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Human gingival fibroblasts release high-mobility group box-1 protein through active and passive pathways

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Introduction: The nuclear protein high-mobility group box-1 (HMGB1) acts as a late mediator of inflammation when secreted in the extracellular milieu. In this study, we examined the effect of lipopolysaccharides from periodontal pathogens and apoptotic and necrotic cell death on HMGB1 production in human gingival fibroblasts (HGF).
Methods: HGF from healthy periodontal tissue were cultured and stimulated with lipopolysaccharides (LPS) from *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Escherichia coli*. We also initiated apoptotic and necrotic cell deaths in HGF. The HMGB1 released in the supernatants from stimulated or dying cells was measured. Immunocytochemical staining against HMGB1 was performed in LPS-stimulated HGF.
Results: A significantly higher amount of HMGB1 was detected from necrotic and apoptotic HGF. LPS from *A. actinomycetemcomitans*, *P. gingivalis*, and *E. coli* significantly induced the production of HMGB1 in a time-dependent manner. After 6 h of LPS stimulation, HMGB1 was present in the cytoplasm of cells whereas its location was mainly nuclear after 24 h.
Conclusions: LPS from two major periodontal pathogens, *A. actinomycetemcomitans* and *P. gingivalis*, induced HMGB1 secretion from HGF. Apoptotic and necrotic cell deaths resulted in the enhancement of HMGB1. Our results suggest that HGF can be a source of HMGB1 by both active secretion and passive release, and that HMGB1 from HGF may contribute to periodontal tissue destruction.

Key words: apoptosis; high-mobility group box 1; human gingival fibroblasts; lipopolysaccharides; necrosis

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High-mobility group box-1 (HMGB1) protein was first characterized as a nuclear protein (6) that binds loosely to the minor groove of DNA and plays an important role in gene transcription and DNA recombination and repair (20). HMGB1 contributes in the inflammation process when secreted in the extracellular milieu (32) and can exit from the cells through at least two distinct pathways: an active secretion by stimulated cells or a passive release from necrotic and damaged cells. The active secretion of HMGB1 has been demonstrated in monocytes/macrophages under certain conditions, such as stimulation with lipopolysaccharides (LPS), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) (15, 32).

The capacity of cells to actively secrete HMGB1 is dependent on cell type. Mast cells do not release HMGB1 upon stimulation, whereas the human colon cancer line HCT 116 can release large amounts of HMGB1 in an unstimulated state (31). Acetylation of HMGB1 is a crucial step in the translocation of HMGB1 from the nucleus to the cytoplasm, where it emigrates into secretory vesicles to reach the extracellular matrix (5). The nucleocytoplasmic shuttling of HMGB1 can also be regulated by phosphorylation (35). In addition to active secretion, HMGB1 can diffuse from necrotic cells, and sometimes apoptotic cells, during the course of tissue destruction by disease (2, 26). Once outside the cells, it triggers

the inflammatory response, in a delayed manner compared to the early effects of other inflammatory mediators such as TNF-α and IL-1β (32). Wang et al. showed that macrophages secreted HMGB1 more than 8 h after stimulation with LPS, IL-1, or TNF-α. In mice, the delayed administration of antibodies against HMGB1 attenuated LPS-induced endotoxemia. However, the injection of HMGB1 caused septic shock and was lethal by itself (32). These interesting findings diverted the focus of researchers towards the role of HMGB1 in the pathogenesis of inflammatory diseases. Effectively, HMGB1 was found to contribute to several inflammatory conditions and diseases such as arthritis, sepsis, acute lung inflammation, endotoxemia, and cancer (1, 3, 9, 32, 36). The mechanisms through which HMGB1 acts are not yet fully understood; however, it is well established that HMGB1 binds to the receptor for advanced glycation end-products (RAGE), a transmembrane receptor belonging to the immunoglobulin superfamily that is expressed on a wide variety of cells, such as smooth muscle cells, neurons, endothelial cells, monocytes, osteoblasts, human gingival fibroblasts (HGF), and epithelial cells (7, 9, 12, 16, 22). Further studies provided evidence that HMGB1 can signal via Toll-like receptor-2 (TLR2) and TLR4 (23, 24, 30); TLR9 has now been added to the receptors interacting with HMGB1 (14, 29).

