Lipopolysaccharide promotes adhesion and migration of murine dental papilla-derived MDPC-23 cells via TLR4

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Abstract. Odontoblasts and/or dental pulp cells are responsible for tooth repair and dentin formation. Furthermore, adhesion and migration are critical processes for tissue regeneration. This study was performed to clarify whether lipopolysaccharide (LPS) modulates adhesion and migration of the murine odontoblast-like cell line MDPC-23, and whether Toll-like receptor 4 (TLR4) signaling is engaged in this process. TLR4 expression in MDPC-23 cells was examined by RT-PCR. Adhesion assay was performed using type I collagen-coated plates. Migration ability was determined by a commercial assay kit. Phosphorylation of IκB-α, FAK, AKT, and ERK was examined by Western blot analysis. TLR4 was functionally expressed in MDPC-23 cells. LPS treatment enhanced adhesion and migration of MDPC-23 cells in a dose-dependent manner. Blockade of TLR4 using its antibody restored LPS-induced adhesion and migration of MDPC-23 cells. These findings indicate that LPS, an immune activator from Gram-negative bacteria, can promote the adhesion and migration ability of MDPC-23 cells via TLR4.

Introduction

Innate immune response is the first line of defense against infection, which is mainly mediated by macrophages and dendritic cells. Pattern recognition receptors (PRRs) initiate the innate immune response through recognition of pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS). Among PRRs, Toll-like receptors (TLRs) are the best characterized receptors. TLRs sense microbial molecules including LPS, lipoproteins, lipoteichoid acid (LTA), dsRNA, ssRNA, and CpG motif from bacterial DNA (1,2). Following recognition, TLRs activate NF-κB and mitogen-activated protein kinases (MAPKs) through the MyD88- or the TRIF-dependent pathway and consequently lead to production of proinflammatory cytokines (1,2).

Odontoblasts and/or dental pulp cells are neural crest-derived mesenchymal cells, which regulate the pulp immune responses triggered by oral bacteria (3,4). A recent study showed that TLR2 and TLR4 are functionally expressed in human odontoblasts and LPS stimulation leads to up-regulation of proinflammatory mediators such as IL-1ß, TNF-α, and human ß defensin-2 (hBD-2). In addition, Durand *et al* revealed that mRNAs of TLR1-6 and TLR9 were expressed in human odontoblasts and LTA stimulation led to an increase in gene and protein expression of TLR2 and to an induction of NF-κB activation (3). These findings suggest that odontoblasts may play an important role for the immune response against oral bacteria through TLR signaling.

In addition to the immune responses, odontoblasts and/or dental pulp cells are also involved in tooth repair and regeneration as well as in dentin formation (dentinogenesis), although the precise mechanism remains to be fully elucidated. Following enamel disruption, odontoblasts and/or dental pulp cells are exposed to bacteria entering dentin from the oral cavity, which may trigger tissue repair processes as well as inflammatory responses of odontoblasts and/or dental pulp cells. It has been known that TLR signaling is involved in tissue repair and regeneration as well as in the immune response (5-7). However, little is known about the role of TLR signaling on the tissue repair process of odontoblasts and/or dental pulp cells. Cell adhesion and migration are the critical processes for tissue repair in a variety of physiological and pathological conditions as well as in tumor metastasis. Therefore, in this study, we examined whether LPS, a Gram-negative bacterial molecule, affects adhesion and migration of a murine dental papilla-derived MDPC-23 cells and whether TLR4 is required for these effects.

Materials and methods

Cell culture and reagents. MDPC-23 cells (8,9), murine dental papilla-derived odontoblast-like cells, were cultured in

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Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 units/ml penicillin and 100 μg/ml non-essential amino acids at 37˚C in a 5% CO_2 humidified incubator. Ultrapure LPS from *E. coli* 0111:B4 was purchased from Invitrogen (San Diego, CA, USA). The TLR4 antibody was from ImGenex (San Diego, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from MDPC-23 cells or mouse spleen tissue, using Qiagen RNeasy mini kit (Valencia, CA, USA). Total RNA (1 μg) was reverse transcribed into cDNA and PCR was performed using the Superscript™ one-step RT-PCR with Platinum® Taq kit (Invitrogen). The following primer sets were used. TLR4: F: 5'-GTGGTACCTGAG AATGATGTGGG-3' and R: 5'-GTTAAGGAAGTCAGGAA CTGGGTG-3'; GAPDH: F: 5'-CCAAGGTCATCCATGAC AACTTTG-3' and R: 5'-GTCATACCAGGAAATGAGCTT GACA-3'. PCR conditions were as follows: 94˚C for 3 min, 35 cycles for TLRs or 25-30 cycles for GAPDH consisting of 94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 30 sec, and 72˚C for 10 min. PCR products were then electrophoresed on a 1.5% acrylamide gel and visualized using a gel documentation system (Bio-Rad, Hercules, CA, USA).

