

# Increase of Grb2 and Ras Proteins and Expression of Growth Factors in LPS Stimulated Odontoblast-like Dental Pulp Cells

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Inflammatory cells express the inflammatory cytokines and growth factors induced by lipopolysaccharide (LPS). Odontoblasts are located at the pulp–dentin interface and extend their cell processes far into the dentin where they are the first cells to encounter microorganisms or their products. Therefore, this study examined the expression of some growth factors related to the signal pathway, such as growth factor receptor binding protein 2 (Grb2)-Ras in odontoblast-like dental pulp cells, after a treatment with LPS. After 60 minutes, the mRNA and protein expression levels of Grb2 and Ras were higher in the LPS-treated cells than in the control cells. The level of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) mRNA expression was increased significantly to a level similar to that of Grb2 and Ras at 60 minutes. The platelet-derived growth factor-AA (PDGF-AA) mRNA level was expressed strongly in the odontoblast like dental pulp cells without an association with LPS stimulation. Scanning electron microscopy revealed many extensions of the cytoplasmic processes and the number of processes increased gradually at 30, 60 and 90 minutes after LPS stimulation. From these results VEGF and bFGF expression might be induced through the Grb2-Ras signal transduction pathway in LPS treated odontoblasts.

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## INTRODUCTION

The cell walls of gram-negative bacteria contain lipopolysaccharide (LPS), which can stimulate most LPS-responsive cells in a mammalian host. Of them, macrophages, causes an inflammatory response by secreting substances, such as protease, to remove the bacteria penetrating into the body (Jin et al., 1998). LPS recognizes the LPS-binding protein (LBP) in the serum of the host (Schumann et al., 1990), and then binds to the cell surface receptor, CD14. This signal is then transferred to Toll-like receptor-4 (TLR-4) (Silva et al., 2001). LPS signaling to TLR-4 can activate the nuclear factor  $\kappa$ B (NF- $\kappa$ B), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathways involved in cell survival and the inflammatory response (Dauphinee & Karsan, 2006).

Growth factor receptor binding protein 2 (Grb2) is an adaptor protein that binds to several receptor tyrosine kinases (RTKs) (Songyang et al., 1994). Moreover, the Grb2-Sos1 complex activates Ras and induces cellular responses, such as survival and growth (Masson et al., 2009). The known Grb2-mediated growth factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF). Ras is activated by undergoing exchange from GDP to GTP by Grb2/Sos1 signaling transduction (Dharmawardana et al., 2006). VEGF and bFGF are essential factors in the regulation of angiogenesis, and several factors are involved in the expression of both genes, whereas some factors are shared reciprocally (Ferrara, 2005). The induction of VEGF receptor 2 (VEGFR-2) phosphorylation recruits Shc, Grb2 and Nck, and forms the Shc-Grb2 complex (Dharmawardana et al., 2006). In addition,

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Grb2 induces the signal transduction of cell cycle progression via the VEGF or bFGF-mediated Sos/Ras pathway (Tammela et al., 2005). PDGF is implicated in the proliferation and migration of several biological processes, such as vascular remodeling, wound healing and cancer (Bornfeldt et al., 1995), and the PDGF receptor (PDGFR) is known to bind to Grb2 (Arvidsson et al., 1994).

Drk, a homolog protein to mammalian Grb2 has been implicated in LPS signal transduction, such as activation of the Ras-MAPK pathway, in insect hemocytes by a treatment with LPS (Foukas et al., 1998). The level of VEGF expression increased in odontoblasts after a treatment with LPS via the CD14-TLR4 signaling pathway (Botero et al., 2003, 2006). On the other hand, there are no reports on the expression of growth factors linked to the Grb2-Ras signal transduction pathway in LPS-responsive odontoblasts. Therefore, this study examined the expression of Grb2 and Ras as a signal transduction molecule after LPS stimulation related to the expression of growth factors, such as VEGF, bFGF and PDGF, using odontoblast-like dental pulp cells.

