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Lipopolysaccharide-induced dental pulp cell apoptosis and the expression of Bax and Bcl-2 *in vitro*

H. Yang[1], Y.T. Zhu[1,2], R. Cheng[1], M.Y. Shao[1], Z.S. Fu[1,3], L. Cheng[1], F.M. Wang[1] and T. Hu[1]

[1]State Key Laboratory of Oral Diseases, West China College of Stomatology, Sichuan University, Chengdu, China
[2]Stomatology Hospital of Guangzhou Medical University, Guangzhou, Guangdong Province, China
[3]The Affiliated Dental Hospital, Chongqing Medical University, Chongqing, China

Abstract

Lipopolysaccharide exerts many effects on many cell lines, including cytokine secretion, and cell apoptosis and necrosis. We investigated the *in vitro* effects of lipopolysaccharide on apoptosis of cultured human dental pulp cells and the expression of Bcl-2 and Bax. Dental pulp cells showed morphologies typical of apoptosis after exposure to lipopolysaccharide. Flow cytometry showed that the rate of apoptosis of human dental pulp cells increased with increasing lipopolysaccharide concentration. Compared with controls, lipopolysaccharide promoted pulp cell apoptosis ($P < 0.05$) from 0.1 to 100 µg/mL but not at 0.01 µg/mL. Cell apoptosis was statistically higher after exposure to lipopolysaccharide for 3 days compared with 1 day, but no difference was observed between 3 and 5 days. Immunohistochemistry showed that expression of Bax and Bcl-2 was enhanced by lipopolysaccharide at high concentrations, but no evident expression was observed at low concentrations (0.01 and 0.1 µg/mL) or in the control groups. In conclusion, lipopolysaccharide induced dental pulp cell apoptosis in a dose-dependent manner, but apoptosis did not increase with treatment duration. The expression of the apoptosis regulatory proteins Bax and Bcl-2 was also up-regulated in pulp cells after exposure to a high concentration of lipopolysaccharide.

Key words: Dental pulp cells; Lipopolysaccharide; Apoptosis; Bax; Bcl-2

Introduction

Apoptosis is a form of programmed cell death that eliminates specific cells without disturbing tissue structure or function and is pivotal for the development and maintenance of multicellular organisms (1). It is necessary for morphogenesis during tooth development (2), and in the dentin formation process, odontoblasts undergo apoptosis to maintain an appropriate dentin deposition rate (3). Apoptosis also occurs in mature dental pulp (4) when the pulp is exposed to extrinsic stimuli such as bacterial infection, ischemia, mechanical stimuli, or dental material (5,6).

Numerous studies have demonstrated that members of the Bcl-2 protein family are involved in cell apoptosis (7-9). The Bcl-2 protein family, which includes pro- and antiapoptotic proteins such as Bax and Bcl-2, respectively, is encoded by the *bcl-2* gene family (10). The balance between pro- and antiapoptotic members of the Bcl-2 protein family determines whether a cell will die or survive. Thus, Bcl-2 family members function as checkpoints through which survival and death signals must pass before the fate of cells is determined (11).

Lipopolysaccharide (LPS), a constituent of the outer cell wall of Gram-negative bacteria and their main virulence factor, has multiple biological effects. It has been shown that LPS induces apoptosis in fibroblasts, macrophages, or endothelial cells (12-14). However, *in vitro* studies have shown that LPS inhibits apoptosis of neutrophils (15,16) and induces the proliferation of rat clonal dental pulp cells (RPC-C2A) (17). Bacterial LPS can also irritate human dental pulp through the dentinal tubules in some cases of mild caries, and pathological pulp changes subsequently

Correspondence: T. Hu, State Key Laboratory of Oral Diseases, West China College of Stomatology, Sichuan University, No. 14, Third Section, Renmin Nan Road, 610041 Sichuan, Chengdu, China. Fax: +86-028-8558-2167. E-mail: acomnet@263.net

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occur (18). Previous studies have demonstrated that LPS affects the odontoblastic properties of odontoblast lineage cell lines (19), vascular endothelial growth factor expression in odontoblasts (20), and gene (21) and adhesion molecule expression (22) in human dental pulp cells. However, there are no reports about the direct effect of LPS on human dental pulp cell apoptosis.

