The Antimicrobial Peptide LL-37 Alters Human Osteoblast Ca^{2+} Handling and Induces Ca^{2+} -Independent Apoptosis

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Key Words

Annexin V · Apoptosis · Calcium · Caspase 3 · LL-37 · Osteoblast

Abstract

The human antimicrobial peptide cathelicidin LL-37 has, besides its antimicrobial properties, also been shown to regulate apoptosis in a cell type-specific manner. Mechanisms involved in LL-37-regulated apoptotic signaling are not identified. Here, we show that LL-37 reduces the human osteoblast-like MG63 cell number and cell viability in the micromolar concentration range with an IC_{50} value of about 5 μM . Treatment with 4 μM LL-37 increased the number of annexin V-positive cells and stimulated activation of caspase 3 showing that LL-37 promotes apoptosis. Treatment with 4 μM LL-37 caused an acute and sustained rise in intracellular Ca^{2+} concentration assessed by laser-scanning confocal microscopy of Fluo-4-AM-loaded MG63 cells. LL-37 increased Ca^{2+} also in the presence of the respective L- and T-type voltage-sensitive Ca^{2+} channel blockers nifedipine and NiCl_2 . LL-37 had no effect on Ca^{2+} in cells incubated with

Ca^{2+} -free solution. LL-37 (4 and 8 μM) reduced the MG63 cell number both in the presence and absence of Ca^{2+} in the medium. In conclusion, LL-37 reduces the osteoblast cell number by promoting apoptosis, and furthermore, LL-37 stimulates Ca^{2+} inflow via a mechanism independent of voltage-sensitive Ca^{2+} channels. Interestingly, LL-37-induced lowering of the cell number seems to be mediated via a mechanism independent of Ca^{2+} .

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Introduction

The human antimicrobial peptide cathelicidin LL-37 is stored as the proprotein form hCAP-18 in neutrophils and epithelial cells until activated by the serine protease proteinase 3 and subsequently released [1, 2]. LL-37 is an amphiphilic α -helical cationic peptide possessing hydrophobic as well as hydrophilic properties [3]. It exerts a direct antimicrobial activity through disrupting the cell wall of both Gram-negative and Gram-positive bacteria causing bacterial cell lysis and by neu-

tralizing lipopolysaccharides [4–6]. The cationic LL-37 molecule binds to negatively charged microbial membrane lipids thereby showing membrane selectivity [3]. Furthermore, LL-37 potentiates chemokine production by microbial stimuli in keratinocytes and other epithelial cells, suggesting that it enhances the immune defense of the skin [7].

LL-37 has, besides its antimicrobial and immune modulator activities, also been shown to affect various cellular functions, such as phagocytosis [8], cell differentiation [9] and apoptosis [10–16]. Importantly, the effects of LL-37 on apoptosis seem to be cell type specific; LL-37 promotes apoptosis in vascular smooth muscle cells [10], periodontal ligament cells [11], neutrophils [12], T cells [13] and airway epithelium [14] but suppresses apoptosis in keratinocytes [15] and dermal fibroblasts [16]. For neutrophils, LL-37 has been reported to exert both pro- and antiapoptotic effects [12, 17].

In periodontitis, which is a progressive inflammatory disease, the end stage is characterized by destruction of the alveolar bone leading to loss of teeth. Interestingly, the LL-37 concentrations are high (μM) in the gingival crevicular fluid from patients suffering from chronic periodontitis [18], suggesting that this peptide may influence the disease process. The bone-forming osteoblasts represent a very important cell type in both periodontal health and disease responsible for maintaining the alveolar bone mass [19].

