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Tumor necrosis factor-α stimulates gingival epithelial cells to release high mobility-group box 1

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Background and Objective: High-mobility-group box 1 functions as a late-phase inflammatory mediator. It can be released extracellularly by macrophages and necrotic cells through lipopolysaccharide and tumor necrosis factor-α. The objective of this study was to clarify the source of high-mobility-group box 1 in chronic periodontitis tissues and tumor necrosis factor-α-stimulated gingival epithelial cells, and subsequently elucidate its inducible inflammatory pathway.

Material and Methods: Chronic periodontitis and healthy gingival sections were stained for high-mobility-group box 1 by immunohistochemistry and immunofluorescence. The amounts of high-mobility-group box 1 released into the gingival crevicular fluid and supernatants from gingival epithelial cells stimulated by tumor necrosis factor-α were examined by western blot. The phosphorylation of mitogen-activated protein kinases (MAPKs) in gingival epithelial cells was also examined.

Results: High-mobility-group box 1 was detected in the cytoplasm and nucleus of gingival epithelial cells with periodontitis. Western blotting revealed a significant increase in high-mobility-group box 1 expression in the gingival crevicular fluid from periodontitis patients. High-mobility-group box 1 production in gingival epithelial cells was increased following stimulation with tumor necrosis factor-α. The molecular dialogue between tumor necrosis factor-α and gingival epithelial cells involved modulation of the activities of p38MAPK, Jun N-terminal kinase and p44/42. Interestingly, only phosphorylation of p38MAPK contributed to more than half of the signaling initiated by tumor necrosis factor-α-elicited high-mobility-group box 1 release.

Conclusion: High-mobility-group box 1 is continuously released from the gingival epithelial cells modulated by tumor necrosis factor-α. These findings imply that high-mobility-group box 1 expression and possibly p38MAPK constitute important features in periodontitis.

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High-mobility-group box 1, a primarily nuclear protein, is present in many eukaryotic cells (1) and has a highly conserved sequence among species. It consists of two tandem domains, designated high-mobility-group boxes A and B, each of which is ≈75 amino acids in length, and a highly acidic carboxyl terminus of 30 amino acids in length. High-mobility-group box 1 appears to have distinct functions in cellular systems. It acts as an

intracellular regulator of transcription and plays a crucial role in the maintenance of DNA functions (2). Extracellular high-mobility-group box 1 released from various cells (i.e. macrophages/monocytes, endothelial cells and pituicytes) or necrotic cells (3–6) and stimulated by lipopolysaccharide or tumor necrosis factor-α acts as a pro-inflammatory cytokine through the multiligand receptor for advanced glycation end-products (7,8) and toll-like receptors 2 and 4 (9). Extracellular high-mobility-group box 1 has been found to play critical roles in the progression of chronic inflammatory diseases, such as septic shock, rheumatoid arthritis and atherosclerotic lesions (3,8,10,11).

Periodontitis is a chronic inflammatory disease in which the production of numerous pro-inflammatory cytokines (i.e. interleukin-1β, interferon-γ and tumor necrosis factor-α) is amplified by several bacteria-derived virulence factors (12), thereby leading to the destruction of soft tissues and bone (13). Among the pro-inflammatory cytokines, tumor necrosis factor-α plays important roles in various inflammatory conditions (14) and has recently gained attention in periodontal diseases as a result of its effects on bone and soft tissue metabolism (15). For example, tumor necrosis factor-α has been reported to have a strong potential for increasing bone resorption (16,17) and to be involved in the degradation of connective tissues (18).

The receptor for advanced glycation end-product expression has been detected in human gingival tissues from subjects with chronic periodontitis, with or without type 2 diabetes (19), and can be induced by advanced glycation end-products and tumor necrosis factor-α (20). Therefore, the pro-inflammatory effects of extracellular high-mobility-group box 1 acting through the receptor for advanced glycation end-products may be involved in the pathogenesis of periodontitis. However, the involvement of extracellular high-mobility-group box 1 in periodontitis remains unknown. In our preliminary study, high-mobility-group box 1 expression was detected in gingival tissues with chronic periodontitis. Interestingly, its expression was also observed in gingival epithelium with periodontitis.

The purpose of the present study was to determine whether gingival epithelial cells express high-mobility-group box 1 with or without tumor necrosis factor-α and release high-mobility-group box 1 into the local microenvironment. Furthermore, the mechanism for high-mobility-group box 1 expression in response to tumor necrosis factor-α stimulation, including the activation of mitogen-activated protein kinase (MAPK) signaling pathways, was also investigated.

