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Expression of high mobility group box 1 in inflamed dental pulp and its chemotactic effect on dental pulp cells

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ABSTRACT

High mobility group box 1 protein (HMGB1) is a chromatin protein which can be released extracellularly, eliciting a pro-inflammatory response and promoting tissue repair process. This study aimed to examine the expression and distribution of HMGB1 and its receptor RAGE in inflamed dental pulp tissues, and to assess its effects on proliferation, migration and cytoskeleton of cultured human dental pulp cells (DPCs). Our data demonstrated that cytoplasmic expression of HMGB1 was observed in inflamed pulp tissues, while HMGB1 expression was confined in the nuclei in healthy dental pulp. The mRNA expression of HMGB1 and RAGE were significantly increased in inflamed pulps. In *in vitro* cultured DPCs, expression of HMGB1 in both protein and mRNA level was up-regulated after treated with lipopolysaccharide (LPS). Exogenous HMGB1 enhanced DPCs migration in a dose-dependent manner and induced the reorganization of f-actin in DPCs. Our results suggests that HMGB1 are not only involved in the process of dental pulp inflammation, but also play an important role in the recruitment of dental pulp stem cells, promoting pulp repair and regeneration.

1. Introduction

High mobility group box 1 (HMGB1) is a non-histone chromosomal protein that is constitutively expressed in the nucleus of eukaryotic cells [1]. The intra-nuclear function of HMGB1 is to maintain the nucleosome structure and regulate gene transcription [2]. However, it is the extracellular actions of HMGB1 that intrigue great interests. HMGB1 could be passively released by necrotic cells or actively secreted by monocytes, macrophages and dendritic cells [3,4]. Once released, extracellular HMGB1 acts as an endogenous signaling molecule by inducing the production of various inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-8 [5,6]. In addition, recent studies showed that extracellular HMGB1 exhibits a strong chemotactic effect on mesenchymal stem cells, smooth muscle cells, and mesoangioblasts by inducing cytoskeletal reorganization and migration [7–9]. In *in vivo* injury models, such as myocardial infarction, hind limb ischemia and diabetic wound healing, HMGB1 has been reported to induce migration and activation of tissue specific cells, leading to tissue repair and regeneration [10–12]. The chemotactic effect of HMGB1 has been found to be mediated by the receptor for advanced glycation end products (RAGE) [2].

Human dental pulp tissue possesses the ability of resisting and repairing injuries caused by trauma or infection [13,14]. Previous studies have isolated stem cells from human dental pulp and identified the multi-differentiation potentials of these dental pulp stem cells (DPSCs) [15]. In pathological conditions, such as deep caries, DPSCs could be activated, migrate to the injury site and further differentiate into odontoblast-like cells which are responsible for reparative dentin formation [16]. Signaling molecules are essential for the recruitment and subsequent functions of stem cells. A recent study has confirmed the expression of HMGB1 and its receptor RAGE in adult dental pulp fibroblasts [17], however, the function of HMGB1 signaling in dental pulp injured state such as pulpitis is still unknown.

In the study reported herein, we investigated the expression of HMGB1 in inflamed and healthy dental pulp tissues, as well as in dental pulp cells (DPCs). In addition, the effects of HMGB1 on the

Abbreviations: DPCs, dental pulp cells; HMGB1, high mobility group box 1; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; RAGE, receptor for advanced glycation end products.

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proliferation, migration abilities and cytoskeleton of DPCs were also investigated.

2. Materials and methods

2.1. Specimen collection

Healthy premolars or third molars were collected from orthodontic patients (age 13–29). In the inflammation group, 15 third molars were collected from patients with symptoms of spontaneous pain or prolonged pain to thermal stimuli and diagnosed as irreversible pulpitis. Informed consent was obtained from each patient, and the research protocol has been approved by ethics committee of Sun Yat-sen university. Healthy and inflamed pulps were then obtained by splitting the teeth lengthwise. Nine healthy and nine inflamed pulpal tissues were fixed in 4% paraformaldehyde for immunohistochemical staining. Six healthy and six inflamed pulpal tissues were preserved at −80 °C for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

2.2. Immunohistochemical staining

Expression of HMGB1 in pulp tissues was identified using immunohistochemical staining. Immunohistochemical staining was performed as previously described [18]. Primary antibodies were rabbit polyclonal HMGB1 antibody (dilution 1:150, Abcam, Cambridge, UK). Images were captured using a digital camera (AxioCam; Zeiss, Germany).