## MATERIALS AND METHODS

### Cell Culture and Treatment of LPS

Pulp cells from the erupted mandibular incisors of ICR mice were isolated and prepared for the primary cultures, as described previously (Balic & Mina, 2010). All animal studies were approved by the 'Institutional Animal Care and Use Committees' at Chosun University, and animal care was carried out according to 'Guide for the Care and Use of Laboratory Animals'. Mineralization was induced in the primary cultures by adding a medium ( $\alpha$ -MEM, 10% FBS, 40 U/mL penicillin, 40  $\mu$ g/mL streptomycin, 50  $\mu$ g/mL ascorbic acid and 4 mM  $\beta$ -glycerol phosphate) to the confluent cultures, which were then assayed and quantified as described previously (Balic et al., 2010). The odontoblast-like dental pulp cells ( $2 \times 10^5$  cells) were plated in 60-mm culture dishes and exposed to 20  $\mu$ g/mL of the *Escherichia coli* k-235 strain LPS (Sigma, St. Louis, MO, USA) for 30 min, 60 min, 90 min, 24 hours and 48 hours.

### Reverse Transcription and Polymerase Chain Reaction

A TRI reagent (MRC, Cincinnati, OH, USA) was used to extract the total RNA of the LPS treated cells according to the manufacturer's instructions. The total RNA was used to synthesize the complementary DNA (cDNA). The synthesis of cDNA and the polymerase chain (PCR) reaction was performed on the AccuPower RT Premix (Bioneer, Daejeon, Korea) and AccuPower PCR Premix (Bioneer). The following primers were synthesized (Bioneer) for reverse transcription-PCR analysis. All the following primers used originated from mice: 1) Grb2 forward: 5'-TAC ATA GAA ATG AAA CCA

CAT CCG-3', reverse: 5'-ACA TAA TTG CGG GGA AAC ATG CC-3', 2) Ras forward: 5'-ATG ACA GAA TAC AAG CTT GTG GTG-3', reverse: 5'-TCT ATA GTG GGA TCA TAC TCG TCC-3', 3) VEGF forward: 5'-GTG GAC ATC TTC CAG GAG TA-3', reverse: 5'-ATC TGC AAG TAC GTT CGT TT-3', 4) bFGF forward: 5'-CCT TGC TAT GAA GGA AGA TG-3', reverse: 5'-TTA TAC TGC CCA GTT CGT TT-3', 5) PDGFAA forward: 5'-AGG AAG CCA TTC CTG CA-3', reverse: 5'-CTT GAC ACT GCG GTG GTG-3', 6) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: 5'-CCA TGG AGA AGG CTG GG-3' and reverse: 5'-CAA AGT TGT CAT GGA TGA CC-3'.

### Western Blotting

The total cytosolic protein was extracted using the NP-40 lysis buffer (150 mM NaCl, 1 % NP-40, 50 mM Tris-Cl (pH 7.4), 2 mM  $\text{Na}_3\text{VO}_4$ , 2 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 50 mM NaF, 2 mM EDTA (pH 7.4) 0.1  $\mu$ g/ml leupeptin and 1  $\mu$ g/ml aprotinin). The lysates were incubated on ice for 30 minutes and centrifuged at 13,000 rpm at 4°C. After protein extraction, the concentration in 30  $\mu$ g was determined using a *Dc* protein assay kit (BioRad Laboratories, Hercules, CA, USA) and loaded onto sodium dodecyl sulfate 12%-polyacrylamide gels. After electrophoresis, the protein was transferred to a nitrocellulose membrane using the transfer buffer (25 mM Tris-base, 192 mM Glycine, 20% methanol) for 90 min at 4°C and blocked with 5% non-fat dry milk for 80 min at room temperature. The membrane was washed in PBS-T (0.15% Tween20 in 1 $\times$ PBS) and blotted with 1:2,500 of anti-mouse Grb2 (Upstate, Lake Placid, NY, USA), anti-rabbit Ras (Upstate) and anti-rabbit  $\beta$ -actin (SantaCruz Biotechnology, Santa Cruz, CA, USA) for 16 hours at 4°C. After washing, the membrane was blotted with 1:5,000 of horseradish peroxidase-conjugated goat anti-mouse or rabbit-IgG (SantaCruz Biotechnology) in a blocking solution for 1 hour at room temperature. The membrane was developed by X-ray film (Fuji Film, Minatoku, Tokyo, Japan) after being treated with an enhanced chemiluminescence (ECL) solution (Amershampharmacia) for 3 min. The density and quantification of the expressed bands was measured using a Science Lab Image Gauge (Fuji Film).

