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Human endogenous peptide p33 inhibits detrimental effects of LL-37 on osteoblast viability

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Background and Objective: High levels of the antimicrobial peptide, LL-37, are detected in gingival crevicular fluid from patients with chronic periodontitis. LL-37 not only shows antimicrobial activity but also affects host-cell viability. The objective of the present study was to identify endogenous mechanisms that antagonize the detrimental effects of LL-37 on osteoblast viability, focusing on the human peptide p33 expressed on the surface of various cell types.

Material and Methods: Human osteoblast-like MG63 cells and human hFOB1.19 osteoblasts were treated with or without LL-37 in the presence or absence of p33. Recombinant human p33 was expressed in an *Escherichia coli* expression system. Lactate dehydrogenase (LDH) was assessed using an enzymatic spectrophotometric assay. DNA synthesis was determined by measuring [^{3}H]-thymidine incorporation. Cell number was assessed by counting cells in a Bürker chamber. Intracellular Ca^{2+} was monitored by recording Fluo 4-AM fluorescence using a laser scanning confocal microscope. Cellular expression of p33 was determined by western blotting.

Results: LL-37 caused a concentration-dependent release of LDH from human osteoblasts, showing a half-maximal response value (EC_{50}) of 4 μM and a rapid and sustained rise in the intracellular Ca^{2+} concentration of osteoblasts, suggesting that LL-37 forms pores in the cell membrane. p33 (10 μM) inhibited the LL-37-induced LDH release and LL-37-evoked rise in intracellular Ca^{2+} concentration, suggesting that p33 prevents LL-37-induced permeabilization of the cell membrane. Moreover, p33 blocked LL-37-induced attenuation of osteoblast numbers. Also, mucin antagonized, at concentrations representative for nonstimulated whole saliva, LL-37-evoked LDH release, whilst cationic endogenous polyamines had no impact on LL-37-induced LDH release from osteoblasts.

Conclusions: The endogenous peptide p33 prevents LL-37-induced reduction of human osteoblast viability. Importantly, this mechanism may protect the osteoblasts from LL-37-induced cell damage in patients suffering from chronic periodontitis associated with high levels of LL-37 locally.

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The human cathelicidin LL-37 is an antimicrobial peptide that plays an important role in the innate immunity (1). LL-37 exerts its antimicrobial activity through neutralizing lipopolysaccharide but also via pore formation, thereby permeabilizing both gram-positive and gram-negative bacteria. Besides its antimicrobial

properties, LL-37 affects different cellular functions, such as phagocytosis (2), cell differentiation (3) and apoptosis (4). Interestingly, the effects of LL-37 on apoptosis seem to be cell-type dependent; LL-37 suppresses apoptosis in keratinocytes (5) and dermal fibroblasts (6), but promotes apoptosis in many cell types such as vascular smooth muscle cells (7), periodontal ligament cells (8) and T cells (9). LL-37, at micromolar concentrations, induces apoptosis and reduces cell viability also in human osteoblasts, as demonstrated by an increased level of caspase-3 and increased stimulation of annexin V expression (10).

Periodontitis is a progressive inflammatory disease that, in its end-stage, leads to loss of teeth through destruction of the alveolar bone. The osteoblasts represent an important cell type for maintaining the alveolar bone mass in both periodontal health and disease, and loss of these cells has a serious, negative impact on tooth attachment (11). Importantly, micromolar concentrations of LL-37 (i.e. concentrations relevant for LL-37-induced negative effects on human osteoblast viability) are demonstrated in the gingival crevicular fluid from patients with chronic periodontitis (12,13), suggesting that LL-37 may influence the progression of the disease by attenuating host-cell viability. Therefore, identifying endogenous factors and mechanisms that counteract the detrimental apoptotic effects of LL-37 on host cells is of great importance. The human endogenous peptide, p33, is expressed on the surface of various cell types, such as endothelial cells, neutrophils, lymphocytes and platelets, and is also referred to as globular C1q receptor (14,15). p33 was originally identified as a complement protein, C1q-binding protein, that prevents complement activation (16). Binding of p33 to antimicrobial peptides such as beta-defensin 3 and LL-37 neutralizes their antimicrobial activity and furthermore prevents antimicrobial peptide-induced endothelial cell and erythrocyte damage (17). Both p33 and mucin are strong anions that probably interact with the polycationic LL-37 through their negative charges. The effects of p33 and mucin on LL-37-induced cell-membrane permeabilization were assessed by measuring lactate dehydrogenase (LDH) release and the intracellular Ca^{2+} concentration. The LL-37-evoked rise in intracellular Ca^{2+} concentration reflects inflow of Ca^{2+} along its gradient from the extracellular space to the intracellular space (10). The objective of the present study was to investigate the impact of p33 on osteoblast viability in the presence of LL-37 and to assess the underlying mechanisms.

Here, we demonstrate that the endogenous peptide, p33, antagonizes LL-37-induced detrimental effects on human osteoblast viability. This mechanism may represent a new pathway that prevents LL-37-evoked osteoblast damage in patients with chronic periodontitis, a condition associated with high levels of LL-37 in the periodontium.