The signaling pathways and mechanisms involved in LL-37-regulated apoptosis are not completely understood [3]. The Ca^{2+} ion is thought to play a role in cell killing and apoptosis, and dysfunctional regulation of Ca^{2+} homeostasis can result in apoptosis [20, 21], suggesting that Ca^{2+} may be involved in LL-37-induced proapoptotic signaling. LL-37 has been reported to interact with different cellular proteins such as the purinergic P2X_7 receptor [17, 22] and the epidermal growth factor receptor [23–25], but LL-37 interacts also with DNA [26]. In the present study, we investigate effects of LL-37 on human osteoblast-like MG63 cell viability and Ca^{2+} signaling demonstrating that LL-37 induces apoptosis also in this cell type. Additionally, we demonstrate that LL-37 causes a rapid and sustained rise in the intracellular Ca^{2+} concentration through inflow of Ca^{2+} from the extracellular space, suggesting that LL-37 alters Ca^{2+} handling. Interestingly, the LL-37-induced reduction of the osteoblast cell number and cell viability seems to be mediated through a mechanism independent of Ca^{2+} .

Materials and Methods

Cell Culture and Experimental Procedure

The human osteoblast-like MG63 cell line and the primary human osteoblast cell line hFOB 1.19 from the American Type Tissue Culture Collection (ATCC, Manassas, Va., USA) were cultured in DMEM/Ham's F12 (1:1) cell culture medium (Life Technologies, Invitrogen, Carlsbad, Calif., USA) supplemented with antibiotics (penicillin 50 U/ml, streptomycin 50 $\mu\text{g}/\text{ml}$) and 10% fetal calf serum in accordance with instructions from ATCC. The cells were kept in a water-jacketed cell incubator at 37°C under 5% CO_2 in air. The MG63 cells express markers for osteogenic differentiation such as alkaline phosphatase enzyme activity and form mineralized nodules showing that they are representative for native osteoblasts [27, 28]. The cells were trypsinized (0.25% trypsin/EDTA) and reseeded upon reaching confluence. Medium was exchanged every second day. Experiments were performed on subconfluent cells (80% confluence). The cells were used for experiments in passages 3–10. Incubation with LL-37 caused similar effects irrespective of passage number. Before experiments fetal calf serum was omitted in order to standardize the experimental conditions. For experiments assessing the importance of Ca^{2+} for LL-37-induced effects on cell number, MG63 cells were incubated in either DMEM cell culture medium containing 1.8 mM Ca^{2+} or Ca^{2+} -free DMEM medium, both from Life Technologies. The Ca^{2+} -free DMEM medium was further supplemented with 1.8 mM MgCl_2 to achieve isoosmolar conditions and to supplement with divalent cations to compensate for those lost by the omission of Ca^{2+} . In order to achieve culture conditions with low Ca^{2+} concentration, the Ca^{2+} -containing DMEM medium was supplemented with 1.8 mM of the Ca^{2+} chelator ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma Chemicals, St. Louis, Mo., USA).

Assessment of Cell Number and Cell Viability

The number of cells was calculated in a Bürker chamber as appropriate. Cell viability was assessed using trypan blue exclusion test. The cells were incubated for 2 min with 0.4% trypan blue (Sigma) and then washed 3 times in 0.9% NaCl. Cells containing trypan blue were counted as a measure of dead/dying cells. Cell morphology was assessed in digital photographs from a Nikon TMS microscope equipped with a digital camera (Pixelink, Nikon, Nikon Nordic AB, Solna, Sweden).

Determination of Active Caspase 3

After washing in phosphate-buffered saline (PBS), cellular proteins were extracted using a protein extraction buffer provided in the active caspase 3 ELISA kit from R&D (R&D Systems, Minneapolis, Minn., USA). The amount of active caspase 3 was determined by ELISA according to the manufacturer's instructions and normalized to total protein in each sample measured by a Bio-Rad protein assay kit (BioRad, Hercules, Calif., USA). Each sample was analyzed in duplicate.