### Microscopic Observation

An inverted microscope (Olympus, Shinjuku-ku, Tokyo, Japan) was used to observe the morphological changes in the odontoblast-like dental pulp cells by exposing them to LPS (100 ng/mL) for 30 min, 60 min, 90 min, 24 hours and 48 hours. Scanning electron microscopy (SEM) was used to observe the ultrastructure of the odontoblast-like dental pulp cells after the LPS treatment. The cells were fixed in a solution containing 2.5% glutaraldehyde (Merck, Darmstadt, Germany) in PBS and post-fixed with 1% osmium tetroxide. After fixation,

the samples were washed three times in PBS and dehydrated in a graded series of ethanol. Hexamethyldisilazane (Sigma Chemical Co., St. Louis, MO, USA) was used to dry the samples. The samples were sputter coated with gold and observed by SEM (H4700; Hitachi, Chiyoda-ku, Tokyo, Japan) at 10 kV.

### Statistical Analysis

All data is reported the mean and standard deviation using the Excel 2007 statistical software (Microsoft, USA). The significant differences ( $n=3$ ,  $*p<0.05$ ,  $**p<0.005$ ) were determined using a Student's t-test.

## RESULTS

### Expression of Grb2 and Ras mRNA in the Odontoblast-like Dental Pulp Cells by a LPS Treatment

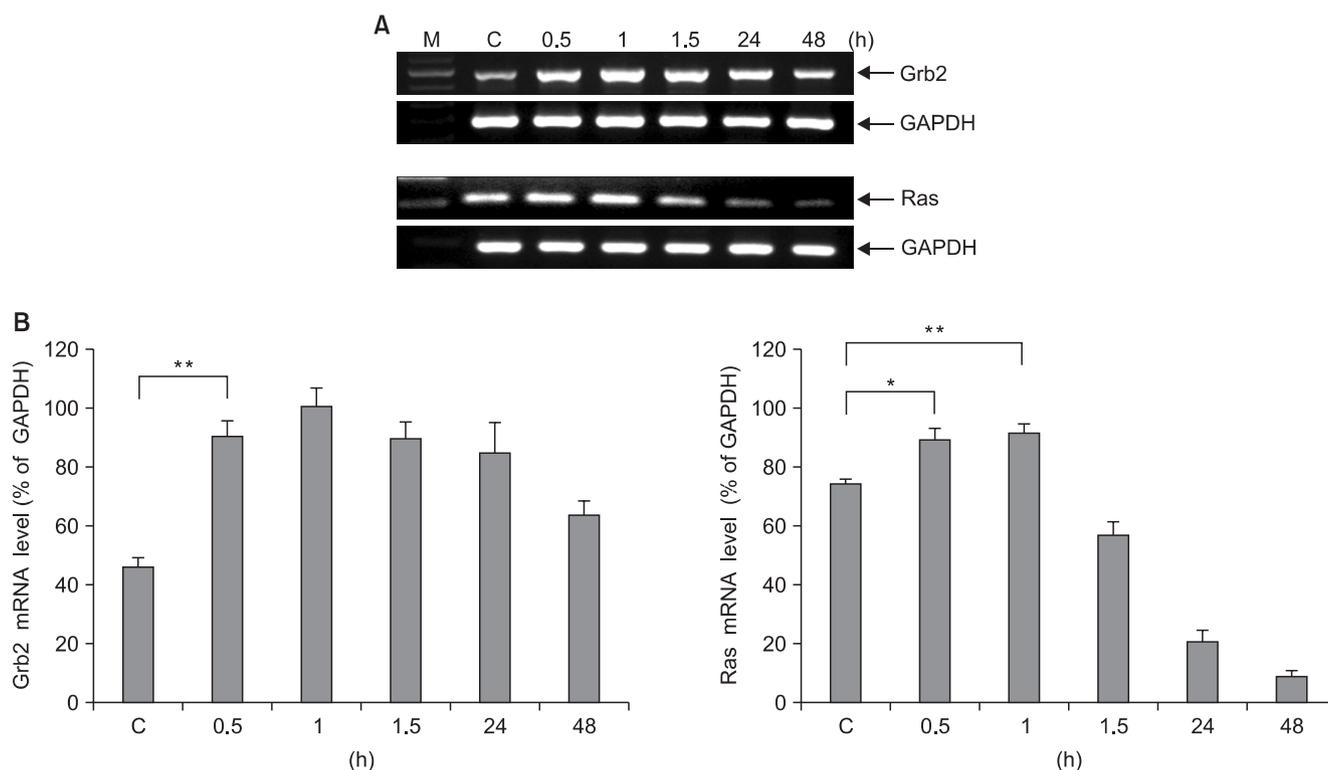
The expression of Grb2 mRNA was increased 1.96 and 2.1-fold after 30 and 60 min, respectively, in the LPS-treated group compared to the control but it decreased gradually thereafter. In addition, the expression of Ras mRNA was increased 1.2-fold after 30 and 60 min in the LPS-treated group compared to the control but it decreased markedly from 90 min to 48 hours (Fig. 1A and B).