Material and methods

Cells

The human osteoblast-like cell line, MG63, and the human osteoblast cell line, hFOB1.19 (both from the American Type Culture Collection (ATCC), Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's minimum essential medium/Ham's F12 (1 : 1, vol/vol) cell culture medium (Life Technologies, Waltham, MA, USA) supplemented with antibiotics (50 U/mL of penicillin and 50 U/mL of streptomycin) and 10% fetal calf serum and kept at 37°C under 5% CO_2 in air in a water-jacketed cell incubator. Both MG63 cells and hFOB1.19 cells show osteoblast-like features, suggesting that they are indeed representative for native human osteoblasts (18,19). The cells were trypsinized (0.25% trypsin/EDTA, 1x, Life Technologies) and reseeded upon reaching confluence. Medium was exchanged every third day. Cells were used at passages 2–10. The cells responded identically to treatment with LL-37 and p33, irrespective of passage number. Experiments were performed on subconfluent cells (80% confluence).

Assessment of cell number and determination of DNA synthesis

After washing in phosphate-buffered saline (PBS), the cells were stained with Trypan Blue (Sigma Chemicals, St. Louis, MO, USA) and counted in a Bürker chamber, as appropriate. Cell morphology was assessed using a Nikon TMS microscope equipped with a digital camera (Pixelink Nikon; Nikon Nordic AB, Solna, Sweden). DNA synthesis was determined by measuring incorporation of [^{3}H]-thymidine into newly synthesized DNA, as previously described by Jönsson *et al.* (20). Briefly, [^{3}H]-thymidine (1 μCi) was included during the last 2 h of the 72-h incubation period. At the end of the 72 h of incubation, the cells were washed in PBS, trypsinized, centrifuged and sonicated in 5 mM NaOH. After centrifugation and washing with 0.5 M trichloroacetic acid, the pellet was dissolved in soluene. Liquid scintillation cocktail was added and the radioactivity measured in a liquid scintillation counter (Beckman, Brea, CA, USA). Radioactivity was expressed as disintegrations per minute and normalized to the concentration of total cellular protein in each sample. Protein concentration was measured using a protein assay kit (Bio-Rad, Hercules, CA, USA).

Measurement of cellular release of LDH

Release of LDH into the cell culture medium from MG63 and hFOB1.19 cells grown in 96-well plates was determined enzymatically, as decribed by Nagant *et al.* (21), by measuring the absorbance in a Multiscan GO Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA). LDH release in response to LL-37 was normalized to total release of LDH obtained by sonication of the cells for 2×10 s.

Determination of intracellular Ca^{2+} concentration

For determination of intracellular Ca^{2+} concentrations, the cells were cultured on glass-bottom cell-culture

Petri dishes (MatTek, Ashland, MA, USA). The cells were washed with a HEPES-buffered physiological salt solution, incubated with the Ca^{2+}-sensitive fluorescent dye, Fluo 4-AM (3 μM, Invitrogen, Carlsbad, CA, USA), for 40 min at room temperature and then washed carefully in HEPES buffer. During the measurement of Ca^{2+}, the cells were incubated in HEPES buffer containing 2.5 mM Ca^{2+}. The experiments were performed at room temperature. Fluorescence was recorded using a laser scanning confocal microscope (LSM 5 PASCAL; Carl Zeiss AG, Jena, Germany). The excitation and emission wavelengths were 488 and 505 nm, respectively. The Ca^{2+} measurements were performed on subconfluent cells as an integrated signal from all cells within the visual field at × 100 magnification. The confocal pinhole setting was kept identical for all experiments.

Purification of recombinant MBP-p33

Recombinant maltose-binding protein (MBP)-p33 was expressed using the pMAL-c2 expression vector (New England Biolabs, Ipswich, MA, USA) and *Escherichia coli* XL1-Blue strain (Stratagene), as previously described (14). Briefly, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to an exponentially growing overnight culture of bacteria to a final concentration of 0.3 mM. Bacteria were harvested after 2 h and the pellet was resuspended in MBP buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4). Bacteria were kept on ice and lysed by sonication. Lysed cells were pelleted and the supernatant was removed and transferred (1 mL/min) to an amylose resin column pre-equilibrated with MBP buffer. After adding the bacterial supernatant, the column was rinsed with 12 column volumes of MBP buffer, then with four column volumes of 10 mM Tris–HCl, pH 7.4 (Tris buffer). Bound MBP-p33 was eluted and fractionated with 10 mM Tris–HCl containing 10 mM maltose. The MBP-p33 was administered to the cells at concentrations from 1 to 20 μM.

