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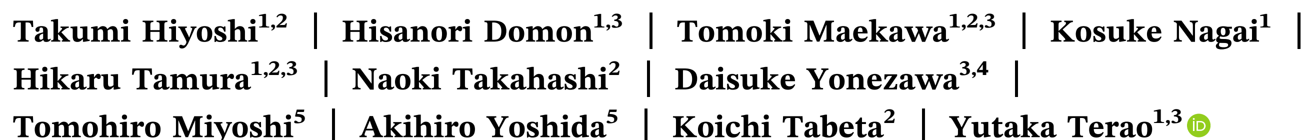
Aggregatibacter actinomycetemcomitans induces detachment and death of human gingival epithelial cells and fibroblasts via elastase release following leukotoxin-dependent neutrophil lysis



Takumi Hiyoshi[1,2] | Hisanori Domon[1,3] | Tomoki Maekawa[1,2,3] | Kosuke Nagai[1] | Hikaru Tamura[1,2,3] | Naoki Takahashi[2] | Daisuke Yonezawa[3,4] | Tomohiro Miyoshi[5] | Akihiro Yoshida[5] | Koichi Tabeta[2] | Yutaka Terao[1,3]

[1]Division of Microbiology and Infectious Diseases, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

[2]Division of Periodontology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

[3]Research Center for Advanced Oral Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

[4]Division of Oral Science for Health Promotion, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

[5]Department of Oral Microbiology, Matsumoto Dental University, Shiojiri, Japan

Correspondence
Yutaka Terao, Division of Microbiology and Infectious Diseases, Niigata University Graduate School of Medical and Dental Sciences, 2-5274, Gakkocho-dori, Chuo-ku, Niigata-shi, Niigata, 951-8514, Japan.
Email: terao@dent.niigata-u.ac.jp

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Abstract

Aggregatibacter actinomycetemcomitans is considered to be associated with periodontitis. Leukotoxin (LtxA), which destroys leukocytes in humans, is one of this bacterium's major virulence factors. Amounts of neutrophil elastase (NE), which is normally localized in the cytoplasm of neutrophils, are reportedly increased in the saliva of patients with periodontitis. However, the mechanism by which NE is released from human neutrophils and the role of NE in periodontitis is unclear. In the present study, it was hypothesized that LtxA induces NE release from human neutrophils, which subsequently causes the breakdown of periodontal tissues. LtxA-treatment did not induce significant cytotoxicity against human gingival epithelial cells (HGECs) or human gingival fibroblasts (HGFs). However, it did induce significant cytotoxicity against human neutrophils, leading to NE release. Furthermore, NE and the supernatant from LtxA-treated human neutrophils induced detachment and death of HGECs and HGFs, these effects being inhibited by administration of an NE inhibitor, sivelestat. The present results suggest that LtxA mediates human neutrophil lysis and induces the subsequent release of NE, which eventually results in detachment and death of HGECs and HGFs. Thus, LtxA-induced release of NE could cause breakdown of periodontal tissue and thereby exacerbate periodontitis.

KEYWORDS
Aggregatibacter actinomycetemcomitans, leukotoxin, neutrophil elastase, periodontitis

Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; DIC, differential interference contrast; HGEC, human gingival epithelial cell; HGF, human gingival fibroblast; LDH, lactate dehydrogenase; LFA-1, leukocyte function antigen-1; LtxA, leukotoxin; NE, neutrophil elastase; PMA, phorbol myristate acetate; TX100, Triton X-100.

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1 | INTRODUCTION

Aggregatibacter actinomycetemcomitans is a gram-negative, facultatively anaerobic, non-motile, and non-sporing bacterium. It has been shown that *A. actinomycetemcomitans* is associated with periodontitis, which often causes rapid bone and tissue destruction and, ultimately, loss of teeth.[1] *A. actinomycetemcomitans* produces several virulence factors, including adherence proteins, biofilm polysaccharides, LPS, cytolethal distending toxin and LtxA.[2] The primary role of LtxA is avoidance of the immune system. LtxA specifically targets LFA-1 on human leukocytes, causing cell death.[3] Leukocytes infiltrating periodontal pockets in response to periodontal disease are killed by LtxA.[4] However, few studies have found that LtxA induces cytotoxicity against human periodontal tissue. Thus, LtxA has the potential to injure periodontal tissues indirectly, the mechanisms currently being unknown.

Neutrophils are the main type of immune cell in adult human blood and the primary mediators of inflammatory responses against invading microorganisms.[5] Circulating neutrophils migrate to peripheral tissues, such as the skin, gut, lungs and periodontal tissue, in response to infection and/or inflammation.[6,7] Indeed, it has previously been reported that the majority of leukocytes recruited into periodontal pockets are neutrophils.[8] Neutrophils bind to and ingest invading microorganisms at sites of infection through a process known as phagocytosis, which is followed by sequential execution of microbicidal processes. The antimicrobial activity of neutrophils is mediated by granules containing antimicrobial peptides, proteins and serine protease.[9]

Neutrophil serine proteases, including NE, are intracellular enzymes that play roles in inflammation, immune responses and coagulation, thus acting as a host defense mechanism.[10–12] Intracellular NE reported directly kills microbes and inactivates bacterial toxins in neutrophils.[13] In experimental gingivitis, there are high NE concentrations in the gingival crevicular fluid and removal of dental plaque decreases these concentrations.[14] Extracellular NE can work as a host defensive and tissue destructive factor.[15] In a previous study, we found that NE release from neutrophils induces alveolar epithelial cell death and disruption of pulmonary immune defense, thereby causing tissue injury.[16,17] Altman *et al.* have also reported that supernatant from PMA-treated neutrophils causes cytotoxicity and detachment of HGECs.[18] In addition, long-term observation of adults with periodontitis undergoing supportive periodontal therapy has revealed a positive correlation between NE in gingival crevicular fluid and clinical detachment.[19] However, the underlying mechanisms by which neutrophils release NE in response to periodontopathic bacteria remain elusive.