### Expression of VEGF, bFGF and PDGF-AA mRNA in the Odontoblast-like Dental Pulp Cells by the LPS Treatment

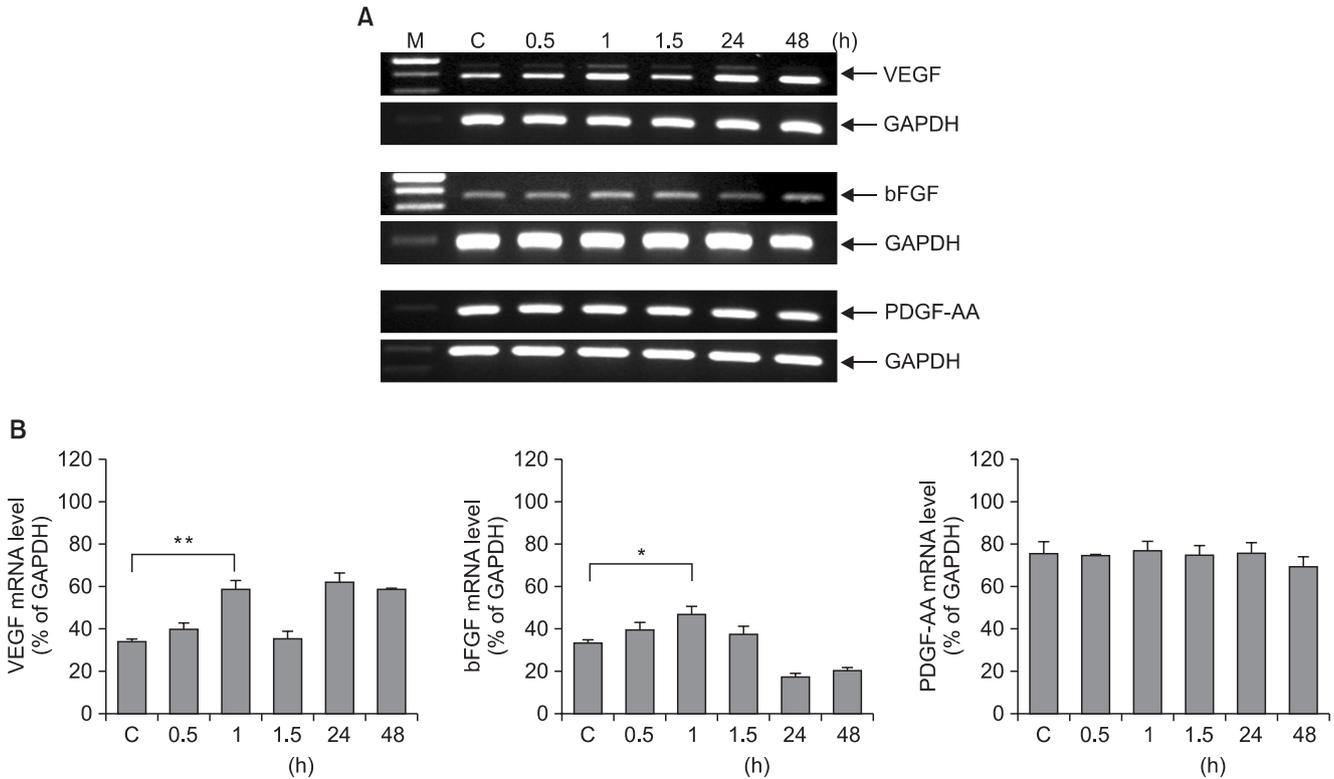
The expression of VEGF mRNA was increased 1.73-fold after 60 min and decreased at 90 min in the LPS-treated group compared to the control, but the expression level at 24 and 48 hours was similar that observed at 60 min. The expression of bFGF mRNA was increased 1.38-fold after 60 min in the LPS-treated group compared to the control, but decreased thereafter. The expression of PDGF-AA mRNA was strong at all times but there was no difference according to the LPS treatment (Fig. 2A and B).

### Expression of Grb2 and Ras Protein in Odontoblast-like Dental Pulp Cells by a LPS Treatment

The expression of the Grb2 protein was increased 1.79-fold after 30 min in the LPS-treated group compared to the control, which was maintained until 90 min but decreased thereafter. Expression of the Ras protein increased 1.79-fold after 60 min in the LPS-treated group compared to the control, was maintained at that level until 90 min, and decreased after 24 and 48 hours (Fig. 3A and B).