Western blotting

Proteins were separated by SDS-PAGE (10% polyacrylamide) then transfererd to a poly(vinylidene difluoride) (PVDF) membrane (Amersham Biosciences, Little Chalfont, UK). The membrane was blocked for 30 min with PBS/0.1% Tween (PBST) containing 5% milk powder (blocking buffer) and incubated for 60 min with mouse anti-p33 (60.11, MMS-606R, Nordic BioSite AB, Täby, Sweden) diluted 1 : 1000 in blocking buffer. The membrane was washed three times in PBST, followed by incubation with a peroxidase-labeled secondary antibody (goat anti-mouse IgG, 1 : 2500 dilution; Bio-Rad Laboratories) in blocking buffer for 60 min at room temperature. The membranes were washed three times in PBST and developed using a SuperSignal West Pico Chemiluminescence kit (Thermo Scientific), according to the manufac-

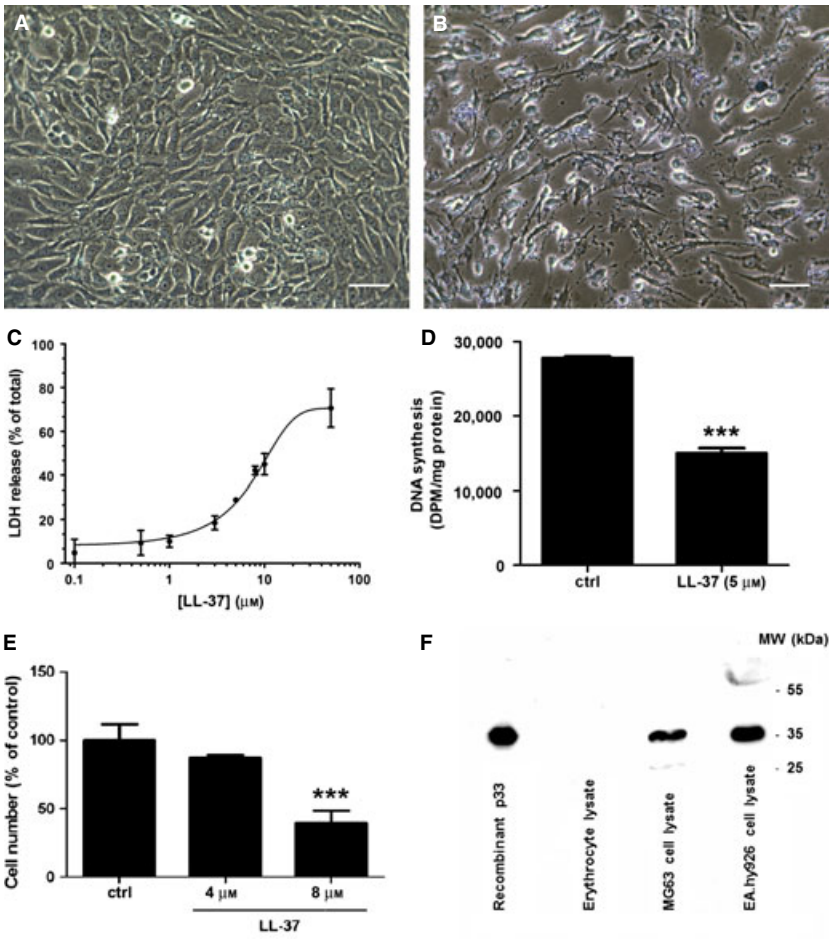

Fig. 1. (B) Phase contrast microscopy image showing MG63 cell shrinkage and membrane blebbing after treatment with 4 μM LL-37 for 24 h. These morphological changes are characteristic for apoptosis. (A) Treatment with vehicle control (0.1% dimethylsulfoxide) has no effect on cell morphology. Panels A and B: bar = 20 μM. (C) Treatment with LL-37 for 30 min stimulates release of lactate dehydrogenase (LDH) from MG63 cells in a concentration-dependent manner. (D) Treatment with 5 μM LL-37 for 3 days reduces MG63 cell DNA synthesis by about 50% and (E) treatment with 8 μM LL-37 attenuates MG63 cell number by about 50%. (F) MG63 cells express p33, as demonstrated by western blotting. Erythocyte lysate and EA.hy926 endothelial cell lysate are included as negative and positive controls, respectively. Summarized data are presented as mean ± standard error of the mean of three to nine observations in each group. ***$p < 0.001$ vs. control (ctrl). d.p.m., disintegrations per minute; MW, molecular weight.

turer's instructions. Cleaved p33 was included as a positive control (17).

p33 small interfering RNA

The MG63 cells were treated for 72 h with p33 small interfering RNA (siRNA) (Hs_C1QBP_6) or noncoding scramble control (All Stars Negative Control) purchased from Qiagen (Valencia, CA, USA) and oligofectamine transfection reagent (Life Technologies). The final concentration of siRNA and scramble control was 20 nM, as recommended by Qiagen. After transfection, the cells were allowed to equilibrate for 24 h before challenge with LL-37. Down-regulation of p33 transcript and protein by the p33 siRNA was confirmed by quantitative real-time PCR and western blotting, respectively. Total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen). The RNA samples were subjected to one-step quantitative real-time PCR measurements using the QuantiFast SYBR Green RT-PCR kit (Qiagen) and QuantiTect primer assays (Qiagen) on a Step One Plus real-time thermal cycler (Applied Biosystems, Carlsbad, CA, USA). Gene expression was calculated using the delta CT method applying glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the housekeeping reference gene (22). Each sample was analyzed in duplicate. The PCR primers (QuantiTect primer assays) for p33 (Hs_C1QBP_1_SG) and GAPDH (Hs_GAPDH_2_SG) were purchased from Qiagen.

