In Vitro Effect of LPS on HMGB1 Expression in Human Periodontal Ligament Fibroblast

นิพนธ์ต้นฉบับ

Original Article

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วารสารไทยเภสัชศาสตร์และวิทยาการสุขภาพ 2554;6(3): 175-181

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บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาการแสดงออกของระดับเอ็มอาร์เอ็นเอและโปรตีนของเอชเอ็มจีบีวัน (high-mobility group box-1; HMGB1) ในเซลล์ไฟโบรบลาสต์เอ็นยึดปริทันต์มนุษย์ เปรียบเทียบกับเซลล์ไฟโบรบลาสต์เนื้อเยื่อในโพรงฟันมนุษย์ เซลล์ไฟโบรบลาสต์เหงือกมนุษย์ และเซลล์ไลน์เคบี โดยเฉพาะในเซลล์ไฟโบรบลาสต์เอ็นยึดปริทันต์มนุษย์ทั้งสภาวะปกติและสภาวะถูกกระตุ้นด้วยไลโปโพลีแซคคาไรด์จากเชื้อ *E. coli* **วิธีการศึกษา:** ศึกษาการแสดงออกของเอชเอ็มจีบีวันในระดับเอ็มอาร์เอนเอโดยวิธี RT-PCR ระดับโปรตีนโดย Western analysis ใช้ไลโปโพลีแซคคาไรด์ในขนาด 25 และ 50 มคก./มล.ในการกระตุ้นเซลล์ไฟโบรบลาสต์เอ็นยึดปริทันต์มนุษย์ **ผลการศึกษา:** พบการแสดงออกของเอชเอ็มจีบีวันในระดับเอ็มอาร์เอนเอ และโปรตีนในไลเซทของเซลล์ทั้ง 4 ชนิด แต่ไม่พบโปรตีนเอชเอ็มจีบีวันในอาหารเลี้ยงเซลล์ เมื่อกระตุ้นด้วยไลโปโพลีแซคคาไรด์จากเชื้อเอสเชอริเชียโคไล พบการเพิ่มระดับของเอ็มอาร์เอนเอของเอชเอ็มจีบีวัน เฉพาะในสภาวะที่มีซีรัมของลูกวัวในอาหารเลี้ยงเซลล์ แต่ไม่พบการเปลี่ยนแปลงระดับโปรตีนเอชเอ็มจีบีวันในเซลล์ไลเซท แสดงว่าเมื่อเอ็มอาร์เอนเอดังกล่าวเพิ่มขึ้น ไม่สามารถผ่านต่อเป็นโปรตีนในสภาวะการทดลองนี้ **สรุป:** เซลล์ไฟโบรบลาสต์เอ็นยึดปริทันต์มนุษย์ เซลล์ไฟโบรบลาสต์เนื้อเยื่อในโพรงฟันมนุษย์ เซลล์ไฟโบรบลาสต์เหงือกมนุษย์ และเซลล์ไลน์เคบี มีการแสดงออกของเอชเอ็มจีบีวันในระดับเอ็มอาร์เอนเอและโปรตีนเฉพาะในเซลล์ไลเซท แสดงว่าเอชเอ็มจีบีวันไม่มีบทบาทเป็นโปรตีนที่หลั่งออกนอกเซลล์ช่องปากเหล่านี้ การเพิ่มระดับเอ็มอาร์เอนเอของเอชเอ็มจีบีวันในเซลล์ไฟโบรบลาสต์เอ็นยึดปริทันต์มนุษย์เฉพาะในสภาวะที่มีซีรัมในอาหารเลี้ยงเซลล์เมื่อได้รับการกระตุ้นด้วยไลโปโพลีแซคคาไรด์ยืนยันความสำคัญของ LPS-binding protein ซึ่งพบในซีรัม บทบาทของเอชเอ็มจีบีวันในโรคปริทันต์อักเสบ การส่งสัญญาณของโปรตีนนี้ และบทบาทภายในเซลล์ของเอชเอ็มจีบีวันต่อเซลล์ไฟโบรบลาสต์เอ็นยึดปริทันต์มนุษย์ต้องศึกษาต่อไป