2.3. Isolation and cultivation of human dental pulp cells (DPCs)

DPCs were isolated and cultured as previously described [18]. Cultures were maintained at 37 °C in an incubator (5%CO_2/20%O_2), with fresh medium provided every 2–3 days. Cells between the third and fifth passages were used in following experimental procedures.

2.4. Immunofluorescence staining

DPCs were seeded at the density of 1×10^5/well in a 48-well plate for 24 h followed by stimulated with 10 μg/mL *Escherichia coli* (*E. coli* 055:B5) lipopolysaccharide (LPS, L4391, Sigma) for 12 h. Cells were fixed in methanol and blocked with 1% BSA. And cells were then incubated with HMGB1 antibody (dilution 1:100) overnight, followed by combined with corresponding FITC-conjugated secondary antibody (Santa Cruz). 4′,6-diamidino-2-phenylindole (DAPI, Beyotime institute of technology, Shanghai, China) was applied to visualize the nucleus. Stained cells were observed under fluorescence microscopy (Carl Zeiss, Jena, Germany).

2.5. Western blotting

DPCs were treated with 10 μg/mL LPS for 6, 12 and 24 h. Cytoplasmic protein in DPCs was then extracted using nuclear and cytoplasmic protein extraction kit (Beyotime). Western blotting was performed as previously described [18]. Primary antibodies were HMGB1 antibody (dilution 1:800), RAGE antibody (dilution 1:800, Santa Cruz, USA) and monoclonal GAPDH antibody (1:500; Beyotime).

2.6. qRT-PCR

Total RNA was exacted from pulp tissues or DPCs using TRIzol reagent (Invitrogen, Carlsbad, CA). The complementary DNA was synthesized using ABI PCR 9700 System (Applied Biosystems, USA). qRT-PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems) using SYBR® Green detection reagent. The sequences of primers were as follows: HMGB1 (forward: 5′-TGCAGAT GACAAGCAGCCTT-3′, reverse: 5′-GCTGCATC AGGCTTTCCTTT-3′), RAGE (forward: 5′-AAACATCACAGCCCGGA TTG-3′, reverse: 5′-TCCGGCCTGTGT TCAGTTTC-3′), and β-actin (forward: 5′-GCATGGGTCAGAAGGATTCCT-3′, reverse: 5′-TCGTCCC AGTTGGTG ACGAT-3′). The mean cycle threshold (Ct) value of target gene was normalized against Ct value of β-actin mRNA.

2.7. Cell proliferation assay

DPCs were seeded in a 96-well plate at a concentration of 5×10^3/well. DMEM composed of 2% FBS served as the control. Graded concentrations of recombinant human HMGB1 (R&D, USA) ranging from 12.5 to 100 ng/mL were then added into the each well with DMEM plus 2% FBS. After incubation for 24 and 48 h, DPCs proliferation was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay, as per the manufacturer's protocol. The optical density (OD) was detected at 450 nm using an UV-spectrophotometer.

2.8. Transmigration assay

Effect of HMGB1 on DPCs migration was evaluated using transwell assay (transwell inserts with 8 μm pore, Corning, New York). DMEM containing 2% FBS (600 μL) with various concentrations of recombinant human HMGB1 (12.5, 25, 50 and 100 ng/mL) was placed in the lower chambers. Wells containing medium only were included as the negative control. DPCs (6×10^4/well) mixed with 200 μL medium were then seeded on the upper chamber. After 10 h incubation, cells remaining on the upper surface of the filters were mechanically removed. DPCs that had migrated to the lower surface were stained with DAPI and observed under fluorescence microscopy (Carl Zeiss). Five fields under a 400-fold magnification were randomly selected and migration index was calculated as the number of migrated cells in HMGB1 group divided by the number of migrated cells in the negative control.