Periodontal disease is characterized by chronic inflammation of periodontal tissues and involves the interaction between bacterial invasion and host response resulting in pocket formation, irreversible bone resorption, and subsequently tooth loss. *Aggregatibacter* (previously *Actinobacillus*) *actinomycetemcomitans* and *Porphyromonas gingivalis* are considered as major periodontal pathogens involved in periodontal disease (4, 21). Inflammation of connective tissues is one of the main features of periodontal disease and gingival fibroblasts have proved their role as immunomodulating cells and not only collagen-producing cells (33). When challenged with LPS, they can produce a wide variety of proinflammatory mediators such as IL-6, IL-8, and prostaglandin E_2 (8, 17, 21), implying their active participation in the inflammation of periodontitis. A recent study showed that gingival crevicular fluid from periodontal patients contained HMGB1 whereas that from healthy patients did not, suggesting a potential role of the HMGB1 protein in sustaining inflammation and hence contributing to disease progression (22). However, the origin and mechanisms of HMGB1 release in periodontal tissues are not fully understood.

We hypothesized that HGF, the main cellular component of periodontal connective tissues, could be one of the sources of HMGB1 in chronic inflammatory periodontal lesions. The purpose of the present study was to investigate whether HGF can produce HMGB1 after cell stress such as stimulation with LPS from periodontal pathogens and necrotic and apoptotic cell deaths.

Materials and methods

Cell culture

Gingival tissue samples were collected from three subjects with clinically healthy periodontium and no history of periodontal disease. The gingival biopsies were obtained at the time of extraction of the lower third molar, at the Periodontal Clinic of Tokyo Medical and Dental University. The experimental protocol was approved by the Ethics Committee of Tokyo Medical and Dental University and informed consent was obtained from all the subjects. The tissue samples were washed several times in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS; Multiser, Melbourne, Australia) and 1% antibiotic–antimycotic (ABAM; Invitrogen, Carlsbad, CA), minced into small fragments and placed in 60-mm tissue culture dishes. HGF from the three different donors were cultured separately in DMEM supplemented with 10% FBS and 1% ABAM until they reached confluence at 37°C in a humidified atmosphere of 5% CO_2. Cells from passages 4 to 10 were detached with 0.05% trypsin and 0.05 mM ethylenediaminetetraacetic acid (Invitrogen) and used throughout the study.

Cell stimulation with LPS

Cells at a density of 5×10^4 cells/ml were plated in 96-well culture dishes (Corning Inc., Corning, NY) and were stimulated with 1, 10, 50, or 75 μg/ml each of *A. actinomycetemcomitans* LPS, *P. gingivalis* LPS (Invivogen, San Diego, CA) and *Escherichia coli* LPS (Sigma) for 48 h. The control group was left unstimulated.

To determine the production of HMGB1 at different time-points, HGF were stimulated with 50 μg/ml *A. actinomycetemcomitans*, *P. gingivalis*, or *E. coli* LPS for 3, 6, 12, 24, and 48 h. Culture dishes were maintained at 37°C under 5% CO_2 humidified atmosphere. Media from conditioned cells were collected and centrifuged, and the cell-free supernatants were assayed for HMGB1 using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (SHINO-TEST, Shiga, Japan).