To understand whether LPS can induce apoptosis of human dental pulp cells and the potential changes of protein expression in this process, we investigated the temporal and dose-related effects of LPS on dental pulp cell apoptosis, Bcl-2 and Bax expression, and the probable interaction between Bcl-2 and Bax expression and dental pulp cell apoptosis *in vitro*.

Material and Methods

Cell culture

Impacted human third molars with no clinical caries or severe abrasions were collected from adults aged 18-25 years with the consent of the patients. Dental pulp cells were extracted and cultured as previously described (23). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% fetal calf serum (Hyclone), 100 U/mL penicillin (Invitrogen, USA) and 100 U/mL streptomycin (Invitrogen), and then incubated in a humidified atmosphere of 5% CO_2 at 37°C. When the cells reached 80%-90% confluence, they were trypsinized with 0.25% trypsin-EDTA (Sigma-Aldrich, USA) and then replated into culture flasks. The cells were confirmed as dental pulp cells using keratin and vimentin as cell markers by immunohistochemistry. *Escherichia coli* LPS (Sigma L6529, USA) was used to stimulate cells between passages five and eight at concentrations of 100, 10, 1, 0.1, and 0.01 µg/mL for 1, 3, or 5 days.

Cell apoptosis

Cells (1 x 10^5) were seeded into six-well plates for 24 h and starved for a further 24 h in serum-free DMEM. After exposure to different concentrations of LPS for 24, 72, and 120 h, respectively, the cells were washed three times with PBS and immediately fixed in 4% paraformaldehyde for 15 min. The cells were then treated with PBS containing 1 mL/L Triton X-100 for 5 min at room temperature and incubated with FITC-phallotoxin (Sigma) for 30 min in the dark at room temperature. Cell nuclei were stained using 10 µg/mL Hoechst 33258 (Sigma) for 5-10 min. Morphological changes were observed using a confocal laser scanning microscope (MRC1024ES; Bio-Rad, USA). Experiments were conducted in triplicate.

Flow cytometry analysis

The annexin V-FITC/propidium iodide (PI) apoptosis assay was conducted using a KeyGEN apoptosis detection kit (China) following manufacturer instructions. Dental pulp cells were harvested, washed, and resuspended in 500 µL of binding buffer. They were then stained with 5 µL annexin V-FITC and 5 µL PI for 15 min at room temperature. Samples were analyzed by flow cytometry using a FACScan instrument (Epics Elite ESP; Beckman Coulter, USA). Fragments of dead cells and debris were gated out by forward- and side-scatter analysis. Cells with negative PI staining and positive annexin V staining were considered to be actively undergoing apoptosis, and the number of these cells and the total number of cells analyzed were determined. Experiments were repeated three times.

Immunohistochemistry

Cells (1 x 10^5) were seeded onto glass slides and treated with various concentrations of LPS for 3 days. The slides were washed three times in PBS for 5 min and subsequently exposed to a fixative containing 4% formaldehyde. After treatment with 0.2% Triton X-100 for 10 min at room temperature, the slides were immersed in 5% bovine serum albumin for 30 min at 37°C. The slides were then incubated with anti-Bax antibody (bs-0127R; Beijing Biosynthesis and Biotechnology, China) or anti-Bcl-2 antibody (bs-0032R; Beijing Biosynthesis and Biotechnology) for 24 h at 4°C. They were then incubated with a biotin-labeled anti-rabbit secondary antibody for 3 h at room temperature followed by incubation with an avidin-biotin-peroxidase complex for 1.5 h at room temperature. Finally, they were exposed to a 3,3'-diaminobenzidine tetrahydrochloride solution and the immunoreactivities of Bax and Bcl-2 were visualized as brown staining. Evaluation of staining intensity and digital documentation were performed using an inverted phase contrast microscope and a photomechanical system (IX50; Olympus, Japan). Histological staining was classified as negative, moderately positive (light brown), or strongly positive (dark brown).

Statistical analysis

Data are reported as means ± SD of 3 replicates. Statistical analyses were performed using one-way analysis of variance (ANOVA), with the level of significance set at $P < 0.05$. Differences between variables within the ANOVA were compared by the Student *t*-test.