In this study, we hypothesized that LtxA induces neutrophil cell death and subsequent release of NE, which eventually damages the periodontal tissue. To investigate our hypothesis, we examined the cytotoxicity of LtxA against primary isolated human neutrophils and investigated whether LtxA induces NE release. In addition, we evaluated the cytotoxicity of NE and supernatant from LtxA-treated human neutrophils against HGECs and HGFs.

2 | MATERIAL AND METHODS

2.1 | Cell lines and growth conditions

HGEC line Ca9-22 (JCRB0625) was obtained from Riken Cell Bank (Ibaraki, Japan). Primary HGFs (ATCC PCS-201-018) were obtained from Summit Pharmaceuticals International (Tokyo, Japan). HGECs and HGFs were grown in minimum essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan), respectively. Both media were supplemented with 10% FBS (Japan Bio Serum, Hiroshima, Japan), 100 U/mL penicillin and 100 μg/mL streptomycin (Wako Pure Chemical Industries). All cells were incubated at 37 °C in the presence of 95% air and 5% CO_2.

2.2 | Purification of LtxA

A. actinomycetemcomitans strain HK1651 was cultured anaerobically in 2x YT medium for 24 hours. Ammonium sulfate solution was added to the supernatant to purify proteins. The pellets were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl and dialyzed using a buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM NaCl and 0.2 mM $CaCl_2$. The dialyzed samples were then applied to a Sephadex G-200 column (General Electric, Boston, MA) pre-equilibrated with a buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl and 0.2 mM $CaCl_2$. The protein fraction (LtxA) was concentrated using Amicon Ultra (Merck, Kenilworth, NJ). There was 4 ng of LPS in 1 μg of LtxA, as detected with an LPS-detection kit (GenScript, Piscataway Township, NJ).

2.3 | Isolation of human neutrophils

Heparinized blood was obtained from four healthy donors and neutrophils isolated by centrifugation of whole blood in the presence of Polymorphprep (Axis Shield, Dundee, UK) according to the manufacturer's instructions. Briefly, whole blood was layered onto

Polymorphprep in a ratio of 1:1 and centrifuged at 500 *g* for 30 mins. Layers containing neutrophils were carefully collected and residual red blood cells lysed by ACK Lysing Buffer (Lonza, Basel, Switzerland). Human neutrophils were counted using a hemocytometer and used for subsequent experiments. The experimental protocol was approved by the Institutional Review Board of Niigata University and the experiments were carried out in accordance with the approved guidelines. Informed consent was obtained from all donors prior to their inclusion in this study (permit # 25-R9-07-13).

2.4 | Cytotoxicity assay

Human neutrophils (2×10^5 cells/100 μL), HGECs (1×10^5 cells/100 μL) and HGFs (5×10^4 cells/100 μL) were cultured in a suitable culture medium, as indicated above. For DIC image analysis, cells seeded onto glass bottom plates were treated with either LtxA (50 fg/cell) or NE (100 mU/mL; Innovative Research, Novi, MI, USA), diluted by the appropriate culture medium, for 3 hrs. The resultant samples were examined under a confocal laser-scanning microscope (Carl Zeiss, Jena, Germany). For the LDH-cytotoxicity assay, cells seeded onto a 96-well plate (Becton Dickinson, Franklin Lakes, NJ) were treated with various concentrations of LtxA (25–500 fg/cell) or LPS (0.4 ng/mL) or Triton X-100 (TX100; 0.2%), diluted in the appropriate culture medium, for 3 hrs, followed by cytotoxicity analysis using an LDH-cytotoxicity test (Wako Pure Chemical Industries). TX100 was used as a positive control. LPS of *Escherichia coli* strain O111:B4 was purchased from Sigma-Aldrich (Saint Louis, MO, USA), the concentration being equivalent in LtxA (500 fg/cell). For the cell viability assay, human neutrophils (2×10^5 cells/100 μL) were treated with LtxA (50 fg/cell) for 3 hrs. HGECs (1×10^5 cells/100 μL) and HGFs (5×10^4 cells/100 μL) were treated with NE (100 mU/mL) or the supernatant obtained from LtxA-treated human neutrophils for 1 hr in the presence or absence of an NE inhibitor (sivelestat; ONO-5046, N-[2-[4-(2,2-dimethylpropionyloxy)phenylsulfonylamino] benzoyl]aminoacetic acid; Ono Pharmaceutical, Osaka, Japan). The cells were stained with Hoechst 33342 and ethidium homodimer III (Apoptotic/Necrotic/Healthy Cells Detection Kit; PromoCell, Heidelberg, Germany) and visualized under a confocal laser-scanning microscope (Carl Zeiss).

2.5 | Immunofluorescence analysis

To analyze the localization of NE, human neutrophils (2×10^5 cells/100 μL) were treated with LtxA (50 fg/cell) for 3 hrs and fixed using 4% paraformaldehyde for 10 mins at room temperature followed by incubation of the cells in a blocking solution (Thermo Fisher Scientific, Waltham, MA) for 30 mins. The samples were then stained with rabbit anti-NE antibody (Abcam, Cambridge, UK) in the blocking solution. After overnight incubation at 4°C, secondary AlexaFluor 594-conjugated goat anti-rabbit IgG antibody (Thermo Fisher Scientific) was added, followed by 2-hr incubation in the dark. For LFA-1 detection, human neutrophils (2×10^5 cells/100 μL), HGECs (1×10^5 cells/100 μL) and HGFs (5×10^4 cells/100 μL) were fixed using 4% paraformaldehyde. The cells were incubated with mouse anti-human CD11+CD18 primary antibody (Abcam) followed by secondary AlexaFluor 488-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific). The samples were then washed with PBS and treated with 4′,6-diamidino-2-phenylindole (DAPI). The samples were then examined under a confocal laser-scanning microscope (Carl Zeiss).