Immunocytochemical staining

For the immunocytochemical staining, HGF were cultured on chamber slides at a density of 5×10^4 cells/ml (200 μl/well), and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. Cells were washed with phosphate-buffered saline (PBS) and fixed with 3–4% paraformaldehyde for 15 min at room temperature. Then cells were washed twice with PBS, after which they were permeabilized by incubation with PBS containing 0.25% Triton-X (PBST) for 10 min at room temperature. Once again, cells were washed three times with PBS, and blocked with PBS containing 3% bovine serum albumin (BSA; Sigma) for 1 h at room temperature. After being washed once in PBS, they were incubated with rabbit anti-human HMGB1 antibody (Abcam, Cambridge, UK) in 0.1% BSA-PBST for 2 h at room temperature. The rabbit anti-human HMGB1 antibody was removed by washing three times with PBS, and cells were incubated with goat anti-rabbit immunoglobulin G Alexa Fluor (Invitrogen) in 0.1% BSA-PBST for 2 h at room temperature, and protected from light. The wells were washed three times with PBS and the nuclei were stained for 1 min with Cell-stain-Hoechst 33258 solution (Dojindo Laboratories, Kumamoto, Japan). Finally, cells were washed three times with PBS and Antifade solution (Invitrogen, Eugene, OR) was added. The cell images were obtained using a fluorescence microscope (Keyence, Osaka, Japan).

Necrosis and apoptosis assays

HGF were necrotized by two different methods, either by immersing them for 30 min in a 56°C preheated bath, or by subjecting them to three consecutive cycles of freeze–thaw.

To induce apoptosis, cells were treated with 50 ng/ml recombinant TNF-α (R&D Systems, Minneapolis, MN) and 10 μM cycloheximide (CHX; Nakarai Chemicals, Kyoto, Japan) for 24 h. To inhibit the effect of apoptosis, cells were preincubated for 30 min with 10 μM caspase inhibitor [Z-Val-Ala-Asp(OMe)-CH_2F (Z-VAD-fmk);

Calbiochem, San Diego, CA] and then incubated with apoptosis inducers for the indicated time. All samples were centrifuged and supernatant was collected for HMGB1 quantification and lactate dehydrogenase (LDH) cytotoxicity assays.

Cell viability assay and LDH cytotoxicity test

Cell viability was determined using cell counting kit-8 (Dojindo Laboratories) and the levels of LDH were assessed using the LDH cytotoxicity detection kit (Takara Bio Inc., Tokyo, Japan) according to the manufacturers' instructions. All assays were performed in triplicate.

Statistical analysis

For each experiment, data are presented as means ± standard deviation of triplicate assays. The statistical differences were analysed by using one-way analysis of variance followed by Dunnett's test. Student's *t*-test was used for paired comparisons. A P value <0.05 was considered to be significant.

Results

Effect of apoptosis on HMGB1 release from HGF

To examine whether HGF can release HMGB1 passively, we first investigated the involvement of apoptosis in HMGB1 release by HGF. We incubated the cells with a combination of CHX and TNF-α, a potent inducer of apoptosis (37). As shown in Fig. 1, CHX and TNF-α stimulation of HGF significantly enhanced HMGB1 release compared to control. Viable cell number was significantly decreased by CHX and TNF-α treatment (Fig. 1). The addition of Z-VAD-fmk, a caspase inhibitor, decreased HMGB1 levels in HGF incubated with apoptotic inducers, suggesting that HMGB1 release by HGF treated with CHX and TNF-α was mediated by apoptosis.

Passive release of HMGB1 by necrotic HGF

To examine another passive pathway of HMGB1 release, we next investigated the effects of necrotic cell death. To study this in HGF, we treated the cells with heat or with three consecutive cycles of freeze–thaw. After inducing cell death by heat or thermal shock, HMGB1 release was significantly higher in these two groups compared with control (Fig. 2B,D). We also assessed LDH, one of the markers of tissue damage, in the same samples to confirm cell death, and the LDH levels followed the same pattern as HMGB1 release from damaged HGF (Fig. 2A,C).