Results

Cell morphology

Various stages of apoptosis were observed in dental pulp cells after exposure to LPS for 1, 3, or 5 days. The characteristics of apoptosis were not usually visible in control groups, whereas the typical morphological changes of apoptosis appeared in the LPS-treated groups at all tested times. The viable pulp cells exhibited an ovoid nucleus (Figure 1A), but cells actively undergoing apoptosis showed lobulated (Figure 1B), crescent, or U-shaped (Figure 1C) nuclei. Nuclear fragmentation (Figure 1D and E, white ar-

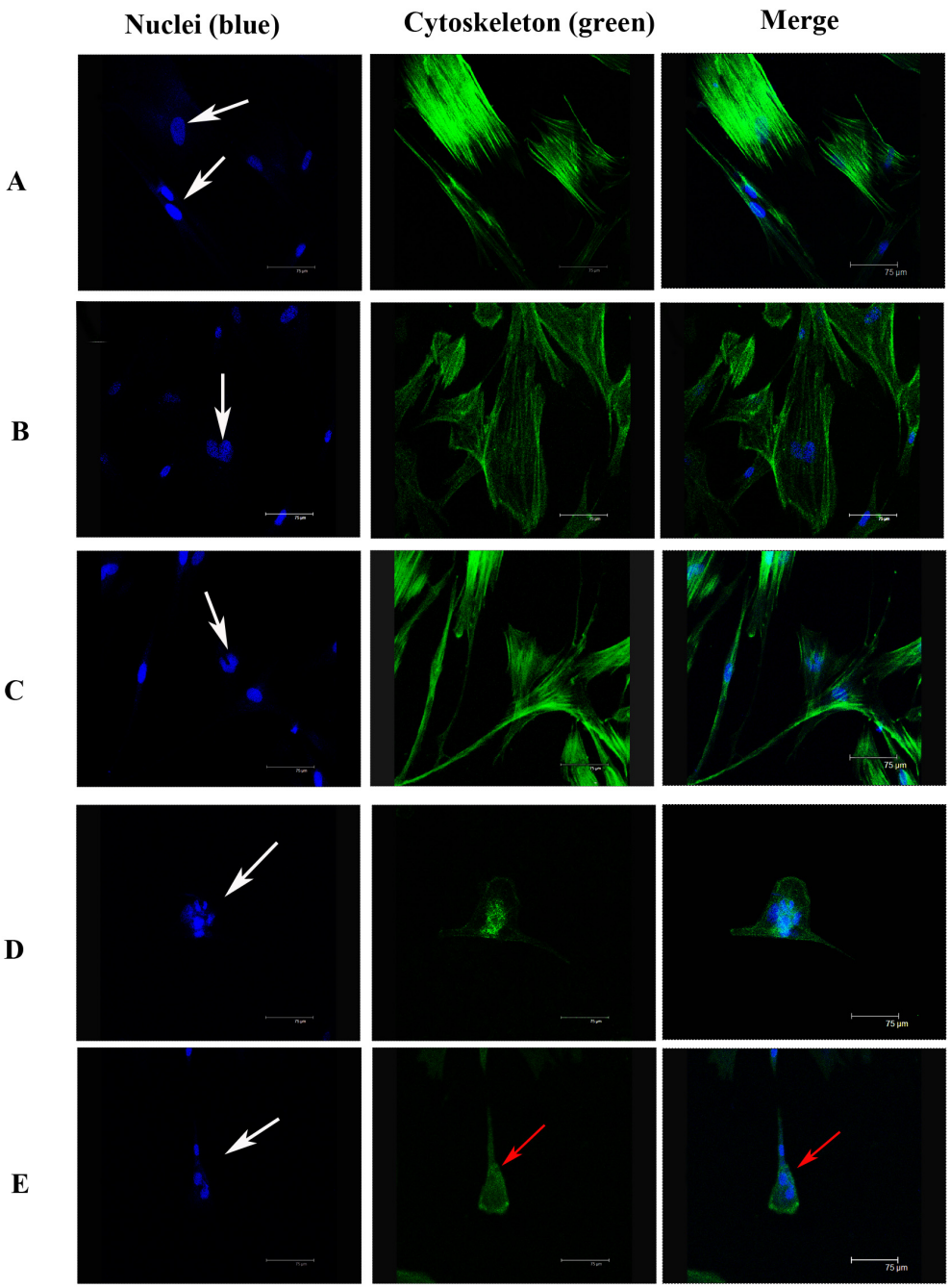

Figure 1. Morphological and ultrastructural changes during lipopolysaccharide-induced dental pulp cell apoptosis. The results were obtained by confocal microscopy. Panels on the left show nuclei (blue fluorescence) and those in the right vertical row show both nuclei (blue fluorescence) and cytoskeletons (green fluorescence). Cells in the control group (A) show oval-shaped nuclei (white arrows in Figure 1A). After exposure to lipopolysaccharide, lobulated-shaped (white arrow in Figure 1B), crescent-shaped, or U-shaped (white arrow in Figure 1C) cell nuclei appeared, and nuclear fragmentation was observed in Figure 1D and E (white arrows). Membrane shrinkage with enclosed nuclear fragmentation (red arrows in Figure 1E) suggests the early formation of an apoptotic body. Scale bar = 75 µm.

rows) and membrane shrinkage (Figure 1E, red arrows) were frequently observed after exposure to LPS. The nuclear fragmentation enclosed by an intact cell membrane (Figure 1E, red arrows) might indicate the early formation of an apoptotic body.

Flow cytometry

Flow cytometry dot plots (Figure 2) showed the percentage of differently staining cells. Cells with positive staining for annexin V-FITC, but negative staining for PI, were considered to be undergoing active apoptosis, and the lower right quadrant of each plot shows the percentage of apoptotic cells. Annexin V-FITC and PI-positive cells in the upper right quadrants revealed the late apoptotic cells or necrotic cells, and to avoid the interference of confounding factors (e.g., necrosis) these cells were excluded from the apoptosis counts. The percentages of viable cells with negative staining for both annexin V-FITC and PI are presented