Material and methods

Chemicals

Human recombinant tumor necrosis factor-α was purchased from PeproTech EC (London, UK). SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA, USA). U0126 was purchased from Promega (Madison, WI, USA). All other reagents were supplied by Sigma-Aldrich Inc. (St Louis, MO, USA).

Antibodies

A high-mobility-group box 1 antibody was obtained from Shino-Test (Tokyo, Japan). A tumor necrosis factor receptor 1 and 2 neutralizing antibody was purchased from R & D Systems Inc. (Minneapolis, MN, USA). A CD68 antibody was purchased from DakoCytomation (Glostrup, Denmark). MAPK assay kits (containing polyclonal antibodies against p38, Jun N-terminal kinase/stress-activated protein kinase and p44/42, phospho-p38, phospho-Jun N-terminal kinase/stress-activated protein kinase and phospho p44/42) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Gingival crevicular fluid sampling

Gingival crevicular fluid samples were obtained from three patients with chronic periodontitis (two men, one woman; average age 63.7 years; probing depth 7–10 mm; alveolar bone loss 24–30%) and from three healthy controls after informed consent was obtained according to guidelines approved by the Ethical Committee at Kagoshima University Graduate School of Medical and Dental Sciences. Gingival crevicular fluid was collected using periopaper (Proflow Inc., Amityville, NY, USA), as previously described (21). Briefly, periopaper was placed into the periodontal pocket for 30 s and then transferred to 50 μL of sodium dodecyl sulfate sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol). Following an interval of 60 s, the procedure was repeated twice. The high-mobility-group box 1 protein levels in the gingival crevicular fluid samples were analyzed by western blotting.

Preparation of gingival tissues

Both healthy ($n = 5$) and chronic periodontitis ($n = 10$; six men/four women; average age 59.2 years; probing depth 7–10 mm; alveolar bone loss 24–30%) tissues were obtained from patients after informed consent was obtained according to guidelines approved by the Ethical Committee at Kagoshima University Graduate School of Medical and Dental Sciences. Diseased sites that exhibited severe periodontal attachment loss were selected at random. The excised tissues were immediately fixed in 4% paraformaldehyde and then embedded in paraffin.

Immunohistochemistry

Paraffin-embedded sections (5 μm) of gingival tissues were deparaffinized in xylene and rehydrated through a series of decreasing concentrations of ethanol. After blocking endogenous peroxidase activity by 3% H_2O_2 for 15 min, the sections were processed for immunostaining using a Histofine simple stain kit (Nichirei Bioscience Inc., Tokyo, Japan). Sections were incubated in each primary antibody (anti-high-mobility-group box 1 rabbit immunoglobulin, 2 μg/mL; and anti-CD68 mouse immunoglobulin, 1 μg/mL) overnight at 4°C. After washing, the sections were incubated in Histo-

fine simple stain MAX-PO for 30 min at room temperature. Sections were washed and further incubated with the 3-amino-9-ethylcarbazole substrate-chromogen system (Nichirei Bioscience Inc.) for 10 min at room temperature. As negative controls, each isotype nonimmune serum (2 μg/mL) of the same species was used instead of the primary antibody. The sections were counterstained with Mayer's hematoxylin, mounted in Aquatex (Merck KGaA, Darmstadt, Germany) and examined with a BH2 light microscope (Olympus, Tokyo, Japan).

Immunofluorescence

To investigate the origin of high-mobility-group box 1 in the periodontitis tissues, paraffin-embedded sections (5 μm) of gingival tissues were deparaffinized in xylene and rehydrated through a series of decreasing concentrations of ethanol. After three washes with phosphate-buffered saline, the sections were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h and then incubated with a rabbit anti-high-mobility-group box 1 polyclonal immunoglobulin (1 μg/mL) and human macrophage marker immunoglobulin (anti-CD68) for 1 h at room temperature. The sections were then washed with phosphate-buffered saline and incubated with fluorescein isothiocyanate-conjugated antirabbit IgG and rhodamine-conjugated antimouse IgG (Immunotech, Marseille, France), diluted 1 : 50 in phosphate-buffered saline, for 30 min at room temperature. Finally, cell nuclei were labeled with 4′,6-diamidino-2-phenylindole (Nakalai Tesque, Kyoto, Japan) and the sections were washed and examined using an Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

Cell culture

Rat gingival epithelial cells were established from 2-wk-old Rowett rats and palatal gingival explants were prepared as described previously (22). Briefly, the palatal gingival explants were placed in tissue culture plates in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% fetal bovine serum. After 2 wk, rat gingival epithelial cells were further cultured in keratinocyte serum-free medium (Life Technologies, Rockville, MD, USA) supplemented with epidermal growth factor (5 ng/mL) and bovine pituitary extract (30–50 μg/mL). The cells were used for the following experiments after four to six passages. A human gingival epithelial cell line (Ca9-22) was obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan) and suspended in Eagle's minimal essential medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum, at 37°C in a humidified 5% CO_2 atmosphere.

