Effects of Leukemia Inhibitory Factor on Proliferation and Odontoblastic Differentiation of Human Dental Pulp Cells

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Abstract

Introduction: The purpose of this study was to determine whether the leukemia inhibitory factor (LIF) is expressed in human dental tissue and exerts its effect on proliferation and odontoblastic differentiation of the dental pulp cells (DPCs). Methods: An immunohistochemical assay was used to detect the expression of LIF and leukemia inhibitory factor receptor (LIFR) in the human dental pulp. The proliferation of DPCs was examined by culturing human primary DPCs in the presence of LIF with different doses or the neutralizing antibody to LIF. Western blot was performed to assay the phosphorylation of Janus kinase 2 (Jak2) and signal transducer and activator of transcription 3 (Stat3) in the presence or absence of LIF and/or AG 490, a specific inhibitor of Jak2. The odontoblastic differentiation of DPCs was determined using the alkaline phosphatase (ALP) activity assay, quantification of bone sialoprotein (BSP) and dentin sialoprotein (DSPP) gene expression, and mineralization nodule formation. Results: LIF and LIFR were present in the odontoblasts and DPCs. LIF induced proliferation of DPCs, which was inhibited by the LIF neutralizing antibody and AG 490. LIF induced phosphorylation of Jak2 and Stat3 but not in the presence of the AG490. ALP activity of DPCs, in the absence or presence of mineralization induction medium, was inhibited by LIF. Furthermore, the mineralization nodule formation and the expression of BSP and DSPP were inhibited by LIF. This inhibition on differentiation was attenuated by the AG490. Conclusions: LIF and LIFR are expressed in the human dental pulp. LIF promotes the proliferation of DPCs, and the odontoblastic differentiation is inhibited via the Jak2-Stat3 signaling pathway. (J Endod 2011;37:819–824)

Key Words
Dental pulp cells, Jak2, leukemia inhibitory factor, odontoblast, Stat3

The dental pulp, a loose connective tissue in the center of teeth, is surrounded by the dentin. Although the dental pulp contains stem cells capable of differentiating into odontoblasts, it is not overmineralized and forms dentin under the physiological condition. When dental caries occur and lead to demineralization of enamel and dentine and subsequent pulp tissue injury, the dental pulp cells (DPCs) are induced to differentiate into odontoblasts that form dentine by the caries or injuries (1). This phenomenon indicates that DPCs are precisely programmed by unknown mechanisms.

Leukemia inhibitory factor (LIF) is a member of the IL-6 family of cytokines expressed by fibroblasts, osteoblasts, endothelial cells, osteoclasts, and bone marrow stromal cells. It is a single-chain glycoprotein that regulates cell growth and the differentiation of a variety of tissues, including bone (2). The local administration of LIF has been shown to strongly stimulate bone formation and osteoclast activation in calvaria of mice (3). The in vivo observation is further confirmed by in vitro studies using LIF-transgenic (4) and the leukemia inhibitory factor receptor (LIFR) gene knockout mice (5).

In patients with cleidocranial dysplasia, which is characterized by developmental defects of bone and tooth caused by runt-related transcription factor 2 (Runx2) mutation, LIF messenger RNA expression in dental pulp is higher than that of controls (6). IL-6, another member of the IL-6 family, regulates the response of DPCs to lipopolysaccharide, cytokines, and dental materials (7–11). Moreover, proinflammatory cytokines, such as IL-1, transforming growth factor-β, and tumor necrosis factor-α, that are elevated in the diseased dental pulp (12, 13) can stimulate LIF secretion from osteoblastic cells (14, 15). However, thus far, the expression or function of LIF in DPCs is unclear.

Taken together with the evidence described earlier, we hypothesized that LIF may be involved in regulating the biological functions of DPCs. The aim of the present study was to investigate the expression pattern of LIF and LIFR in human dental pulp and the effects of LIF on proliferation and odontoblastic differentiation of DPCs.

Materials and Methods

Immunohistochemical Analysis

The human teeth extracted for the orthodontic treatment purpose were obtained from 10- to 14-year-old children. These teeth have not been orthodontically moved. Each tooth was immediately sectioned, fixed with 10% buffered formalin overnight, and decalcified in buffered 10% EDTA solution for 3 months. The specimens were dehydrated in an ascending series of graded alcohol and embedded in paraffin. Consecutive sections with 5-μm thickness were performed using a rotary microtome and
collected on poly-L-lysine-coated slides. The sections were used for immunohistochemical staining in a sequence to examine the presence and localization of LIF and LIFR. The mouse monoclonal antibody against human LIF (1/50 dilution; R&D, Fremont, CA) and the polyclonal goat antibody against human LIF-R (1/50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) were purchased. The staining was performed using the biotin-streptavidin kit (ZhongShan Biotech, Beijing, China). Sections incubated with preimmune serum or phosphate-buffered saline (PBS) lacking the first antibody were used as negative controls. The slices were counterstained with hematoxylin, a blue dye. The protocol and procedures were reviewed and approved by the Review Board of the School and Hospital of Stomatology, Wuhan University, China.