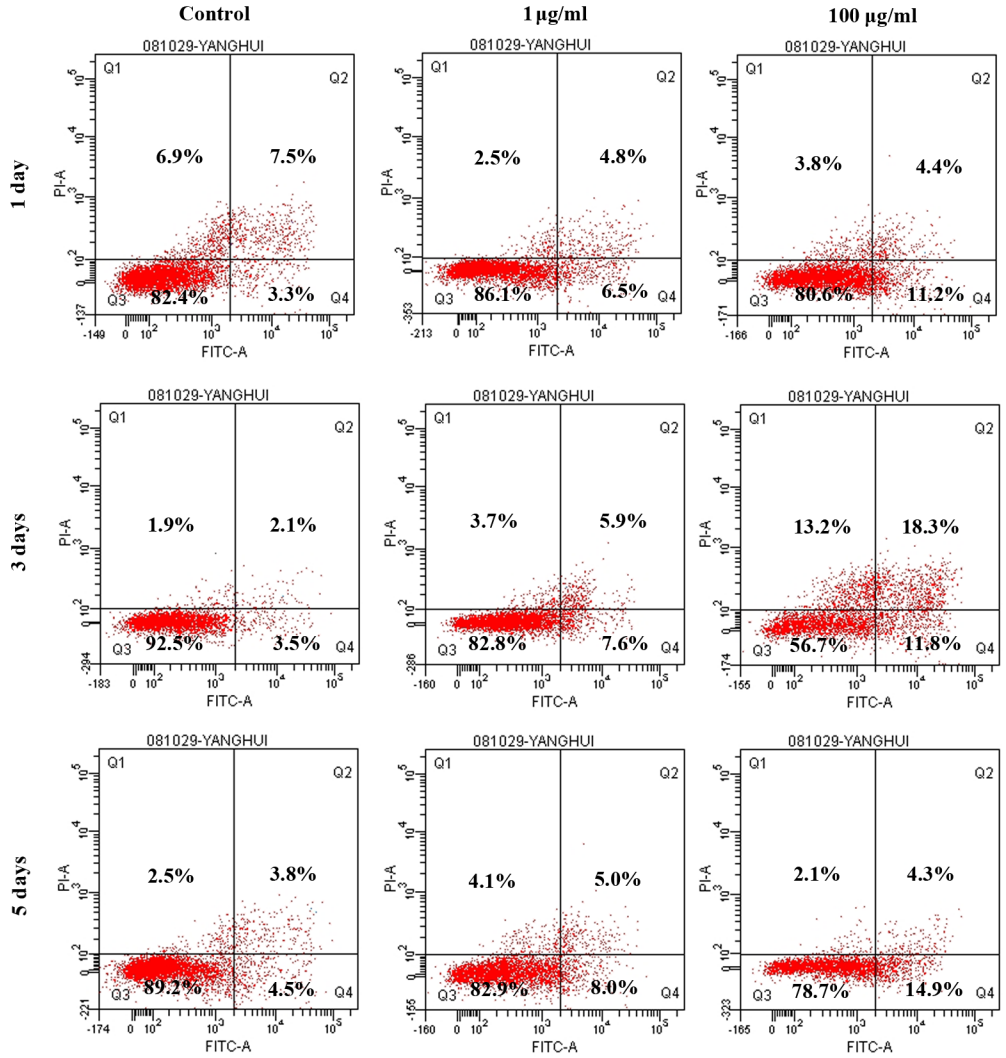

Figure 2. Flow cytometry of dental pulp cell apoptosis after lipopolysaccharide (LPS) treatment. After starvation for 24 h, the cells were treated with different concentrations of LPS for 1, 3, or 5 days. The left panels show the rate of apoptosis in control groups at different times, and the middle and right panels show the 1- and 100-μg/mL LPS-treated groups, respectively. The lower right quadrant indicates the rate of early apoptotic cells that are only annexin V-FITC positive, which indicates the percentage of active apoptotic cells. The upper right and the lower left quadrants indicate the rates of necrotic cells or late apoptotic cells with both annexin V-FITC and PI-positive staining and viable cells with both annexin V-FITC and PI-negative staining, respectively. The data presented here do not show the results obtained with other concentrations of LPS because of space limitations.

in the lower left quadrants. Apoptosis was enhanced with increasing concentrations of LPS at different times (Figure 2). The increasing trend of apoptosis in the 1- and 100-µg/mL groups was notable compared with control groups. The effects of stimulation concentration and stimulation time were also analyzed. Paired comparisons between the LPS-treated groups at each time resulted in significant differences ($P < 0.05$). Although most cells in the 0.01-µg/mL groups and control groups were viable, the number of apoptotic cells increased in a dose-dependent manner with increasing LPS concentration. Apoptosis was greater than that of the control group in all LPS groups except for the 0.01-µg/mL groups (Figure 3A). Apoptosis on the third and fifth days differed from that on the first day after exposure to LPS, but no significant difference was observed between that on the third and fifth days (Figure 3B). Apoptosis did not increase with stimulation time. The effect of LPS on dental pulp cell apoptosis was not time dependent.

