense peptide LL-37 is active against both gram-positive and gram-negative bacteria, but it has also been shown to reduce human host cell viability. However, the mechanisms behind LL-37-induced human host cell cytotoxicity are not yet fully understood. Here, we assess if LL-37-evoked attenuation of human osteoblast-like MG63 cell viability is associated with apoptosis, and if the underlying mechanism may involve LL-37-induced plasma membrane permeabilization. MG63 cell viability and plasma membrane permeabilization were investigated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and by measuring lactate dehydrogenase (LDH) release, respectively. Apoptosis was assessed by the terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) assay and Annexin V flow cytometry, and caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage were determined by Western blot. LL-37 (4 and 10 μM) reduced both cell number and cell viability, and these effects were associated with a pro-apoptotic effect demonstrated by positive TUNEL staining and Annexin V flow cytometry. LL-37-induced apoptosis was not coupled to either caspase-3 or PARP cleavage, suggesting that LL-37 causes caspase-independent apoptosis in MG63 cells. Both LL-37 and the well-known plasma membrane permeabilizer Triton X-100 reduced cell viability and stimulated LDH release. Triton X-100-treated cells showed positive TUNEL staining, and the detergent accumulated cells in late apoptosis/necrosis. Similar to LL-37, Triton X-100 caused no PARP cleavage. We conclude that LL-37 promotes caspase-independent apoptosis, and that this effect seems coupled to plasma membrane permeabilization in human MG63 cells.

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Interestingly, the globular C1q receptor (p33), a protein associated with the complement system, has been reported to reduce the LL-37-induced cytotoxic effects, and the cellular expression levels of p33 seem to negatively correlate to LL-37-induced cytotoxicity [20,21]. We have recently demonstrated that LL-37 is internalized by MG63 cells, and that LL-37-induced cytotoxicity occurs independent of LL-37 import through endocytosis [22]. Importantly, LL-37-induced host cell cytotoxicity is observed in a similar concentration range of LL-37 as that detected locally in skin lesions of psoriasis (> 100 μM), and in the gingival crevicular fluid collected at diseased sites in patients with periodontitis (~ 1 μM), showing that LL-37-evoked cytotoxicity is indeed relevant for the in-vivo situation [23–25]. In fact, the local cellular concentration of LL-37 facing the osteoblasts of the periodontal tissue at diseased sites in periodontitis may be even higher than that in the gingival crevicular fluid. Notably, periodontitis is an inflammatory condition associated with degradation of the bone tissue surrounding the teeth, and thereby this disease leads to loss of tooth attachment. Taken together, it is of great importance to further investigate the mechanisms behind LL-37-induced human osteoblast cytotoxicity.

The aim of this study was to assess LL-37-induced human osteoblast cytotoxicity and its underlying mechanisms of action, and to compare the effects of LL-37 with those of the well-known plasma membrane permeabilizer Triton X-100. We demonstrate that LL-37 reduces MG63 cell viability and causes caspase-independent apoptosis, and show that these effects are associated with plasma membrane permeabilization.

2. Material and methods

2.1. Cells and cell culture

Human osteoblast-like MG63 cells (ATCC) were cultured in Dulbecco’s DMEM/Ham’s F12 medium (1:1, Life Technologies), supplemented with 10 % fetal bovine serum (FBS) and antibiotics (penicillin 50 units/mL and streptomycin 50 μg/mL) and kept in a water-jacketed cell incubator at 37 °C under 5% CO₂ in air. The cells were reseeded twice a week upon reaching confluence by using 0.25 % trypsin/EDTA and counted using a LUNA automated cell counter (Logos Biosystem). The LDH measurement was performed in Opti-MEM medium lacking phenol red (Life Technologies) to remove optical disturbances when absorbance was measured. All other experiments were performed in DMEM/Ham’s F12 medium supplemented with 1 % FBS and antibiotics to achieve sub-maximal growth conditions.

2.2. Assessment of cell morphology, cell number and cell viability

Cell morphology was investigated using a phase-contrast microscope (Olympus CXX41, Olympus), equipped with a SC50 5-megapixel digital camera (Olympus). The cells were stained with Trypan-blue (0.2 %, Sigma-Aldrich) and viable cells counted using a LUNA automated cell counter system. Cell viability was assessed using a MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich). The cells were incubated with MTT solution in DMEM (0.5 mg/mL) for 1 h in a cell incubator at 37 °C. The formazan product was dissolved in dimethyl sulfoxide (DMSO) before reading the absorbance at 540 nm in a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific).