2.9. Actin cytoskeleton staining

DPCs were seeded at the density of 1×10^4/well in a 48-well plate and cultured for 24 h followed by 12 h starvation. Cells were then stimulated with 50 ng/mL HMGB1 for 30 min, fixed in 4% paraformaldehyde for 15 min and further treated with 0.3% Triton-X100 for 8 min. Fibrous actin (F-actin) was stained with FITC-phalloidin (Sigma–Aldrich, USA) to identify cell cytoskeleton and visualized under fluorescence microscope (Carl Zeiss).

2.10. Statistical analysis

All data were presented as mean ± SEM. Data analysis was performed using SPSS13.0. Statistical significance between groups was determined using one-way analysis of variance followed by the Student–Newman–Keuls test. A p value of less than 0.05 was considered as significant.

3. Results

3.1. Expression of HMGB1 and RAGE in healthy and inflamed pulpal tissues

Immunostaining showed that HMGB1 expression was moderate to low in healthy dental pulp where it was restricted in the nuclei of odontoblasts, fibroblasts and endothelial cells (Fig. 1Aa–c). In

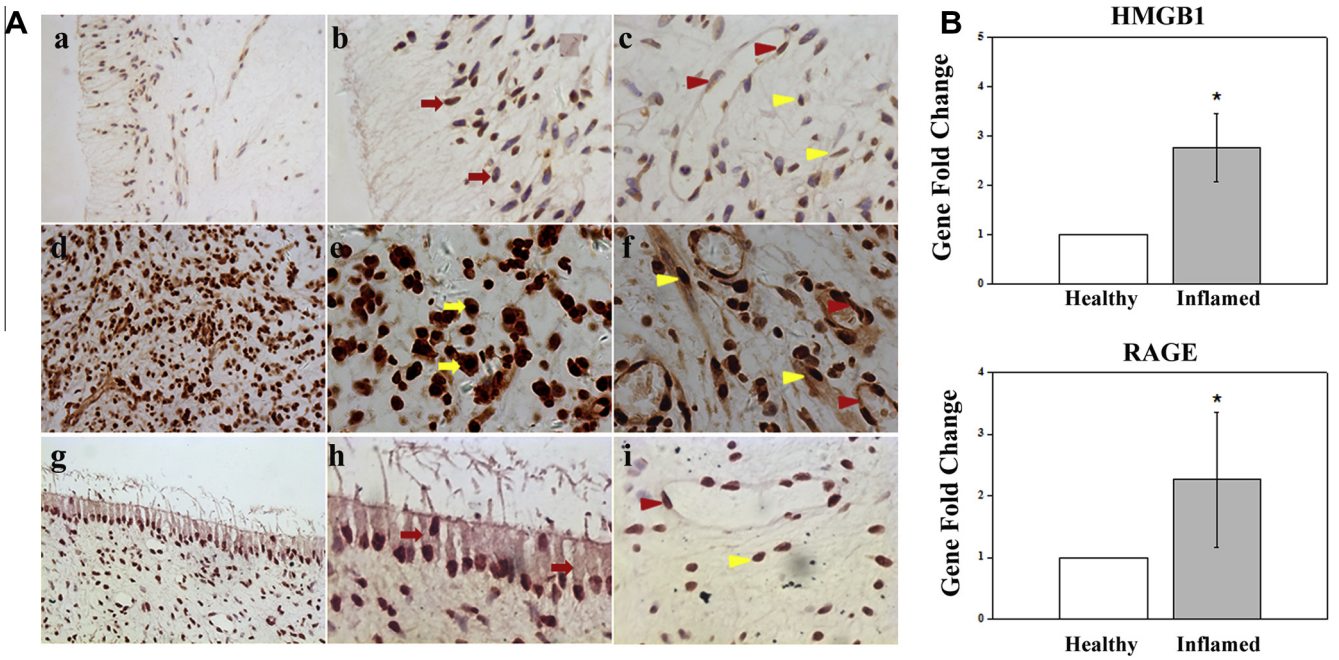

Fig. 1. Expression of HMGB1 and RAGE in inflamed and healthy dental pulp. (A) Immunolocalization of HMGB1 in human healthy and inflamed dental pulp tissues. (a) Nuclear staining of HMGB1 in mature healthy dental pulp (×400). (b) HMGB1 expression in the nuclei of odontoblasts of mature healthy dental pulp (×1000). (c) Nuclear expression of HMGB1 in fibroblasts and endothelial cells of mature healthy dental pulp (×1000). (d) Expression of HMGB1 in inflamed dental pulp (×400). (e) Expression of HMGB1 in inflammatory cells of inflamed dental pulp (×1000). (f) Expression of HMGB1 in the cytoplasm of microvascular endothelial cells of inflamed dental pulp (×1000). (g) Expression of HMGB1 in immature healthy dental pulp (×400). (h) Expression of HMGB1 in odontoblasts of immature dental pulp (×1000). (i) Expression of HMGB1 in endothelial cells and fibroblasts of immature dental pulp (×1000). (B) mRNA expression of HMGB1 and RAGE in human healthy and inflamed dental pulp tissues. Each sample was performed in triplicate. Error bars indicate mean +/− SEM ($n = 6$). $^{*}p < 0.05$ versus the healthy pulp. Red arrow, odontoblast; red arrowhead, endothelial cells; yellow arrow, inflammatory cells; yellow arrowhead, fibroblast. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inflamed pulp samples, however, enhanced HMGB1 expression was detected in the regions containing infiltrative inflammatory cells (Fig. 1Ad–f). It appeared to be both nuclear and cytoplasmic positive in inflammatory cells, as well as in fibroblasts and in microvascular endothelial cells. However, in healthy immature pulp tissues (from patients of 13–15 years old), HMGB1 staining was positive in both cytoplasm and nucleus in odontoblasts (Fig. 1Ag–i). In addition, inflamed pulps have a higher HMGB1 and RAGE mRNA expression compared to the healthy group ($p < 0.05$) (Fig. 1B). These data indicate that the increased cytoplasmic expression of HMGB1 as well as up-regulated mRNA level of HMGB1 and its receptor RAGE in inflamed dental pulp.

3.2. Expression of HMGB1 and RAGE in DPCs

Immunofluorescence staining showed that cytoplasmic expression of HMGB1 was almost undetectable in DPCs. However, highly expressed HMGB1 was observed in cytoplasm of DPCs after treated with LPS (Fig. 2A). Western blot results showed that cytoplasmic HMGB1 expression was significantly up-regulated in DPCs after treated with LPS for 6, 12 and 24 h compared to the control ($p < 0.05$) (Fig. 2B). RAGE expression in DPCs was also increased after treated with LPS for 6 and 24 h compared to the control ($p < 0.05$), however, no effect of LPS on RAGE expression was observed at 12 h (Fig. 2B). In addition, LPS has been found to up-regulate the mRNA expression of both HMGB1 and RAGE in DPCs in a time and dose-dependent manner compared to the control ($p < 0.05$) (Fig. 2C).

3.3. Effects of HMGB1 on the proliferation of DPCs

To identify the effects of HMGB1 on cell proliferation, DPCs were treated with different concentrations of HMGB1 (12.5, 25, 50 and 100 ng/mL) for 24 h and 48 h. As shown in Fig. 3, no inhibitory effect of HMGB1 (12.5, 25 and 50 ng/mL) on DPCs proliferation was detected at either 24 h or 48 h. However, DPCs proliferation was 48.87% ± 17.45% and 34.83% ± 5.48% below the control ($p < 0.05$), when exposed to 100 ng/mL HMGB1 for 24 h and 48 h, respectively, indicating that HMGB1 of high concentration has suppressive effect on cell proliferation of DPCs.

3.4. HMGB1 enhances DPCs migration

To investigate whether HMGB1 promotes DPCs migration, chemotaxis assay was performed using 8 μm polycarbonate filters. As shown in Fig. 4A, HMGB1 at concentrations of 12.5, 25 and 50 ng/mL promoted DPCs migration by 276.47% ± 88.42%, 561.67% ± 117.53% and 603.65% ± 148.10% compared to the control ($p < 0.05$), respectively. Although 100 ng/mL HMGB1 also increased DPCs migration by 242.45% ± 55.90% compared to the control ($p < 0.05$), HMGB1 at this concentration was less effective on improving DPCs migration compared to 50 ng/mL HMGB1.