Sample preparation for western blot analysis

All samples were prepared as described previously (8), with slight modifications. Briefly, cells (8×10^5 cells/well) were stimulated, for 0–20 h, with tumor necrosis factor-α at 0, 1, 5 or 10 ng/mL in Eagle's minimal essential medium containing 1% fetal bovine serum. High-mobility-group box 1-containing supernatants (1 mL), in the presence or absence of tumor necrosis factor-α, were collected and further incubated overnight with 50 μL of heparin–Sepharose 6B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After three washes with phosphate-buffered saline, sodium dodecyl sulfate sample buffer was added. For MAPK assays, the cells were lysed, as previously described (23). Briefly, 120-μL aliquots of cell suspensions (5×10^5 cells/dish) were seeded into 60-mm cell culture dishes, and cell lysates were obtained by adding 120 μL of sodium dodecyl sulfate sample buffer containing 50 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride and 0.5 mM Na_2VO_3. The supernatants and lysates were assayed for their protein concentrations using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA) and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All experiments using inhibitors were performed as described above, except that the cells were pre-incubated with a tumor necrosis factor receptor 1-neutralizing antibody or respective MAPK inhibitors for 1 h before exposure to tumor necrosis factor-α.

Western blot analysis

After subjecting all the samples to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (pH 7.4), containing 0.02% Tween 20, for 1 h at room temperature and then incubated with a primary antibody (anti-high-mobility-group box 1 immunoglobulin at 10 μg/mL or anti-MAPK immunoglobulins diluted 1 : 3000) in Tris-buffered saline (pH 7.4) containing 0.02% Tween 20 and 1% nonfat dry milk, overnight at 4°C. After three washes with Tris-buffered saline (pH 7.4) containing 0.02% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated antirabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1 : 3000 in Tris-buffered saline (pH 7.4) containing 0.02% Tween 20 and 2.5% nonfat dry milk, for 1 h at room temperature. Finally, the membranes were washed with Tris-buffered saline (pH 7.4) containing 0.02% Tween 20, and developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biosciences, Bucks., UK).

Cell viability assay

The cell viabilities were analyzed by the mitochondrial respiratory activity, which was measured using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenol tetrazolium bromide (MTT) cleavage assay (Boehringer Mannheim, Indianapolis, IN, USA). This assay was performed using a slight modification of the method described by Twentyman *et al.* (24). Briefly, cells cultured in 96-well plates (with 100 μL of medium per well) were incubated with MTT (20 μL of 2.5 μg/mL per well) at 37°C for 3 h. The formazan product was solubilized by the addition of 100 μL of dimethylsulfoxide and 100 μL of 10% sodium

dodecyl sulfate (in 0.01 M HCl) for 16 h at 37°C. The dehydrogenase activity was expressed as the absorbance (read with an Immuno Mini NJ-2300 (Inter Medical, Tokyo, Japan)) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Statistical analysis

Inhibition of individual MAPKs study and cell viability test were calculated from three separate experiments and expressed as the mean ± standard deviation. The Bonferroni correction was used for multiple *t*-test comparisons, and *p*-values were determined using STATVIEW version 5.0 for Macintosh. Values of $p < 0.05$ were considered statistically significant.

