Lipopolysaccharide stimulation improves the odontoblastic differentiation of human dental pulp cells

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Abstract. Lipopolysaccharide (LPS) is one of the causative agents of pulpitis and previous studies have demonstrated that the LPS stimulation of human aortic valve interstitial cells induces inflammatory mediators and the gene expression of osteogenic factors. Therefore, in the present study, it was hypothesized that LPS affects the odontoblastic differentiation of human dental pulp cells (hDPCs). In order to investigate this, an *in vitro* study using hDPCs was performed. Increased alkaline phosphatase (ALP) activity was observed in the hDPCs treated with LPS, which was more marked when the cells were costimulated with odontogenic induction medium (OM). LPS also appeared to increase the gene expression levels of dentin sialophosphoprotein and dentin matrix protein-1 and the protein expression level of dental sialoprotein in the hDPCs, particularly in combination with OM. In addition, the size and the number of nodules formed in the hDPCs exposed to OM and LPS were increased compared to those stimulated by OM alone. To determine the role of nuclear factor κB (NF-κB) during the LPS-induced odontoblastic differentiation of hDPCs, immunofluorescence was performed. The nuclear translocation of NF-κB, induced by LPS was confirmed, suggesting its involvement in the LPS-induced increase in odontoblastic differentiation of hDPCs. In conclusion, there may be an association between LPS stimulation, with or without OM, and odontoblastic differentiation.

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Introduction

Dental pulp tissues have the ability to repair and regenerate following injury. In the presence of an infection within the dentin-pulp complex, oral microorganisms and their components diffuse into the pulp through the dentinal tubules. This type of stimulation induces the mixed population of pulp cells (1,2), which include stem cells and odontoblast progenitor cells, to differentiate and form a dentin-like mineralized matrix. This is marked by an increase in the expression of hard-tissue-forming proteins, including alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1) (3,4).

Lipopolysaccharides (LPS) comprise a major molecular component of the outer cell wall of Gram-negative bacteria and are potent virulence factors that contribute to bacterially induced pathology, including general sepsis (5), lung disease (6), periodontal disease (7) and pulpitis (8). In several experiments, LPS has been used to form a model of inflammation and the protective inflammatory reaction evoked by LPS is considered to induce signal transduction through Toll-like receptor 4 (TLR4) (9,10). In our previous study, the mRNA and protein expression of TLR4 was observed in the cells of the odontoblast layer and pulp tissues (11). TLRs, broadly distributed pattern recognition receptors, are also involved in the pathogenesis of chronic inflammatory diseases. LPS and peptidoglycans, which are TLR4 and TLR agonists, have been observed to induce the upregulation of osteogenesis-associated factors in human aortic valve interstitial cells (hAVICs) (12,13). To date, few studies have examined the effects of LPS or TLRs on hDPC differentiation.

Furthermore, LPS is known to activate NF-κB, predominantly through the TLR4 downstream pathway (14). Increased expression levels of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 are associated with the translocation of NF-κB from the nucleus to the cytoplasm in hDPCs following incubation with LPS (15).

hDPCs can promote reparative dentin formation by odontoblastic differentiation, resulting in the production of mineralized matrix (1,3). This property of differentiation by the pulp cells is critical for its repair following injury or inflammation and is similar to the processes of osteogenic differentiation and bone formation. In addition to LPS and TLR4, NF-κB may also contribute to osteogenic differentiation (12,13) and the present study hypothesized that they may be involved in the odontoblastic differentiation of hDPCs. In the present study,

hDPCs were treated with *Escherichia coli* LPS in growth medium and odontogenic induction medium (OM), respectively. Subsequently, an ALP enzymatic assay, reverse transcription quantitative polymerase chain reaction (RT-qPCR), western blotting and alizarin red staining were used to investigate and confirm the upregulation of mineralization indicators in the hDPCs following stimulation. The translocation of NF-κB in the cells was evaluated using immunofluorescence microscopy.