Annexin V Flow Cytometry

Apoptosis was assessed by flow cytometry using Annexin V staining. Cells were washed with PBS and then incubated with FITC-labeled Annexin V and the fluorescent viability dye 7-aminoactinomycin D (7-AAD) using the FITC Annexin V apoptosis detection kit 1 (BD, Franklin Lakes, N.J., USA) according to the

Fig. 1. a Treatment with LL-37 for 24 h reduces the MG63 osteoblast cell number in a concentration-dependent manner. LL-37 at 4 and 8 μM reduces the osteoblast cell number by 40 and 75%, respectively, while lower concentrations of LL-37 lack an effect. **b** Treatment with 4 μM LL-37 for 1 and 3 days reduces the MG63

cell number by about 40%. A lower concentration (0.1 μM) of LL-37 has no effect on the cell number neither at 1 nor 3 days of treatment. Values are means ± SEM of 4–15 observations in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. controls.

manufacturer's instructions. The proportion of Annexin V-positive/negative and 7-AAD-positive/negative cells was determined by flow cytometry using an Accuri C6 flow cytometer (BD). Cells in early stages of apoptosis are Annexin V positive and 7-AAD negative, whereas cells in later stages of apoptosis and necrosis stain positive for both Annexin V and 7-AAD. Gates for Annexin V- and 7-AAD-positive cells were set using fluorescence minus one control. 20,000 events were recorded in each sample.

Measurement of Intracellular Ca^{2+} Concentration

For determination of intracellular Ca^{2+} concentration, the cells were cultured on glass-bottom cell culture Petri dishes (MatTek, Ashland, Mass., USA). The cells were washed with PBS, incubated with the Ca^{2+} -sensitive fluorescent dye Fluo-4-AM (3 μM, Invitrogen) for 40 min at room temperature, and then washed carefully. During the Ca^{2+} measurements the cells were incubated in an N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered salt solution containing 2.5 mM Ca^{2+} . This Ca^{2+} concentration is somewhat higher than the plasma concentration of Ca^{2+} in healthy adults. We used HEPES-buffered salt solution with 2.5 mM Ca^{2+} only in these acute experiments. The composition of the HEPES-buffered salt solution was (mM): NaCl 135.5, KCl 5.9, CaCl_2 2.5, MgCl_2 1.2, HEPES 11.6 and glucose 11.5. In some experiments measurement of Ca^{2+} was performed under Ca^{2+} -free conditions, i.e. in HEPES-buffered salt solution without CaCl_2 and with the addition of 2 mM EGTA (Sigma). The experiments were performed at room temperature. Fluorescence was recorded using a laser-scanning confocal microscope (LSM 510 PASCAL, Carl Zeiss AG, Göttingen, Germany). The excitation and emission wavelengths were 488 and 505 nm, respectively. The Ca^{2+} measurements were performed on subconfluent (80% confluence)

cells as an integrated signal from all cells (about 300 cells) within the visual field at $\times 100$ magnification. The confocal pinhole setting was kept identical for all experiments.

Drugs

LL-37 was purchased from Bachem AG (Bubendorf, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions. The L-type Ca^{2+} channel blocker nifedipine (Sigma), the thromboxane A_2 analogue U46619 (Tocris Bioscience, Bristol, UK) and the P2X_7 receptor antagonist AZ11645373 (Tocris) were dissolved in DMSO. The controls received vehicle, DMSO, as appropriate. The final concentration of DMSO was 0.1%.

Statistics

Values are presented as means ± SEM. Statistical significance was calculated using ANOVA and Student's two-tailed t test for unpaired comparisons with Bonferroni correction for post hoc analysis as appropriate. p values < 0.05 were regarded to denote statistical significance.

Results

LL-37 Reduces MG63 Cell Number and Cell Viability

Treatment with LL-37 for 24 h reduced the number of human MG63 osteoblasts by 40 and 75% at 4 and 8 μM, respectively (fig. 1a). Lower concentrations of LL-37 (0.1–2 μM) had no effect on the number of cells (fig. 1a). LL-37 caused a concentration-dependent decrease in the

Fig. 2. Staining with trypan blue, assessing cell viability, shows that treatment with 8 μM LL-37 for 24 h reduces the MG63 cell number and causes alteration of cell morphology characterized by shrinkage of the cells. Furthermore, nearly all LL-37-treated cells contain trypan blue indicating that these cells represent dead/dying cells. No trypan blue-positive cells were observed in response to a lower concentration (1 μM) of LL-37. Control cells show normal morphology with no trypan blue-positive cells. Bars represent 25 μm .