Cell adhesion assay. Type I collagen (50 μl) diluted in PBS was added to each well of 96-well plates and placed at 4˚C overnight. Subsequently, each well was rinsed with PBS and non-specific binding sites were blocked with 1% BSA at 37˚C for 1 h. Various doses of LPS were added to each well for 30 min and subsequently $5x10^4$ cells were placed in each well and allowed to adhere at 37˚C for 30-90 min. Non-adherent cells were rinsed off with PBS, and the remaining cells were fixed with 4% paraformaldehyde for 5 min. The cells were stained with 0.5% toluidine blue in 4% para-formaldehyde for 5 min and finally rinsed in water. The cells were solubilized with the addition of 100 μl of 1% SDS and the optical density was measured by a Microplate Autoreader ELISA (Bio-Tek Instruments Inc., Winooski, VT, USA) at 595 nm.

Cell migration assay. The cell migration assay was performed using a Chemotaxis Cell Migration Assay kit (Chemicon) according to the manufacturer's instructions. The cells were collected by trypsinization and resuspended in serum-free medium at a density of $2.5x10^4$/ml. The cells were added onto the insert and treated with various doses of LPS in the media. The cells were then allowed to migrate for 24 h at 37˚C. The cells that migrated to the lower surface of the membrane were fixed with methanol and stained with hematoxylin for 5 min. The number of migrated cells on the lower side of the membrane was counted from five randomly selected high power fields (x200).

Western blot analysis. The cells treated with LPS were lysed with 2X SDS sample buffer. The proteins were resolved by SDS-PAGE and transferred onto PVDF membrane. After blocking with 5% skim milk in TBS at room temperature for 1 h, the membranes were incubated with IκB-α, phospho-IκB-α, phospho-ERK (Cell Signaling, Danvers, MA, USA), phospho-focal adhesion kinase (FAK), phospho-AKT, and actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4˚C overnight. The membranes were then washed three times with TBS supplemented with 0.05% Tween-20 (T-TBS), followed by incubation with secondary antibody at room temperature for 1 h. Finally, the membranes were visualized with West-Zol® (plus) (iNtRON Biotechnology Inc., Seongnam, Korea) detection reagent using the LAS-1000 Image Reader of the Luminescent Image Analyzer (FujiFilm LifeScience, Tokyo, Japan).

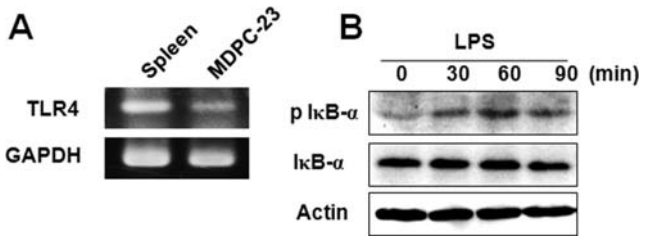

Figure 1. (A) Gene expression of TLR4 was examined by RT-PCR in mouse spleen and in MDPC-23 cells. (B) MDPC-23 cells were treated with LPS (1 μg/ml) and phosphorylation of IκB-α was examined by Western blot analysis.

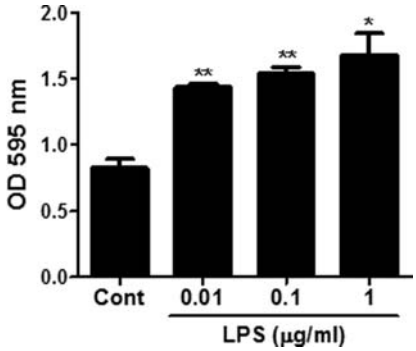

Figure 2. MDPC-23 cells were incubated in type I collagen-coated plate at the absence or presence of LPS. The cells adherent to type I collagen were stained with toluidine blue and solubilized with the addition of 100 μl of 1% SDS. The optical density was measured at 595 nm. Results are expressed as the means ± SD; $^*P<0.05$, $^{**}P<0.01$.

Statistical analysis. Statistical significance between the groups was determined by a two-tailed Student's t-test (Excel, Microsoft). Differences were considered significant when p-values were <0.05.

Results

Functional expression of TLR4 in MDPC-23 cells. The gene expression of TLR4 in MDPC-23 cells was examined by RT-PCR. mRNA from mouse spleen tissue was used as positive control. The TLR4 gene was expressed in MDPC-23 cells, although its expression level was less than that in spleen (Fig. 1A). In addition, LPS led to phosphorylation of IκB-α after 30 min of stimulation (Fig. 1B), although IκB-α degradation was not detected. These findings suggest that TLR4 is functionally expressed in MDPC-23 cells.