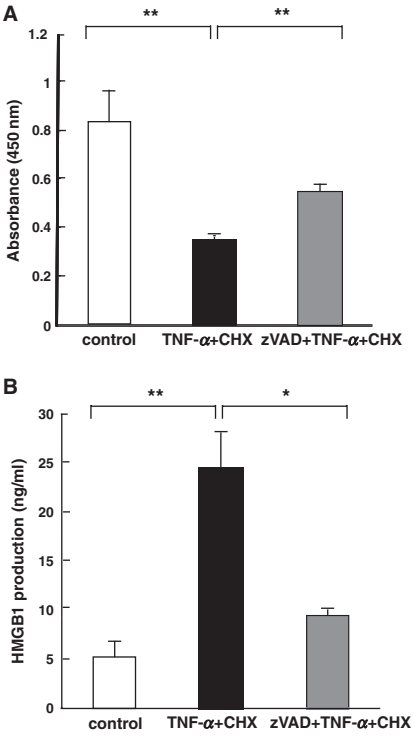

Fig. 1. Effect of apoptosis on high-mobility group box-1 (HMGB1) release by human gingival fibroblasts (HGF). HGF were treated with 50 ng/ml tumour necrosis factor-α (TNF-α) and 10 μM cycloheximide (CHX) for 24 h to induce apoptosis. To inhibit the effect of apoptosis, cells were incubated with 10 μM Z-VAD-fmk for 30 min, before the addition of the inducers of apoptosis. (A) Cell viability. (B) HMGB1 amounts in the supernatants. Data are presented as means ± standard deviations of triplicate assays. $*P < 0.05$, $**P < 0.005$.

Active secretion of HMGB1 from HGF stimulated by LPS

To examine the active secretion of HMGB1 by HGF, we investigated if LPS from periodontal pathogens could induce HMGB1 release from HGF. Cultured HGF were subjected to increasing amounts of LPS from two periodontal pathogens, *A. actinomycetemcomitans* and *P. gingivalis*; *E. coli* LPS was used as a control LPS.

In preliminary experiments, we used concentrations ranging from 0.001 to 1 μg/ml LPS to stimulate HGF, however, we did not find any significant HMGB1 release from cells at any concentration we tested (data not shown). For this reason, we increased the concentration of LPS, and used concentrations between 1 and 75 μg/ml. As shown in Fig. 3, addition of the three LPS increased HMGB1 levels in most of the cases. We selected the concentration of 50 μg/ml LPS as our inducing dose and used it in a time–course experiment (Fig. 4). The three LPS showed a time-dependent increase in HMGB1 production. Significant HMGB1 release from HGF was first observed 6 h after stimulation with LPS and slowly increased until 48 h. The release patterns of HMGB1 were similar among the three LPS. Since the amount of LPS used in