3.5. Effects of HMGB1 on the cytoskeleton of DPCs

Cytoskeleton reorganization and cell morphology change are important features of chemoattractant-induced cell mobility. As shown in Fig. 4B, the distribution of f-actin exhibited a uniform appearance within the cytoplasm of DPCs (Fig. 4Ba and b). However, stimulation of HMGB1 (50 ng/mL) caused a dramatically reorganized f-actin in DPCs. In addition, polarized morphology, accumulation of actin at the cell periphery and the formation of filopods (Fig. 4Bc and d), were observed in DPCs after treated with HMGB1. Taken together, these data indicate that HMGB1 increases DPCs mobility.

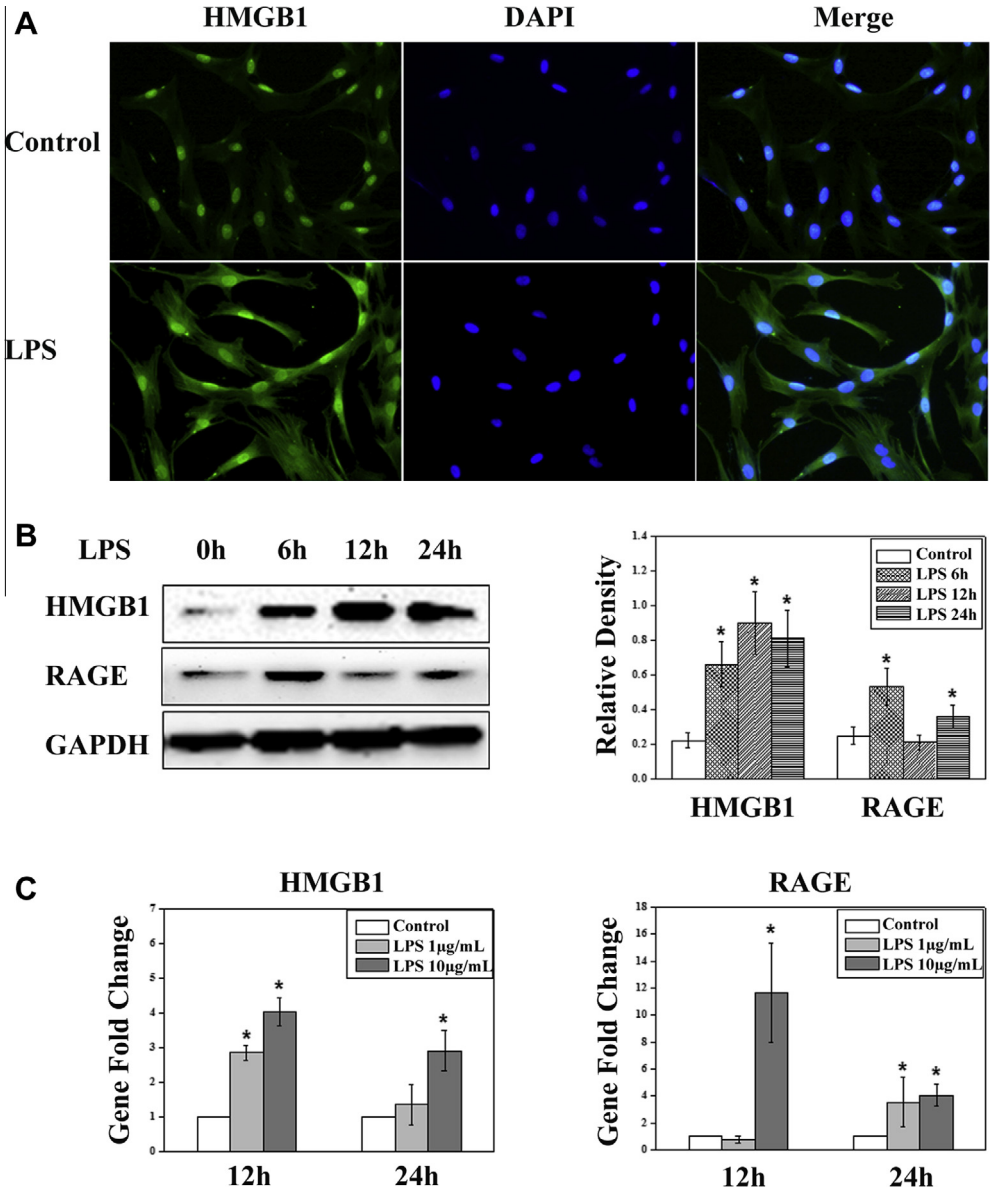

Fig. 2. Effects of LPS on HMGB1 and RAGE expression in cultured DPCs. (A) Immunofluorescence staining with anti-HMGB1 antibody (green) was performed in cultured DPCs. DAPI (blue) staining indicated cell nuclei, (200× magnification); (B) Protein expression of HMGB1 and RAGE in DPC; (C) mRNA expression of HMGB1 and RAGE in DPCs. Expression of target protein was measured by quantifying the intensity of the bands and further normalized to the expression of GAPDH. The target gene was normalized to β-actin and then converted to the percentage of the control. Error bars indicate mean +/− SEM ($n = 3$). $^{*}p < 0.05$ versus the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Dental pulp tissues are capable to initiate innate and adaptive immune responses under inflammatory conditions [19]. Recently, it is of particular interest to discover several endogenous alarmins participating in both infectious and sterile inflammation, such as HMGB1, heat shock proteins (HSPs) and calcium regulators S100s [2,20]. HMGB1 has been firstly