cell number with an IC_{50} value of about 5 μM . Treatment with 4 μM LL-37 for 1 and 3 days reduced the MG63 cell number by about 40% (fig. 1b). A lower concentration (0.1 μM) of LL-37 had no effect on the cell number neither at 1 nor 3 days of treatment (fig. 1b). Staining with trypan blue to assess cell viability showed that treatment with LL-37 (8 μM) for 24 h reduced the cell number and caused alteration of cell morphology characterized by shrinkage of the cells representing classical signs of apoptosis (fig. 2). Furthermore, almost all cells remaining after treatment with 8 μM LL-37 contained trypan blue, which indicates that they represent dead/dying cells (fig. 2). No trypan blue-positive cells were observed in response to a lower concentration (1 μM) of LL-37 (fig. 2). The positive trypan blue staining observed in response to 8 μM LL-37 in MG63 cells was also confirmed in another human osteoblast cell line, hFOB 1.19 cells (fig. 3), demonstrating that micromolar concentrations of LL-37 reduce cell viability in two different human osteoblast cell lines.

LL-37 Increases the Number of Annexin V-Positive Cells and Elevates Active Caspase 3 in MG63 Cells

Treatment with LL-37 (4 μM) for 6 h increased the proportion of Annexin V-positive MG63 cells by about 70% as demonstrated by flow cytometry (fig. 4a). The well-known proapoptotic agent staurosporine (0.5 μM) was included as a positive control [29]. Treatment with 0.5 μM staurosporine for 6 h increased the number of Annexin V-positive cells by about 9 times (fig. 4a). Staurosporine (0.5 μM) but not LL-37 (4 μM) increased the proportion of Annexin V-positive cells also at a shorter time point, i.e. 2 h (fig. 4a). Stimulation with LL-37 (4 μM) for 24 h increased the amount of active caspase 3 between 2 and 3 times in MG63 cells (fig. 4b). The positive control staurosporine (0.5 μM) increased active caspase 3 by about 2-fold (fig. 4b). Taken together these data show that LL-37 is proapoptotic for osteoblasts.

LL-37 Increases MG63 Intracellular Ca^{2+} Concentration

Defective cellular Ca^{2+} handling is thought to be involved in proapoptotic signaling [21], and therefore we assessed the effects of LL-37 on the intracellular Ca^{2+} concentration. Treatment with LL-37 (4 μM) caused an acute (within about 60 s) and sustained rise in intracellular Ca^{2+} concentration demonstrated by laser-scanning confocal microscopy of Fluo-4-AM-labeled MG63 cells incubated in Ca^{2+} -containing (2.5 mM) HEPES-buffered salt solution (fig. 5a, b). A lower concentration of LL-37 (0.4 μM) also elevated Ca^{2+} , but the Ca^{2+} response to 0.4 μM LL-37 was small compared to that of 4 μM (fig. 5b). The addition of vehicle control (0.1% DMSO) had no effect on Ca^{2+} (fig. 5a). LL-37 (0.4 and 4 μM) had no effect on intracellular Ca^{2+} concentration in Ca^{2+} -free solution (fig. 5c), suggesting that the LL-37-induced increase in Ca^{2+} depends on inflow of extracellular Ca^{2+} . Treatment with 100 nM and 1 μM of the L-type voltage-sensitive Ca^{2+} channel blocker nifedipine had no effect on the LL-37-induced Ca^{2+} response (4 μM LL-37), suggesting that LL-37 acts via another mechanism than by stimulation of Ca^{2+} inflow through L-type Ca^{2+} channels (fig. 6, 7a). The T-type Ca^{2+} channel blocker NiCl_2 (100 μM) had no effect on the LL-37-induced (4 μM) Ca^{2+} response (fig. 7b), suggesting that inflow of Ca^{2+} by LL-37 is mediated through another pathway than via T-type Ca^{2+} channels. Furthermore, inclusion of the P2X₇ receptor antagonist AZ11645373 (10 μM) had no impact on the rise in Ca^{2+} evoked by 4 μM LL-37 (fig. 7c). The thromboxane A₂ analogue U46619 was included as positive control causing a rapid and powerful rise in intracellular Ca^{2+} concentration (fig. 7d).