คำสำคัญ: เอชเอ็มจีบีวัน, ไลโปโพลีแซคคาไรด์, เซลล์ไฟโบรบลาสต์เอ็นยึดปริทันต์มนุษย์

Abstract

Objective: To investigate an *in vitro* mRNA and protein expression of high-mobility group box-1 (HMGB1) in human periodontal ligament fibroblast (HPDLF) in comparison to human pulpal fibroblast (HPF), human gingival fibroblast (HGF) as well as human KB carcinoma cell line (KB). For HPDLF, investigation was performed in normal condition as well as after challenged by lipopolysaccharide from *Escherichia coli* (*E. coli* LPS). **Method:** For all four cell types, reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine HMGB1 mRNA expression. Western analysis was used to detect protein expression. *E. coli* LPS at 25 and 50 µg/ml was treated to HPDLF. **Results:** For all cell types, HMGB1 mRNA and protein expressions in cell lysates were found, but not in conditioned culture media. HMGB1 mRNA was up regulated after treated with *E. coli* LPS only in the condition with fetal calf serum added. However, HMGB1 in HPDLF cell lysate remained unchanged indicating that the increased RNA expression of HMGB1 could not proceed through protein production in our experimental condition. **Conclusion:** HPDLF, HPF, HGF and KB cell express HMGB1 mRNA and protein only in cell lysate. This seems to exclude the roles of HMGB1 as a secreted protein in these oral cells. Up-000regulation of HMGB1 mRNA in HPDLF only in the presence of serum confirmed the stimulation of LPS to cell required LPS-binding protein presence in serum. The role of HMGB1 in periodontitis and signaling pathway of this protein expression, and intracellular role of HMGB1 in HPDLF is subject to further study.

Keywords: high mobility group box 1 (HMGB1), lipopolysaccharide (LPS), human periodontal ligament fibroblasts (HPDLF)

Introduction

High mobility group box protein 1 (HMGB1), a small protein of 215 residues, was first identified over 30 years ago.[1] Previously, HMGB1 was known as a nuclear component with its role involving maintenance of chromatin structure and regulation of gene transcription.[1,2] Later studies showed extracellular role of HMGB1 as a proinflammatory cytokine.[3] This role has opened up a new field of research of HMGB1 in inflammatory diseases.

HMGB1 can be released by some 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, e.g. tumor necrosis factor (TNF), interleukin 1 β (IL-1β) and interferon gamma (IFN-γ).[4-6] In addition to being actively released from activated innate immune cells, HMGB1 can also be passively released from necrotic or damaged cells. Once released, HMGB1 is able to alert other

cells involved in the immune response or inflammatory reaction. Therefore, HMGB1 might be a critical molecule that allows innate immune cells both to respond to injury, and to further induce inflammatory reaction. HMGB1 has been termed as a late mediator of endotoxin lethality, because its releasing is delayed by several hours compared with other proinflammatory cytokines that mediate shock and tissue injury.[7] The cytokine activity of HMGB1 has been well-documented in many cell types and tissues such as macrophages/monocytes, endothelial cells, neutrophils, epithelial cells, dendritic cells, smooth muscle cells, brain, lung, joint, etc.[8] However, most cells including lymphocytes, adrenal or kidney cells, are unable to secrete HMGB1.[4]

HMGB1 has an important role in several acute and chronic inflammatory diseases such as sepsis, acute lung injury and arthritis.[9] Periodontitis is an infectious disease that leads to inflammation of the gingiva and destruction of periodontal tissues.[9] Oral microbes are associated in the initiation and progression of periodontal disease that eventually cause tooth loss. Gram negative bacteria have become widely recognized as the periodontopathic bacteria.[10] The bacteria are capable of producing virulent substances that directly cause periodontal tissue breakdown and alveolar bone resorption.[11]

