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Original

Role of HMGB1 in proliferation and migration of human gingival and periodontal ligament fibroblasts

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Abstract: High mobility group box 1 (HMGB1) was originally defined as a nuclear protein. However, later studies showed that HMGB1 was released from damaged cells into the extracellular milieu and functioned as a danger signaling molecule. HMGB1 has also been shown to exert proliferative and chemoattractant effects on many cell types. In this study, we investigated the *in vitro* effect of human recombinant HMGB1 on the proliferation and migration of human gingival fibroblasts (HGF) and human periodontal ligament fibroblasts (HPDLF). For the proliferation assay, HGF and HPDLF were cultured in the presence of 5, 10, and 50 ng/mL HMGB1. After a period of 6 days, cell proliferation was determined by MTT assay. The migration assay was performed by culturing the two cell types in Transwells with HMGB1 in the lower chamber as a chemoattractant. Cell migration during 16 h was determined by crystal violet staining of the cells that migrated across the membrane. The results showed that HMGB1, at 50 ng/mL, was able to significantly induce proliferation of HGF by up to 171.4 ± 17.1%. No such proliferation induction was seen for HPDLF. In the migration assay, however, 100 ng/mL HMGB1 induced migration of both cell types. The counts of cells that migrated across the membrane, as compared with the control, were increased to 273 ± 24.1% and 410.3 ± 158% for HGF and HPDLF, respectively. Since proliferation and migration are basic abilities of cells required for proper tissue repair, these data suggest that HMGB1 plays an important role in these functions of periodontal cells.
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Keywords: HMGB1; proliferation; migration; human gingival fibroblasts; human periodontal ligament fibroblasts.

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Introduction

HMGB1, high mobility group box 1, was discovered over 30 years ago as a non-histone chromatin-binding protein present in almost all cell types (1,2). For a long time its major role was considered to be maintenance of nucleosome structure and regulation of gene transcription. Later studies revealed an extracellular role of HMGB1 as a proinflammatory cytokine (2-5), and HMGB1 is now known to be a key player in inflammatory diseases such as sepsis, acute lung injury and arthritis (3,6,7). HMGB1 can be secreted by certain cell types. It can be actively released by macrophages/monocytes in response to stimulation with exogenous bacterial endotoxin, e.g. lipopolysaccharide (LPS), or endogenous proinflammatory cytokines (2,3,5). In addition, HMGB1 can also be passively released from necrotic or damaged cells (2,4,8). Once released, HMGB1 is able to activate several other cell types involved in immune responses or inflammatory reactions. Therefore, HMGB1 might be a critical molecule that allows innate immune cells to respond to injury, and to further induce inflammation (3-5,8,9).

Apart from its cytokine activity, HMGB1 has recently been shown to recruit cells and promote regeneration in many tissues, such as heart, skeletal muscle and skin wounds (10). HMGB1 is known to affect cell behavior,

including proliferation as well as migration. In a transwell experiment, Ranzato et al. (11) showed that HMGB1 stimulated keratinocyte proliferation and promoted cell migration and in a scratch wound assay it also accelerated *in vitro* wound closure. Palumbo et al. (12) reported that extracellular HMGB1 induced both migration and proliferation of vessel-associated stem cells (mesoangioblasts) and played a role in muscle tissue regeneration. Decreased expression of T160, a member of the HMGB1 group, by antisense RNA resulted in impairment of mouse fibroblast proliferation, providing evidence of its involvement in a function that is basic to all cells (13). HMGB1 has also been reported to inhibit the proliferation of human mesenchymal stem cells, but to promote their migration and differentiation into osteoblasts (14). HMGB1 has also been shown to be important for skin wound repair, conferring chemotactic activity on skin fibroblasts and keratinocytes *in vitro*. In an experiment using diabetic mice, application of topical HMGB1 to wounds enhanced arteriole density and granulation tissue deposition, and accelerated wound healing (15). Furthermore, HMGB1 has been shown to stimulate the migration of several cell types including human dendritic cells (16), rat smooth muscle cells (17), human monocytes (18), mouse neuroblastoma cells, mouse myoblasts, mouse melanoma cells, rat glioma cells and human fibrosarcoma cells (19).

Periodontal disease, including gingivitis and periodontitis, is a common oral problem, especially among the elderly. The disease causes inflammation and destruction of periodontal tissues, eventually leading to tooth mobility and tooth loss. Attempts have been made to repair and regenerate these destroyed tissues, including the use of specific bioactive substances or surgical bone grafts (20). For periodontal regenerative therapy, attachment gain is the most important event, and this requires migration of periodontal cells to cover the formerly disease-affected cementum or dentin. In the present study, we investigated the *in vitro* effect of HMGB1 on proliferation and migration of human gingival fibroblasts (HGF) and human periodontal ligament fibroblasts (HPDLF) in order to clarify the response of periodontal tissues to HMGB1. Any positive effect of HMGB1 on periodontal cells would suggest its potential utility for induction of periodontal attachment.