Results

High-mobility-group box 1 expression in human inflamed gingival crevicular fluid and chronic periodontitis tissues

Gingival crevicular fluid contains a rich array of biochemical factors that reflect the metabolic status of the periodontal tissue component (25). By western blot analysis, high-mobility-group box 1 was detected in the gingival crevicular fluid from periodontitis subjects, but not in that from control subjects (Fig. 1). To explore high-mobility-group box 1 expression in chronic periodontitis tissues, we performed immunohistochemical staining. As seen for gingival crevicular fluid (Fig. 1), most of the inflamed gingival epithelial cells stained positive for high-mobility-group box 1 in the periphery of the nucleus together with some translocation from the nucleus to the cytoplasm in all specimens (Fig. 2A, a,d), whereas staining showed the high-mobility-group box 1 to be localized only in the nuclei of the control cells (Fig. 2A, g,j). Cells were negatively stained with nonspecific IgG (Fig. 2A, b,e,h,k). Recent studies have demonstrated active secretion of high-mobility-group box 1 by macrophages and monocytes during tissue injury (3,8,26). Therefore, we confirmed the origin of the high-mobility-group box 1 by double-immunostaining the same tissue sample with a high-mobility-group box 1 antibody and a macrophage marker antibody (anti-CD68). Only macrophage-like cells showed positive staining for CD68, whereas no positive signals for CD68 were detected in the high-mobility-group box 1-positive gingival epithelial cells (Fig. 2B), indicating that gingival epithelial cells are the source of high-mobility-group box 1 in periodontitis.

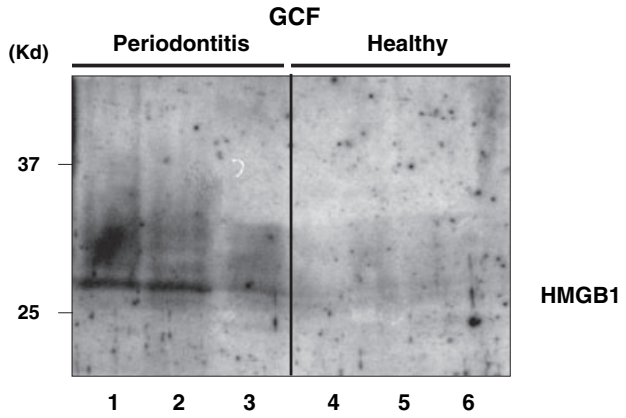

Fig. 1. High-mobility-group box 1 release into gingival crevicular fluid. Western blot analyses with a high-mobility-group box 1 antibody were performed on gingival crevicular fluid samples from three separate patients with periodontitis (lanes 1–3) and from three healthy control subjects (lanes 4–6). High-mobility-group box 1 is present in the gingival crevicular fluid from the periodontitis patients, but absent from that from the control subjects. GCF, gingival crevicular fluid; HMGB1, high-mobility-group box 1.

Tumor necrosis factor receptor 1-dependent high-mobility-group box 1 release into supernatants from rat gingival epithelial cells and Ca9-22 cells

Tumor necrosis factor-α plays a central role in gingival inflammation and is predominantly produced by activated macrophages that invade a lesion following dental plaque or bacteria becoming trapped in a periodontal pocket (27). As described above, we found that high-mobility-group box 1 was localized in both the nucleus and the cytoplasm of epithelial cells in gingival tissues of chronic periodontitis. Thus, we next explored high-mobility-group box 1 release from gingival epithelium stimulated with tumor necrosis factor-α *in vitro*. High-mobility-group box 1 was released constitutively and abundantly into culture medium from both rat gingival epithelial cells and Ca9-22 cells in a dose-dependent (Fig. 3A) and time-dependent (Fig. 3B) manner. No high-mobility-group box 1 was detected in the media from either cell type before stimulation. High-mobility-group box 1 release increased in proportion to the tumor necrosis factor-α concentration until 10 ng/mL and was present at similar levels for samples from both cell types (Fig. 3A). Beyond 20 h, the trend of increasing high-mobility-group box 1 release by tumor necrosis factor-α reached a plateau, probably because of saturation (Fig. 3B).

High-mobility-group box 1 is passively released upon necrotic cell death (6). It was therefore important to ascertain that high-mobility-group box 1 release was from viable rat gingival epithelial cells and Ca9-22 cells, rather than a result of the cytotoxicity of tumor necrosis factor-α. To address this issue, rat gingival epithelial cells and Ca9-22 cells were exposed to various concentrations of tumor necrosis factor-α for up to 24 h, and the cell viabilities were analyzed by the MTT assay. No tumor necrosis factor-α concentration up to 10 ng/mL (i.e. the highest concentration tested in this study) exerted cytotoxic effects on the cells (Fig. 3C), indicating direct dependency of the high-mobility-group