2.3. Western blot

Cells were lysed in sodium dodecyl sulfate (SDS)-buffer (SDS/phosphate-buffere saline 1:1, 1 % protease inhibitor) and sonicated once for 10 s on ice. The homogenates were boiled for 5 min, centrifuged at 16,000 g for 15 min at 4 °C and the supernatants collected. The total protein concentration in each sample was determined using a DC protein assay kit (Bio-Rad) to ensure equal loading. Proteins were separated using Criterion TGX 4–15 % precast gels from Bio-Rad and transferred to nitrocellulose membranes using a Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were blocked in Tris-buffered saline (TBS) with 0.5 % casein for 2 h, followed by incubation with primary antibodies for poly (ADP-ribose) polymerase (PARP, Cell Signaling, #9542, rabbit, diluted 1:3000), cleaved caspase-3 (Cell Signaling, #9664, rabbit, diluted 1:500), GAPDH (Merck Millipore, #MAB374, mouse, diluted 1:5000) and histone H3 (Cell Signaling, #4499S, rabbit, diluted 1:2000) at 4 °C either overnight (PARP, GAPDH, histone H3) or for 3 days (cleaved caspase-3). All primary antibodies were diluted in 0.5 % casein/TBS-Tween (TBS-T). The membranes were incubated with horseradish peroxidase conjugated secondary rabbit or mouse antibodies (Cell Signaling), diluted 1:5000 in 0.5 % casein/TBS-T for 2 h and the immunoreactivity was assessed using the SuperSignal West Femto chemiluminescence substrate (Thermo Fisher Scientific). The immunoreactive bands were visualized and analyzed in a LiCOR Odyssey FC instrument (LI-COR Biosciences). Either GAPDH or histone H3 was included as internal control.

2.4. TUNEL assay

For analysis of apoptosis using the terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) assay, cells were cultured on cover glasses, fixed with ice-cold methanol for 5 min and then permeabilized by 33 % acetic acid in ethanol for 5 min at ~20 °C. Cells were labeled with the Apotag Red In Situ Apoptosis Detection Kit (EMD Millipore), according to manufacturer’s instructions. The cover glasses were mounted on microscopic slides using DAPI loaded histological mounting medium (Fluoroshield, Sigma-Aldrich). DAPI was included as a nuclear marker. Pictures of TUNEL and DAPI staining were acquired by a Zeiss Axiolab 200 M microscope (Zeiss) equipped with a Basler aca1440-220um camera and a 10x objective for morphological assessment. Quantitative assessment of the images was performed using the Image J software. For each cover glass, apoptotic index (the percentage of apoptotic cells) was calculated in images from 3 different areas and the average value was used.

2.5. Flow cytometry

Apoptosis was determined by flow cytometry of Annexin V positive cells. Cells were harvested, washed in PBS and incubated with a FITC-labeled Annexin V and the fluorescent viability marker propidium iodide (PI) kit from BD Biosciences in accordance with manufacturer’s instructions. Cells in early apoptosis are Annexin V positive and PI negative, whereas cells in late apoptosis and necrotic cells are Annexin V positive and PI positive. Gates for Annexin V and PI positive cells were set using fluorescence minus one controls. For each sample, about 2000 events were recorded. The proportion of early apoptotic and late apoptotic/necrotic cells was obtained as percentage of total number and data are normalized to control.

2.6. LDH assay

Plasma membrane permeabilization was assessed by measuring release of lactate dehydrogenase (LDH) as previously described [21]. Briefly, cells were seeded in 96-well plates in Opti-MEM culture medium and at the end of the incubation, cells were centrifuged at 1,200 g for 10 min. The supernatants were transferred to a new plate and incubated with NADH solution (56 mM Tris, 5.6 mM EGT, 170 μM β-NADH) and sodium pyruvate solution (14 mM). The absorbance of non-oxidized NADH was determined at 340 nm in a Multiskan GO Microplate spectrophotometer (Thermo Fisher Scientific) at 30 °C. Opti-MEM culture medium was used as blank. The release of LDH was normalized to the total release of LDH for each sample. Total release of LDH was obtained by sonicating cells for 10 s on ice.
2.7. Agents

LL-37 (Bachem), thapsigargin (Sigma-Aldrich) and staurosporine (Sigma-Aldrich) were dissolved in DMSO. Triton X-100 (Sigma-Aldrich) was dissolved in PBS. For both TUNEL assay and flow cytometry, we stimulated cells with 90 μM Triton X-100, whereas for western blot analysis, cells were stimulated with 130 μM Triton X-100. Notably, both these concentrations of Triton X-100 reduce MG63 cell viability. LL-37 was reconstituted in plastic tubes and stored in aliquotes at a concentration of 10 mM. In order to ensure that all groups in one experiment received the same volume of dissolvent, the agents were diluted appropriately. DMSO vehicle was added to all groups that did not receive treatment with either LL-37, thapsigargin or staurosporine as appropriate.