**Fig. 1.** Analysis of Grb2 and Ras mRNA expression. (A) Grb2 and Ras mRNA expression in odontoblast-like dental pulp cells treated with 20  $\mu\text{g}/\text{mL}$  of LPS for 30, 60, 90 min, 24 h, and 48 h. (B) The level of Grb2 and Ras mRNA expression was increased at 30 min compared to the control group. The Grb2 and Ras mRNA level was normalized to the GAPDH mRNA level.  $*p<0.05$ ,  $**p<0.005$ ; M, 100 bp DNA size marker; C, control; Grb2, growth factor receptor binding protein 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide.



**Fig. 2.** Analysis of VEGF, bFGF, and PDGF-AA mRNA expression. (A) VEGF, bFGF and PDGF-AA mRNA expression in odontoblast-like dental pulp cells treated with 20 µg/ml of LPS for 30, 60, 90 min, 24 h and 48 h. (B) The level of VEGF and bFGF mRNA expression was increased at 60 minute compared to the control group. The expression of PDGF mRNA was unaffected by the LPS treatment. The Grb2 and Ras mRNA level was normalized to the GAPDH mRNA. \* $p < 0.05$ , \*\* $p < 0.005$ ; M, 100 bp DNA size marker; C, control; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; PDGF-AA, platelet-derived growth factor-AA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide.