discovered as a ubiquitous and highly-conserved nuclear protein [1]. Besides its nuclear function, extracellular HMGB1 also acts as pro-inflammatory cytokines. Recent studies have focused on the chemoattractant effect of HMGB1 and its potential application in myocardial infarction and skeletal muscle ischemia [10,12], indicating that HMGB1 may contribute to the tissue repair and regeneration process.

In the present study, we investigated the expression pattern of HMGB1 and its receptor RAGE in inflamed dental pulp. HMGB1 was positively stained in healthy dental pulps but was confined in nuclear, which is consistent with the expression pattern of "classical" nucleus protein [20]. In contrast, in inflamed pulps, the intensive cytoplasmic staining of HMGB1 was observed in infiltrated inflammatory cells, fibroblasts and endothelial cells, indicating that HMGB1 can be released in response to pulp inflammation and may be involved in the inflammatory progression and repair process of dental pulp injuries. In addition, elevated mRNA level of HMGB1 has been found in inflamed pulp tissue, indicating that the pulp infection may not only induce translocation of HMGB1 from nucleus to cytoplasm, but also stimulate the transcription and newly synthesis of HMGB1. The expression of HMGB1 has been reported to be complexly regulated. Evidences showed that serum level of HMGB1 were elevated in infectious and autoimmune diseases, such as rheumatoid arthritis, ischemia and reperfusion injury, and acute lung

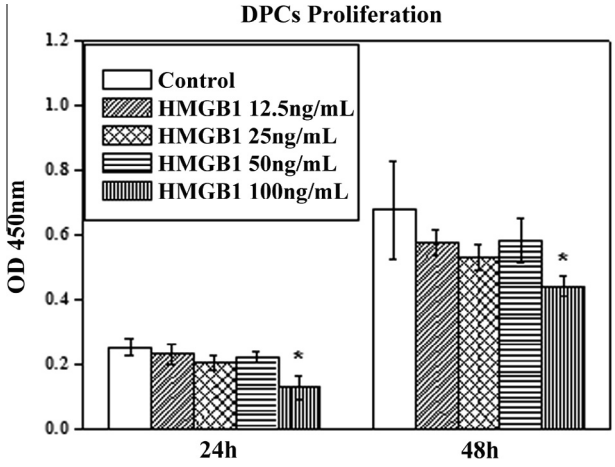

Fig. 3. Effects of exogenous HMGB1 on DPCs proliferation. Data are presented as means ± SEM ($n = 3$). $^{*}p < 0.05$ versus the control group. OD, optical density.