2.8. Statistics

Summarized data were analyzed in SPSS Statistics for Windows, version 25 (SPSS Inc) and in GraphPad Prism7 (GraphPad Software). The summarized data are presented as mean ± SEM. Each experiment was repeated at least three times, and n values presented in the figure legends represent the number of independent experiments. For each experiment, multiple measurements in duplicate or triplicate were performed. Statistical significance was calculated by ANOVA followed by Fisher’s LSD, Dunnett’s or Tukey’s multiple comparison post hoc tests as appropriate.

3. Results

3.1. High concentrations of LL-37 reduce MG63 cell number and viability

MG63 cell morphology was assessed by phase-contrast microscopy after stimulation with or without LL-37 (1–10 μM) for 24 h. The images showed a concentration-dependent reduction in cell density, but morphological changes were only observed in cells treated with the highest concentration (10 μM) of LL-37 (Fig. 1 A-D). Cells treated with 10 μM LL-37 displayed membrane blebbing, cellular shrinkage and loss of cellular protrusions, which are morphological characteristics representative for apoptotic cells (Fig. 1D).

Next, we investigated the effects of LL-37 on cell number. Treatment with high concentrations of LL-37 (4 and 10 μM) for 24 h reduced cell number compared to control cells, whereas only 10 μM LL-37 significantly decreased number of cells at 48 h (Fig. 1E). Treatment with 1 μM LL-37 for 24 or 48 h had no effect (Fig. 1E). As seen in Fig. 1E, number of control cells and cells treated with a low concentration of LL-37 (1 μM) increased with time. Notably, cell number tended to increase between 24 and 48 h also for cells treated with high concentrations of LL-37 (4 and 10 μM), indicating that LL-37 is metabolized within this time frame (Fig. 1E). Metabolism of LL-37 may involve proteases produced by the cells themselves and/or present in the FBS. In the next set of experiments, we assessed effects of LL-37 on cell viability by using the MTT assay which monitors cellular metabolic activity. Treatment with LL-37 (4 and 10 μM) for 4 h reduced cell viability in a concentration-dependent manner compared to control cells, whereas low concentrations of LL-37 (0.1 and 1 μM) had no effect (Fig. 1F).

3.2. LL-37 is pro-apoptotic but does not trigger cleavage of PARP or caspase-3 in MG63 cells

Next, we investigated if LL-37-induced down-regulation of MG63 cell number and cell viability is associated with apoptosis. Apoptosis was assessed by the TUNEL assay measuring DNA fragmentation, with the pro-apoptotic protein kinase inhibitor staurosporine (0.2 μM) included as a positive control. Cells stimulated with LL-37 (4 μM) for 24 h showed positive staining for apoptotic cells (Fig. 2A). For staurosporine, a
somewhat different staining pattern was observed with less intensive and more diffuse nuclear staining compared to cells stimulated with LL-37 (Fig. 2A). Both cells stimulated with LL-37 and cells treated with staurosporine had a higher apoptotic index (percentage of apoptotic cells) compared to control cells (Fig. 2B). Apoptosis was also assessed by Annexin V flow cytometry analysis. Treatment with LL-37 (4 μM) for 1 and 4 h increased the number of early apoptotic cells (Annexin V positive/PI negative) by 2–3 times (Fig. 3A-C). Stimulation with staurosporine (0.2 μM) for 4 h enhanced the number of apoptotic cells by about 60 %, whereas staurosporine had no effect already at 1 h (Fig. 3A-C). Thus, LL-37-induced apoptosis was demonstrated both by positive TUNEL staining and accumulation of cells in early apoptosis by Annexin V flow cytometry.