Agents

LL-37 was purchased from Bachem AG and dissolved in dimethylsulfoxide according to the manufacturer's instructions. Mucin from bovine submaxillary gland, spermidine and poly-L-lysine (molecular mass 4000–15,000 Da) were purchased from Sigma Chemicals and dissolved in PBS. Controls received PBS and/or dimethylsulfoxide (0.1%) as vehicle, as appropriate.

Statistics

Summarized data are presented as mean ± standard error of the mean. All experiments were performed at least twice. Statistical significance was calculated using ANOVA and the Student's two-tailed *t*-test for unpaired comparisons with Dunnett's test for *post-hoc* analysis as appropriate. $p < 0.05$ was regarded as being statistically significant.

Results

LL-37 induces LDH release and reduces DNA synthesis and cell number in MG63 cells

Treatment with 4 μM LL-37 for 24 h altered MG63 cell morphology (Fig. 1A and 1B). The MG63 cells treated with LL-37 were characterized by shrinkage of the cells and membrane blebbing representative for apoptosis (Fig. 1A and 1B). Stimulation with LL-37 for 30 min caused a concentration-dependent release of LDH from MG63 cells (Fig. 1C). The half-maximal response value (EC_{50}) for LL-37-stimulated LDH release was 4 μM (Fig. 1C). Treatment with 5 μM LL-37 for 72 h reduced MG63 cell DNA synthesis by about 50% (Fig. 1D), and furthermore, 8 μM LL-37 reduced the number of MG63 cells by about 50% (Fig. 1E). Moreover, the MG63 cells expressed p33, as demonstrated by western blotting (Fig. 1F). In order to investigate the reversibility of LL-37-induced LDH release from MG63 cells, we assessed LDH release at different time-points after removing 4 μM LL-37. As demonstrated in Fig. 2, LDH release evoked by treatment with LL-37 for 30 min had decreased to the control level 5 min after removal of LL-37, showing that the effect of LL-37 is reversible. In fact, LDH release increased slightly with time from the sudden decrease after removal of LL-37 until reaching that of untreated controls (Fig. 2).

p33 antagonizes LL-37-evoked stimulation of LDH release and attenuation of MG63 cell number

Treatment with 8 μM LL-37 for 24 h reduced the number of MG63 cells by about 50% (Fig. 3). LL-37-induced attenuation of cell number was completely reversed by 10 μM human

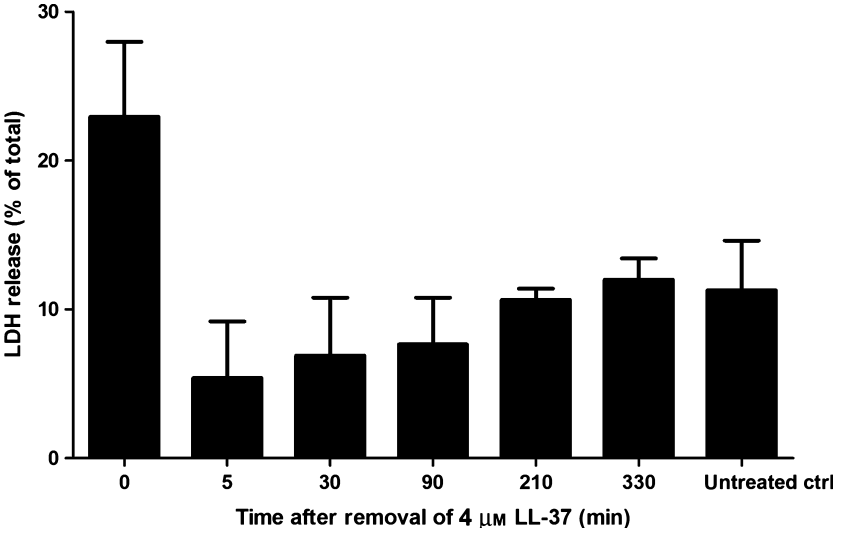

Fig. 2. MG63 cells were stimulated with 4 μM LL-37 for 30 min, then the LL-37 was removed, the cells were washed carefully with phosphate-buffered saline (PBS) and lactate dehydrogenase (LDH) release was measured at specific time points 5–330 min after the removal of LL-37. Five minutes after the removal of LL-37, LL-37-evoked (4 μM) LDH release from MG63 cells had decreased to a value lower than the control value. Thereafter, LDH release showed a slight increase with time after the removal of LL-37 until reaching that of the untreated control. Values are presented as the mean ± standard error of the mean of four observations in each group.