Culture of Human Primary DPCs

DPCs were isolated from the healthy dental pulp of premolars undergoing tooth extraction for orthodontic treatment. All donors gave their informed consent. The dental pulp tissues were harvested and minced and then transferred to plastic flasks (Costar, Cambridge, MA) for culture in α-modified essential medium (α-MEM; HyClone Laboratories, Inc, Logan, UT) supplemented with 10% fetal bovine serum (FBS; GIBCO, Sydney, Australia), with 100 U/mL penicillin and 100 μg/ml streptomycin. Cultured cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The primary cells were analyzed under the microscope. Before experimental analyses, alkaline phosphatase (ALP) staining and mineralization nodule formation assays were performed to characterize the phenotype of DPCs in the third to fourth passages cells pooled from three donors.

Cell Proliferation Assay

The effects of LIF on DPC proliferation were studied using the Cell Counting Kit-8 (CCK-8; Dojindo Kagaku Co, Kumamoto, Japan) according to the manufacturer’s protocols. The CCK-8 is a very sensitive assay using highly water-soluble tetrazolium salt (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. The cells were seeded at a density of 1 × 10⁴ cells/well in a 24-well plate and cultured for 24 hours, and then the medium was replaced by fresh medium with 1% FBS containing human recombinant LIF (Chemicon, Temecula, CA) with different concentrations (0, 0.1, 1, 10, 20, and 100 ng/mL). The cells were cultured for further 5 days, and then the number of cells was assessed by the cell counting kit. The absorbance at 450 nm was measured to calculate the number of vital cells in each well. The well with medium and CCK-8 solution but without cells was used as a baseline. Cell proliferation was represented as the mean ± standard deviation of absorbance for five wells for each group.

Proliferation Inhibition Test

To confirm the effects of LIF on proliferation, the neutralizing monoclonal antibody against LIF was used to block the binding of LIF to LIFR. The CCK-8 assay was conducted for DPCs cultured in the presence or absence of LIF (10 ng/mL) or the neutralizing monoclonal antibody against LIF (40 μg/mL, R&D) or AG 490 Jak2 inhibitors (3 μmol/L; BioSource, Camarillo, CA).

Western Blot

DPCs were cultured in the medium with or without AG 490 (30 μmol/L) for 1 hour and then treated with LIF (10 ng/mL) for 15 minutes. The protein extraction kit (Chemicon) was used to isolate total proteins. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL); 50 μg of protein in each sample was boiled for 5 minutes and then separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and transferred onto a PVDF membrane (Bio-Rad...
Labs, Hercules, CA). Nonspecific reactivity was blocked by 5% bovine serum albumin in a Tris-Buffered Saline Tween-20 (TBST) buffer and subsequently incubated with primary antibodies for Jak2, Stat3 (polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Jak2 (phospho-Jak2), or phosphorylated Stat3 (phospho-Stat3) monoclonal antibody (Cell Signaling Technology, Irvine, CA) overnight at 4°C. The membranes were also probed with anti–β-actin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to ensure an equal amount of protein. Bound antibodies were detected by a horseradish peroxidase-conjugated secondary antibody (Pierce). Western blot experiments were repeated at least twice to confirm the results.

**Mineralization Induction**

After cell counting with a cell counter (Beckman), cells were cultured in a six-well plate in 5% CO₂ at 37°C. After an attachment period of 24 hours, the medium was changed to the mineralization induction medium, containing α-MEM, 5% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L β-glycerophosphate, 100 μg/mL ascorbic acid, 10 mmol/L dexamethasone (Sigma-Aldrich Co, St Louis, MO), and 1.8 mmol/L KH₂PO₄. Cultures were maintained for an additional 21 days with a medium change every 3 days. At the end of the culture period, cells were rinsed in PBS, fixed in a solution of 70% ethanol for 1 hour, and stained with alizarin red for mineralized nodules. The wells were washed with PBS four times to remove the nonspecific staining. After the photos of the nodules were taken, the nodule formation 10% cetylpyridinium chloride was used to dissolve the nodules, and the absorbance was examined at 562 nm.

**ALP Activity Assay**

We examined the ALP activity of DPCs treated with or without LIF under the circumstances of normal culture or mineralization induction. First, the cells treated with or without LIF (20 ng/mL) after overnight incubation were examined. Second, after an attachment period of 24 hours, the cells were cultured in the mineralization induction medium in the presence or absence of LIF (20 ng/mL).