2.6 | NE activity assay

Human neutrophils (2×10^6 cells/200 μL) were cultured in serum-free RPMI-1640 medium, after which they were exposed to LtxA (25–50 fg/cell), LPS (0.4 ng/mL) or PMA (40 nM) for 3 hrs. PMA was used as a positive control. The LPS concentration was equivalent in LtxA (50 fg/cell). NE activity in the culture supernatant was evaluated using an elastase activity assay kit (Cayman Chemical, Ann Arbor, MI, USA), according to manufacturer's instructions.

2.7 | Cytotoxicity of the supernatant obtained from LtxA-treated human neutrophils

Human neutrophils (2×10^6 cells/200 μL) were treated with LtxA (50 fg/cell) for 3 hrs and supernatants collected by centrifugation at 500 *g* for 10 mins. HGECs (1×10^5 /100 μL) and HGFs (5×10^4 cells/100 μL) were cultured in the collected supernatant for 3 hrs, followed by DIC image analysis and cell viability assay.

2.8 | Transcription analysis of *CD11a* and *CD18*

RNA was extracted from cell lysates using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and quantified by spectrometry at 260 and 280 nm. The RNA was reverse transcribed using a SuperScript VILO Master Mix (Thermo Fisher Scientific). The PCR cycling conditions were 94°C for 5 mins followed by 35 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1 min and finally at 60°C for 10 min. The internal fragments of the *CD11a*, *CD18*, and

GAPDH genes were amplified using 5′-CACATCTTTC ACACTTCCACCA-3′ and 5′-AGCCTTTAC CCTCACAG TTCACT-3′; 5′-AGGCTCTGATCCACCTGAGC-3′ and 5′-TCACCAACCTCAAGCCCTCC-3′; and 5′-GATGACATC AAGAAGGTGGTG-3′ and 5′-GCTGTAGCCAAATTCGT TGTC-3′ primers, respectively.

2.9 | Statistical analysis

Data were analyzed by ANOVA with either the Dunnett multiple-comparisons test or Student's *t*-test using Graph Pad Prism Software version 7.03 (GraphPad Software, La Jolla, CA, USA).

3 | RESULTS

3.1 | Cytotoxicity of LtxA against HGECs and HGFs

We investigated whether LtxA induces cytotoxicity against HGECs and HGFs. Figure 1A,B show that LtxA treatment did not induce any morphological changes in either HGECs or HGFs compared with untreated cells. In addition, LtxA treatment did not induce release of LDH in either HGECs or HGFs at concentrations of 25–500 fg/cell (Figure 1C,D). These findings suggest that LtxA does not induce cytotoxicity at concentrations of up to 500 fg/cell against either HGECs or HGFs.

3.2 | Cytotoxicity of LtxA against human neutrophils

Amounts of NE are reportedly greater in severe periodontitis than in chronic periodontitis.[20] Thus, we hypothesized that *A. actinomycetemcomitans* destroys human neutrophils by LtxA and induces NE release in periodontal tissue. To investigate this possibility, we investigated whether LtxA demonstrates cytotoxicity against human neutrophils. As shown in Figure 2A, LtxA induced cell lysis in isolated primary human neutrophils. A cell viability assay also showed that LtxA caused death of neutrophils (Figure 2B). Furthermore, LtxA-treated human neutrophils exhibited significant release of LDH in a dose-dependent manner, whereas LPS did not induce LDH release (Figure 2C).