### Morphological Change in the Odontoblast-like Dental Pulp Cells by a LPS Treatment

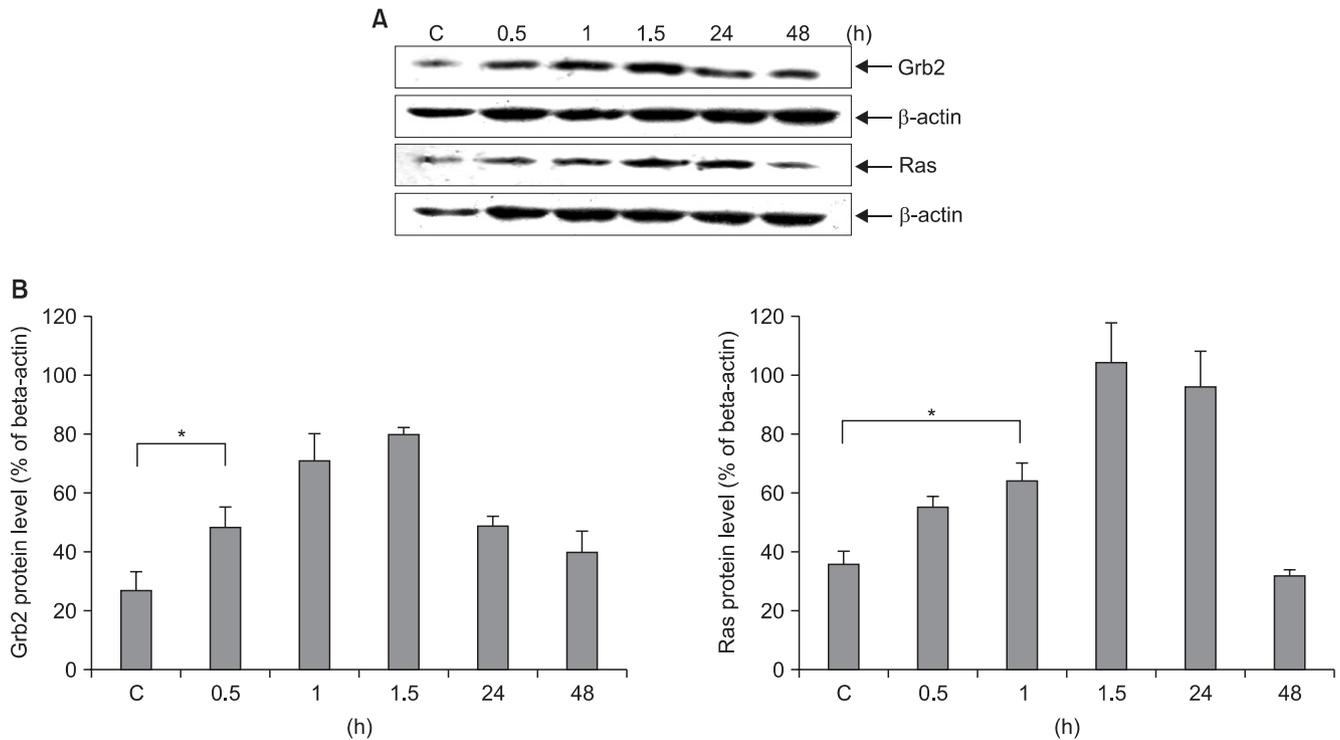
Phase-contrast microscopy was used to observe the morphological changes in the cells after a treatment with LPS. The results showed no specific changes in the morphology of the LPS treated group compared to the control (Fig. 4A1-4). SEM was performed to observe the ultrastructure of the LPS-treated cells. The membrane was smooth in the control group but many extensions of the cytoplasmic processes were observed at 30 min after LPS stimulation, which were extended further at 60 and 90 min (Fig. 4B1-4).