The cultured cells described earlier were maintained for an additional 5 days. At the end of the culture period, cells were rinsed in PBS and treated with 1% Triton X-100. The ALP activity of the cell lysate was tested with the ALP Activity Assay Kit (Nanjing, JS, China). The protein concentration was examined using the BCA protein assay kit (Pierce) following the manufacturer’s instructions.
Real-time Polymerase Chain Reaction

After 5 days of incubation in mineralization induction medium with or without LIF (20 ng/mL) and AG490 (30 μmol/L), total RNA was isolated using the Trizol reagents (Invitrogen, Carlsbad, CA). First-strand complementary DNA syntheses were performed by reverse transcription with the SuperScript preamplification system (Invitrogen). The design of the oligonucleotide primers was based on published complementary DNA sequences (Table 1). Real-time polymerase chain reaction for bone sialoprotein (BSP), dentin sialophosphoprotein, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with the SYBR Green Kit (TOYOBO, Tokyo, Japan) using the Thermal cycler (ABI, Foster City, CA). Data were analyzed using ABI Prism 7000 SDS Software Version 1.1 (ABI).

Statistics

All experiments were repeated at least in triplicate, and data were presented as mean ± standard error. Analysis of variance tests, Student Newman-Keuls tests, and the homogeneity of variance tests were used for statistical analysis. P values less than .05 were considered statistically significant.

Results

Expression of LIF and LIFR in Human Dental Pulp Tissue

Immunohistochemical staining using antibodies to LIF and LIFR were positive in DPCs and strongly positive in the odontoblasts (Figs. 1 and 2), showing the expression of LIF and its receptor in DPCs and odontoblasts.

LIF-regulating Proliferation of DPCs

The result of the CCK8 assay showed that LIF increased the proliferative response of DPCs in a dose-independent manner when the cells were cultured with a low concentration of 1% FBS. LIF-treated DPCs (10 or 20 ng/mL) had a higher proliferative rate than untreated DPCs (P < .01), suggesting that LIF promoted the proliferation at the concentration of 10 and 20 ng/mL (Fig. 3).

To test whether the effect of LIF on proliferation of DPCs was blocked, the neutralization antibody to LIF was used. As shown in Figure 4, LIF-induced proliferation of DPCs (10 ng/mL) was abolished by the antibody, confirming that LIF regulates the proliferation of DPCs in vitro.

LIF-inducing Proliferation of DPCs via the Jak2-Stat3 Signaling Pathway

AG490, a specific Jak2 inhibitor, blocked the proliferation of DPCs induced by LIF (Fig. 5). LIF was likely to induce cell proliferation through Jak2-Stat3 axis.

We tested the phosphorylation of Jak2 and Stat3 in DPCs stimulated with or without LIF and AG490. The results showed that LIF indeed induced the expression of phospho-Jak2 (Tyr1007/1008) and phospho-Stat3 (Tyr705). This phosphorylation of Jak2 and Stat3 in DPCs was abolished by the AG490 (Fig. 6).

LIF Inhibiting the Osteoblastic Differentiation of DPCs via Jak2-Stat3 Signaling Pathway

The ALP activity assay showed that LIF inhibited the ALP activity, regardless of the presence or absence of the mineralization induction medium (Fig. 7). LIF treatment also inhibited the osteogenic induction medium–induced nodule formation (Figs. 8 and 9). Meanwhile, there was a statistically significant difference of DSPP and BSP messenger RNA expression between the LIF-treated and untreated group (Fig. 10).

Interestingly, AG490 rescued the nodule formation that was inhibited...
by LIF (Figs. 8 and 9). This was confirmed by the result that AG490 up-regulated DSPP and BSP expression inhibited by LIF (Fig. 10).

**Discussion**

In the present study, we first showed the presence of LIF and LIFR in human DPCs and odontoblasts by immunohistochemical assay using specific antibodies. Then, we found that LIF induced the proliferation of DPCs at 10 and 20 ng/mL but not at lower or higher concentrations. Our observation is in agreement with the results from studies using primary cultured osteoblasts, including primary mouse (16) and rat calvariae osteoblasts (17) and human bone-derived osteoblast-like cells (18), but it is in contrast to studies using the osteogenic sarcoma cell line UMR-106 (17) and the mouse osteoblast cell line MC3T3-E1 (19).

LIF has either a positive or negative effect on osteoblast proliferation, depending on the osteoblastic cell types (16, 17). In our case, it is unclear whether the discrepancy among studies is caused by the cell types used (eg, heterogeneous primary cells and homogeneous cell lines), the stage of differentiation represented by a given cell preparation and original location, or even inherent differences in the proliferative potential of transformed cell lines.