LPS, a component of the bacterial outer membrane, is the pathogenic factor in a wide variety of periodontopathic bacteria. The LPS molecule consists of three parts: LIPID A, core polysaccharide, and O-specific chains (O antigens). When periodontopathic bacteria proliferate in periodontal pockets, it is suggested that LPS is released, causing resident fibroblasts as well as immune cells to synthesize inflammatory cytokines including IL-1, IL-6 and TNF-α.[12] These cytokines, in turn, aggravate inflammation, destroy periodontal tissues, and induce alveolar bone resorption.[13,14] When derived from *Escherichia Coli* (*E. Coli*), LPS serves as polyclonal B-cell mitogens commonly used in immunology laboratory. Treatment of mice with LPS which causes liver injury induces a threefold increase of HMGB1 protein in liver within 1 - 2 hours.[15]

There had been some studies regarding HMGB1 roles in oral diseases. Morimoto et al (2006) showed expression of HMGB1 in gingival epithelium of patients with periodontitis as well as human gingival carcinoma cell line *in vitro*.[16] Feghali et al showed expression of HMGB1 in human gingival fibroblast (HGF) and its released after stimulation with periodontopathic bacteria LPS.[17] Our present study aimed to characterize the mRNA and protein expression profile of HMGB1 in primary human periodontal ligament fibroblast (HPDLF) under normal condition and after stimulation by *E. coli* LPS. The findings from this study could help understand more on the biologic basis of periodontal pathogenesis, which might lead to new strategies for the treatment of periodontitis.

Materials and Method

Cell culture and activation by LPS

Human periodontal ligament fibroblasts (HPDLF), human gingival fibroblasts (HGF) and human pulpal fibroblasts (HPF) were cultured from the explants obtained from the non-carious, freshly extracted third molars, or teeth removed for orthodontic reason with informed consent. The periodontal ligament (PDL) was scraped from the middle third of the root. Pulpal tissue was obtained from the pulp chamber after separated the tooth. Gingival tissue was obtained from healthy gingiva attached to extracted tooth which was removed for orthodontic reason. At least 2 sets for each tissue were used in this study. All of the tissue were washed with sterile phosphate buffer saline (PBS) and cut into small pieces, then plated into 60-mm tissue cultured plates (Nunc, Napervile, IL, USA). All explants as well as KB cells line (originally from ATCC NO.CCL-17: oral epidermal carcinoma cell) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum (5%FCS-DMEM), 2mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in humidified atmosphere of 5% CO_2. Media and the supplements were from Gibco BRL (Carlsbad, CA, USA). Cells from the third to eighth passages were used in this study. Lipopolysaccharide (LPS) from *E.Coli* (0111:B4 serotype) (Sigma) was dissolved in distilled water and its stock was prepared at 10 µM.

Cells were seeded in 6-well plates ($2x10^5$ cells/well) (Nunc, Napervile, IL, USA) or in 96-well plates (15,000 cells/well) in 5% FCS–DMEM and allowed to attach overnight. After attachment, cells were washed 3 times with DMEM and media changed to serum free DMEM (SF-DMEM) as well as 2.5% fetal calf serum (2.5%FCS-DMEM). In LPS treatment group, LPS was added to a final concentration of 25 µg/ml and 50 µg/ml and sterile distilled water was used as a control. Durations of LPS treatment were 24 hours for RNA extraction, and 48 and 72 hours for

cell lysate extraction and conditioned media collection respectively. The experiments were performed twice with duplication in each experiment. To determine HMGB1 expression, total RNA analysis was performed by reverse-transcriptase polymerase chain reaction (RT-PCR) and protein analysis was investigated both in cell lysate and cultured media by Western analysis.

The determination of HMGB1 expression by RT-PCR

Total RNA was extracted using TRIzol reagent (Life Technologies), as recommended by the manufacturer. Briefly, 1 ml of TRIzol reagent was added to each well for 5 minutes. Then 200 µl of chloroform were added and centrifuged at 10,000 rpm, 4 °C, for 15 min. The upper aqueous part was collected, 500 µl of isopropanol was then added and centrifuge at 10,000 rpm, 4 °C, for 10 min. The cell pellet was collected and washed with 75% ethanol, mixed and spun down. The pellet was air dried at room temperature. Total RNA was dissolved by adding 15 µl of RNase-free water, warmed at 55 °C for 10 min. and stored at -70 °C until used.