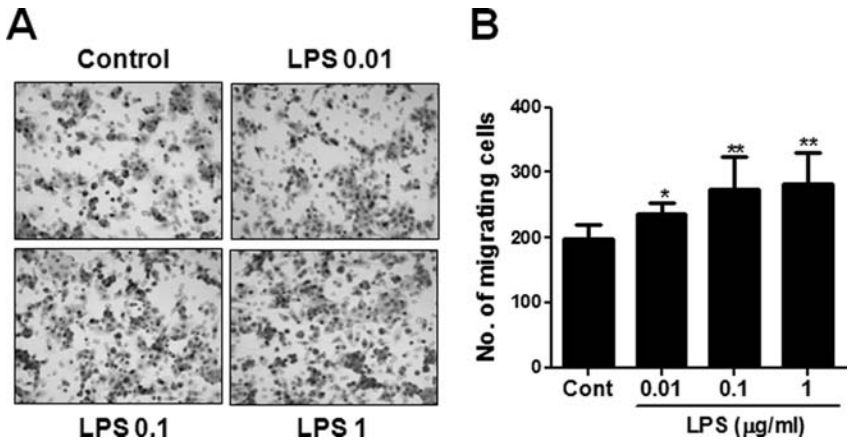

Figure 3. The migration assay was performed as described in Materials and methods. Migrating cells were fixed with methanol and stained with hematoxylin (A). The number of migrating cells was counted from five randomly selected areas (x200 magnification) (B). Results are expressed as the means ± SD; *P<0.05, **P<0.01.

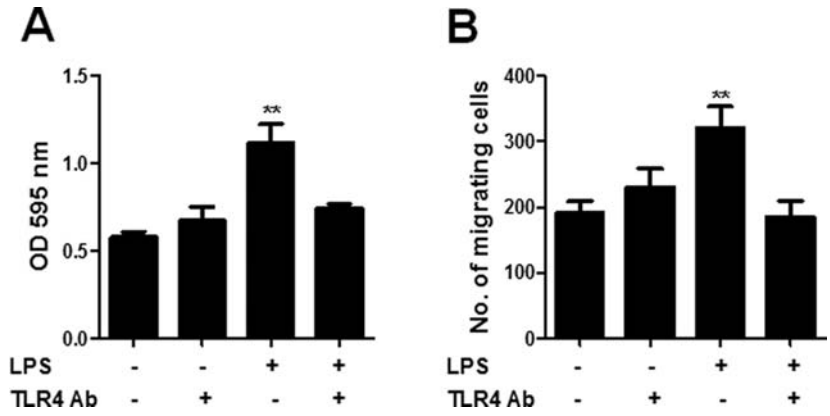

Figure 4. MDPC-23 cells were pretreated with TLR4 antibody (10 μg/ml) 2 h before LPS treatment. Subsequently, adhesion (A) and migration (B) assays were performed as described in Materials and methods. Results are expressed as the means ± SD; **P<0.01.

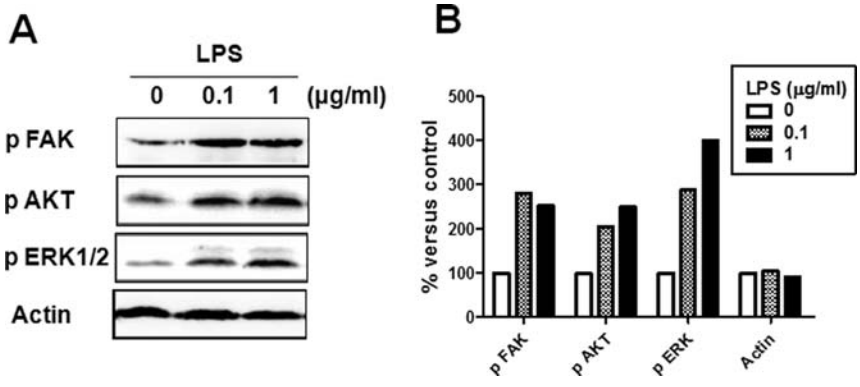

Figure 5. MDPC-23 cells were treated with LPS at indicated doses for 24 h and the cellular proteins were used for Western blot analysis to detect phosphorylation of FAK, AKT and ERK (A). The band densities were measured and the percentage of the value vs. control was calculated (B).

Adhesion and migration ability of MDPC-23 cells enhanced by LPS. It was examined whether LPS affects the adhesion ability of MDPC-23 cells using type I collagen-coated plate. As shown in Fig. 2, LPS stimulation significantly enhanced the adhesion ability of MDPC-23 cells on type I collagen in a dose-dependent manner. Moreover, LPS stimulation led to significant dose-dependent increase in the number of cells that migrated to the lower surface of the membrane (Fig. 3).