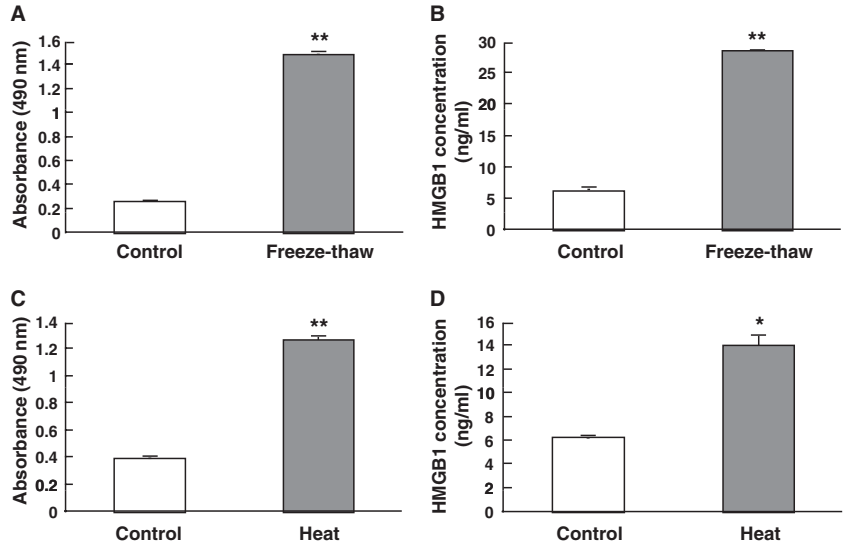

Fig. 2. Effect of necrosis on high-mobility group box-1 (HMGB1) release by human gingival fibroblasts (HGF). Necrosis was induced in HGF using two different methods: by three consecutive cycles of freezing and thawing (A, B) or by immersing the cells for 30 min in a 56°C preheated bath (C, D). (A, C) Lactate dehydrogenase (LDH) levels. (B, D) HMGB1 amounts in the supernatants. Data are presented as means ± standard deviations of triplicate assays. $*P < 0.005$, $**P < 0.0005$.

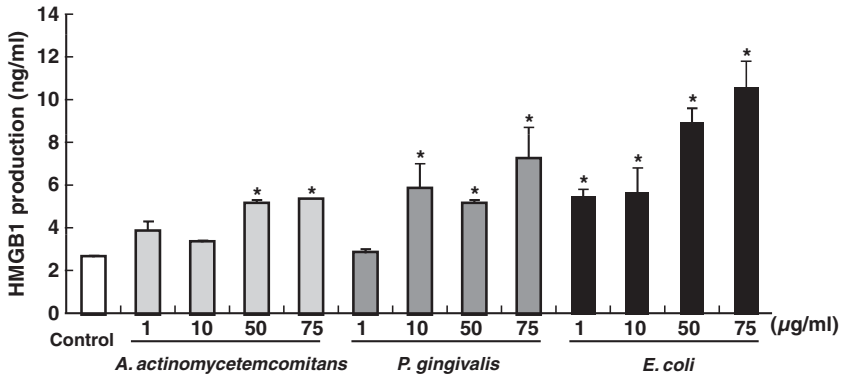

Fig. 3. Human gingival fibroblasts (HGF) release high-mobility group box-1 (HMGB1) after stimulation with lipopolysaccharide (LPS). HGF were stimulated with 1, 10, 50, or 75 μg/ml *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Escherichia coli* LPS for 48 h. In the control group, cells were cultured for 48 h without any stimulation. HMGB1 amounts in the supernatants were measured by enzyme-linked immunosorbent assay. Data are presented as means ± standard deviations of triplicate assays. *Significant difference from control ($P < 0.05$).

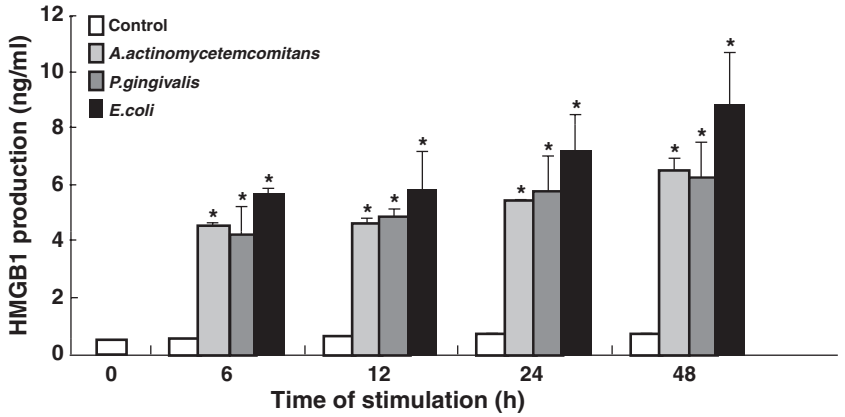

Fig. 4. Time–course stimulation of human gingival fibroblasts (HGF) with lipopolysaccharide (LPS). HGF were stimulated with 50 μg/ml of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Escherichia coli* LPS for 6, 12, 24, and 48 h. For the 0-h samples, supernatants were collected at time 0 without any stimulation with LPS. High-mobility group box-1 (HMGB1) amounts in the supernatants from conditioned cells were measured by enzyme-linked immunosorbent assay. Data are presented as means ± SD of triplicate assays. *Significant difference from control at time 0 ($P < 0.05$).

this study was relatively high for cell stimulation *in vitro*, we investigated the possible toxicity of LPS to HGF by using LDH and cell viability assays. As shown in Fig. 5, the two tests did not show any significant difference between the groups. Both cytotoxicity and viability tests showed that the doses of LPS used in our study were not toxic to cells, reflecting that HMGB1 was secreted by activated cells and did not diffuse as a result of necrosis or apoptosis. These results strongly suggest that HGF can actively secrete HMGB1 in response to LPS stimulation.