Materials and methods

hDPC cultures. Extracted premolars or third molars were collected from patients (13-25 years old) at the Department of Oral and Maxillofacial Surgery, Guanghua School and Hospital of Stomatology, Sun Yat-sen University (Guangzhou, China). Informed consent was obtained from each patient and the study was performed under the approval of the ethics committee of Guanghua School and Hospital of Stomatology, Sun Yat-sen University (Guangzhou, China). The hDPCs were isolated, as previously reported (16) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA) and 10% fetal bovine serum (FBS; Gibco-BRL). The cultures were maintained at 37˚C in an incubator (5% CO_2/20% O_2). The cells obtained between passages three and four were used in the subsequent experiments.

ALP. The hDPCs ($5x10^5$ cells per well) were seeded into 12-well culture plates. The cells in the experimental groups were treated with either 0.1, 1.0 and 10.0 μg/ml LPS *(E. coli* 0111:B4; Sigma) in growth medium or odontogenic induction medium (OM; 2%FBS, 10 mmol/l β-glycerophosphate, 50 μmol/l ascorbic acid and 100 nmol/l dexamethasone in DMEM) for 1, 3, 5 and 7 days, respectively, while those belonging to the control group were incubated with growth medium and OM alone. The cultures were maintained at 37˚C in an incubator (5% CO_2/20% O_2). Following treatment at each time point, the plates were washed twice with phosphate-buffered-saline (PBS) and lysed with 200 μl 1% Triton X-100. After 16 h, 30 μl aliquots of cell lysate per well were subjected to ALP activity and protein content measurement using an ALP kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and a bicinchoninic acid assay kit (Boshide Bioengineering Co., Ltd., Wuhan, China). All results were normalized by total protein content.

RT-qPCR. The cells from the experimental groups were treated with 0.1 μg/ml LPS, in growth medium and OM for 3 days, while the control groups were incubated with growth medium and OM alone. The cultures were maintained at 37˚C in an incubator (5% CO_2/20% O_2). Following the treatment, total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). Quantification of the mRNA levels of DSPP, DMP-1 and GAPDH were performed based on previously described methods (17). Complementary DNA was synthesized from 2 μg total RNA using Revert AidTM First Strand cDNA Synthesis kit (Fermentas, Ottawa, Canada) according to the manufacturer's instructions. RT-qPCR was performed to detect genes of DSPP, DMP-1 on the Chromo4 four-color Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR Green Realtime PCR Master mix (Toyobo, Osaka, Japan). The sequences of the primers used are listed in Table I. The conditions for RT-qPCR were as follows: 95˚C for 10 min for activation, followed by 40 cycles of denaturation at 95˚C for 15 sec and primer extension at 60˚C for 1 min. All reactions were performed in triplicate for the independent experiments. The mean cycle threshold (ΔCt) value of each target gene was normalized against ΔCt value of house-keeping gene GAPDH and the relative expression calculated using the following formula: $2^{-(\text{normalized average } \Delta Cts)} x10^4$. The expression of the target gene was subsequently converted to the fold-change of the control gene.

Western blotting. The cells from the experimental groups were treated with 0.1, 1.0 and 10.0 μg/ml LPS, in growth medium and in OM for 3 days, while the control group cells were incubated with growth medium and OM alone. The cultures were maintained at 37˚C in an incubator (5% CO_2/20% O_2). Following treatment, the hDPCs were harvested in lysis buffer containing 1% Igepal CA-630 (Sigma-Aldrich), 0.5% sodium deoxycholate and 0.1% sodium dodecylsulfate-polyacrylamide. Protease inhibitor cocktail (Merck Millipore, Darmstadt, Germany), containing AEBSF, hydrochloride, aprotinin, bovine lung, crystalline, E-64 protease inhibitor, EDTA, disodium, leupeptin and hemisulfate, was added prior to cell lysis. Western blotting was performed, as described previously (11). The proteins were detected using mouse polyclonal antibodies against human dental sialoprotein (DSP; 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonal antibodies against human GAPDH (1:3,000; EarthOx, San Francisco, CA, USA).