In the next set of experiments, the expression of cleaved PARP and caspase-3 was examined by western blot in cells stimulated with or without LL-37. PARP is a substrate of caspases and cleaved PARP is both an inducer and a marker of caspase-mediated apoptosis. No immunoreactive band for cleaved PARP (expected molecular weight of 89 kDa) was observed neither in control cells nor in cells stimulated with LL-37 (4 μM) for 24 h (Fig. 4A). However, immunoreactivity for cleaved PARP was detected in cells treated with the pro-apoptotic agents thapsigargin (1 μM), acting as inhibitor of endoplasmic reticulum Ca\(^{2+}\) ATPase, and staurosporine (0.2 μM), both used as positive controls (Fig. 4A). Immunoreactive bands for the non-cleaved PARP protein (expected molecular weight of 116 kDa) were observed in all groups (Fig. 4A). Cleaved caspase-3 products (expected molecular weight of 17 and 19 kDa) were not observed in control cells or cells stimulated with LL-37 (4 μM) for 24 h, but immunoreactive bands representing cleaved caspase-3 were detected in cells stimulated with either thapsigargin (1 μM) or staurosporine (0.2 μM) (Fig. 4A). We also examined PARP and caspase-3 cleavage at a shorter time-point (6 h) to rule out rapid effects of LL-37. No immunoreactive band for cleaved PARP was observed in either control cells or in cells stimulated with LL-37 (4 μM) for 6 h, though cleaved PARP was detected in cells stimulated with staurosporine (0.2 μM) as expected (Fig. 4B). No immunoreactive band for cleaved PARP was detected in cells stimulated with thapsigargin (1 μM) for 6 h (Fig. 4B). The non-cleaved PARP protein was observed in all groups (Fig. 4B). We were unable to demonstrate staurosporine-induced caspase-3 cleavage already at 6 h (data not shown). Notably, cleaved caspase-3 protein levels are probably below detection limit at 6 h of treatment. Overall, cells stimulated with LL-37 show positive TUNEL staining and are accumulated in early apoptosis (Annexin V positive/PI negative), but they display no cleavage of PARP or caspase-3, suggesting that LL-37 may induce apoptosis through a caspase-independent pathway.

### 3.3. Both LL-37 and Triton X-100 reduce MG63 cell viability and induce plasma membrane permeabilization

To assess if LL-37-induced apoptosis is associated with plasma membrane permeabilization, we compared the effects of LL-37 on MG63 cell viability and LDH release with those of the well-known plasma membrane permeabilizer Triton X-100. Treatment with Triton X-100 (18–180 μM) for 4 h reduced cell viability in a concentration-dependent manner (Fig. 5A). Stimulation with LL-37 (4 μM), which was included as a reference, also attenuated cell viability (Fig. 5A). To investigate if Triton X-100 and LL-37 trigger plasma membrane permeabilization, we performed measurement of LDH release. Treatment with Triton X-100 (130 or 180 μM) for 30 min increased the release of LDH by 3 and 4 times, respectively, compared to control (Fig. 5B). Stimulation with LL-37 (4 μM) for 30 min increased LDH release by about 4 times (Fig. 5B). Overall, both LL-37 and Triton X-100 reduce viability of MG63 cells and permeabilize their plasma membranes.

### 3.4. Triton X-100 accumulates cells in late apoptosis/necrosis but does not trigger PARP cleavage in MG63 cells

To assess if Triton X-100 induces apoptosis, analysis of TUNEL staining was performed. MG63 cells treated with Triton X-100 (90 μM)
for 24 h showed positive TUNEL staining representative for apoptotic cells (Fig. 6A). Treatment with staurosporine (0.2 μM) for 24 h was included as positive control (Fig. 6A). Cells stimulated with either Triton X-100 or staurosporine had higher apoptotic index (percentage of apoptotic cells) compared to control cells (Fig. 6B). Notably, almost all Triton X-100-treated cells were Annexin V positive/PI positive at both 1 and 4 h (77 % of total number of cells). Hence, stimulation with Triton X-100 initiates transition of cells into late apoptosis/necrosis.

Fig. 3. LL-37 increases the proportion of MG63 cells in early apoptosis. (A-C) Cells were treated with or without LL-37 (4 μM) or staurosporine (0.2 μM) for 1 or 4 h, and apoptosis was assessed by Annexin V flow cytometry. (A) Proportion of Annexin V positive/PI negative cells representing cells in early apoptosis. The values are presented as means ± SEM. Each value was normalized to control, and control was set to 100 %. n = 3 in each group. * , ** and *** represent P < 0.05, P < 0.01 and P < 0.001, respectively, vs. control. (B-C) Representative scatter plots for cells treated with or without LL-37 or staurosporine for 1 (B) or 4 h (C). a, b and c represent viable cells (Annexin V negative/PI negative), early apoptotic cells (Annexin V positive/PI negative) and late apoptotic/necrotic cells (Annexin V positive/PI positive), respectively.