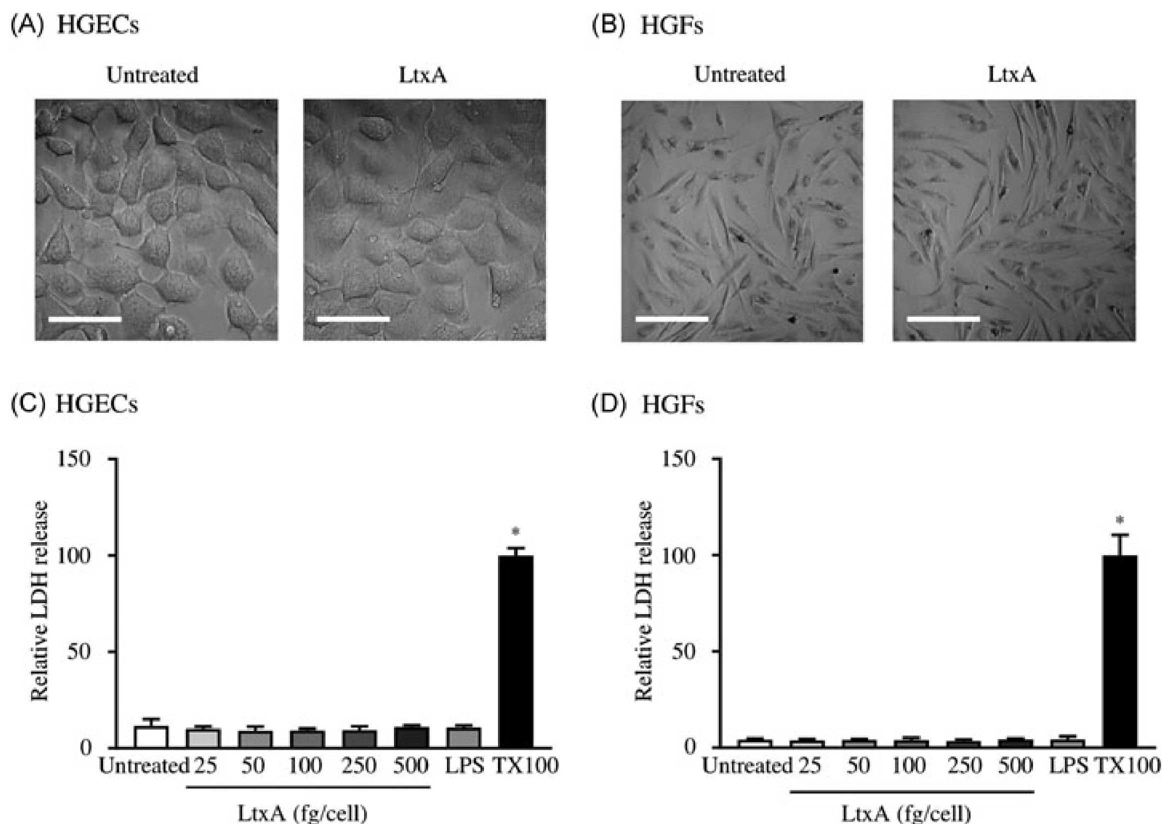


FIGURE 1 LtxA does not induce cytotoxicity against both HGECs and HGFs. A, HGECs (1×10^5 cells/100 μL) and (B) HGFs (5×10^4 cells/100 μL) were exposed to LtxA (50 fg/cell) for 3 hrs. Representative DIC images are shown. C,D, HGECs and HGFs were exposed to various concentrations of LtxA (25–500 fg/cells), LPS (0.4 ng/mL) or TX100 (0.2%) for 3 hrs followed by evaluation of cytotoxicity by LDH assay. Data are presented as the mean ± SD ($n = 4$ per group) and were evaluated using ANOVA with the Dunnett multiple-comparisons test. *Significantly different from the control group at $P < 0.01$. Scale bar = 50 μm for HGECs and 200 μm for HGFs