Immunofluorescence. The hDPCs ($5.0x10^5$ cells per well) were seeded into 12-well culture plates and cultured overnight. The experimental group was then treated with 0.1 μg/ml LPS, with/without OM, for 16 h, while the control group was incubated with growth medium and OM alone. The cultures were maintained at 37˚C in an incubator (5% CO_2/20% O_2). Following treatment, the cells were fixed with methanol for 15 min and washed with PBS. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min, incubated in 3% bovine serum albumin in PBS for 30 min and then with the primary monoclonal anti-NF-κB antibody (1:50; Cell Signaling Technology, Inc., Boston, MA, USA) and primary monoclonal anti-c-Jun antibody (1:50; Millipore, Temecula, CA, USA) simultaneously at 4˚C overnight. The cells were rinsed and then incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:200; Cell Signaling Technology, Inc.). Detection was then performed using a fluorescence microscope (Axio Observer Z1; Zeiss, Oberkochen, Germany).

Alizarin red stain. The hDPCs ($1.0x10^6$ cells per well) were seeded into 6-well culture plates and cultured overnight. The experimental group was then treated with 0.1 μg/ml LPS in growth medium and OM for 14 days and the control group was incubated with growth medium and OM alone. Following treatment, at each time point, the mineralized cells were washed with 1X PBS and fixed using 95% ethanol at room temperature for 10 min. Calcium accumulation was detected using 40 mM alizarin red stain solution (Sigma-Aldrich). Following washing with distilled water, images of the dried-culture dishes were captured

Table I. Primer sequences used in reverse transcription quantitative polymerase chain reaction.

Gene	Primer sequence	Length (bp)
DSPP	Forward: 5'-GCCACTTTCAGTCTTCAAAGAGA-3' Reverse: 5'-GCCCAAATGCAAAAATATGTAA-3'	130
DMP1	Forward: 5'-TGGGCATAGATTTCCTCTTTG-3' Reverse: 5'-TGAGCAGGATGCTGATCTTC-3'	121
GAPDH	Forward: 5'-AAGGTGAAGGTCGGAGTCAA-3' Reverse: 5'-AATGAAGGGGTCATTGATGG-3'	108

DSPP, dentin sialophosphoprotein; DMP1, dentin matrix protein-1; bp, base pairs.

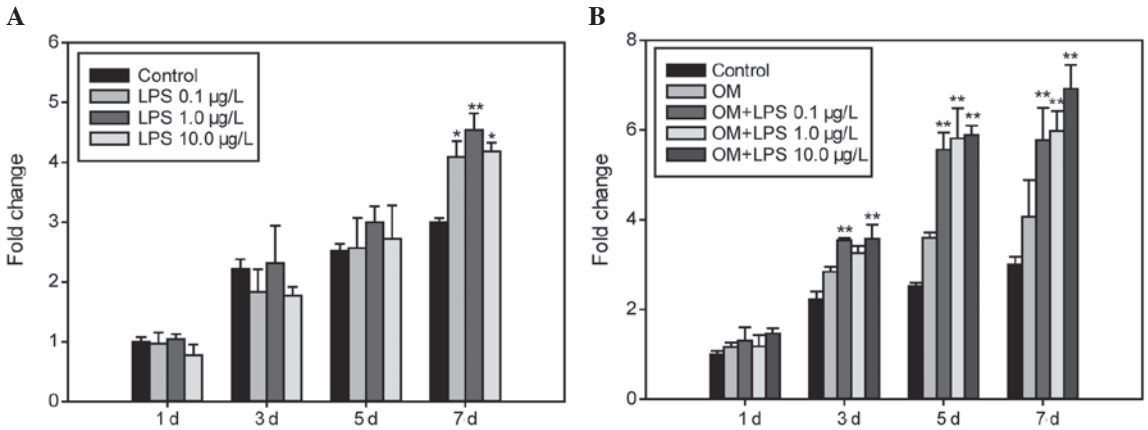

Figure 1. Effect of LPS components on the ALP activity of hDPCs. (A) ALP activity was increased in the hDPCs treated with LPS in growth medium on day 7 (*P<0.05 and **P<0.01, compared with the control group). (B) In the OM group, the ALP activity of the cells exposed to LPS increased at each time point with the exception of the 1st day and at the final concentration of 1.0 μg/ml LPS on the 3rd day (**P<0.01, compared with the group treated with OM only). The fold increase of ALP activity relative to the control group on the day 1 is also shown. Values are expressed as the mean ± standard deviation. hDPCs, human dental pulp cells; ALP, alkaline phosphatase; LPS, lipopolysaccharide; OM, odontogenic induction medium.

under a fluorescence microscope (Axio Observer Z1; Zeiss). The positively stained nodules appeared orange/red.