Fig. 3. Treatment with LL-37 (8 μ M) for 24 h causes accumulation of trypan blue in primary human osteoblast hFOB 1.19 cells. Control cells show no trypan blue-positive cells. Trypan blue-positive cells represent dead/dying cells. Bars represent 20 μ m.

Fig. 4. a Treatment with 4 μ M LL-37 for 6 h increases the proportion of apoptotic MG63 cells. Apoptosis was determined by flow-cytometric analysis of Annexin V-positive cells. Staurosporine (0.5 μ M) was included as positive control. **b** Treatment with 4 μ M LL-37 for 24 h increases MG63 cellular active caspase 3 (aCasp-3) level 2–3 times. The amount of aCasp-3 was determined by ELISA and normalized to total protein in each sample. Values are means $\pm$ SEM of 3–4 observations in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. controls. NS = Not significant.

LL-37 Reduces MG63 Cell Number Both in the Presence and Absence of Extracellular Ca^{2+}

Treatment with LL-37 (8 μ M) for 24 h reduced the number of MG63 cells by about 55% for cells cultured in DMEM culture medium containing 1.8 mM Ca^{2+} and by about 85% for cells cultured in Ca^{2+} -free DMEM culture medium (fig. 8a). In fact, the LL-37-induced reduction of the cell number was more powerful ($p < 0.01$) in the absence than in the presence of extracellular Ca^{2+} (fig. 8a). A lower concentration of LL-37 (4 μ M) reduced the cell number by about 15% in the presence ($86 \pm 2\%$ for LL-

37-treated cells vs. $100 \pm 5\%$ for control cells, $p < 0.05$, $n = 3$ in each group) and by about 35% in the absence ($66 \pm 7\%$ for LL-37-treated cells vs. $100 \pm 16\%$ for control cells, $p < 0.05$, $n = 3$ in each group) of extracellular Ca^{2+} . Omitting Ca^{2+} for 24 h reduced slightly, but not significantly, the number of cells as demonstrated when cell counts are plotted as absolute data (fig. 8b). The MG63 cells showed a similar morphology in the presence and absence of Ca^{2+} (fig. 8c). Treatment with LL-37 (4 μ M) for 24 h reduced the cell number by about 60% when the Ca^{2+} chelator agent EGTA (1.8 mM) was included in the Ca^{2+} -

Fig. 5. **a** Treatment with LL-37 (4 μM) causes an acute and sustained rise in intracellular Ca^{2+} concentration assessed by laser-scanning confocal microscopy of Fluo-4-AM-loaded MG63 cells incubated in Ca^{2+} containing (2.5 mM) HEPES-buffered salt solution. No treatment (left panel) represents the Ca^{2+} signal in the presence of vehicle control (0.1% DMSO). Addition of 0.1% DMSO has no effect on Ca^{2+} . The Ca^{2+} indicator Fluo-4-AM fluorescence is shown in red. **b** Line trace showing that both 0.4 and 4 μM LL-37 elevates Ca^{2+} . **c** LL-37 (0.4 and 4 μM) has no effect on intracellular Ca^{2+} concentration in Ca^{2+} -free solution. Ca^{2+} -free conditions were achieved by removing CaCl_2 from the HEPES-buffered salt solution and by inclusion of 2 mM EGTA. Each experiment was repeated at least twice.

Fig. 6. LL-37 (4 μM) increases MG63 intracellular Ca^{2+} concentration monitored by laser-scanning confocal microscopy of Fluo-4-AM-loaded cells in the presence of the L-type Ca^{2+} channel blocker nifedipine (100 nM). Nifedipine was included at the arrow and present throughout the experiment. This trace shows one representative experiment out of two.

containing DMEM culture medium in order to lower Ca^{2+} (fig. 9). LL-37 (4 μM) also reduced the cell number by about 60% when extra Ca^{2+} (final Ca^{2+} concentration 3.6 mM) was included in the EGTA-containing medium (fig. 9). For these experiments EGTA was administered in an equimolar concentration to extracellular Ca^{2+} (1.8 mM) in order to bind most of the Ca^{2+} ions. Thus, LL-37-induced lowering of the osteoblast cell number is observed both when the cells are cultured under Ca^{2+} -free conditions and when Ca^{2+} ions are absorbed with EGTA.