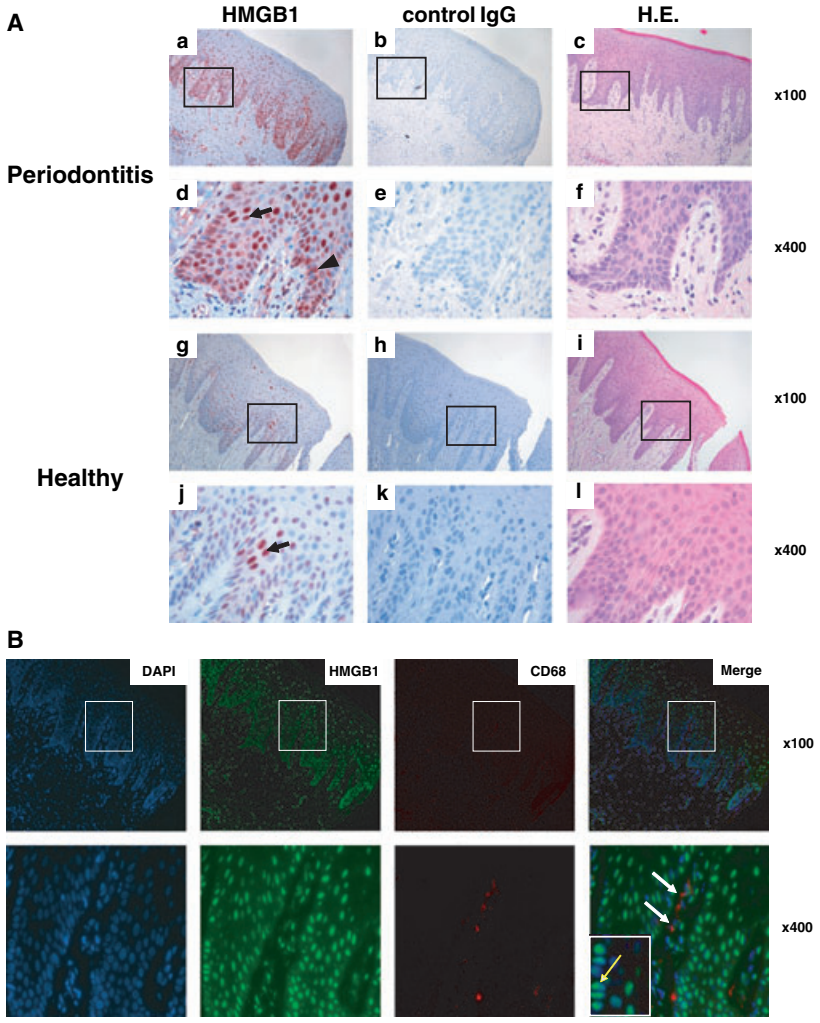

Fig. 2. Cytoplasmic high-mobility-group box 1 expression in inflamed gingival tissues. (A) High-mobility-group box 1 expression in inflamed gingival tissues. The arrow and arrowhead indicate high-mobility-group box 1 positivity in both the nucleus and cytoplasm of gingival epithelial cells, respectively (a and d). Healthy gingival tissues show high-mobility-group box 1 localization in the nucleus (arrow, g and j). Hematoxylin and eosin staining was employed at the same time (c, f, i and l). Magnifications: ×100 (rows 1 and 3) and ×400 (rows 2 and 4). (B) Double immunostaining of high-mobility-group box 1 antibody (green) and a human macrophage marker (CD68; red). High-mobility-group box 1 shuttled to the periphery of the nucleus (yellow arrow). The white arrows indicate CD68-positive staining. Magnifications: ×100 (upper row of panels) and ×400 (lower row of panels). DAPI, 4′, 6-diamidino-2-phenylindole; H.E., hematoxylin and eosin; HMGB1, high-mobility-group box 1.

box 1 release from viable gingival epithelial cells upon tumor necrosis factor-α exposure. The similar high-mobility-group box 1 up-regulation patterns between primary cell cultures of rat gingival epithelial cells and the cell line Ca9-22 (Fig. 3A,B) suggest that Ca9-22 cells can serve as a suitable model for further studies on the regulation of high-mobility-group box 1 synthesis.