Statistical analysis. The SPSS 16.0 software package (SPSS, Inc, Chicago, IL, USA) was used to perform statistical analyses. The ALP activity, protein expression of DSP and gene expression of DSPP in the different treatment groups were assessed using the Least Significant Difference test. The gene expression of DMP-1 was assessed using the Kruskal-Wallis and Bonferronni's test due to heterogeneity of variance. P<0.05 and P<0.01 were considered to indicate statistically significant differences.

Results

Effect of LPS on ALP activity. The activity of ALP was examined at different time points using an ALP enzymatic assay and was found to be higher in the hDPCs treated with LPS at all concentrations in the growth medium on the 7th day (P<0.05; Fig. 1A), while no changes were observed on the 3rd and 5th days. In OM, the ALP activity of cells exposed to three concentrations of LPS increased at each time point (P<0.01; Fig. 1B) with the exception of the 1st day and at the final concentration of 1.0 μg/l LPS on the 3rd day. Exposure of the stimulated cells to 10.0 μg/l resulted in the highest ALP activity on day 7.

Effect of LPS on the gene expression levels of DSPP and DMP-1. The gene expression levels of DSPP and DMP-1 were examined using RT-qPCR on the 3rd day of treatment with 0.1 μg/ml LPS in the growth medium and OM. The expression levels of DSPP increased significantly in the hDPCs treated with LPS compared with the control group in growth medium (P<0.05) and in OM (P<0.01). The expression levels of DMP-1 were significantly higher in the hDPCs treated with LPS compared with OM alone (P<0.05; Fig. 2).

Effect of LPS on the expression of DSP. After treatment with LPS for 3 days, the protein levels of DSP were increased in the hDPCs at concentrations of 0.1, 1.0 and 10.0 μg/ml compared with the control group in growth medium and OM (P<0.01). The highest expression level of DSP was observed at a concentration of 0.1 μg/ml LPS in OM (P<0.01; Fig. 3).

LPS induces the nuclear translocation of NF-κB, but not c-Jun, in the hDPCs. The nuclear and cytosolic expression levels of

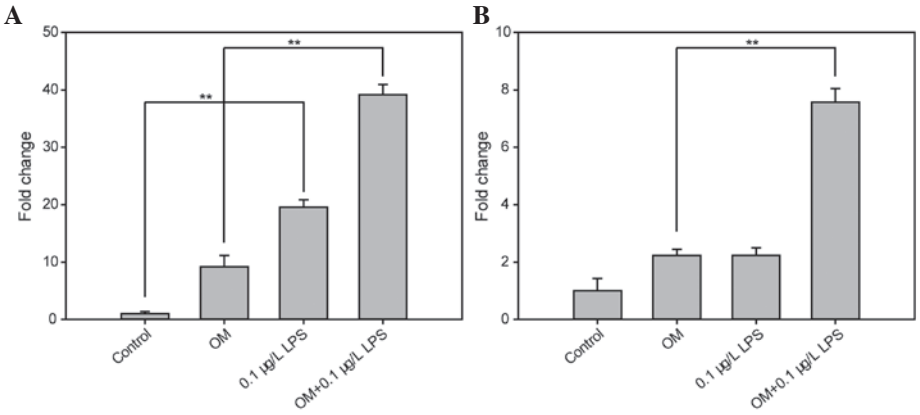

Figure 2. Effect of LPS on the gene expression levels of (A) DSPP and (B) DMP-1 in the hDPCs. mRNA levels of DSPP and DMP-1 were detected on the 3rd day following exposure to LPS in the growth medium and OM, respectively. LPS upregulated the expression levels of DSPP and DMP-1 in the hDPCs cultured in OM and upregulated the expression of DSPP cultured in the growth medium compared with medium alone. Values are expressed as the mean ± standard deviation. (**P<0.01). DSPP, dentin sialophosphoprotein; DMP-1, dentin matrix protein-1; hDPCs, human dental pulp cells; LPS, lipopolysaccharide; OM, odontogenic induction medium.