injury, etc [20,21]. In mice model of sepsis, Wang et al. showed that increased serum level of HMGB1 was detected from 8 to 32 h after endotoxin exposure [22]. Apart from immune system, HMGB1 is also secreted by somatic cells, including pituicyte, enterocytes and hepatocytes, in response to IL-1 or TNF-α stimulation or under hypoxic condition [2]. The present study showed that bacterial endotoxin LPS from *E. coli* could induces cytoplasmic expression and up-regulates mRNA level of HMGB1 in cultured DPCs, indicating that DPCs can be a source of HMGB1 in pulpal inflammation. It has been reported that extracellular HMGB1 increases cytokines including TNF-a, IL-1 and IL-6 expression [5] and these cytokines are highly expressed in inflamed pulp tissues [14,23]. It is therefore reasonable to speculate that in inflamed dental pulp, bacterial endotoxins LPS, hypoxia-induced oxidative stress and released cytokines could induce the cytoplasmic expression and release of HMGB1, initiating the immune response. However, excessive HMGB1 in pulp may create a positive feedback loop and contribute to the pathogenesis of dental pulp inflammation. Therefore, the pro-inflammatory effects of HMGB1 during pulpitis progression and the underlying mechanisms still need to be further investigated.

Human dental pulp cells (DPCs) are heterogeneous population containing a variety of potential stem/progenitor cells and have been used as the *in vitro* model in tissue repair and regeneration of dental pulp [16]. The migration and differentiation of dental pulp cells are critical for repair of pulp tissue. HMGB1–RAGE axis has been reported to be involved in the migration of stem/progenitor cells and development of neurite outgrowth [6,24]. Degryse et al. showed that HMGB1 stimulates migration of rat smooth muscle cells and induces rapid and transient structural changes, leading to morphology typical of motile cells [8]. In our study, HMGB1 was found to significantly promote DPCs migration and this chemoattraction effect of HMGB1 seems to be dosage-dependent. It was interesting to discover that DPCs migration index peaks at the 50 ng/mL HMGB1 treatment, while increased HMGB1 concentration at 100 ng/mL has no further effect on cell migration. This result might be due to the limited number of HMGB1 receptors-RAGE on cell surface of DPCs. On the other hand, CCK-8 assay showed that 100 ng/mL HMGB1 inhibited DPCs proliferation. This result indicates HMGB1 of high concentration possesses cytotoxic effect on DPCs, which may hamper cell viability and migration of DPCs. Moreover, HMGB1 induces f-actin reorganization, accumulation of actin at the cell periphery and the formation of filopods in