Fig. 4. LL-37 does not induce PARP or caspase-3 cleavage in MG63 cells. (A-B) Western blot analysis was used to determine expression of cleaved PARP and cleaved caspase-3 in cells stimulated with LL-37 (4 μM) for 24 h (A). PARP cleavage was also investigated in cells stimulated with LL-37 (4 μM) for a shorter time (6 h) (B). Treatment with pro-apoptotic thapsigargin (1 μM) and staurosporine (0.2 μM) was used as positive control for all blots. The non-cleaved PARP (expected molecular weight 116 kDa), cleaved PARP (89 kDa), cleaved caspase-3 (17 and 19 kDa) and GAPDH (37 kDa) were examined on the same membrane. GAPDH served as an internal loading control. The blots depicted here show one representative replicate out of four for each type of experiment. For each type of experiment, three independent experiments were performed.
In the next experiments, we analyzed PARP cleavage by western blot in MG63 cells treated with or without Triton X-100 and LL-37. No immunoreactive band for cleaved PARP (expected molecular weight of 89 kDa) was observed in cells treated with either Triton X-100 (130 μM) or LL-37 (4 μM) for 6 h, whereas cleavage of PARP was observed in cells stimulated with staurosporine (0.2 μM) included as positive control (Fig. 8). We noted that Triton X-100 reduced GAPDH expression, and therefore we instead used histone H3 as internal loading control (Fig. 8). We used histone H3 as internal loading control for assessment of PARP expression, since they are both nuclear proteins. In summary, cells treated with Triton X-100 display positive TUNEL staining and accumulation of cells in late apoptosis/necrosis but no expression of cleaved PARP.

Fig. 5. Both Triton X-100 and LL-37 reduce MG63 cell viability and trigger release of LDH in MG63 cells. (A) Treatment with Triton X-100 (18-180 μM) for 4 h reduces cell viability in a concentration-dependent manner assessed by the MTT method. LL-37 (4 μM) was included as a reference. (B) Treatment with Triton X-100 (18-180 μM) for 30 min increases LDH release in a concentration-dependent manner. LL-37 (4 μM) was included as a reference. The values are presented as means ± SEM. For the MTT analysis, each value was normalized to control, and control was set to 100 %, whereas for the LDH release assay, each value was normalized to total LDH release. n = 3-4 in each group. *, ** and *** represent P < 0.05, P < 0.01 and P < 0.001, respectively, vs. control.

Fig. 6. MG63 cells treated with Triton X-100 show positive TUNEL staining, indicating apoptosis. (A-B) Cells were treated with Triton X-100 (90 μM) and the positive control staurosporine (0.2 μM) for 24 h. (A) Positive cells stain red and DAPI (blue) was used as nuclear marker. The bar in panel A represents 30 μm and applies to all images in panel A. (B) Apoptotic index was calculated using 2-3 cover glasses for each measurement, and for each cover glass an average value was calculated from 3 different areas. The values are presented as means ± SEM. n = 3 in each group. *** represents P < 0.001 vs. control.

4. Discussion

Here, we show that LL-37 is pro-apoptotic, reduces cell viability and triggers LDH release in human osteoblast-like MG63 cells, suggesting that LL-37-induced apoptosis is associated with plasma membrane permeabilization. Notably, we observe LL-37-evoked apoptosis by different techniques in independent experiments. LL-37-treated cells show morphological signs of apoptosis, they show positive TUNEL staining, and they are accumulated in early apoptosis demonstrated by Annexin V flow cytometry. Interestingly, LL-37-treated cells seem to be permeabilized and apoptotic, but they show no PARP cleavage, indicating that LL-37-evoked plasma membrane permeabilization is coupled to the caspase-independent apoptotic pathway. The well-known plasma
It is interesting to speculate that this phenomenon is relevant also in other context where host cells and bacteria are present. The fact that LL-37 and Pseudomonas aeruginosa act in synergy to trigger caspase-independent apoptosis, and that LL-37 induces apoptosis in non-infected airway epithelial cells, whereas it activated caspase-3 in cells incubated with Pseudomonas aeruginosa, suggesting that LL-37 and Pseudomonas aeruginosa act in synergy to trigger caspase-dependent apoptosis, and that LL-37-induced caspase-dependent apoptosis in airway epithelial cells is critically dependent on the presence of bacteria. It is interesting to speculate that this phenomenon is relevant also in other context where host cells and bacteria are present together.