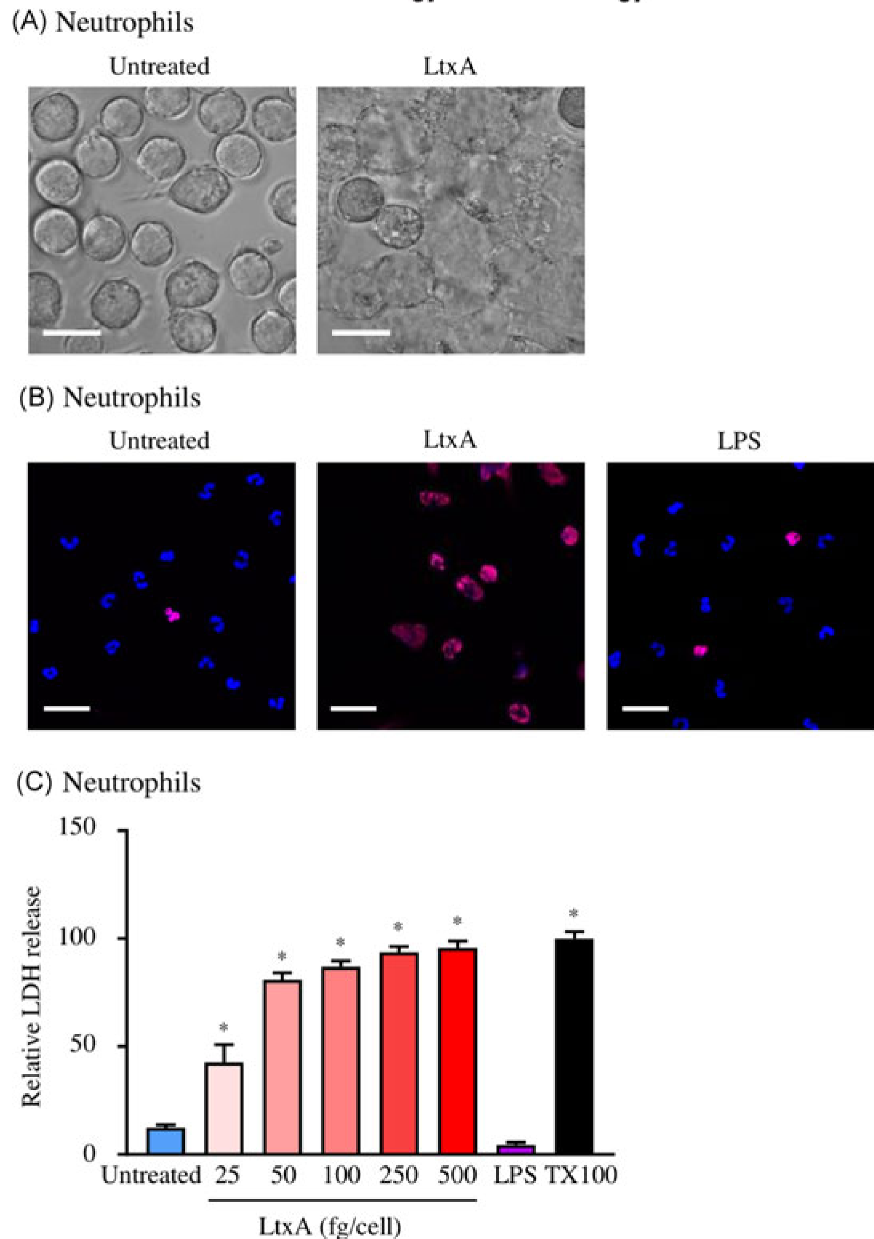


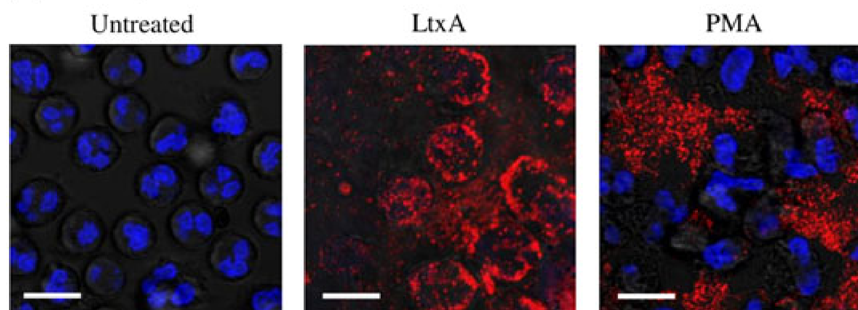
FIGURE 2 LtxA induces cytotoxicity against neutrophils and disrupts cell membrane. A, Human neutrophils were exposed to LtxA (50 fg/cell) for 3 hrs. Representative DIC images are shown. B, Human neutrophils (2×10^5 cells/100 μL) were exposed to LtxA (50 fg/cell) for 3 hrs. Human neutrophils were stained with Hoechst 33342 (stains live cell nuclei a blue) and ethidium homodimer III (stains dead cell nuclei red). Representative fluorescence images observed using a confocal laser-scanning microscope are shown. C, Human neutrophils were exposed to various concentrations of LtxA (25–500 fg/cells), LPS (0.4 ng/mL) or TX100 (0.2%) for 3 hrs followed by evaluation of cytotoxicity by LDH assay. Data are presented as mean ± SD ($n = 4$ per group) and were evaluated using ANOVA with the Dunnett multiple-comparisons test. *Significantly different from the control group at $P < 0.01$. Scale bar = 10 μm for A and 20 μm for B

3.3 | Release of NE from LtxA-treated human neutrophils

To investigate whether LtxA-induced neutrophil lysis causes NE release, we performed immunofluorescence confocal microscopy analysis. As shown in Figure 3A, LtxA-treatment induced release of NE into the extracellular space. In addition, NE activity was significantly greater in the supernatant obtained from LtxA-treated human neutrophils than in the supernatant obtained from the untreated neutrophils, whereas LPS did not enhance NE activity (Figure 3B).

3.4 | Cytotoxicity of NE against both HGECs and HGFs

In our previous study, we found that NE causes detachment of alveolar epithelial cells.[17] Therefore, we speculated that release of NE from LtxA-treated dead neutrophils leads to destruction of periodontal tissues. To determine the effects of NE in periodontitis, we investigated whether NE induces detachment and cytotoxicity of HGECs and HGFs. On the basis of the results of NE activity assay (Figure 3B), the concentration of NE was adjusted to 100 mU/mL. NE-treated HGECs and HGFs had become round and detached from the bottoms of the dishes within 3 hrs (Figure 4A,B). In addition, a cell viability assay showed that NE treatment induced cell death in both HGECs and HGFs within 1 hr (Figure 4C,D; Figure S1A,B). There was significantly more detachment of NE-treated HGECs and HGFs than of untreated control HGECs and HGFs (Figure S1C,D).



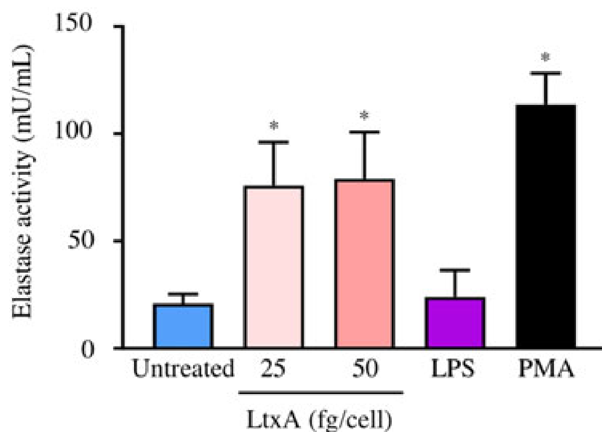


FIGURE 3 Neutrophils release NE in response to LtxA. A, Human neutrophils (2×10^6 cells/100 µL) were exposed to either LtxA (50 fg/cells) or PMA (40 nM) for 3 hrs, after which they were stained with DAPI (stains nuclei blue) and anti-NE antibody (stains NE red). Representative fluorescence images observed using a confocal laser-scanning microscope are shown. B, Human neutrophils were exposed to two concentrations of LtxA (25 and 50 fg/cells), LPS (0.4 ng/mL) or PMA (40 nM) for 3 hrs. NE activity in the culture supernatant was evaluated using an elastase activity assay kit. Data are presented as the mean ± SD ($n = 4$ per group) and were evaluated using ANOVA with the Dunnett multiple-comparisons test. *Significantly different from the control group at $P < 0.01$. Scale bar = 10 µm

3.5 | Cytotoxicity of supernatant from LtxA-treated human neutrophils against HGECs and HGFs

We subsequently investigated whether the supernatant from LtxA-treated human neutrophils induced detachment and cell death in HGECs and HGFs. As shown in Figure 5A,B, the supernatant obtained from LtxA-treated human neutrophils induced detachment of HGECs and HGFs within 3 hrs. A cell viability assay revealed that the supernatant also induced death of HGECs and HGFs (Figure 5C,D; Figure S2A,B). These findings were further validated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure S2C,D). Furthermore, these effects were inhibited by administration of an NE inhibitor, sivelestat, suggesting that the NE in the supernatant plays a vital role in the detachment and death of both HGECs and HGFs.