Materials and Methods

Cell culture and reagents

HGF and HPDLF were obtained from caries-free third molars extracted for orthodontic reasons at the Faculty of Dentistry, Srinakharinwirot University, with the patients' informed consent. The teeth were washed with sterile phosphate-buffered saline (PBS) several times. The gingival tissues were gently removed from the cervical area and the periodontal tissues were scraped from the middle third of the roots using sterile surgical blades. HGF and HPDLF were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (10% FCS-DMEM), 2 mM L-glutamine, 100 IU/mL penicillin G and 100 IU/mL streptomycin. Cultures were maintained at 37°C under 5% CO_2. The medium and all supplements were obtained from Gibco BRL (Carlsbad, CA, USA). HGF and HPDLF were prepared from three donors, and cells between passages 3-8 were used. This study had been approved by the Research Ethics Committee, Faculty of Dentistry, Srinakharinwirot University. Human recombinant HMGB1 (HMGB1) was purchased from Sigma, USA, solubilized in sterile distilled water, and kept at -20°C until use.

MTT cell proliferation assay

Cells (3,000 per well) were plated in 96-well plates in 10% FCS-DMEM overnight to allow attachment. The next day, the wells were washed 3 times with DMEM and the medium was changed to serum-free DMEM (SF-DMEM). HMGB1 was added to the cells at 1, 5, 10, and 50 ng/mL (in triplicate). Sterile distilled water was used as a negative control. On day 6, the medium was changed to fresh DMEM (100 µL per well). Ten microliters of methyl thiazolyl-diphenyl-tetrazolium bromide (MTT) solution (5 mg/mL in PBS) was added to each well and incubated for an additional 30 min at 37°C, 5% CO_2. The colored formazan product was then dissolved in 100 µL of dimethyl sulfoxide (DMSO), and the optical density (OD) was read at 550 nm. Cell numbers were calculated using a standard curve generated between the cell count and the OD550 read. Before analyzing the data, the cell counts were converted to percentages relative to the control group, which was regarded as 100%.

Migration assay

Cell migration assay was performed in a 24-Transwell chemotaxis chamber (6.5 mm in diameter, 8 µm pore size, Corning Life Sciences, USA). Cells were harvested using PBS containing 0.2 mg/mL EDTA and resuspended in DMEM containing 1% fetal calf serum (1% FCS-DMEM). The upper chamber of the Transwell contained 120,000 cells, and HMGB1 dissolved in 1% FCS-DMEM was put into the lower chamber (100 ng/mL in 500 µL of medium). Fibroblast-conditioned medium was used as a positive control, and sterile distilled water added to 1%

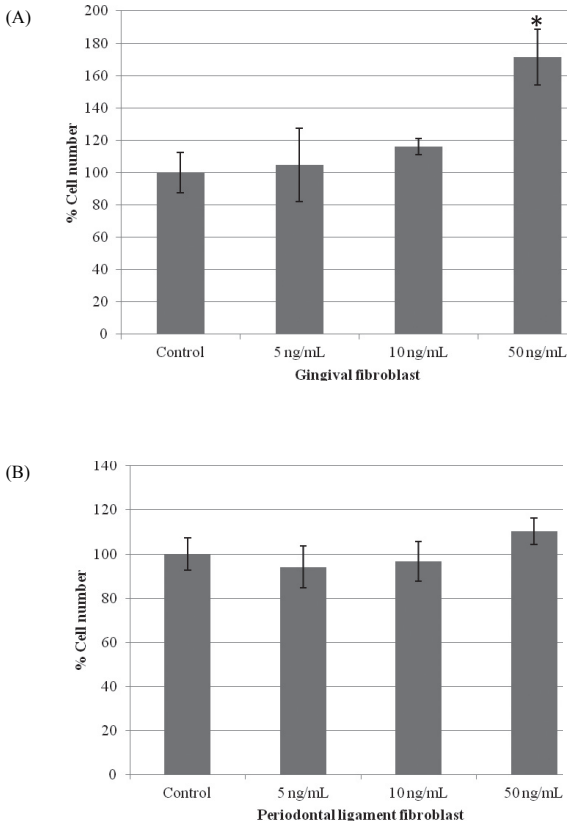

Fig. 1 Determination of proliferation by the MTT assay for HGF (A) and HPDLF (B). Cells (3,000 per well) were plated overnight in a 96-well-plate. The following day, the conditioned medium was changed to SF-DMEM (100 µL/well). HMGB1 was added to the cells at concentrations of 5, 10 and 50 ng/mL. Sterile distilled water was used as a control. After 6 days, the cultured medium was discarded and the MTT assay was performed. (*statistically significant at $P < 0.05$).