Discussion

In the present study, we demonstrate that the human antimicrobial peptide LL-37 induces apoptosis in human osteoblast-like MG63 cells and that this effect is associated with an elevated intracellular Ca^{2+} concentration. LL-37 causes, in the micromolar concentration range, an acute and sustained rise in intracellular Ca^{2+} in Ca^{2+} -containing but not in Ca^{2+} -free solution showing that the LL-37-induced increase in intracellular Ca^{2+} is due to an in-flow of Ca^{2+} along its gradient from the extracellular space to the cytosol. The increase in Ca^{2+} by LL-37 was much stronger at 4 than at 0.4 μM, suggesting a concen-

Fig. 7. Treatment with 1 μ M nifedipine (**a**), 100 μ M $NiCl_2$ (**b**) and 10 μ M AZ11645373 (**c**) has no effect on the LL-37-induced (4 μ M) Ca^{2+} response in MG63 cells. **d** The thromboxane A_2 analogue U46619 (10 μ M) was included as positive control causing a rapid and powerful rise in intracellular Ca^{2+} concentration. The intracel-

lular Ca^{2+} concentration was monitored by laser-scanning confocal microscopy of Fluo-4-AM-loaded MG63 cells. Nifedipine, $NiCl_2$ and AZ11645373 were included at the arrow and present throughout the experiment. Each experiment was repeated at least twice.

tration-dependent effect, although it is difficult to come to firm conclusions about the concentration dependence since we have investigated only two concentrations of LL-37. Membrane depolarization results in an inflow of Ca^{2+} from the extracellular space, causing a sustained and long-lasting increase in intracellular Ca^{2+} [30] similar to that observed in response to LL-37 in the present study. The pattern of LL-37-induced rise in intracellular Ca^{2+} concentration is thus compatible with inflow of Ca^{2+} from the extracellular space. Osteoblasts (MC3T3-E1 cells and MG63 cells) have been reported to express volt-

age-sensitive Ca^{2+} channels [31, 32]. Our data show that neither the L-type Ca^{2+} channel blocker nifedipine nor the T-type Ca^{2+} channel blocker $NiCl_2$ have an impact on the LL-37-evoked rise in Ca^{2+} in MG63 cells, suggesting that the inflow of Ca^{2+} triggered by LL-37 is through another mechanism than via voltage-sensitive L-type and T-type Ca^{2+} channels. We used relevant concentrations of nifedipine (0.1 and 1 μ M), which fully inhibit the inflow of Ca^{2+} via L-type Ca^{2+} channels in cultured vascular smooth muscle cells [30], and $NiCl_2$ (100 μ M) inhibiting T-type but not L-type Ca^{2+} channels [33]. Furthermore,

Fig. 8. **a** Treatment with 8 μM LL-37 for 24 h reduces the MG63 cell number by about 55% for cells cultured in DMEM culture medium containing 1.8 mM Ca^{2+} and by 85% for cells cultured in Ca^{2+} -free DMEM culture medium. **b** Omitting Ca^{2+} for 24 h reduces slightly, but not significantly, the number of cells as demonstrated when cell count data are plotted as absolute data. **a, b** Values are means $\pm$ SEM of 4–5 observations in each group. * $p < 0.05$, ** $p < 0.001$ vs. controls; ** $p < 0.01$ for LL-37-treated groups as indicated. **c** The MG63 cells show similar morphology in the presence and absence of Ca^{2+} . Bars represent 20 μm .