First strand cDNA was generated according to RevertAid™ first strand cDNA synthesis kit (Fermentas, Life Sciences, USA). The RNA was first DNase-treated in a 20 µl reaction containing 10 µl of RNA, 2 µl PCR buffer, 10 U DNase, and 40 U RNase inhibitor. Reverse transcription was conducted in a 25 µl reaction of 2 µg of DNase-treated RNA, 0.5 µg of oligo d(T) 12-18 and DEPC-treated water. The reaction was heated to 70 °C for 10 mins then chilled on ice for 5 min. The content of 5X reaction buffer, 40 U of ribonuclease inhibitor and dNTP was added into the mixture and incubated at 37 °C for 5 min. before the addition of 1 µl of RevertAid™ M-MuLV reverse transcriptase. The mixture was mixed and incubated at 42 °C for 1 hour. The mixture was then collected and stored at -20 °C until used.

HMGB1 primers were synthesized by OPERON (Qiagen). The sequence of primers was determined using the clustalx 1.6b. Primer's length was between 18 and 25 bases and melting temperature ™ was between 50 and 65 °C (Oligo v.3.4). To determine specificity, all sequences were compared with the Genbank using the program Blast available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

The sequences of primers are as follows:

HMGB1: Forward; 5'-CTCTTCTGCTCTGAGTATCGC-3'
Reverse; 5'-CAACTGAAGATGAAAAAACTACC-3'
GAPDH: Forward; 5'-TGAAGGTCGGAGTCAACGGAT-3'
Reverse 5'-TCACACCCATGACGAACATGG-3'.

The PCR was performed using Taq polymerase with a PCR volume of 25 µl. The mixtures contained 10 pg – 1 µg of cDNA template, 0.2 mM of each of dNTPs, 10x PCR buffer, $MgCl_2$ concentration between 1 - 5 mM, 0.6 U of Taq DNA polymerase, 0.2 - 0.3 µM of HMGB1 and GAPDH primers. The PCR working conditions were set for initial activation at 95 °C, for 5 min., a denaturation at 94 °C, for 15 sec, primer annealing at 53 °C for 1 min, chain elongation at 72 °C for 1 min. and final extension at 72 °C for 10 min. on a DNA thermal cycler (ICycler, BioRad). The amplified DNA was proceeded to electrophoresis on a 1.6% agarose gel and visualized by ethidium bromide staining.

HMGB1 protein determination by Western analysis

Conditioned media were collected and spun down to remove cell debris. Supernatant was kept for extracellular protein measurement. Cells were washed with PBS and protein extracted by 100 µl of 1% SDS for cell lysate analysis. The total proteins were measured by Bradford reagent. All samples were fractionated by 10% SDS-PAGE. Equal amount of sample loadings were calibrated based on amount of total protein in cell lysate. The proteins were then transferred to a (poly) vinylidene difluoride immunoblot membrane (PVDF) (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked for 1 hour with blocking solution (5% skim milk in TBS-T) and then incubated with HMGB1 antibodies (Monoclonal Anti-HMG-1 Clone HAP46.5, Sigma, USA) for 1 hour, at 1:1,000 dilution. The membrane was washed for 10 min. 5 times in washing solution and then incubated with ECL Anti-Mouse IgG, Horseradish Peroxidase-Linked Species-Specific whole Antibody (from sheep) (Amersham Bioscience, UK) as the secondary antibody, 1:50,000 dilution for 1 hour at room temperature. Signals were developed with an enhanced chemiluminescence kit (ECL plus) (Amersham Pharmacia Biotech) according to manufacturer's protocol. For detection of the chemiluminescent signal, the membrane should be exposed to standard Hyperfilm™ ECL (Amersham Bioscience, UK). The several exposures may be required in order to generate the appropriate signal strength.