Immunocytochemical staining

To further confirm the active release of HMGB1 from LPS-challenged HGF, we investigated the localization of HMGB1 in HGF after different periods of stimulation. Without any stimulation, HMGB1 was located in the nucleus (Fig. 6). Upon stimulation with LPS, HMGB1 changed localization toward the cytoplasm after 6 h. After 12 h HMGB1 could be detected in the cytoplasm of most of the cells. However, after 24 h HMGB1 staining was mainly nuclear.

Discussion

In this study we show that HGF can actively secrete HMGB1 when stimulated with LPS from two major periodontal pathogens, *P. gingivalis* and *A. actinomycetemcomitans*. On the other hand, HGF also proved to be an important source of HMGB1 when undergoing apoptosis and necrosis. Some studies have stated that HMGB1 can be released passively following necrosis but not after apoptosis, in which HMGB1 remains adherent to the chromatin inside the nucleus even in the late stage of apoptosis also known as secondary necrosis (25, 26). However, more recent studies succeeded in showing that HMGB1 release by apoptotic cells can effectively occur depending on cell types (2). In our results, incubation of HGF with a combination of TNF-α and CHX increased HMGB1 levels and reduced cell viability and the enhanced HMGB1 release was diminished by the addition of Z-VAD-fmk, a potent inhibitor of caspase-2 and caspase-3 (Fig. 1). These results strongly suggest that HMGB1 is released when apoptosis occurs in HGF. Moreover, as shown in Fig. 2, necrotic cell death by heat and freeze–thaw cycles also resulted in increased levels of HMGB1. Our data suggest that HMGB1 can be released from HGF following cell death, whether it is apoptotic or necrotic. HMGB1 is recognized as a danger signal molecule which can evoke an immune response to infection and tissue damage. Danger-signaling molecules function extracellularly by alerting the body of an impending danger and initiating an appropriate host response toward tissue repair. HMGB1 can promote stem cell migration to the damaged area and cell differentiation following cardiac infarction and neural injury (11, 13). It is also possible that HMGB1 from necrotic and apoptotic HGF death may function as a danger signal molecule in periodontal tissues by transmitting information related to bacterial infection and tissue damage in periodontitis.

Previous studies have demonstrated the active release of HMGB1 from monocytes and macrophages upon stimulation with inflammatory mediators. In contrast to findings in other cell types (22, 32), TNF-α failed to generate HMGB1 release from HGF (data not shown). However, HGF produced HMGB1 at significant levels when challenged with LPS. As far as we know, our study is the first to show an active release of HMGB1 from fibroblasts. HMGB1 is a member of a category of proteins known as 'leaderless' proteins that do not have a signal peptide at the C-terminal end of the protein sequence (10). A leaderless protein has unique secretion pathways different from the classical protein secretion mechanism through endoplasmic reticulum and Golgi apparatus. Upon stimulation in monocytes, HMGB1 translocates from the nucleus to