3.6 | *CD11a* and *CD18* gene transcription and *LFA-1 protein* expression in human neutrophils, HGECs and HGFs

It has been previously reported that LtxA binds specifically to LFA-1, which is a heterodimer of CD11a and CD18, causing rapid death of leukocytes.[21] To confirm *CD11a* and *CD18* transcription in human neutrophils, HGECs and HGFs, we performed PCR analyses. As shown in Figure 6A, *CD11a* and *CD18* gene transcription was detectable in human neutrophils. In contrast, *CD11a* and *CD18* bands were not observed in either HGECs or HGFs. Furthermore, as shown in Figure 6B, human neutrophils expressed LFA-1 on the cell surface, whereas HGECs and HGFs did not. These data suggest that LFA-1 expression on the cell surface is a potential mechanism for the higher sensitivity of neutrophils than HGECs and HGFs to LtxA treatment.

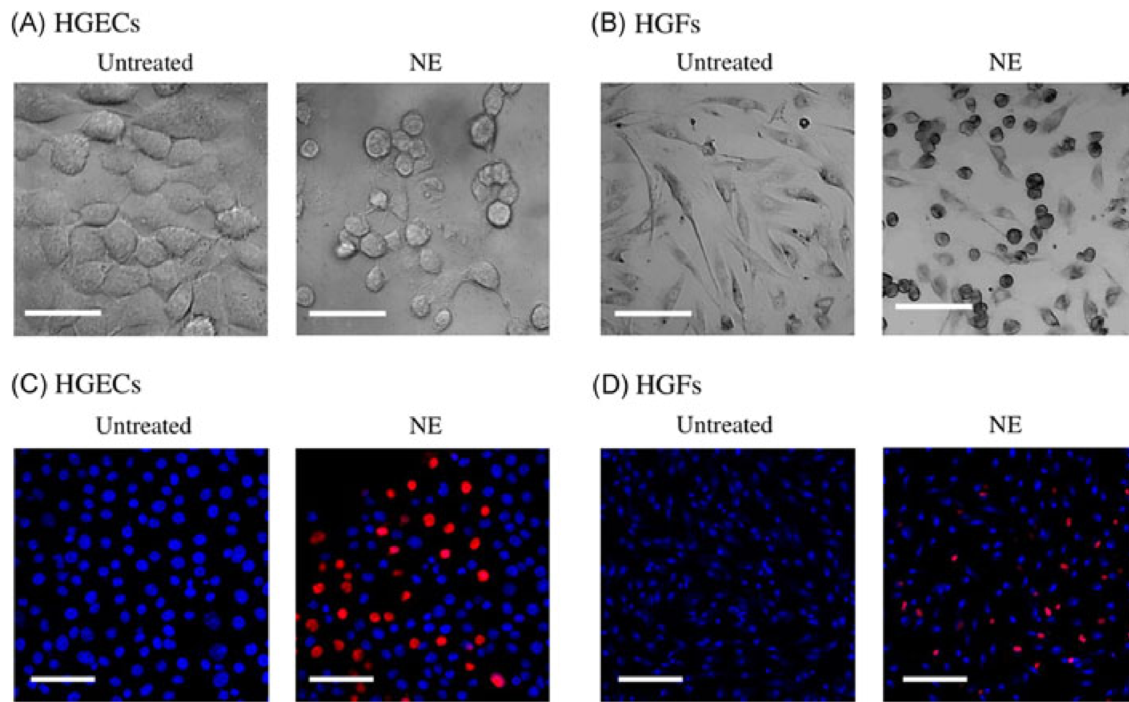


FIGURE 4 NE induces detachment and death of both HGECs and HGFs. A, HGECs (1×10^5 cells/100 μL) and (B) HGFs (5×10^4 cells/100 μL) were exposed to NE (100 mU/mL) for 3 hrs. Representative DIC images are shown. C, HGECs and (D) HGFs were exposed to NE (100 mU/mL) for 1 hr, after which the cells were stained with Hoechst 33342 (stains live cell nuclei blue and ethidium homodimer III (stains dead cell nuclei red). Representative fluorescence images observed using a confocal laser-scanning microscope are shown. Scale bar = 50 μm for (A), 100 μm for (B,C), and 200 μm for (D)

4 | DISCUSSION

In the present study, we found that LtxA does not induce significant cytotoxicity against either HGECs or HGFs. However, the supernatant obtained from LtxA-treated human neutrophils causes detachment and death of HGECs and HGFs. In addition, these effects are inhibited by administration of an NE inhibitor, sivelestat. These findings indicate that *A. actinomycetemcomitans* exploits NE release from human neutrophils to promote periodontal tissue destruction.

A. actinomycetemcomitans strain JP2 exhibits significantly greater LtxA activity than other strains of *A. actinomycetemcomitans*[22,23] and is closely associated with severe periodontitis.[24–26] The *A. actinomycetemcomitans* strain HK1651 used in this study belongs to JP2 clone[27]; additionally, this strain is reportedly associated with severe periodontitis.[28] Evidence for a correlation between LtxA-activity and loss of periodontal attachment has previously been published.[29] In the present study, biochemical and morphological analyses showed that LtxA exhibits cytotoxicity against human neutrophils using, suggesting that LtxA exacerbates periodontitis via a neutrophil-dependent pathway.