Results

Expression of HMGB1 in HPDLF, HPF, HGF and KB cell line

HMGB1 RNA, protein expression in HPDLF and their responses by LPS activation were investigated in this study. It was of interest to determine the expression of HMGB1 in primary HPDLF compared to other oral cells including primary HPF, HGF as well as KB cell line. We performed RT-PCR and western analysis for RNA and protein detection, respectively. It was found that all cells expressed HMGB1 mRNA (Figure 1A) as well as HMGB1 protein in cell lysate (Fig. 1B). HMGB1 was not found in conditioned media for all cell types in this study (data not shown).

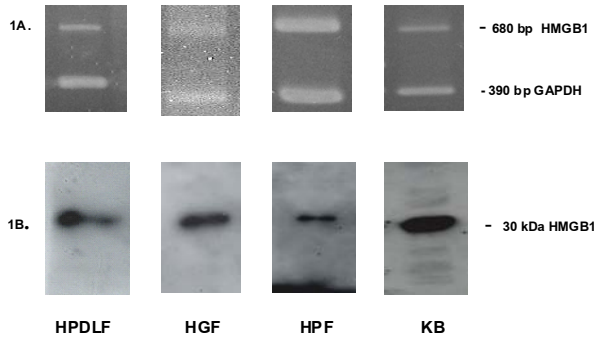

Figure 1 Expression of HMGB1 in various oral fibroblasts and KB cells.

Note: Cells ($2x10^5$ cells/well) were plated in a 6-well plate. After attachment, media were changed to DMEM with 2.5% FCS. At 24 and 48 hours, RNA and cell lysate were collected respectively. HMGB1 RNAs were shown by RT-PCR along with GAPDH (1A). The levels of cellular HMGB1 contained in the cell lysate were determined by Western analysis of the cell lysate (1B).

HMGB1 RNA expression after LPS activation

In this study *E. coli* LPS was used to induce HPDLF injury. Previous study suggested that activation by LPS required specific plasma protein such as LPS-binding protein (LBP) in order to mediate attachment to receptors on cells.[18] Thus, we treated HPDLF with *E. coli* LPS in both serum-free and in 2.5% FCS media condition.

After 24-hour incubation, expression of HMGB1 RNA by semi-quantitative RT-PCR was analyzed. GAPDH as a house keeping gene was used for an internal control. Appropriate cycle number was initially tested and cycle 24 was selected as it had started to show HMGB1 expression in LPS treated group but not in a control group (Fig. 2A). However, this up-regulation only occurred in a condition with 2.5% FCS but not in a serum free condition, confirming the requirement of LPS on LPS-binding protein (LBP) presence in serum.[18,19] In SF-DMEM, HMGB1 expression started to show around cycle 26 and there was no difference in levels between the control and LPS-treated group even when we delayed the PCR cycle to 30 (figure 2B.). The data here suggested that the expression of HMGB1 RNA in HPDLF has been up-regulated by 25 and 50 µg/ml of LPS, only in 2.5% FCS-DMEM condition.

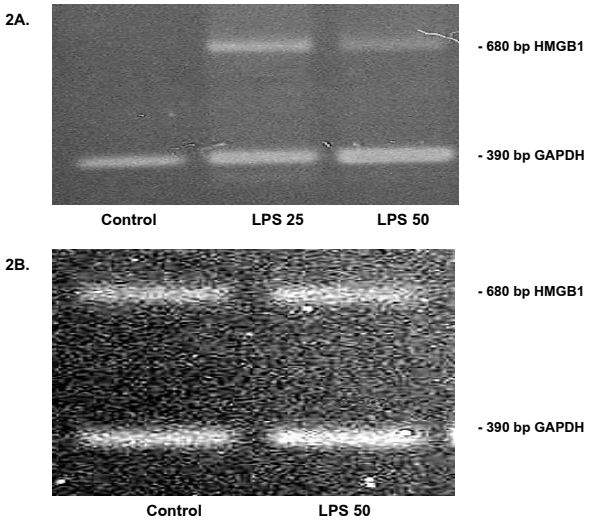

Figure 2 Up regulation of HMGB1 RNA after LPS treatment.