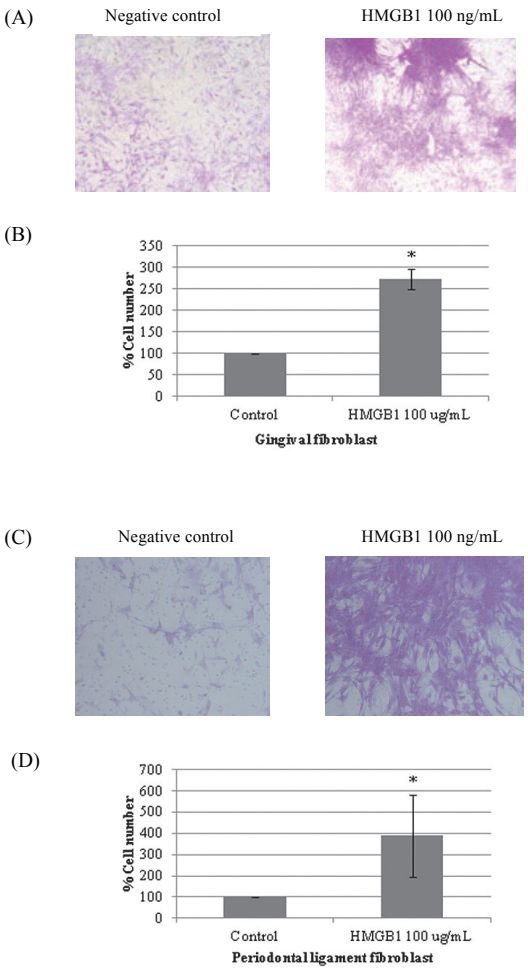

Fig. 2 Migration assay using the transwell method for HGF (A, B) and HPDLF (C, D). Cells (120,000) were plated into the upper chamber of a 6.5-mm transwell (8 µm pore size). Five hundred microliters of chemoattractant (100 ng/mL HMGB1 in 1% FCS-DMEM) was added to the lower chamber. Sterile distilled water in 1% FCS-DMEM was used as a negative control, and fibroblast-conditioned medium was used as a positive control (data not shown). After 16 h, cells on the upper side were removed and cells in the lower chamber were fixed with methanol and stained with 0.1% crystal violet. (A) and (C) show examples of HGF and HPDLF, respectively, on the stained membrane viewed at 40× magnification with an inverted microscope (*statistically significant at $P < 0.05$).

FCS-DMEM was used as a negative control. The cultures were maintained at 37°C under 5% CO_2 for 16 h. Cells on the upper surface of the membrane were removed with a cotton swab and cells that migrated to the lower side were fixed with methanol and stained with 0.1% crystal violet. The membrane was visualized with an inverted microscope at x40 magnification. Cell counting was performed using the Motic image analysis program. Four representative visual fields were selected for each membrane. Three separate experiments were performed.

Statistical analysis

Data were presented as mean ± SD for triplicate assays. The significance of differences was analyzed by one-way ANOVA, and Student's *t*-test was used for paired comparisons. Differences were considered to be significant at $P < 0.05$.

Results

HMGB1 induces proliferation of HGF but not HPDLF

HGF and HPDLF appeared morphologically normal in the presence of HMGB1 at 5, 10, and 50 ng/mL (data not shown). After 6 days, 50 ng/mL HMGB1 induced significant proliferation of HGF as determined by the MTT assay (Fig. 1A). The HGF cell count was increased

to 171.4 ± 17.1% relative to the control (100 ± 12.6%). At a lower concentration of HMGB1, slight but non-significant stimulation of cell proliferation was observed (104.7 ± 22.6% and 116.3 ± 4.9% in the presence of 5 and 10 ng/mL HMGB1, respectively). HPDLF, however, did not show any significant changes in cell count relative to the control group, being 100 ± 4.4%, 94.1 ± 10.2%, 96.7 ± 3.5%, and 110.3 ± 7.2% for the control preparation, and in the presence of 5, 10, and 50 ng/mL HMGB1, respectively (Fig. 1B).