Involvement of TLR4 in adhesion and migration of MDPC-23 cells is enhanced by LPS. Because TLR4 is required for the LPS-induced immune response, we examined whether TLR4

mediates the adhesion and migration of MDPC-23 cells enhanced by LPS. When TLR4 was blocked by its antibody, LPS did not promote the adhesion ability of MDPC-23 cells (Fig. 4A). Similarly, blocking of TLR4 restored the migrating ability of MDPC-23 cells enhanced by LPS (Fig. 4B). These findings indicate that LPS can promote adhesion and migration of MDPC-23 cells through a TLR4-dependent pathway.

Enhanced activation of FAK, AKT, and ERK by LPS in MDPC-23 cells. There are various mechanisms involved in cell migration. FAK is found to promote cell migration and invasion through the ERK and PI3/AKT signaling pathways (10). Therefore, we examined whether LPS induces the activation of these factors in MDPC-23 cells. At 24 h after stimulation, LPS induced the phosphorylation of FAK, AKT, and ERK in MDPC-23 cells (Fig. 5).

Discussion

TLR activation in wound healing appears to be triggered by two classes of ligands including microbial products and endogenous ligands (5). In organs such as the intestine and the skin, tissue damage results in destruction of the protective barrier and subsequent TLR activation by bacterial products, such as LPS and lipoprotein. In addition, in many organs such as the liver, kidney and the heart, tissue injury leads to the release of endogenous ligands such as heat shock proteins, high mobility group box 1 (HMGB1) and uric acid from dying and dead cells, which results in TLR activation and sterile inflammation.

The effect of TLRs on tissue injury seems to be a double-edged sword. In ischemia-reperfusion and alcoholic liver injury, TLR2 or TLR4 are involved in the exacerbation of epithelial injury (11,12). On the other hand, in the intestine, TLR2/TLR4-MyD88 signaling is required for the epithelial regeneration following DSS-induced injury (13,14). In addition, LPS signaling enhances hepatic fibrogenesis by modulating TGF-ß signaling through a TLR4/MyD88-dependent pathway (15). These findings suggest that TLR signaling may be closely involved in tissue repair with distinct mechanisms.

Regeneration of a functional and living tooth is considered the most promising therapeutic strategy for the replacement of a damaged tooth (16-18). Odontoblasts that are a part of the outer surface of the dental pulp play a role in tooth repair and the regeneration process as well as in the inflammatory response. Although TLR signaling was shown to be involved in the immune response in odontoblasts (3), it remains to be clarified how TLR signaling mediates tissue repair processes of odontoblasts and/or dental pulp cells. Wound healing processes are divided into three distinct phases: first is the inflammatory and fibrogenic phase, second is the regenerative phase, and third is a remodeling phase (19). Among those phases, cell adhesion and migration are essential processes in tissue regeneration. Therefore, in this study, we examined the effect of TLR4 activation on cell migration and adhesion of MDPC-23 cells.

In human odontoblasts, the genes of TLR1-6 and 9 were functionally expressed (3). LTA led to an increase in TLR2 mRNA and protein expression and to the induction of the nuclear translocation of NF-κB. In this study, we used a murine dental papilla-derived odontoblast-like cell line, MDPC-23. The TLR4 gene was detectable and LPS treatment led to phosphorylation of IκB-α, suggesting that TLR4 is functional in MDPC-23 cells. In adhesion and migration assay, LPS stimulation enhanced the adhesion and migration ability of MDPC-23 cells, which was restored by TLR4 blocking. These finding suggest that TLR4 may play a pivotal role in the tissue repair process of odontoblasts and/or dental pulp cells.

A previous study showed that LPS enhanced tumor cell adhesion and invasion through ß1-integrin-dependent mechanism (20). FAK has been shown to regulate integrin-mediated signaling (21,22). After ligand stimulation or integrin engagement, FAK becomes phosphorylated, which leads to the phosphorylation of downstream regulators such as AKT and ERK. In the present study, even though ß1-integrin expression wan not determined, LPS stimulation led to phosphorylation of FAK, AKT, and ERK in MDPC-23 cells. The precise mechanism remains to be elucidated.

In conclusion, in the present study we have shown that LPS enhanced the cell adhesion and migration of MDPC-23 cells, a process that requires TLR4, suggesting that TLR4 may play a pivotal role for tooth repair and regeneration. Because odontoblasts are found to express various TLRs and TLR signaling displayed a double-edged effect on tissue injury, it is recommended that whether and how other types of TLRs mediate tissue repair processes of odontoblasts and/or dental pulp cells should be further evaluated.

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