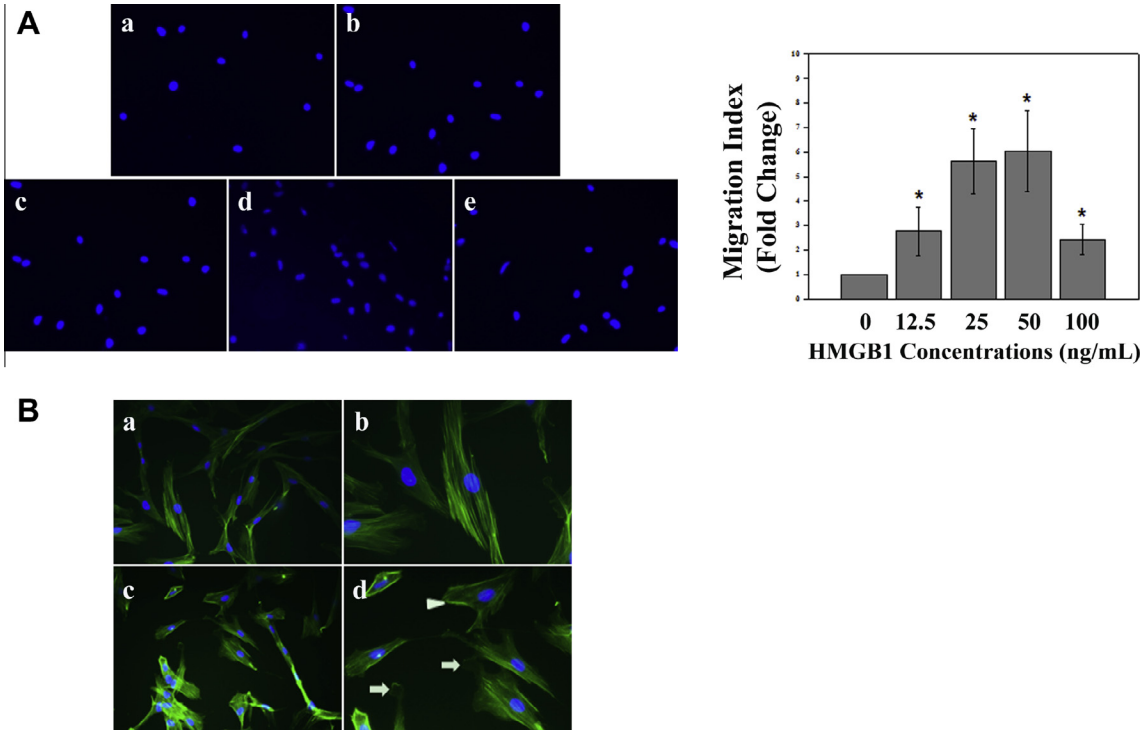

Fig. 4. Effects of HMGB1 on DPCs migration and cytoskeleton organization. (A) Cell migration in a transwell assay: (a) transmigrated DPCs in control group; (b–e) migration of DPCs under the stimulation of HMGB1; (b) 12.5 ng/mL HMGB1; (c) 25 ng/mL HMGB1; (d) 50 ng/mL HMGB1; (e) 100 ng/mL HMGB1. Migration index are calculated as the fold increase in the number of migrated cells relative to the number in the negative control. Error bars indicate mean +/− SEM ($n = 3$). $^{*}p < 0.05$ versus the control group, (200× magnification). (B) Effects of HMGB1 on DPCs morphology and actin cytoskeleton formation. (a–b) The f-actin distribution in DPCs. (c and d) f-actin distribution in DPCs after treated with HMGB1. Arrowhead indicates the accumulation of actin at the cell periphery; arrow suggests the formation of filopods. (A and C, 200× magnification; B and D, 400× magnification.)

DPCs. It has been established that HMGB1–RAGE axis activates Rho family of GTPases (such as RhoA, Rac and Cdc42) in diverse cell types, leading to the reorganization of actin cytoskeleton, neurite extension and tumor metastasis [24–26]. Accordingly, our findings suggest the chemotactic effect of HMGB1 on dental pulp cells is associated with the activation of Rho signaling pathway and cytoskeleton reorganization.

Several receptors, including RAGE and the Toll-like family of receptor (TLR) 2, TLR4 and TLR9, have been identified to mediate HMGB1 signaling [5]. The chemotactic effect of HMGB1 has been reported to be RAGE-dependent *via* activating CDC42/Rac1 and/or mitogen-activated protein kinases (MAPKs) signaling pathway [24,27]. Expression of RAGE is very low in normal tissues but can be up-regulated after the stimulation of its ligands [2]. Our results suggest that the up-regulated expression of RAGE in pulpitis tissue and LPS-stimulated DPCs may be result of the accumulated HMGB1 and the chemotactic effect of HMGB1 on DPCs is possibly due to its interaction with RAGE.

When tissue injury occurs, immune regulation plays an integral role in the progression of inflammatory reaction as well as wound healing process via secretary molecules [28]. Therefore, it is not surprising that the pro-inflammatory cytokine HMGB1 could not only serve as signal to initiate the inflammation, but also promote tissue repair. In myocardial infarction model, it has been reported that low doses administration of HMGB1 (200 ng/mouse) is beneficial, while high doses (10 μg/mouse) is deleterious, suggesting that the effects of HMGB1 are dose-dependent [10,27,29]. Our study showed that up-regulation of HMGB1 in pulpitis and its chemotactic effect on dental pulp fibroblasts. It is therefore reasonable to speculate that during dental pulp injury, locally produced HMGB1 induces cell migration to the injured site and contributes to wound healing, but excessive HMGB1 may cause amplification of inflammation cascade and eventually leading to tissue damage. In addition, a recent study showed HMGB1 promoted odontoblastic differentiation of DPCs, which also indicates the potential of HMGB1 in dental pulp regeneration [30]. Further investigations are needed to elucidate the underlying mechanism of HMGB1 in inflammation and tissue repair and its therapeutic potential in dental pulp regeneration.

In summary, our study showed the increased cytoplasmic expression of HMGB1 and up-regulation of its receptor RAGE in dental pulp during inflammation. Exogenous HMGB1 enhances DPCs migration and induces cytoskeleton reorganization. Our study suggests that HMGB1 are not only involved in the process of dental pulp inflammation, but also play an important role in the recruitment of dental pulp stem cells and may therefore promote pulp repair and regeneration.

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