Note: HPDLF ($2x10^5$ cells/well) were plated in a 6-well plate and treated with 25 and 50 µg/ml of *E. coli* LPS in 2.5% FCS-DMEM (2A) and SF-DMEM (2B) for 24 hours. Sterile dH_2O was used as a control. After 24-hr RNA extraction, RT-PCR was performed to analyze HMGB1 (680 bp) and GAPDH (390 bp), internal control. Only in media with 2.5% FCS that up regulation of HMGB1 RNA could be seen (cycle 24 for 2A. and cycle 30 for 2B.).

HMGB1 protein expression and secretion after LPS activation

In this study HMGB1 protein expression in HPDLF, both in cell lysate and in conditioned media, was investigated. It was found that cellular HMGB1 was unchanged after LPS treatment for 48 - 72 hours, as shown in Fig. 3A and 3B. HMGB1 secretion in conditioned media also was investigated since there are studies showing induction of HMGB1 secretion with LPS, IL-1β and TNF-α stimulation in some cell types.[4-6] However, after 48-, 72- and 96-hour LPS treatment to HPDLF, HMGB1 was still undetectable in conditioned media as shown in Fig. 3C for a 96-hour incubation.

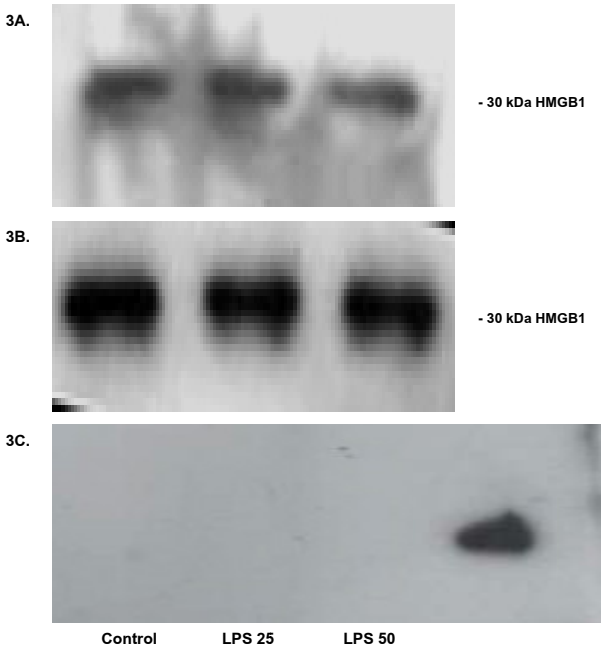

Figure 3 Western analysis of HMGB1 protein in cell lysate and conditioned media.

Note: HPDLF ($2x10^5$ cells/well) were plated in a 6-well-plate and treated with 25 and 50 µg/ml of *E. coli* LPS in 2.5% FCS-DMEM. Sterile dH_2O was used as a control. Cellular HMGB1 was not up-regulated after LPS treatment for 48 and 72 hour (3A and 3B respectively). In Fig. 3C, HMGB1 was undetectable in conditioned media after 96 hour LPS treatment. KB cell lysate was used as a positive control for western analysis.

Discussions and Conclusion

Many basic and clinical studies support the concept that human periodontitis is a disease caused by several types of gram-negative bacteria, mainly existing in dental plaque [20]. It has also been clarified that periodontal disease is the end result of the host immune response to bacterial product such as capsular polysaccharide, outer membrane protein, enzymes and LPS, rather than the result of one sided invasion of a periodontopathic bacterium. HPDLF may interact directly with bacteria and their products and it is suggested that these cells play important roles in the host responses of patients. Previous studies showed that, upon stimulation by LPS, HPDLF produced various inflammatory cytokines including IL-6 and IL-8.[15] These cytokines may then initiate and exacerbate periodontal inflammatory diseases.