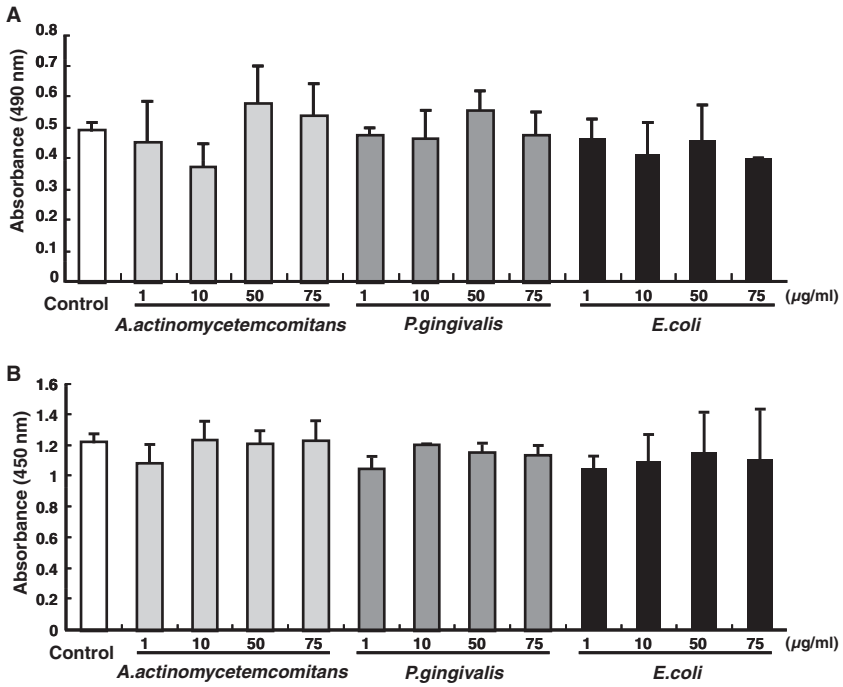

Fig. 5. Lactate dehydrogenase (LDH) cytotoxicity assay and cell viability in lipopolysaccharide (LPS)-stimulated human gingival fibroblasts (HGF). The HGF were stimulated with 1, 10, 50, or 75 μg/ml of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Escherichia coli* LPS for 48 h. In the control group, cells were cultured for 48 h without LPS stimulation. (A) LDH levels. (B) Cell viability. Data are presented as means ± standard deviations of triplicate assays. The differences between the groups were not statistically significant compared to control.

the cytoplasm by acetylation and is loaded into secretory lysosomes to be released outside the cells. HMGB1 in HGF changed its localization toward the cytoplasm after 6–12 h of stimulation with LPS (Fig. 6). Our results are consistent with previous findings in monocytes (5). Furthermore, messenger RNA levels of HMGB1 were not enhanced by LPS stimulation in HGF (data not shown), whereas protein levels in supernatant were increased dramatically.

One interesting point in this study is the relatively higher concentrations of LPS that were needed to initiate the secretion of HMGB1 by HGF compared to monocytes. According to our results, HGF produced HMGB1 at significant levels when stimulated with 10 μg/ml *P. gingivalis* LPS and 50 μg/ml *A. actinomycetemcomitans* LPS. The reason for the difference in LPS concentrations required to stimulate HGF or monocytes to produce HMGB1 is still elusive. As mentioned earlier, it is interesting to consider the unique mode of secretion of HMGB1 compared with other inflammatory mediators. This might partly explain this phenomenon. Further investigations are needed to give a plausible explanation of the mechanism behind this observation.

The results of our study showed that HGF could contribute to HMGB1 release in periodontal lesions through active release by LPS-stimulated cells and diffusion from dead cells, whether necrotic or apoptotic. Morimoto et al. have shown histological sections of chronic periodontitis tissues where most of the gingival epithelial cells stained positive for HMGB1 around their nucleus together with some translocation towards the cytoplasm (22). The presence of similarly stained cells was observed in some areas of the connective tissue. The nature of these cells was not identified in the paper. The authors also found a significant increase of HMGB1 expression in the gingival crevicular fluid from periodontal patients. Although it has been suggested that gingival epithelial cells were the main origin of HMGB1 in periodontal tissues, our data that HGF release HMGB1 following LPS stimulation or cell death may also explain the higher HMGB1 amounts in the gingival crevicular fluid from patients and the positive staining in the connective tissue. In periodontal disease, the invasion of connective tissues by periodontal pathogens such as *A. actinomycetemcomitans* and *P. gingivalis* was frequently observed and this might suggest