### DISCUSSION

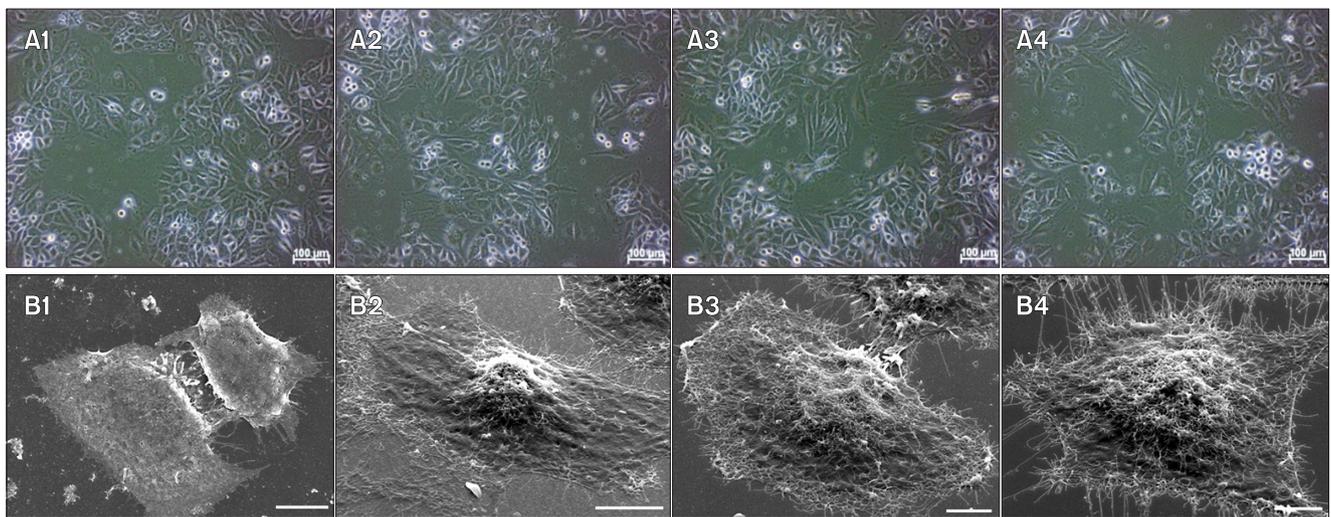
The main function of odontoblasts is the secretion of several collagenous and noncollagenous proteins to form dentin. These cells are the first pulpal cells to encounter dental pathogens through their cellular process in the dentin (Butler & Ritchie, 1995). In addition, odontoblasts secrete the interleukin (IL)-8 and secretory leucocyte protease inhibitor (SLPI) as a proinflammatory cytokine in response to LPS (Levin et al., 1999; Choi et al., 2009). Therefore, odontoblasts

play an important role in the inflammatory response by cariogenic bacteria (Staquet et al., 2008).

LPS signaling is initiated by binding the LPS/LBP complex to the glycosylphosphatidyl inositol (GPI)-anchored membrane receptor CD14. LPS signaling binding to CD14 is transferred to TLR-4, which induces the proinflammatory cytokines, such as IL-1 $\beta$ , IL-6 and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), in monocytes, macrophages and neutrophils, respectively. This signal transduction pathway is activated through phosphorylation of the MAPKs, such as p42/44 MAPK and p38 MAPK (Guha & Mackman, 2001; Saluk-Juszczak & Wachowicz, 2005). Moreover, expression of the Grb2 protein was increased 4~5-fold in B-lymphocytes after a LPS treatment compared to the control and proliferation was induced through the RasRaf/MAPK pathway (Zamorano et al., 2001). In this study, Grb2 and Ras expression in LPS treated odontoblast-like dental pulp cells were increased the most at 60 min compared to the control. Moreover, the expression of the two proteins was increased at 90 min after the LPS treatment. This suggests that stimulation by LPS can induce the expression of proinflammatory cytokines and growth factors through the Grb2-Ras signal transduction



**Fig. 3.** Analysis of Grb2 and Ras protein expression. (A) Grb2 and Ras protein expression was identified in odontoblast like dental pulp cells treated with 20 µg/ml of LPS for 30, 60, 90 min, 24 h, and 48 h. (B) The levels of Grb2 and Ras protein expression was increased at 30 min and 60 min compared to the control group, respectively. The Grb2 and Ras protein level was normalized to the β-actin. \*p<0.05; C, control; Grb2, growth factor receptor binding protein 2.