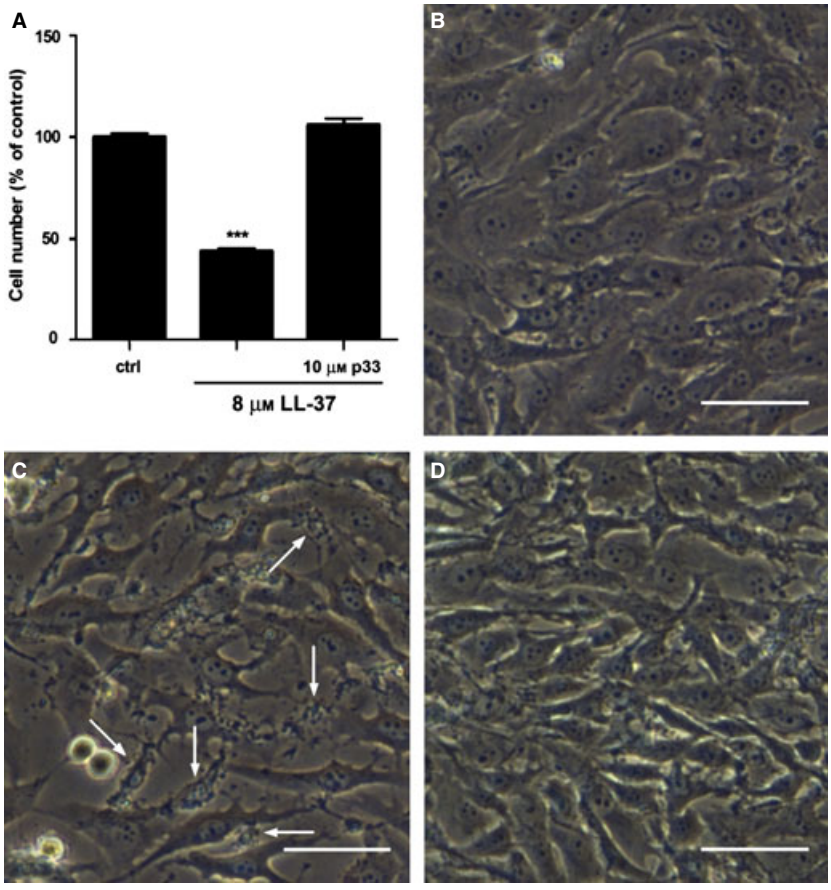

Fig. 3. (A) Reduction of MG63 cell number after 24 h of treatment with 8 μM LL-37 is completely reversed by 10 μM p33. (B) MG63 cells treated with vehicle control (0.1% dimethylsulfoxide), (C) MG63 cells treated with 8 μM LL-37 and (D) MG63 cells treated with 8 μM LL-37 + 10 μM p33. Cells treated with LL-37 alone (C) display shrinkage and membrane blebbing representative for apoptosis (arrows). Panels B–D: bar = 20 μm. Summarized data are presented as mean ± standard error of the mean of four observations in each group. ***$p < 0.001$ vs. control.

endogenous peptide p33 (Fig. 3). The p33 peptide attenuated LL-37-induced LDH release from MG63 cells (Fig. 4A). LDH release by 4 μM LL-37 was completely reversed by 10 μM p33 (Fig. 4A). p33 (4 and 10 μM) also attenuated LL-37-evoked (4 μM) LDH release in human hFOB1.19 osteoblasts (Fig. 5). Decreased LL-37 antibacterial activity has been observed in the presence of mucin, as demonstrated by Bucki *et al.* (23), suggesting that mucin also affects LL-37-induced attenuation of host cell viability. Therefore, we investigated the effects of mucin on LL-37-induced LDH release in MG-63 cells. Mucin (0.1–5 mg/mL) attenuated 4 μM LL-37-evoked LDH release in a concentration-dependent manner (Fig. 4B). The cationic LL-37 (which contains six positively charged amino acids) interacts with negatively charged membrane lipids (1). We hypothesized that other polycationic substances may compete with LL-37 for these binding sites in the cell membrane, preventing LL-37-induced LDH release. Therefore, we assessed the effects of the endogenous polyamine spermidine (which has three positive charges) and poly-L-lysine (which has > 20 positive charges), representing two other polycationic substances, on LL-37-induced LDH release in MG63 cells. Neither spermidine (50–1000 μM) nor poly-L-lysine (1 and 5 μM) influenced the LDH release evoked by stimulation with 4 μM LL-37 (Fig. 4C), even though the number of positive charges available from 50–1000 μM spermidine and 1 and 5 μM poly-L-lysine was in great excess compared with the number from 4 μM LL-37.

p33 inhibits the LL-37-evoked increase in MG63 intracellular Ca^{2+} concentration

LL-37 (4 μM) caused a rapid and sustained rise in intracellular Ca^{2+} concentration, as demonstrated by laser-scanning confocal microscopy of Fluo 4-AM-labeled MG63 cells incubated in HEPES buffer containing 2.5 mM Ca^{2+} (Fig. 6). The LL-37-induced Ca^{2+} response was delayed and strongly reduced in the presence of 10 and 20 μM p33, suggesting that p33 blocks LL-37-induced inflow of extracellular Ca^{2+} (Fig. 6).