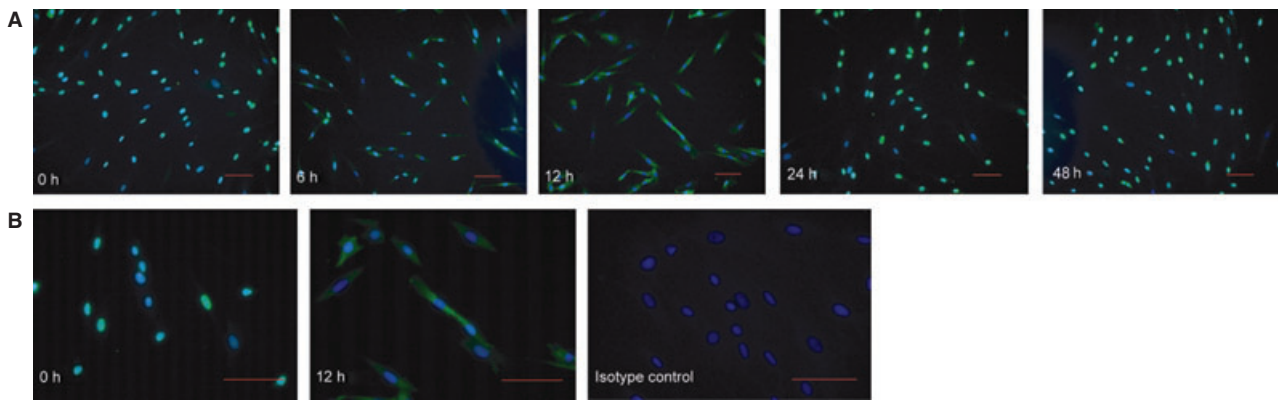

Fig. 6. Effect of lipopolysaccharide (LPS) stimulation on high-mobility group box 1 (HMGB1) relocation in human gingival fibroblasts (HGF). (A) 200× magnification, (B) 400× magnification. Cells were stimulated with 50 μg/ml of *Aggregatibacter actinomycetemcomitans* LPS for 6, 12, 24, and 48 h. Immunocytochemical staining with anti-HMGB1 antibody (green) and Hoechst 33258 (blue) was performed and the cells were observed under a fluorescence microscope. Negative control staining was performed with immunoglobulin G control antibody. Bar, 100 μm.

high concentrations of LPS from these bacteria in the inflamed tissues. Furthermore, apoptosis of gingival tissue cells had been demonstrated in experimental periodontitis (19), and extracellular metabolites from periodontal pathogens such as butyric acid and gingipain have been reported to induce apoptosis *in vitro* (18, 28). Combining reports in the literature with our data, we can suggest that HGF can be a source of HMGB1 in acute inflammation, when the bacterial invasion of connective tissue is severe, causing more cell damage. HMGB1 from HGF through active release in response to LPS and diffusion following apoptotic or necrotic cell death may contribute to the pathogenesis of periodontal disease. Further studies are needed to establish the exact role of HMGB1 in periodontal disease. As reported previously, extracellular HMGB1 regulates proinflammatory cytokine production including TNF-α, IL-1α, IL-1β, and IL-6, and these cytokines are highly expressed in inflamed gingival tissues (17). HMGB1 can bind to other molecules such as LPS and cytokines and probably enhance their activity when these complexes bind to their specific receptors (27, 34).

The present study shows evidence of the ability of HGF to actively and passively release HMGB1 when challenged or damaged.

In conclusion, HGF release HMGB1 through an active pathway after stimulation with LPS from two major periodontopathogens, *A. actinomycetemcomitans* and *P. gingivalis*, and also through a passive pathway following apoptotic and necrotic cell deaths. Our results suggest that HMGB1 from HGF may contribute to the pathogenesis of periodontal disease.

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