Immunohistochemistry

Bcl-2 and Bax immunostaining was mainly present in the cytosol and was occasionally observed on the nuclear membrane. According to the results of flow cytometry, pulp cells were exposed to LPS for 3 days. In the control groups (Figure 4), immunostaining for Bcl-2 and Bax was weak, and the staining in the 0.01- and 0.1-µg/mL groups was also very weak (data not shown). In the 1-µg/mL groups (Figure 4), some light brown staining appeared, but was not very strong. However, strongly positive dark brown immunostaining appeared in the 100-µg/mL groups (Figure 4), indicating an enhanced expression of Bcl-2 and Bax. We expected that Bcl-2 and Bax expression might differ between control groups and low LPS concentration groups (0.01 and 0.1 µg/mL), and the expression level might be different between Bcl-2 and Bax in apoptotic cells, but our data did not demonstrate this. It may be that the detection method was not sufficiently sensitive to differentiate small changes in protein expression. Further studies should be conducted to obtain accurate information related to the expression of the target proteins.

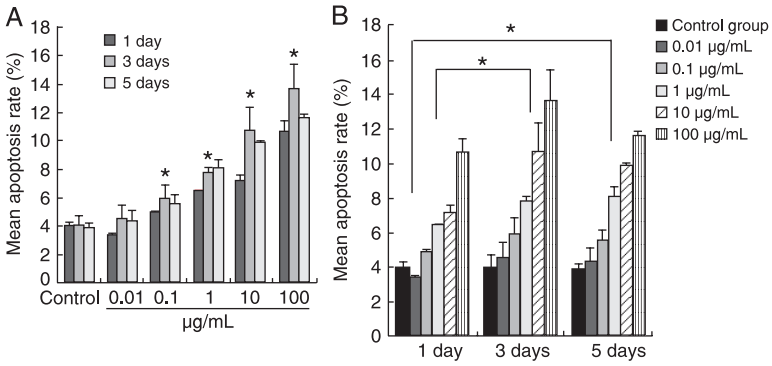

Figure 3. Lipopolysaccharide (LPS)-promoted dental pulp cell apoptosis. After starvation for 24 h, the cells were treated with different concentrations of LPS for 1, 3, and 5 days. Data are reported as means ± SD of triplicate experiments. *A*, LPS induced dental pulp cell apoptosis in a dose-dependent manner. Apoptosis in each treatment group was statistically different from unstimulated control cells except for the 0.01 µg/mL group. Paired comparisons between treated groups showed significant differences. *B*, Comparisons at different times revealed a statistically significant difference between 1 and 3 days and 1 and 5 days, but no difference was observed between 3 and 5 days. *P < 0.05, Student *t*-test.