Down-regulation of p33 by siRNA has no impact on LL-37-induced LDH release in MG63 cells

Treatment with p33 siRNA reduced the expression of *p33* mRNA and p33 protein by 90% and 60%, respectively, in MG63 cells (Fig. 7A and 7B). The down-regulation of p33 had no effect either on LL-37-induced attenuation of MG63 cell number or on LL-37-evoked LDH release (Fig. 7C and 7D).

Discussion

Here, we show, by different techniques and read-outs, that the human endogenous peptide p33 reverses the negative effects of LL-37 on human osteoblast viability. p33 antagonizes LL-37-induced release of LDH and reverses LL-37-evoked reduction in cell number. Furthermore, p33 inhibits the LL-37-induced rise in intracellular Ca^{2+} concentration. LL-37 is thought to reduce host-cell viability and induce apoptosis through permeabilization of the cell membrane followed by lysis of the cell (i.e. via a mechanism similar to that observed in LL-37-induced permeabilization and lysis of bacteria) (1). We demonstrate that p33 prevents LL-37-induced release of LDH and inflow of Ca^{2+},

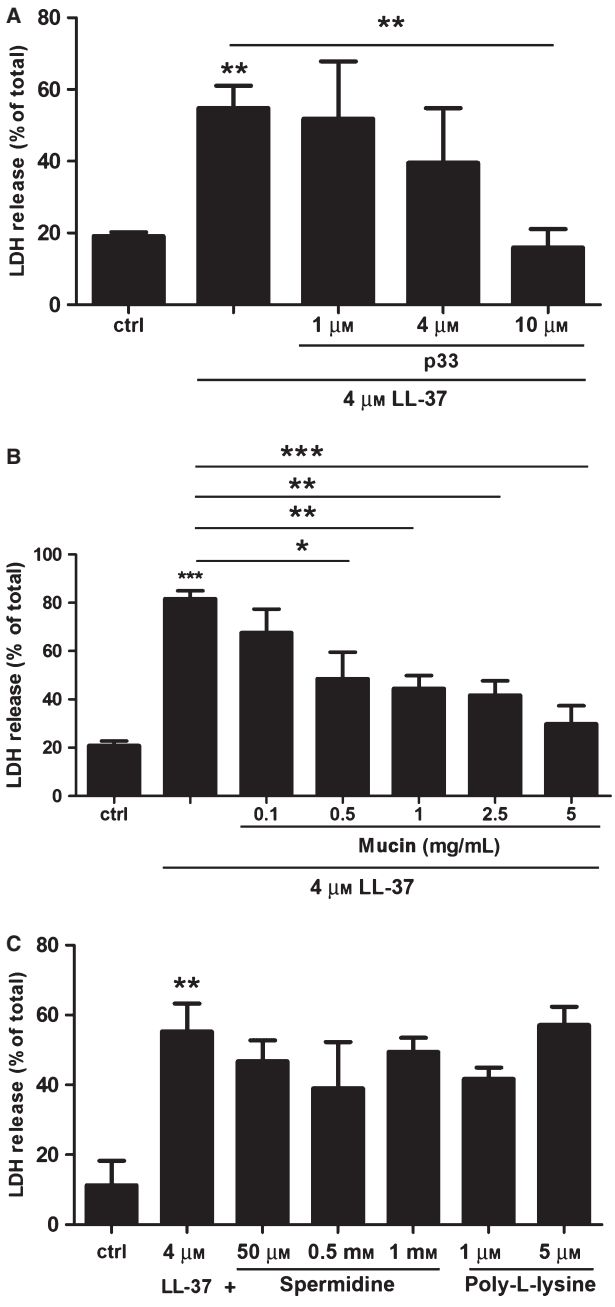

Fig. 4. MG63 cells were preincubated for 60 min with 4 μM LL-37, and lactate dehydrogenase (LDH) release was subsequently assessed in a 30-min time period in the presence of LL-37 only or in the presence of LL-37 together with different concentrations of (A) p33, (B) mucin and (C) spermidine and poly-L-lysine. (A) p33 and (B) mucin inhibit LL-37-induced LDH release from MG63 cells in a concentration-dependent manner. (C) The polycations spermidine and poly-L-lysine have no effect on LL-37-evoked LDH release from MG63 cells. Values are presented as mean ± standard error of the mean of three to four observations in each group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