**Fig. 4.** (A) and (B) phase contrast microscopy and scanning electron microscopy (SEM) images of the odontoblast-like dental pulp cells exposed to lipopolysaccharide (LPS) (20 µg/ml) for the control (A1), 30 (A2), 60 (A3), and 90 (A4) min, respectively. SEM images show the smooth membrane in the control group but many cytoplasmic processes from 30 to 90 min after LPS stimulation. The scale bars of (B1) and (B2) indicate 10 µm. The scale bars of (B3) and (B4) indicate 5 µm.

pathway in odontoblast-like dental pulp cells. The signaling pathway of the VEGF gene expression exists through the Ras/MAPK or phosphoinositide 3-kinase (PI3-K)/Akt, which induces cell survival and proliferation

(Berra et al., 2000). In addition, the expression of VEGF was increased in the LPS-treated pulp cells and odontoblasts (Matsushita et al., 1999; Botero et al., 2003). The expression of PDGFR and PDGF was increased in the myofibroblast and

embryonic fibroblast cell line, NIH3T3, by a treatment with LPS (Coin et al., 1996; Lee et al., 2006). In the inflammatory response, bFGF promotes angiogenesis and the formation of granulation tissue (Gospodarowicz et al., 1990), and an infection of *Mycoplasma pneumoniae* increases the expression of bFGF (Liu et al., 1998). In this study, the expression of the VEGF gene was increased in the LPS-treated odontoblast-like dental pulp cells compared to the control. In addition, the expression of the bFGF gene was increased at the same time as the expression of VEGF, Grb2 and Ras. On the other hand, the PDGF-AA expression pattern was unaffected because PDGF is more involved with the formation of dentin than the inflammatory response in odontoblasts at an injury site (Yokose et al., 2004). Therefore, LPS stimulates an increase in VEGF and bFGF, suggesting that the Grb2-Ras transduction pathway might be involved in LPS signaling in odontoblast-like dental pulp cells.

Odontoblasts are epithelial-like cells that exhibit morphological and functional behavior (Jontell et al., 1987). In addition, odontoblasts synthesize sclerotic dentin in the distal portion of the odontoblastic process against an invasion of cariogenic bacteria (Frank & Veogel, 1980). In previous studies, another odontoblast cell line, MDPC-23 cells, formed numerous cellular processes on the cell surface due to cytotoxic effects and LPS stimulation (Costa et al., 1999; Choi et al., 2009). In addition, LPS induced a rearrangement of the cytoskeleton

in insect hemocytes due to the phagocytosis of *E. coli* via the expression of  $\beta$ 3-Integrin link to Drk signaling, such as the Drk-Ras/P44MAPK signal transduction pathway (Foukas et al., 1998). In particular, in the inflammatory response, irregular and elongated cellular processes were observed on the cell surface of macrophages (Botero et al., 2003; He et al., 2008). In this study, phase-contrast microscopy showed no detectable changes in the morphology of the odontoblast-like dental pulp cells and LPS treated cells. In contrast, SEM revealed a smooth cytoplasmic membrane in the control group but many extensions of the cytoplasmic processes in the LPS treated group, and the number of these processes increased with time. These results suggest that the morphological changes in the odontoblast-like dental pulp cells by LPS are a cellular response against an inflammatory response.

## CONCLUSIONS

An increase of VEGF and bFGF expression by LPS stimulation of odontoblast like dental pulp cells is accompanied by Grb2 and Ras induction as well as by morphological changes similar to inflammatory cells, suggesting that the induction VEGF and bFGF by LPS stimulation occurs through the Grb2-Ras signaling pathway in odontoblasts.

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