The biological effects of tumor necrosis factor-α are mediated through its interaction with two distinct receptors, tumor necrosis factor receptor 1 (p55) and tumor necrosis factor receptor (p75), on target cells (28). High-mobility-group box 1 up-regulation is correlated with the extent of chronic diseases, such as rheumatoid arthritis (8). We performed blocking studies using neutralizing antibodies against tumor necrosis factor receptor 1 and tumor necrosis factor receptor 2 overlaid on the cells before stimulation with tumor necrosis factor-α, and detected high-mobility-group box 1 release into the supernatants. These blocking studies revealed inhibition of tumor necrosis factor-α-induced high-mobility-group box 1 release by the tumor necrosis factor receptor 1-neutralizing antibody (Fig. 3D), whereas the tumor necrosis factor receptor 2 neutralizing antibody had no effect (data not shown). Simultaneous incubation with both anti-tumor necrosis factor receptor immunoglobulin showed no cumulative inhibitory effect (data not shown). Taken together, these results indicate that high-mobility-group box 1 release from Ca9-22 cells can be induced through tumor necrosis factor-α/tumor necrosis factor receptor 1 ligation.

P38MAPK-mediated tumor necrosis factor-α-induced high-mobility-group box 1 release

MAPKs are involved in tumor necrosis factor receptor 1-initiated signal transduction in some cells (29,30), resulting in increased high-mobility-group box 1 release (10,31). To clarify the pathway recruited in Ca9-22 cells in response to the persistent presence of tumor necrosis factor-α, we investigated the activation of Jun N-terminal kinase 1/2, p38MAPK and p44/42 by western blot analyses with antibodies that specifically recognize each kinase or its phosphorylated form (Fig. 4A). Phosphorylation of Jun N-terminal kinase 1/2 (Fig. 4A, top panel) and p38MAPK (Fig. 4A, middle panel) was detected within 3.5 min of exposure to tumor necrosis factor-α and sustained until 30 min after exposure. Tumor necrosis factor-α also induced the phosphorylation of p44/42 within 15 min of exposure, followed by a sudden decrease (Fig. 4A, bottom panel). In parallel, our results also revealed specific inhibition of all MAPK isoforms following pre-incubation with their respective inhibitors (Jun N-terminal kinase1/2: SP600125; p38MAPK: SB203580; p44/42: U0126; Fig. 4A, last lanes of each panel).

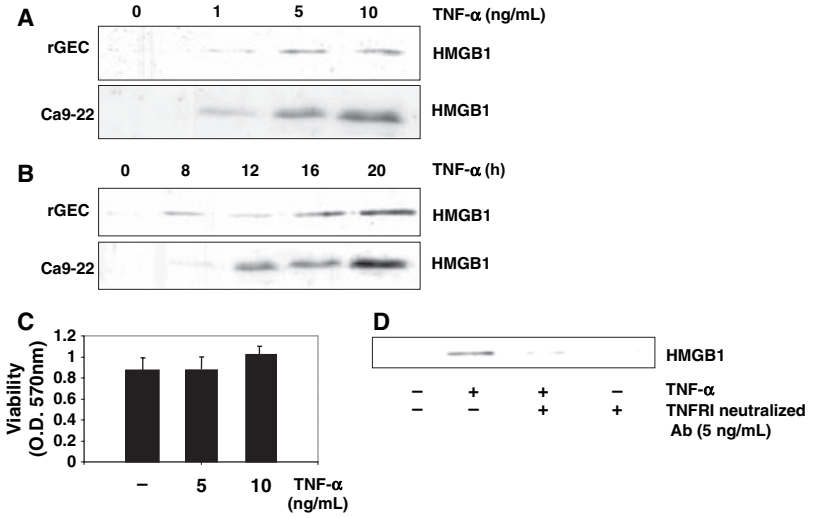

Fig. 3. Tumor necrosis factor receptor 1-dependent high-mobility-group box 1 release into the supernatants of cultured gingival epithelial cells. (A) Rat gingival epithelial cells (upper panel) and Ca9-22 cells (lower panel) were incubated with various concentrations of tumor necrosis factor-α (1, 5 or 10 ng/mL) for 12 h. (B) Rat gingival epithelial cells (upper panel) and Ca9-22 cells (lower panel) were incubated with 5 ng/mL of tumor necrosis factor-α for 0, 8, 12, 16 or 20 h, before aliquots of the supernatants containing equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by western blotting. (C) Cell viability as a function of tumor necrosis factor-α concentration. Cells were incubated with various concentrations of tumor necrosis factor-α (1–10 ng/mL). Cell viability was measured, after 24 h, by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenol tetrazolium bromide (MTT) assay, as described in the Material and methods. (D) Inhibition of high-mobility-group box 1 by a tumor necrosis factor receptor 1-neutralizing antibody. Ca9-22 cells were pre-incubated with a tumor necrosis factor receptor 1-neutralizing antibody for 1 h before being stimulated with 5 ng/mL of tumor necrosis factor-α for 12 h. Aliquots of the supernatants containing equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by western blotting. The results of duplicate experiments are shown. Ab, antibody; HMGB1, high-mobility-group box 1; rGEC, rat gingival epithelial cells; TNF-α, tumor necrosis factor-α; TNFRI, tumor necrosis factor receptor 1.