Our data suggest that LL-37-induced down-regulation of MG63 cell number and viability is associated with caspase-independent apoptosis as demonstrated by positive TUNEL staining, increased proportion of Annexin V positive/PI negative, and lack of cleavage of caspase-3 and PARP in cells treated with LL-37. Caspase-independent apoptosis is a well-known cell death program that seems to involve permeabilization of mitochondria and translocation and activation of the mitochondrial pro-apoptotic apoptosis-inducing factor (AIF) to cytosol and nucleus [27]. Besides mitochondria, also other organelles, such as lysosomes, are associated with caspase-independent apoptosis, and this form of apoptosis may substitute for lack of caspase-dependent apoptosis in cells subjected to cytotoxic agents [28]. Interestingly, LL-37-induced caspase-independent apoptosis of Jurkat T leukemia cancer cells has been shown to be critically dependent on AIF activation, indicating that LL-37 affects mitochondrial functional properties [16]. Furthermore, Ren et al. have demonstrated that LL-37 induced caspase-independent apoptosis in colon cancer cells is associated with translocation of both AIF and endonuclease G, the latter another pro-apoptotic factor associated with caspase-independent apoptosis, from cytosol to nucleus [29].

In the present study, we demonstrate that LL-37 (4 μM) reduces MG63 osteoblast-like cell viability through a process involving apoptosis. Periodontitis is an inflammatory condition that leads to degradation of the alveolar bone tissue of the jaws causing loss of bone support for the teeth and finally tooth loss. Indeed, high concentrations of LL-37 (1 μM) have been observed locally in the gingival crevicular fluid of diseased sites in patients suffering from periodontitis [25]. These data may indicate that the local periodontal tissue concentrations of LL-37 are even higher than 1 μM. Thus, although the gingival crevicular fluid concentrations of LL-37 are lower than those used here to show reduced MG63 cell viability, it may suggest that destruction of bone tissue and loss of tooth attachment in periodontitis may involve LL-37-induced osteoblast cytotoxicity. Hence, it is of human in-vivo relevance to study mechanisms behind LL-37-induced attenuation of LL-37 cell viability. Periodontitis is an inflammatory condition that leads to degradation of the alveolar bone tissue of the jaws causing loss of bone support for the teeth and finally tooth loss.
a local impact on osteoblast viability. We assessed LL-37 and Triton X-100-evoked plasma membrane permeabilization by measuring LDH release. Interestingly, we observed increased LDH release in response to 4 μM LL-37, whereas Triton X-100 enhanced LDH release at higher concentrations (130 μM), suggesting that LL-37 and Triton X-100 may permeabilize the plasma membrane via different mechanisms. LL-37 is thought to permeabilize plasma membranes either via oligomerization and formation of pores (barrel-stave model) or via the carpet model, where the peptide assembles at the outer surface of the plasma membrane and reaches a critical concentration which allows LL-37 to permeabilize the membrane similar to detergents [6]. Based on our present data, we may assume that LL-37 and the detergent Triton X-100, at least partly, permeabilize MG63 cells via different mechanisms. Interestingly, our data show that Triton X-100 permeabilizes human host cell plasma membranes in the 130–200 μM concentration-range which confirm findings by Koley and Bard [32], assessing Triton X-100-induced permeabilization in HeLa cells.

In conclusion, our data show that LL-37 induces caspase-independent apoptosis in human osteoblast-like MG63 cells, and that this process is associated with plasma membrane permeabilization. We suggest that LL-37-induced cytotoxicity may play a role for the tissue reactions occurring locally in inflammatory diseases such as periodontitis. However, in the present study we do not consider the interplay between different types of cells occurring in the in-vivo situation which may represent a weakness of the study.

Credit author statement

Elisabeth Bankell: Designed and performed the experiments. Sara Dahl: Designed, performed the experiments and wrote the manuscript. Olof Gidlöf: Designed and performed Annexin V flow cytometry. Daniel Svensson: Designed and performed TUNEL analysis. Bengt-Olof Nilsson: Contributed to the design of the experiments and wrote the manuscript.

All authors contributed with input and feedback on the experiments, data and the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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