To further clarify the signaling pathway of the cell proliferation activated by LIF in DPCs, our results showed that LIF (10 ng/mL) led to the phosphorylation of Jak2/Stat3. Furthermore, pharmacologic inhibition of the Jak2 pathway attenuated both cell proliferation and Jak2/Stat3 phosphorylation induced by LIF. It suggested that LIF might initiate the proliferation of DPCs at least through the Jak2-Stat3 signaling pathway. The Jak2-Stat3 axis is an important signaling pathway that regulates cell growth and apoptosis as its downstream target genes mediate the cell cycle progression (ie, Fox, cyclin-D, CDC25A, c-Myc, or Pim1), cell growth, and survival (ie, BCL2, BCL-xl, and β2-microglobulin) (20). LIF exerts its action via binding to the receptor complex of LIFR and glycoprotein 150 (gp150), which is located in the cell membrane, leading to the phosphorylation and dimerization of Jak2. The activated Jak2 induces the recruitment, phosphorylation, and nuclear translocation of Stat3 and then the transcription of target genes.

The differentiation of DPCs to odontoblasts is a pivotal process for regenerative dentin formation. In the present study, LIF inhibited the differentiation of DPCs by suppressing ALP activity and down-regulating expression of DSPP and BSP. This was confirmed by the results that LIF inhibited the mineralization nodule formation. Our finding is consistent with the result that LIF messenger RNA expression in dental pulp from patients with cleidocranial dysplasia associated with Runx2 mutation leading to defects of bone and tooth development was higher than that in controls (21). As we mentioned previously, the results of the immunohistochemical assay showed that LIF and LIFR immunoreactivity within odontoblasts (odontoblasts represent a differentiated phenotype in the dental pulp) was stronger than that in dental pulp core. This phenomenon seems somewhat contradictory. Other growth factors, such as transforming growth factor-β and epidermal growth factor, also inhibit the expression of ALP (22, 23) and DSPP (24), whereas they have a stronger expression in odontoblasts (25). Actually, the differentiation of DPCs is controlled by a balance of promotion and inhibition by numerous cytokines, growth factors, and hormones. None of the single factors can decide the effect or function of DPCs. We think that it is possible that both of the factors promoting and inhibiting DPCs differentiation are up-regulated in functional odontoblasts when compared with pulp cells. The eventual effects may depend on whether the balance leans to promotion or inhibition.

The inhibition of Jak2 phosphorylation attenuated LIF-induced ALP activity, DSPP/BSP expression, and mineralization nodule formation, suggesting that the Jak2-Stat3 signaling pathway modulates the odontoblastic differentiation of DPCs. The conditional knockout of Stat3 using the α1(1)-collagen promoter Cre transgenic mice resulted in an osteoporotic phenotype because of a reduced bone formation rate (26). Bellido et al (19) found that a nonspecific Stat3 inhibitor, staurosporine (a tyrosine kinase inhibitor), attenuated the differentiation of MG63 and MC3T3-E1. Our data provide direct evidence that the Jak2-Stat3 signaling regulates the odontoblastic differentiation of DPCs, but the underlying mechanism is still unclear. Cheng et al (27) reported that Stat3 directly binds to the Twist promoter and activates its transcriptional activity, so that IL-6 can induce the activation of Stat3 and Twist expression in human breast cancer cell lines. Twist is a strong osteoblastic differentiation inhibitor (28). Therefore, it is possible that LIF-induced activation of the Jak2-Stat3 may lead to Twist expression, which inhibits the odontoblastic differentiation of DPCs.

In human dental pulp tissue, the undifferentiated mesenchymal stem cells are capable of forming dentin under the pathological conditions (29). LIF has been shown to maintain the self-renewal of the

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Figure 9. The quantification of the mineralization nodules. *P < .01, there were statistically significant differences of optical density (OD) values between the osteogenic induction group and the osteogenic induction/LIF group and between the osteogenic induction group/LIF and the osteogenic induction/LIF group.

Figure 10. Real-time reverse-transcription PCR results of messenger RNA expressions of DSPP and BSP. *P < .01, there were statistically significant differences between the control group and the LIF group and between the LIF group and the LIF/AG490 group.
embryonic and postnatal stem cells through the Jak2-Stat3 signaling pathway (30). Recent studies showed that LIF is identified as one of the potential reliable markers for human bone marrow stromal cells (31), which are similar to dental pulp stem cells (32). Taken together with the results of the present study and others, LIF may play a role in promoting the proliferation of DPCs and maintaining the undifferentiated states of DPCs.

**Acknowledgments**

The authors deny any conflicts of interest related to this study.

**References**


8. Paatkonen V, Vuoristo J, Salo T, Tjaderhane L. Effects of interleukin-8 in exudates from embryonic and postnatal stem cells through the Jak2-Stat3 signaling pathway. Basic Research——Biology