Next, we endeavored to confirm the specificity of the U0126 inhibitory effects. We showed, in Fig. 4B (top panel), that U0126 selectively ablated the tumor necrosis factor-α-activated phosphorylation of p44/42 (top panel), but did not affect the levels of phospho-p38 or phospho Jun N-terminal kinase 1/2. Interestingly, high-mobility-group box 1 protein was reduced to about 30% ($P < 0.05$) following SB203580 treatment, whereas U0126 and SP600125 had no significant effect (Fig. 4C). These results clearly suggest that the phosphorylation of p38MAPK contributes to more than half of the signaling initiated by tumor necrosis factor-α-elicited high-mobility-group box 1 release.

Discussion

The present study has shown that high-mobility-group box 1 is present in gingival tissues with chronic periodontitis, released from gingival epithelial cells and involved in excessive inflammation regulated by the tumor necrosis factor-α/p38MAPK pathway. These results suggest that continued release of high-mobility-group box 1 over time following stimulation can act, at least in part, as an important amplification signal for progressive periodontal destruction.

In the present study, we found that human gingival crevicular fluid in chronic periodontitis contained high-mobility-group box 1, whereas that from healthy control subjects did not (Fig. 1). In addition, high-mobility-group box 1 was located in the nucleus in healthy tissues, but translocated from the nucleus to the cytoplasm of epithelial cells in the chronic periodontal tissues (Fig. 2A). This finding implies that high-mobility-group box 1 is a highly motile protein that can shuttle to the cytosol via nuclear pores and be released from the cells into the gingival crevice in the inflammatory state. In Fig. 2, macrophages in the connective tissue also expressed high-mobility-group box 1, as reported previously (8,10). Interestingly, high-mobility-group box 1 was strongly detected in gingival epithelial cells. Therefore, we focused on the regulation and expression of high-mobility-group box 1 in gingival epithelial cells. To the best of our knowledge, this is the first report to demonstrate that gingival epithelial cells are the source of high-mobility-group box 1. Immunohistochemical results suggest that translocation of high-mobility-group box 1 in epithelial cells with periodontitis lesions may be affected by stimulation with inflammatory mediators.

Accordingly, we next examined the possibility of bridge formation between tumor necrosis factor-α and high-mobility-group box 1 in stimulated gingival cells, and found a direct dependency of high-mobility-group box 1 extracellular release upon tumor necrosis factor-α exposure. Specifically, tumor necrosis factor-α induced high-mobility-group box 1 release from rat gingival epithelial cells and Ca9-22 cells in a dose- and time-dependent manner. Previous studies have reported delayed kinetics of high-mobility-group box 1 secretion (8–24 h after stimulation), compared with early kinetics, such as tumor necrosis factor-α and interleukin-1, which are secreted within minutes of stimulation with lipopolysaccharide (3,32,33). In keeping with these *in vitro* secretion kinetics, high-mobility-group box 1 was found to increase in serum at 16–32 h after treatment with lipopolysaccharide in an experimental model (3). In the present study, high-mobility-group box 1 release into the supernatants from rat gingival epithelial cells and