suggesting that p33 inhibits LL-37-evoked permeabilization of the cell membrane. Although p33 completely abolishes both LL-37-induced LDH release and attenuation of cell number, a small, LL-37-evoked intracellular Ca^{2+} response is still observed in the presence of p33, probably indicating that some small pores, allowing Ca^{2+} influx along its concentration gradient from the extracellular to the intracellular space, remain in the plasma membrane of cells treated with the combination of LL-37 and p33. Importantly, p33 strongly reduces and delays the Ca^{2+} response to LL-37, suggesting that p33, at least partially, prevents LL-37-induced pore formation. The concentration of p33 needed to achieve complete inhibition of 4 μM LL-37-induced cell-damage effect is 10 μM (i.e. 2.5 times higher than the concentration of LL-37), indicating that p33 is a potent inhibitor of LL-37. Exogenous LL-37 exerts a harmful effect on MG63 cells, although these cells express endogenous p33, as demonstrated by western blotting. The lack of protection by the endogenous p33 may be caused by the unfavorable cellular localization of p33 and/or that the cellular concentration of p33 is too low to antagonize micromolar concentrations of LL-37.

We show that down-regulation of expression of endogenous MG63 cell *p33* mRNA and protein by p33 siRNA does not affect LL-37-evoked LDH release or an LL-37-induced decrease in cell number (i.e. the sensitivity of the MG63 cells to treatment with LL-37 is not influenced by lowering endogenous p33 expression), suggesting that p33 affects LL-37 signaling at the outside of the cell membrane rather than from the intracellular side. Probably, negatively charged p33 binds the polycationic LL-37 at the extracellular space and/or at the outer surface of the cell membrane, inhibiting membrane permeabilization by LL-37, disclosed by p33-induced inhibition of the LL-37-evoked rise in intracellular Ca^{2+} concentration. The LL-37-evoked pore formation has previously been reported to be associated with the well-known LL-37-induced antimicrobial effect (1). Importantly, it has been stated that the cytotoxic concentration of LL-37 for mammalian cells is three times higher than that needed to kill bacteria, suggesting that LL-37 reduces bacterial viability through a selective mechanism (24).

The presence of LL-37 in the gingival crevicular fluid of patients with chronic periodontitis, and the high expression level of *LL-37* mRNA in

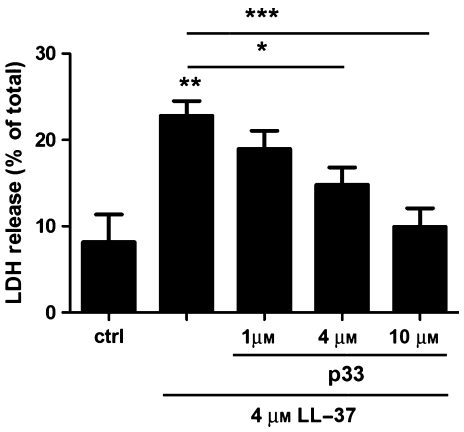

Fig. 5. p33 inhibits LL-37-induced lactate dehydrogenase (LDH) release from human hFOB1.19 osteoblasts in a concentration-dependent manner. The hFOB1.19 osteoblasts were preincubated for 60 min with 4 μM LL-37 in the presence or absence of p33, and then the LDH release was assessed during a 30-min time period in the continuous presence of LL-37, with or without addition of p33. Values are presented as mean ± standard error of the mean of six observations in each group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

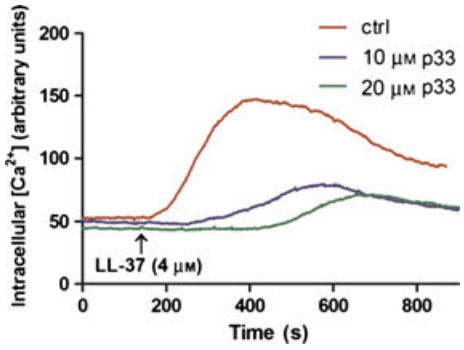

Fig. 6. Treatment with 4 μM LL-37 causes an acute and sustained rise in the intracellular Ca^{2+} concentration, as demonstrated by laser-scanning confocal microscopy of Fluo 4-AM-labeled MG63 cells incubated in HEPES-buffered solution containing 2.5 mM Ca^{2+}. In contrast, 10 μM p33 delays and reduces the LL-37-induced Ca^{2+} response, and 20 μM p33 inhibits the LL-37-evoked Ca^{2+} response more strongly compared with 10 μM p33. The cells were preincubated with p33 for 20 min and p33 was retained in the culture medium for the duration of the experiment. LL-37 was added (arrow) and was also retained in the culture medium for the duration of the experiment. These traces show one representative experiment out of three.

gingival tissue samples from such patients, suggest that LL -37 may influence the progression of the disease by attenuating host cell viability (12,13,25). However, down-regulation of *LL-37* mRNA has been reported in gingival tissue samples of patients with generalized aggressive periodontitis, representing a confounding association between weak expression of *LL-37* mRNA and rapid destruction of periodontal tissues in generalized aggressive periodontitis (25). In the present study performed on cultured cells, we show that the endogenous peptide p33 reduces the negative effects of LL-37 on osteoblast viability. Importantly, future studies on the impact of p33 and LL-37 on bone homeostasis are needed to clarify the association between these endogenous peptides and periodontal disease in more complex experimental systems, such as animal models of periodontal disease.