Di Franco *et al.* have reported that LtxA induces lysosomal-mediated cell death in LFA-1-expressing cells.[30] LFA-1 is a heterodimer of CD11a and CD18 and plays an important role in inflammatory and immune responses. LFA-1 binds to intercellular adhesion molecules -1, -2 and -3 located on vascular endothelial cells.[31] After binding, leukocytes extravasate from the blood vessels into inflammatory sites. Although a few studies have found that either CD11a or CD18 is responsible for binding to LtxA,[32,33] it has been confirmed that LtxA targets LFA-1. In the present study, we found that HGECs and HGFs are less susceptible than neutrophils to LtxA, suggesting that LtxA does not directly promote periodontal tissue destruction. Furthermore, anti-LFA-1 antibodies are reportedly capable of inhibiting LtxA-mediated cytotoxicity.[3] Thus, LFA-1 inhibition by antibodies or antagonists may become a novel therapeutic strategy against periodontitis caused by *A. actinomycetemcomitans.*

Neutrophils are equipped with several microbicidal and proinflammatory mechanisms and form the first line of defense against pathogenic infections.[6,9] In periodontitis, neutrophils appear in the affected tissues during the very early stages of tissue breakdown.[34,35] Gram-positive oral bacteria and gram-negative endotoxins can trigger sequential activation of the complement system,[36,37] which affects neutrophil migration in periodontal tissue. It has been proven that patients

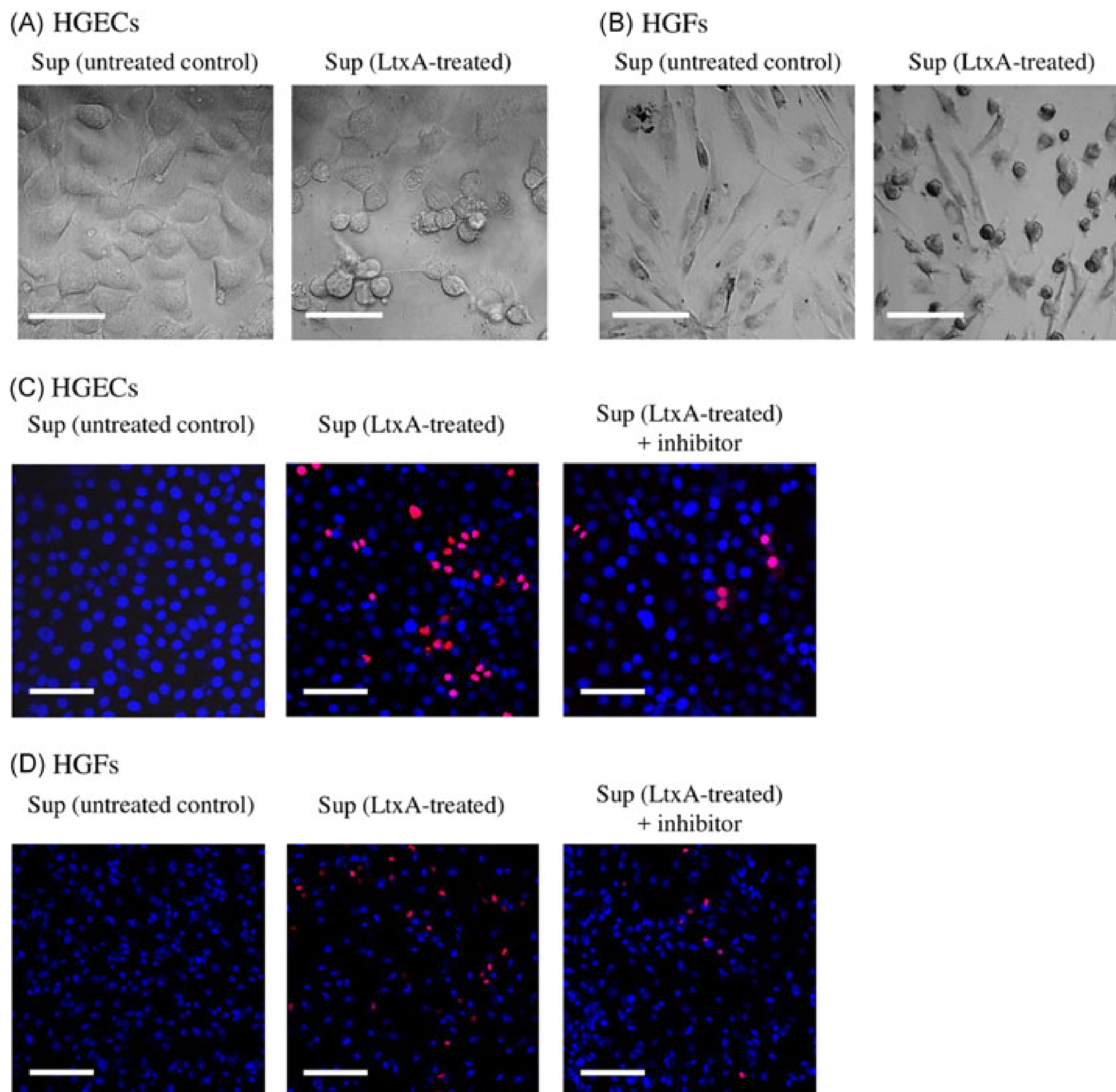


FIGURE 5 Supernatant from LtxA-treated human neutrophils induces cytotoxicity against both HGECs and HGFs. A,B, Human neutrophils (2×10^6 cells/200 µL) were incubated in the presence (Sup [LtxA-treated]) or absence (Sup [untreated control]) of LtxA (50 fg/cell) for 3 hrs and the supernatant collected. Both (A) HGECs (1×10^5 cells/100 µL) and (B) HGFs (5×10^4 cells/100 µL) culture media were changed to the collected supernatant followed by incubation for 3 hrs. Representative DIC images are shown. C, HGECs and (D) HGFs were cultured in the supernatant in the presence or absence of an NE inhibitor, sivelestat (100 µg/mL), for 1 hr, after which the cells were stained with Hoechst 33342 (stains live cell nuclei blue) and ethidium homodimer III (stains dead cell nuclei red). Representative fluorescence images observed using a confocal laser-scanning microscope are shown. Scale bar = 50 µm for (A), 100 µm for (B,C), and 200 µm for (D). Sup, supernatant