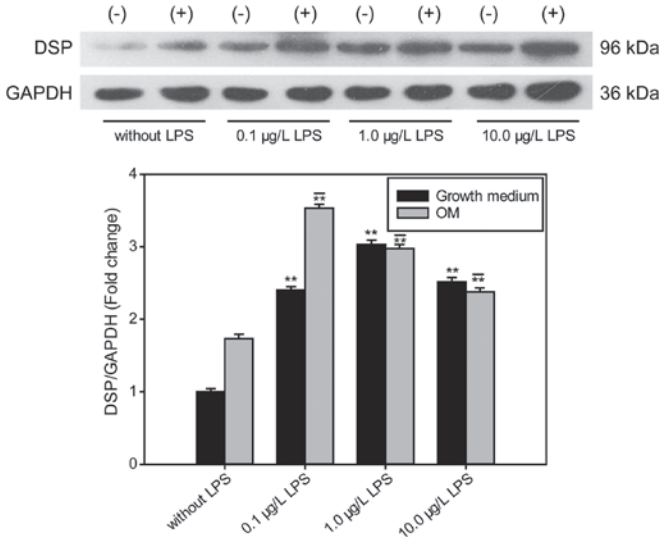

Figure 3. Effect of LPS on the protein expression of DSP in the hDPCs. The cells were exposed to LPS for 3 days in the growth medium and OM groups. DSP was increased in the hDPCs treated with LPS compared with the control group in growth medium (**P<0.01, compared with the control group). Following stimulation with OM and LPS, the expression of DSP increased compared to the group treated with OM only. Stimulation with 0.1 μg/ml LPS demonstrated the most significant upregulation (**P<0.01, compared with the group treated with OM only). Values are expressed as the mean ± standard deviation. DSP, dentin sialoprotein; OM, odontogenic induction medium; LPS, lipopolysaccharide.

NF-κB and c-Jun were detected using immunofluorescence after 16 h treatment with LPS (0.1 μg/ml) with or without OM. NF-κB was localized predominantly in the cytoplasm of the cells treated with 2% FBS in DMEM alone and in the nuclei of the cells in the experimental groups treated with LPS wit or without OM (Fig. 4). However, no change was noted in the expression of c-Jun in the cells in either the control or experimental group.

Effect of LPS on the mineralized nodule formation of hDPCs. To determine whether *E. coli* LPS stimulation increased cellular calcification, the cells were treated in OM with or without 0.1 μg/ml LPS for 14 days (Fig. 5). The results revealed that LPS promoted calcified nodule formation when the hDPCs were stimulated. As shown in Fig. 5, nodules of increased size and number were formed in the hDPCs exposed to OM and LPS compared with those stimulated by OM alone.

Discussion

Subsequent to injury or inflammation, reparative dentin is formed by the odontoblastic differentiation of hDPCs leading to increased production of mineralized matrix (1,3). As bacteria are one of the most important causative agents of pulpitis, LPS, which comprises a major molecular component of the outer cell wall of Gram-negative bacteria, is often used to establish a model of inflammation for the investigation of dental pulp cells (8). The present study used *E. coli* LPS, as it is a putative inflammation inducer, evokes a protective inflammatory reaction and is hypothesized to induce signal transduction through TLR4 (9,10). The present study hypothesized that LPS and TLR4 may be involved in the odontoblastic differentiation of hDPCs.

ALP, DSPP and DMP-1 are mineralization markers for odontoblast and osteoblast-like differentiation of hDPCs (3,18). ALP activity is higher in odontoblast-like cells compared to undifferentiated dental mesenchymal cells and is closely associated with dentin formation (19). In the present study, increased ALP activity was observed in the hDPCs treated with LPS, which was more marked when the cells were simultaneously stimulated with OM. The rise in ALP activity was time-dependent, but not dose-dependent, indicating that treatments with OM and LPS, with or without OM, may enhance odontoblastic differentiation. DSP, which promotes predentin to transform into mature dentin (20), is considered to be a specific labeled protein for odontoblastic differentiation (21). DSPP and DMP-1 are also expressed by differentiating odontoblasts. Western blot analysis revealed that the protein levels of DSP were similar to those of ALP following treatment. Similarly, the mRNA levels of DSPP and DMP-1 were reconfirmed by western blotting. Furthermore, larger and increased numbers of nodules were formed in the hDPCs exposed to OM and LPS compared with those stimulated by OM only. These observations suggested that