HMGB1 induces migration of HGF and HPDLF

Both HGF and HPDLF show cell motility. By 16 h in the Transwell chamber, many of the cells had migrated through the 8-µm pores to the lower side of the membrane, where they were stained dark purple with crystal violet (Figs. 2A and 2C). Fibroblast-conditioned medium, which was used as a positive chemoattractant, induced a very strong migratory response. Both HGF and HPDLF migrated through the membrane and appeared as large clumps of cells with very dark crystal violet staining (data not shown). HMGB1, at 100 ng/mL, also elicited a marked migratory response of both HGF and HPDLF as compared with the negative control (Fig. 2). The counts of cells that migrated across the membrane were increased to 273 ± 24.1% and 410.3 ± 158% relative to the control for HGF and HPDL, respectively.

Discussion

Peridontal disease involves a complex interaction between causative periodontopathic bacteria and host immune responses. Contemporary periodontal therapy is aimed at controlling the infection and regenerating lost periodontium, i.e. cementum, periodontal ligament, alveolar bone and gingiva (20). Basic wound healing requires migration, adhesion, proliferation and differentiation of several cell types (21). These processes involve cytokines and inflammatory mediators in the periodontal tissues. In the present study, we tested the proliferative and migratory activities of two cell types from periodontal soft tissues, HGF and HPDLF, after stimulation with HMGB1.

HMGB1 is now widely accepted as a multifunctional cytokine involved in inflammatory responses and tissue repair (4,5,9,15). Many studies have shown that HMGB1 is secreted from many types of cells, including HGF (22), under stressed conditions (2,8-10). The gingival crevicular fluid (GCF) of patients with periodontal disease contains high levels of HMGB1 (23), possibly released from inflamed epithelium in periodontal pockets (24), as well as activated immune cells (2,5). Extracellular HMGB1, apart from its cytokine activity, affects the proliferation and/or migration of many cell types (11,12,15,17,25). A role of HMGB1 in wound closure has been demonstrated in an *in vitro* study (11), and its reported ability to act as a chemoattractant for fibroblasts, endothelial and smooth muscle cells lends support for its role in wound healing (12,15,17,18).

In the present study, HGF and HPDLF showed different responses to HMGB1 in terms of proliferation. Only HGF showed a proliferative response to HMGB1. This difference between the two fibroblast lines was not surprising. Although both are fibroblastic in nature, there is evidence to support some characteristic differences between the two cell lines. Somerman et al. (26) compared the *in vitro* characteristics of HGF and HPDLF. Although both lines are able to stimulate periodontal regeneration, they differ in terms of protein pattern, collagen production and alkaline phosphatase levels (27). Previous work has shown that HMGB1 induces proliferation of keratinocytes and mesoangioblasts (11,12). By contrast, HMGB1 has been reported to inhibit the proliferation of human mesenchymal stem cells (14). With regard to cell migration, various studies have demonstrated mostly a stimulatory effect of HMGB1 on skin fibroblasts, keratinocytes, mesoangioblasts, monocytes, human mesenchymal stem cells and human dendritic cells (12,14,15,25,28). A migration-inhibitory effect of HMGB1 has been reported only for enterocytes (29). In the present study, HMGB1 stimulated the migration of both HGF and HPDLF. Interestingly, HPDLF, which showed no proliferative response to HMGB1, showed a stronger migratory response. A previous study by Basdra and Komposch (30) showed that HPDLF exhibited the phenotypic characteristics of osteoblast-like cells *in vitro*, and suggested that they had the potential to differentiate into osteoblasts. In support of this, HMGB1 has been shown to inhibit the proliferation of human mesenchymal stem cells and promote their migration and differentiation along the osteoblastic pathway (14).

However, the difference in the responses between HGF and HPDLF observed in the present study might have been due to the presence of fibroblast subpopulations. As reviewed by MuCulloch and Bordin (31), fibroblasts *in vitro* contain heterogeneous populations that differ in terms of matrix biosynthesis, enzyme activities, protein expression and proliferative potential. The various effects of HMGB1 on soft tissues are an interesting aspect that requires further *in vitro* and *in vivo* studies before its clinical application can be considered.

In conclusion, we have demonstrated that HMGB1 stimulates the proliferation of HGF and the migration of HGF and HPDLF. Since both proliferation and migration

are cell properties essential for repair and regeneration, HMGB1 would seem a promising molecule for therapeutic use in periodontal regeneration. Further studies of its mechanism of action and the cell receptor(s) involved will be required. However, since extracellular HMGB1 also acts as a proinflammatory cytokine (4,5), its overexpression might lead to inflammatory reactions and tissue destruction. Another important issue is that periodontium also contains hard tissues: cementum and alveolar bone. Yamoah et al. (32) have reported an interrelationship between HMGB1, TNF-α expression and osteoclast formation. A thorough understanding of the molecular mechanism of HMGB1 and its effects on various local cells is essential before this molecule can be applied clinically.

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