Fig. 9. Treatment with 4 μM LL-37 for 24 h reduces the cell number by about 60% for cells grown in culture medium (DMEM + 1.8 mM Ca^{2+}) containing either 1.8 mM EGTA alone or 1.8 mM EGTA in combination with an excess of Ca^{2+} (3.6 mM Ca^{2+}). Values are means $\pm$ SEM of 3 observations in each group. ** $p < 0.01$ vs. controls.

inclusion of the selective and highly potent P2X_7 receptor antagonist AZ11645373 [34] had no effect on the rise in Ca^{2+} evoked by LL-37, suggesting an alternative mechanism. Based on our findings presented here, we suggest that the LL-37-induced inflow of extracellular Ca^{2+} represents a novel signaling pathway for LL-37 that may involve LL-37-induced permeabilization of the cell membrane causing formation of transmembrane pores and/or that LL-37 acts as a detergent. These data implicate that LL-37 may work through a similar mechanism in human osteoblasts as in LL-37-induced permeabilization of the bacterial cell wall [3].

Here, we show for the first time that the antimicrobial peptide LL-37 reduces the human MG63 osteoblast cell number by promoting apoptosis. LL-37-induced apoptosis was demonstrated by both an enhanced proportion of Annexin V-positive cells and by an elevated active caspase 3 level in response to LL-37 treatment. Furthermore, LL-37-treated cells showed altered morphology such as cell shrinkage representing a classical sign of apoptosis. Previously, proapoptotic effects of LL-37 have been demonstrated in vascular smooth muscle cells, periodontal ligament cells, neutrophils, T cells and

airway epithelial cells [10–14]. We demonstrate a reduction of the osteoblast cell number by LL-37 in the micromolar concentration range, while no effect on the cell number is observed at lower concentrations of LL-37. We have previously shown that LL-37 reduces lipopolysaccharide-induced MCP-1 and IL-6 expression at low concentrations (0.1 and 1 μM) and induces apoptosis only at high ($>5 \mu\text{M}$) concentrations in human periodontal ligament cells [11]. LL-37-induced proapoptosis is thus observed in different cell systems but only in the micromolar concentration range. Very high levels of LL-37 have been demonstrated in lesional tissue from patients suffering from autoimmune diseases such as psoriasis, rosacea and ulcerative colitis [35–37]. For example, the median concentration of LL-37 is 304 μM in psoriatic skin lesions [35]. The LL-37 levels are elevated locally in chronic periodontitis; in fact, they are well within the micromolar concentration range [18], suggesting that LL-37-induced apoptosis of osteoblasts may have an impact on alveolar bone homeostasis in patients suffering from this disease. Thus, we may conclude that the LL-37-induced apoptosis of osteoblasts, observed in the micromolar concentration range in the present study, is relevant for the in vivo situation considering the very high levels of LL-37 observed in various autoimmune and inflammatory diseases.

Ca^{2+} governs many important cellular processes and is thought to be involved in apoptosis [21, 38]. A rise in intracellular Ca^{2+} may originate from intracellular stores such as the endoplasmic reticulum but also from the extracellular space [21]. Here, we show that LL-37 elevates intracellular Ca^{2+} concentration in human MG63 osteoblasts through stimulation of Ca^{2+} inflow. The LL-37-evoked inflow of Ca^{2+} seems not to be critically important for the LL-37-induced reduction of the MG63 cell number, since LL-37 lowers the cell number both in the presence and absence of Ca^{2+} in the cell culture medium and, furthermore, LL-37 causes a rise in intracellular Ca^{2+} but no apoptosis at a low concentration (0.4 μM). Ca^{2+} -independent apoptosis has been described in different experimental systems [39, 40]. Interestingly, the LL-37-evoked inward flow of Ca^{2+} , occurring independently of LL-37-induced apoptosis, may represent an important mechanism regulating Ca^{2+} -dependent cellular processes governed by LL-37. Thus, we demonstrate here that LL-37 alters human osteoblast Ca^{2+} handling and induces Ca^{2+} -independent apoptosis.

In summary, we show that LL-37 alters cellular Ca^{2+} homeostasis by causing stimulation of Ca^{2+} inflow

through a voltage-sensitive L- and T-type Ca^{2+} channel-independent mechanism in human osteoblasts. Furthermore, we demonstrate that LL-37 induces apoptosis of osteoblasts via a mechanism that is independent of Ca^{2+} .

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