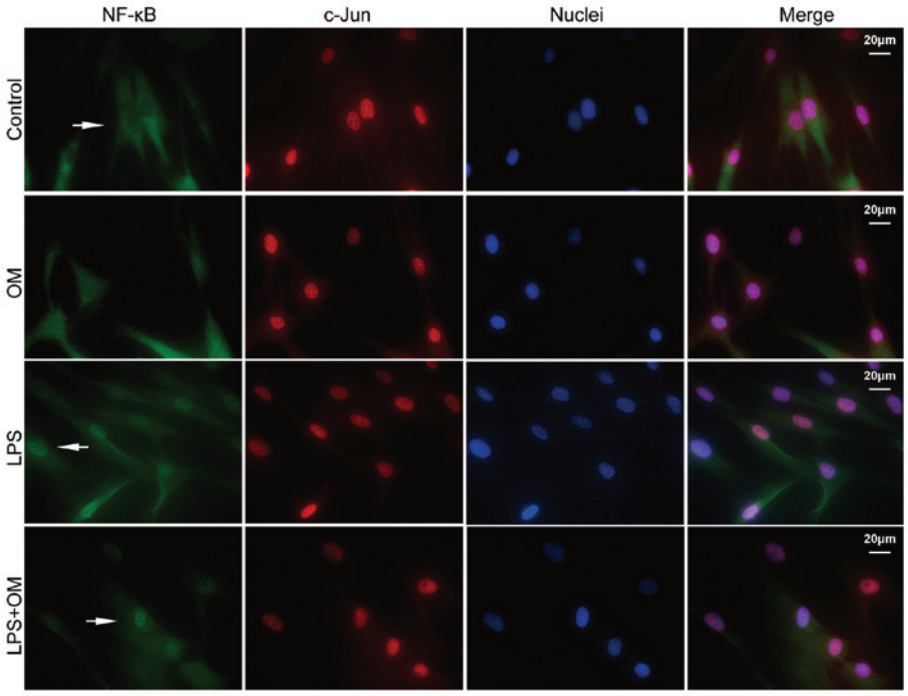

Figure 4. Effect of LPS on the nuclear translocation of NF-κB and c-Jun in the hDPCs. NF-κB was localized predominantly in the cytoplasm, but not in the nucleui of the cells treated with 2% fetal bovine serum in Dulbecco's modified Eagle's medium alone (arrow). NF-κB was also observed primarily in the nuclei of the cells in the experimental groups treated with LPS, with or without OM (arrow). The expression of c-Jun did not change in the nuclei or cytoplasm of the cells in the control or experimental groups. LPS, lipopolysaccharide; NF-κB, nuclear factor-κBl; hDPCs, human dental pulp cells; OM, odontogenic induction medium.