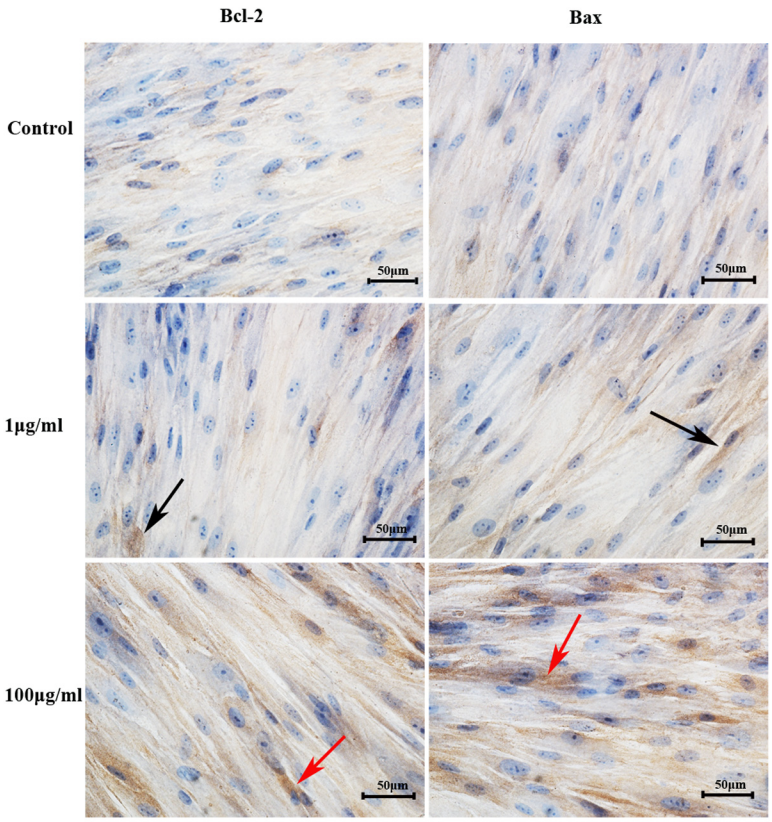

Figure 4. Bcl-2 and Bax immunoreactivity in dental pulp cells. Serum-starved cells were stimulated with different concentrations of lipopolysaccharide (LPS) for 3 days. The upper two panels show the basal expression level of Bcl-2 and Bax in control groups. The immunoreactivity of Bcl-2 and Bax in the 1-µg/mL groups (black arrows) was weakly positive. Increased dark brown staining indicated that the expression of both Bcl-2 and Bax was enhanced (red arrows) in cells receiving 100 µg/mL. Scale bar = 50 µm.

Discussion

Dental pulp can be invaded by bacterial LPS through dentinal tubules following caries infection (18). Apoptosis of dental pulp cells can be triggered by a wide variety of internal and external stimuli (5,6). In the present study, LPS-stimulated dental pulp cells underwent morphological and ultrastructural changes typical of apoptosis. Apoptotic bodies, a special characteristic of apoptosis, were formed after exposure to LPS, which would facilitate the elimination of unnecessary components to stabilize the internal pulp environment (24). Irritation of pulp releases growth factors that regulate pulp cell apoptosis, migration, and differentiation (25). Apoptosis of dental pulp cells plays a pivotal role when the pulp is insulted by harmful stimuli. Apoptotic cells may transmit a death signal to facilitate prompt clearance of cell debris to prevent inflammation. Dental pulp cell apoptosis induces subsequent changes in the pulp and the secretion of growth factors after the death signal, which may activate or inactivate signaling pathways that promote mesenchymal cell migration and differentiation to initiate the pulp reparative process (25). Therefore, pulp cell apoptosis could be considered to be cytoprotective against bacterial infection and clearance of infected cells could be regarded as a means of restricting the spread of infection (26). Cell apoptosis in dental pulp may actively reduce pulp injury to a minimum. This may explain why pulp does not undergo irreversible changes in the presence of bacteria and their metabolites in some cases of slow, progressive caries. However, further studies are needed to confirm this conclusion.