We demonstrate that not only p33 but also mucin inhibits LL-37-induced LDH release from osteoblasts, suggesting that mucin represents another endogenous protein, besides p33, that antagonizes the detrimental effects of LL-37 on osteoblast viability. Both p33 and mucin are strong anions that probably interact with the polycationic LL-37 through electrostatic interactions. Thus, both p33 and mucin probably interact with LL-37 through their negative charges, inhibiting the biological activity of LL-37. The cationic LL-37 molecule carries six positive charges and binds to negatively charged membrane lipids, thereby showing membrane selectivity (1), suggesting that other polycationic substances may compete with LL-37 for its negatively charged binding sites in the cell membrane. However, we were unable to reduce LL-37-induced LDH release from osteoblasts by inclusion of polycationic polyamines and poly-L-lysine carrying a surplus positive charge, suggesting that LL-37 permeabilizes the osteoblast cell membrane also in the presence of a high number of positive charges.

Mucins are highly glycosylated, high-molecular-weight gel-forming proteins secreted onto the mucosal surface of the intestinal tract and airways and into the primary saliva. Previously, mucin has been shown to inhibit the antibacterial effects of LL-37 (23), and here we show that mucin prevents LDH release from osteoblasts. Thus, mucin seems to antagonize the effects of LL-37 in both eukaryotic and prokaryotic systems. Importantly, mucin inhibits LL-37-induced LDH release in osteoblasts at concentrations representative of those in unstimulated whole saliva from healthy subjects (26), suggesting that the inhibitory effect of mucin on LL-37-evoked LDH release observed here is relevant for the *in-vivo* situation. In unstimulated whole saliva from subjects with severe periodontitis, the mucin concentration is even higher than in that of healthy subjects (26), indicating that mucin modulates LL-37-evoked effects on host-cell viability in patients with severe periodontal disease.

In summary, we demonstrate that the endogenous peptide, p33, antago-

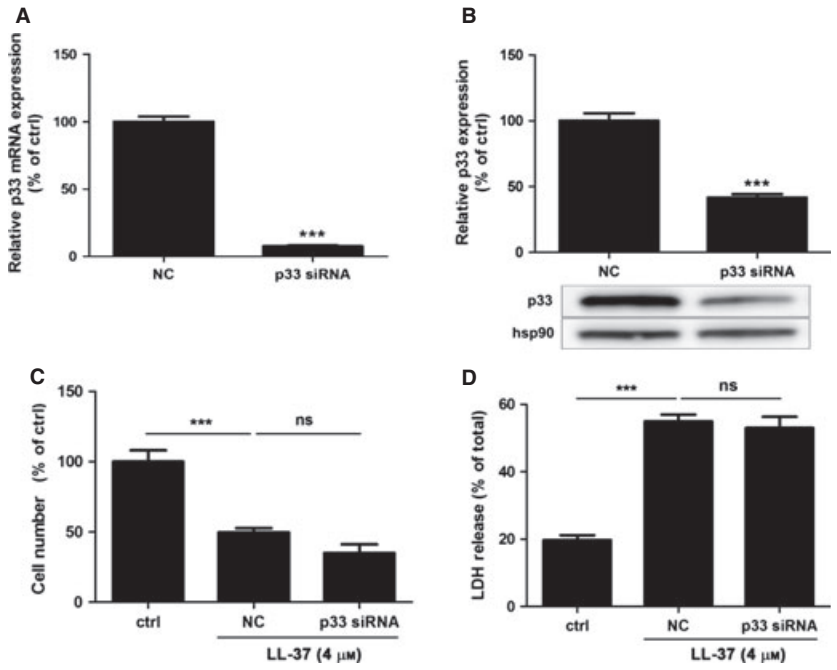

Fig. 7. p33 small interfering RNA (siRNA) reduces (A) *p33* mRNA, analyzed by quantitative real-time PCR, by about 90% and (B) p33 protein expression, analyzed by western blotting, by about 60%, in MG63 cells. Down-regulation of p33 has no impact on either (C) LL-37-induced attenuation of cell number or (D) LL-37-evoked lactate dehydrogenase (LDH) release in MG63 cells. For western blotting, the absorbance of the p33 immunoreactive band was normalized to that of the housekeeping reference protein, heat shock protein 90 (hsp90), run in parallel for each lane. Values are presented as mean ± standard error of the mean of six observations in each group. Controls were treated with a noncoding scramble construct (NC). ns, not statistically significant. ***$p < 0.001$.

nizes LL-37-induced detrimental effects on human osteoblast viability. p33 probably acts by preventing LL-37-induced permeabilization of the cell membrane. This mechanism may represent a new protective pathway that opposes LL-37-evoked osteoblast damage in patients with chronic periodontitis, a condition associated with high levels of LL-37 in the periodontium.

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Conflict of interests

None declared.

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