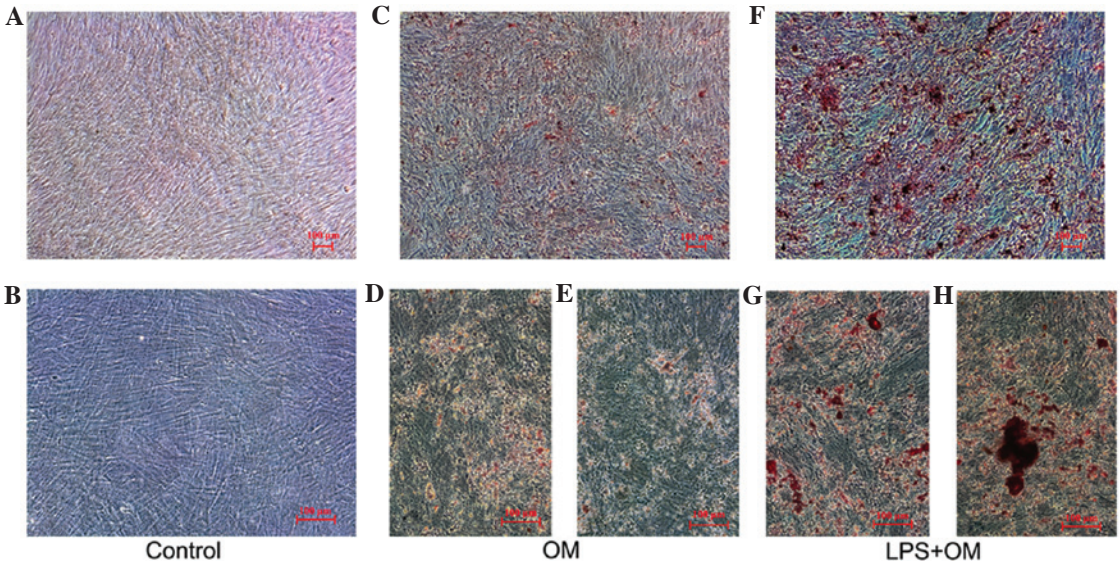

Figure 5. Effect of LPS on calcified nodule formation of the hDPCs. The hDPCs were cultured in (A and B) growth medium, (C, D and E) OM and (F, G and H) OM and LPS for 14 days, respectively. (A, C and F, x50; B, D and G, x100). Alizarin red staining revealed that the mineral nodule accumulation of the hDPCs treated by OM and LPS was higher compared with the hDPCs treated with OM only. LPS, lipopolysaccharide; hDPCs, human dental pulp cells; OM, odontogenic induction medium.

there may be an association between LPS stimulation, with or without OM, and odontoblastic differentiation.

The results of the present study are in agreement with those of calcific aortic valve cells (hAVICs). Previous studies on calcific aortic valve stenosis have revealed that LPS stimulation of hAVICs induces inflammatory mediators and the gene expression of osteogenic factors, similar to those induced by OM (22,23). Osteogenic changes have been associated with high expression levels of TLR4 (13). In addition, the combined effects of LPS and OM have been demonstrated on the osteogenic differentiation of hAVICs (12). As fibroblasts form the major bulk of hDPCs, it they may have similarities to hAVICs, which are myofibroblasts. It has been previously reported that human dental pulp stem cells exposed to LPS, obtained from

Porphyromonas gingivalis, exhibit a reduction in the expression levels of DSPP and osteocalcin and this effect can be moderated by TLR2 inhibition (24). Based on this finding and the results of the present study, it can be concluded that LPS from different bacteria, including *P. gingivalis* and *E. coli,* may not simulate the same receptor and may exert different effects with different receptors.

NF-κB activation is known to contribute to cytokine induction in response to bacterial products and is necessary for innate immunity and inflammation (25,26). It has been demonstrated that NF-κB is required for the LPS-induced expression of interleukin-8 in the hDPSCs and NF-κB is important in mediating the intracellular signaling generated by LPS, which induces the expression of pro-inflammatory genes (27). LPS has also been demonstrated to cause NF-κB intranuclear translocation and rapid phosphorylation in hAVICs (12). In the present study, the LPS-induced translocation of NF-κB was demonstrated using immunofluorescence and was in accordance with the results of western blot analysis reported in a previous study (15), indicating its role during the LPS-induced odontoblastic differentiation of hDPCs. However, LPS did not affect the translocation of c-Jun. The results suggested that NF-κB was involved in the transcriptional activation of various downstream genes and may have a similar role in the odontoblastic differentiation of hDPCs, improved by OM and LPS with or without OM.

In the present study, the expression of mineralization markers for the odontoblast and osteoblast-like differentiation of hDPCs stimulated by LPS, with or without OM, increased, suggesting the involvement of TLR4 and NF-κB. To further understand the roles of LPS, TLR4 and NF-κB in the odontoblastic differentiation of hDPCs, further studies examining the involvement of the TLR4 inhibitor, NF-κB inhibitor and the relevant signal pathways involved are required.

In addition to inducing ALP activity, LPS was observed to increase the gene expression levels of DSPP and DMP-1 and the protein expression of DSP in the hDPCs, particularly in combination with OM. The nuclear translocation of NF-κB, induced by LPS suggested that NF-κB is involved in the LPS-induced increase in odontoblastic differentiation of hDPCs.

Acknowledgements

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