Moreover, flow cytometry demonstrated that LPS-induced apoptosis occurs in a dose-dependent manner. This is consistent with the results of Hamada et al. (27) who showed that LPS induced a dose-dependent increase in the number of TUNEL-positive hepatocytes. Another study demonstrated that LPS indirectly induced apoptosis in osteoblasts and periodontal ligament cells in a dose-dependent manner (28). In our study, the rate of apoptosis differed in each treatment group after exposure to LPS. When compared with the control group, a notable difference also existed except for the 0.01-µg/mL group. However, a previous study initiated by Kitamura et al. (17) found that LPS induced the proliferation of pulp cells in a dose-dependent manner. In our study, pulp cells preexposed to a low concentration (0.01 µg/mL) of LPS showed the baseline apoptosis rate, but higher concentrations (0.1, 1, 10, and 100 µg/mL) induced obvious apoptosis. Further studies are needed to verify the cytoprotective or cytotoxic role of LPS at concentrations lower than 0.01 µg/mL in human dental pulp cells. Kitamura et al. (17) conducted an experiment using clonal dental pulp cells (RPC-C2A). Thus, a different biological response and sensitivity of cells exposed to LPS might have occurred because of species specificity. Our study also found that the rate of apoptosis differed at different times. The 3rd day and the 5th day rates were much higher than that on the first day, but no significant difference was observed between the 3rd and 5th day. The results indicated that LPS-induced dental pulp cell apoptosis did not show a time-dependent effect for different exposure times, and that apoptosis did not increase constantly with stimulation time. Although apoptosis was enhanced on the 3rd day when compared with the 1st day, the evidence was not strong enough to illustrate a temporal effect of LPS in our study.

To obtain further information regarding apoptotic modulation, we examined the immunoreactivity of Bax and Bcl-2 in dental pulp cells after exposure to LPS. The two Bcl-2 family proteins acted as pro- and antiapoptotic modulators in cell apoptosis, respectively. Our findings indicate that both proteins were up-regulated after stimulation with high LPS concentrations. This was consistent with a previous report that showed increased expression of Bcl-2 and Bax accompanying increased apoptosis (29). Several studies (29-31) have demonstrated that pro- and antiapoptotic members of the Bcl-2 family might interact with each other to form homodimers and heterodimers in regulating apoptosis. Our results showed that high concentrations of LPS corresponded to an increased expression of Bcl-2 and Bax. Although the exact dose-response relationship could not be inferred from the results, the tendency for LPS-induced apoptosis to be increased with LPS concentration was obvious. However, the evidence of direct involvement of Bcl-2 and Bax in dental pulp cell apoptosis in our study is insufficient, but may indicate a role for the two proteins in mediating LPS-induced dental pulp cell apoptosis and provide some clues for understanding the mechanisms of dental pulp cell apoptosis after exposure to LPS. Moreover, Bcl-2 and Bax expression was not different between control groups and low concentration groups; it may be that immunohistochemistry could not detect the small changes in protein expression and immunoreactivity could only be observed after strong expression in groups treated with high LPS concentrations.

Interestingly, in our study, the apoptosis rate did not differ between the 0.01-µg/mL LPS group and the control group. Therefore, the lowest concentration of LPS was insufficient to induce notable dental pulp cell apoptosis. This may indicate that pathogenic material and bacteria of restricted virulence may not reach the intensity required to induce meaningful pulp repair responses such as apoptosis. Considering the limited repair capacity of pulp, acquiring information regarding the threshold level of irritation to induce biological pulp responses appears to be crucial in understanding the modulation of the pulp repair process.

Our study showed that LPS induces a significant degree of dental pulp cell apoptosis in a dose-dependent manner, but a temporal effect was not demonstrable. Dental pulp cells showed increased expression of Bcl-2 and Bax after exposure to high LPS concentrations compared to the control groups and low concentration groups. The increased expression of Bcl-2 and Bax suggests that they might play a role in LPS-induced dental pulp cell apoptosis.

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