HMGB1, a small protein discovered 30 years ago[1], was first described as a nuclear protein but later found to be extracellular in some cells types. Previous studies showed that, HMGB1 is produced in many cell types including monocytes, macrophages, endothelial cells, dendritic cells and smooth muscle cells etc.[21,22] Lymphoid cells contain HMGB1 in both cytoplasm and nucleus, whereas cells in liver and brain tissues contain HMGB1 predominantly in the cytoplasm.[23] Under inflammatory or injurious conditions, HMGB1 can be actively released by innate immune cells such as monocytes, macrophages and passively released by necrotic or damaged cells.[7] This extracellular HMGB1 triggers an inflammatory response.[7] Extracellular roles of HMGB1 include stimulation of cytokine release in monocyte/macrophage[21,24], and endothelial cells.[25] It also induces chemotaxis and cytoskeleton reorganization in rat smooth muscle cells.[22] However, most cell including kidney and adrenal cells are not able to secrete HMGB1.[4]

So far, there had been very few reports on HMGB1 expression in human fibroblasts and studies on HMGB1 in oral cells are still limited. Recently, Morimoto et al found the expressions of HMGB1 in the gingival epithelial tissue of the patients with periodontitis.[16] Feghali et al found HMGB1 released from apoptotic and necrotic HGF.[17] In this study, the expression of HMGB1 mRNA and cellular protein from primary oral fibroblast cells and KB cell had been found. However, the secreted HMGB1 either in normal HPDLF or stimulated with LPS were unable to be detected, suggesting that HPDLF might be one of the cells lacking extracellular HMGB1.

From previous knowledge, HMGB1 has a very strong TATA-less promoter, and 18-fold more active than the SV40 promoter.[26] *Hmgb1* gene is transcriptionally controlled by steroid hormone and Hmgb1 up-regulation in rodent are likely to be cytokine-dependent, and channeled through the JAK/STAT signaling pathway.[27] However, in our present study, the increased HMGB1 mRNA was not accompanied by increased in protein production, either cellular or secreted. The reason for this discrepancy is still not known. Possible explanation might be the limitation of translation or protein degradation process in our particular *in vitro* condition. There has been no previous study on HMGB1 mRNA stability. However, HMGB1 protein appears very stable: with half-life of more than two-cell generations.[28] Also, no data exist on post-translationally modified HMGB1.

There had been a few studies of LPS role on HMGB1. Wang et al reported HMG-1 mRNA levels were unaffected by

LPS treatment.[4] However, they found increased cellular and extracellular HMGB1 protein in macrophages. Sass et al demonstrated up regulation of HMGB1 protein after LPS treatment in liver of experimental mice.[15] Feghali et al showed LPS from *Aggregatibacter actinomyctemcomitans* and *Porphyromonas gingivalis* induced HMGB1 secretion from HGF.[17] However, they could not detect any changes in HMGB1 mRNA levels. The mechanism of HMGB1 induction seems complicate and requires further clarification. The difference might be partly due to cell types. However, the results from our present study suggested periodontal ligament cells might not be a source of secreted HMGB1. Another interesting work by Hertal et al found that decreased expression of HMG protein T160 by antisense RNA impaired growth in mouse fibroblasts.[29] As HMGB1 has long been known as a nuclear protein in almost all mammalian cells and many evidences suggested its involvement in a basic cell function, e.g. DNA replication.[30,31] An up-regulation of its mRNA might suggest its intracellular role which needs further investigation.

Conclusion

This study found primary oral fibroblasts and KB cell line to contain HMGB1 RNA and cellular protein, but they are unable to secrete HMGB1. In the presence of serum, LPS is able to up-regulate HMGB1 transcription but not protein production in HPDLF. The role of HMGB1 in periodontitis is still unclear and requires further investigation. The LPS-induced increase in HMGB1 protein was markedly smaller than the increment in HMGB1 RNA. The reason for this apparent discrepancy between RNA and protein levels for HMGB1 is not known. Further investigation is required for an interesting intracellular role of HMGB1 to some basic cell function of HPDLF, rather than in its secreted form.

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