with low counts of circulating neutrophils associated with rare conditions such as cyclic neutropenia exhibit a pattern and progression of loss of periodontal attachment that is similar to that observed in individuals with severe periodontitis.[38] In contrast, some authors have reported increased numbers of neutrophils in patients with severe periodontitis.[39] In addition, LFA-1-dependent excessive neutrophil infiltration induces periodontitis in animal models.[40] Although the presence of neutrophils cannot define the pathobiological significance of local leukocytosis in the causation and development of periodontal diseases[41], they are undeniably conspicuous elements in periodontitis.

Neutrophil granule proteases include members of the serine protease family (NE, cathepsin G and proteinase 3) and matrix metalloproteinases (neutrophil collagenase and gelatinase)[42] and the activity of these enzymes is tightly controlled by endogenous inhibitors. However, excessive protease activity can potentially destroy human tissues.[43] In fact, accumulation of large amounts of NE has been implicated in the pathogenesis of a wide range of disorders characterized by severe, progressive or

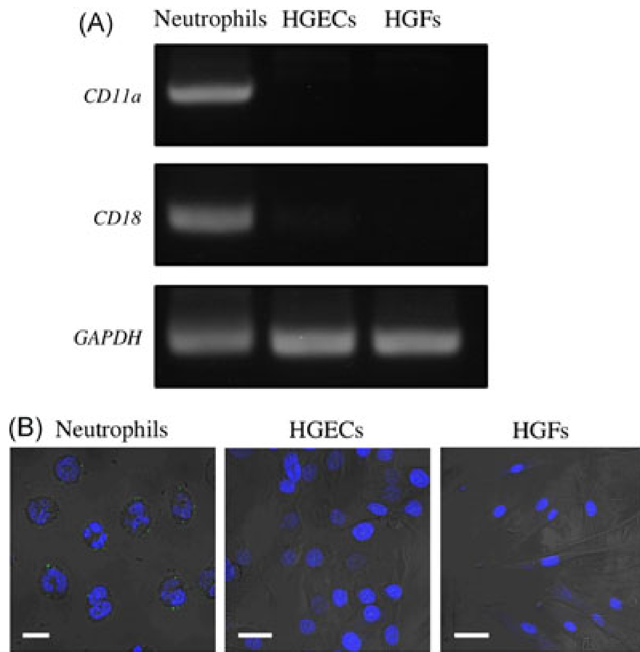


FIGURE 6 Human neutrophils transcribe *CD11a* and *CD18* and express their respective proteins. A, RNA was extracted from human neutrophils, HGECs and HGFs, and degree of gene transcription analyzed by RT-PCR. B, Human neutrophils (2×10^5 cells/100 μL), HGECs (1×10^5 cells/100 μL) and HGFs (5×10^4 cells/100 μL) were stained with the anti-LFA-1 antibody (stains LFA-1 green) and DAPI (stains nuclei blue). Representative merged fluorescence and DIC images are shown. Scale bar = 10 μm for neutrophil, 20 μm for HGEC, 100 μm for HGF

chronic inflammation, such as cystic fibrosis, chronic obstructive pulmonary disease and acute respiratory distress syndrome.[44–46] Furthermore, neutrophil degranulation is readily observable in inflamed gingival tissues and gingival crevicular fluids.[47] Although local NE activity (enzyme units) has not been demonstrated to be associated with inflamed periodontal tissue, there are significantly greater amounts of NE in periodontitis.[14] Therefore, it is likely that NE release caused by LtxA, as demonstrated in the present study, induces destruction of periodontal tissue *in vivo*.

We have previously reported that release of NE into the extracellular space promotes lung injury caused by alveolar epithelial cell detachment.[17] In the present study, NE and the supernatant from LtxA-treated human neutrophils induced detachment of HGECs. Although the molecular mechanisms associated with NE-induced cell detachment are complex and have not yet been fully elucidated, NE is thought to be involved in disruption of cell-matrix adhesion and cell-cell junctions.[48,49] The gingival epithelia form physical, chemical and immunological barriers against invading plaque-associated bacteria and provide the first line of host defense.[50] Breakdown of the barriers produced by NE may contribute to spread of periodontal pathogens followed by periodontal tissue destruction. In addition, several studies have shown that NE inhibition ameliorates lung injury in animal models.[15,51] In the current study, NE inhibition significantly suppressed detachment and death of HGECs, suggesting that inhibition of NE may act as a novel therapeutic strategy against periodontitis.

In conclusion, our *in vitro* study demonstrated that LtxA is a mediator of neutrophil cell death and subsequent release of NE, which eventually induces detachment and death of HGECs and HGFs. Furthermore, an NE inhibitor, sivelestat, was found to inhibit these effects. Therefore, preventing binding of LtxA to LFA-1 and thus regulating NE activity presents a novel therapeutic technique for preventing breakdown of periodontal tissues due to periodontitis caused by *A. actinomycetemcomitans*.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests regarding this article.

ORCID

Yutaka Terao http://orcid.org/0000-0002-4450-5583

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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