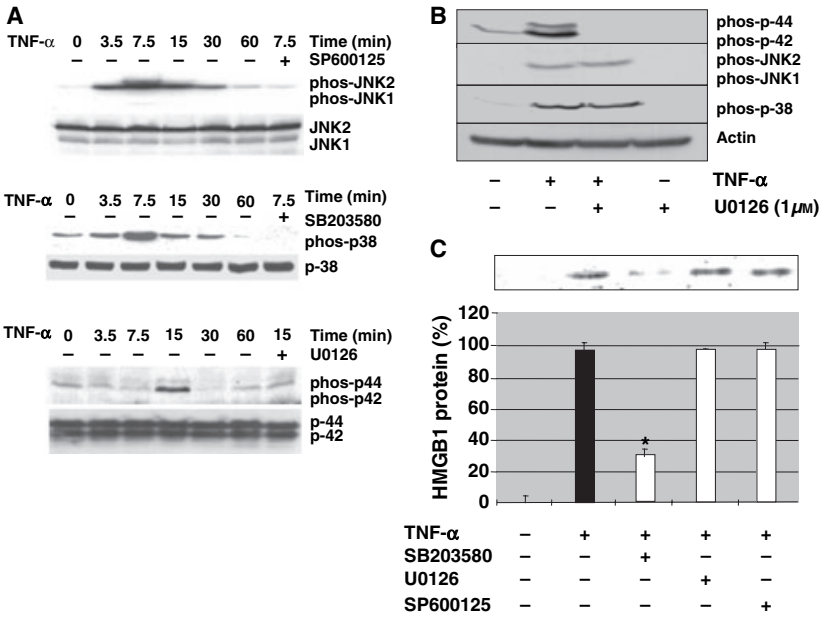

Fig. 4. p38 Mitogen-activated protein kinase (MAPK) phosphorylation in Ca9-22 cells treated with tumor necrosis factor-α, and its contribution to high-mobility-group box 1 release. (A) Cells were incubated with 5 ng/mL of tumor necrosis factor-α for 0–60 min, and the activation of Jun N-terminal kinase 1/2 (top), p38MAPK (middle) and p44/p42 (bottom) was determined by western blot analyses using antibodies that specifically recognize the activated or inactivated forms of these kinases. In the last lanes of panel A, cells were premixed for 1 h with 1 μM SB203580, U0126 or SP600125, and were then incubated in the presence of 5 ng/mL of tumor necrosis factor-α for 7.5 min (top and middle) or 15 min (bottom). (B) Cells were pre-incubated for 1 h with 1 μM U0126 before the addition of 5 ng/mL of tumor necrosis factor-α for 15 min, and then were evaluated by western blot analysis using phospho-p44/p42 (top panel), phospho-Jun N-terminal kinase 1/2 (second panel) and phospho-p38MAPK (third panel) antibodies. (C) Cells were premixed for 1 h with 1 μM SB203580, U0126 or SP600125 and were then incubated in the presence of 5 ng/mL of tumor necrosis factor-α for 12 h. Aliquots of the supernatants containing equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by western blotting. The high-mobility-group box 1 suppression levels were evaluated using computer-generated images and are shown relative to the highest high-mobility-group box 1 concentration, which was set as 100. $*p < 0.05$, $n = 3$ in each group. The values are presented as means ± standard deviation. JNK, Jun N-terminal kinase; TNF-α, tumor necrosis factor-α.

Ca9-22 cells was only detected after the cells had been exposed to tumor necrosis factor-α for at least 8 h, and the secretion continued for an unusually long period (20 h).

The signaling pathways of MAPKs (p38MAPK, p44/42 and Jun N-terminal kinase) play important roles in inflammatory diseases, such as septic shock, rheumatoid arthritis, atherosclerosis and periodontitis, as well as in other physiological processes (10,34–38). We found that p38MAPK-mediated high-mobility-group box 1 secretion is stimulated by tumor necrosis factor-α, consistent with a previous study showing that lipopoly saccharide-induced high-mobility-group box 1 release is mediated through the p38MAPK signaling pathway (31). However, another recent study reported that high-mobility-group box 1 release occurs independently of p38MAPK (10). This discrepancy may be caused by differences in the cell types and stimulants examined.

In previous studies, tumor necrosis factor-α was reported to induce cytokine release (39,40), and the addition of high-mobility-group box 1 was reported to induce *de novo* cytokine synthesis [e.g. tumor necrosis factor-α, interleukin-1α, interleukin-1β, interleukin-6, interleukin-8, macrophage-inflammatory protein-1α and macrophage-inflammatory protein-1β but not interleukin-10 or interleukin-12 (41)]. In the present study, we demonstrated that high-mobility-group box 1 release was promoted by tumor necrosis factor-α. Therefore, both tumor necrosis factor-α and the endogenous high-mobility-group box 1 may be involved in the up-regulation of cytokine production. Accordingly, cytokine release by tumor necrosis factor-α should be considered to be a result of the involvement of endogenous high-mobility-group box 1. Further studies using neutralizing anti-high-mobility-group box 1 immunoglobulin are needed to confirm this.

Taken together, we have demonstrated that high-mobility-group box 1 release from gingival epithelial cells stimulated by tumor necrosis factor-α may be involved in the progression of periodontitis. Therefore, understanding the mechanisms of high-mobility-group box 1 may lead to novel therapeutic approaches in chronic periodontitis. Further studies are still required to examine the roles of high-mobility-group box 1 in periodontal pathology.

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