Regulation of osteoprotegerin and receptor activator of nuclear factor-kappa B ligand by interleukin-10 in human dental follicle cells

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Purpose: This study was undertaken to determine the expression of interleukin-10 at protein level in human dental follicle cells and the effect of interleukin-10 on gene expression of osteoprotegerin and receptor activator of nuclear factor-kappa B ligand.
Materials and Methods: Primary human dental follicle cells were cultured. The expression of interleukin-10 at protein level was detected by immunocytochemistry. The gene expression of osteoprotegerin and receptor activator of nuclear factor-kappa B ligand treated with 0, 5, 10, 25, 50, and 100 ng/mL interleukin-10 for 1 hour (for osteoprotegerin) or for 6 hours (for receptor activator of nuclear factor-kappa B ligand) was examined by reverse transcription-polymerase chain reaction. Osteoprotegerin secretion treated with 25 ng/mL interleukin-10 at 0, 2, 4, 6, and 8 hours was examined by enzyme-linked immunosorbent assay.
Results: Interleukin-10 was expressed in human dental follicle cells. Interleukin-10 enhanced osteoprotegerin gene expression and secretion. In contrast, interleukin-10 suppressed receptor activator of nuclear factor-kappa B ligand gene expresson. Protein kinase C inhibitors, myristoylated protein kinase C peptide inhibitor and Go6983, decreased interleukin-10-induced osteoprotegerin, whereas protein kinase A inhibitors, cAMP-dependent protein kinase peptide inhibitor and KT5720, did not decrease it.
Conclusion: The molecular basis for interleukin-10 on the inhibition of osteoclastogenesis is by enhancement of osteoprotegerin gene expression and suppression of receptor activator of nuclear factor-kappa B ligand gene expression in human dental follicle cells. Interleukin-10-induced osteoprotegerin secretion was dependent on protein kinase C pathway. Interleukin-10 signaling may play an important role in the tooth eruption process.
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Key Words: dental follicle cell, interleukin-10, osteoprotegerin, protein kinase A, protein kinese C.

Introduction

Dental follicle, a loose connective tissue sac surrounding the unerupted teeth, is necessary for tooth eruption to occur.[1] Alveolar bone resorption is also required for tooth eruption to form an eruption pathway. The dental follicle regulates the cellular events of tooth eruption, including recruitment of mononuclear cells to the follicle and directing the subsequent osteoclastogenesis needed for alveolar bone resorption.[2] Receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) are two essential factors acting as positive and negative regulators in osteoclast formation. RANKL, a member of tumor necrosis factor (TNF) ligand family, is expressed on osteoblast/stromal cell membranes. This ligand binds to the receptor activator of nuclear factor-kappa B (RANK), which is a receptor on the membrane of osteoclastic precursors and on mature osteoclasts, and induces osteoclast differentiation and activity.[3-5] In contrast, OPG, a member of the TNF receptor family expressed by osteoblasts, strongly inhibits bone resorption by binding with high affinity to its ligand RANKL, thereby preventing RANKL from engaging its receptor RANK.[3,6-9] It has been reported that rat dental follicle cells express both OPG[10] and RANKL gene[11] and secret OPG.[12] In earlier experiments, we also found the gene expression of OPG and RANKL and OPG secretion in human dental follicle cells (HDFCs),[13] suggesting that HDFCs is critical for osteoclastogenesis to occur during tooth eruption.

Interleukin-10 (IL-10), initially described as cytokine synthesis inhibitory factor, is synthesized by T cells, macrophages, monocytes and B cells and dampens many inflammatory responses,[14] e.g. inhibiting TNF-α

production from LPS-activated monocytes, macrophages.[15] IL-10 is expressed in human periodontal ligament cells[16] and rat dental follicle cells as well.[17] IL-10 not only suppresses inflammation but also inhibits bone resorption,[18-20] so it may be necessary in both periodontitis and tooth eruption.

Liu et al.[17] have reported the effect of IL-10 on gene expression of OPG and RANKL in rat dental follicle cells. However, whether IL-10 is expressed in HDFCs and regulates gene expression of OPG and RANKL in HDFCs remains unknown. And the signal transduction pathways through which IL-10 regulates OPG secretion have not been elucidated. In the present study, we examined IL-10 expression at protein level and the effect of IL-10 on gene expression of OPG and RANKL, as well as its effect on OPG secretion in HDFCs. In addition, the signal transduction pathways to regulate IL-10-induced OPG secretion treated with specific protein kinase A (PKA) and protein kinase C (PKC) inhibitors were also investigated.

Materials and Methods

Culture of human dental follicle cells

Prior to commencement, the study protocol was approved by Ethics Committee of the Fourth Military Medical University. The third mandibular molars were surgically removed from adolescents for the need of orthodontic treatment with informed consent. HDFCs were cultured as previously described.[13] Briefly, the dental follicle tissues were cleaned after isolation and cut into pieces using a sterilized scalpel and were digested. Then the minced and digested tissues were seeded into culture dishes in Dulbecco's modified Eagle medium (DMEM, Hyclone, Logan, UT, USA) with 10% fetal bovine serum and 1mmol/L sodium pyruvate, in a humidified incubator with 5% CO_2 at 37˚C. When the cells had reached confluence, they were detached and subcultured.

Immunocytochemistry

Cells of the fifth passage were grown on coverslips at a density of $2x10^5$ cells/mL, maintained for another 2 days, and then fixed with 4% polyoxymethylene for 10 minutes. Next, the fixed cells were permeabilized with 0.3% TritonX-100 in phosphate buffered saline (PBS) for 10 minutes. After endogenous peroxidase activity was suppressed with 3% H_2O_2 for 10 minutes, and after non-specific binding sites were blocked with 10% normal goat serum for 30 minutes, the fixed cells were incubated with rabbit anti-human IL-10 polyclonal antibody (primary antibody, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) at 4˚C overnight. Staining was performed with goat anti-rabbit IgG (secondary antibody) conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc.) and developed with 3,3'-diaminobenzidine-HCl (Dako, Carpinteria, CA, USA). For control experiment, immunostaining with PBS omitting the primary antibody was performed. Cellular nuclei in all experiments were counterstained with hematoxylin.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Cells of the fifth passage were grown in 25-cm^2 T-flasks until confluent. Then, the cells were incubated with 0, 5, 10, 25, 50, and 100 ng/mL IL-10 (Pepro Tech, Rock Hill, NJ, USA) for 1 hour (for OPG gene expression) or for 6 hours (for RANKL gene expression). After incubation, the cells were harvested for RNA extraction. Total RNA was extracted from HDFCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). To remove contaminating genomic DNA, the RNA was treated with RNase-free DNase I (Qiagen, Chatsworth, CA, USA) at 37˚C for 30 minutes. Then the RNA concentrations were calculated spectrophotometrically. The RNA samples had an optical density ratio (OD_{260}/OD_{280}) of at least 1.8.

First-strand cDNA was synthesized using 2 μg of total RNA with Superscript II reverse transcriptase

(Invitrogen) in a volume of 20 μL, according to the manufacturer's instructions. For each PCR, 2 μL cDNA was mixed with PCR buffer, dNTP, primers and Taq DNA polymerase (Invitrogen) to make a total reaction volume of 25 μL. The following primers were used: OPG (GenBank Accession No. NM002546; forward primer: 5'-TCAAGCAGGAGTGCAATCG-3'; reverse primer: 5'-AGAATGCCTCCTCACACAGG-3'; expected product size 342 bp); RANKL (GenBank Accession No. NM003701; forward primer: 5'-GGCTCATGGTTAGATCTGGC-3'; reverse primer: 5'-TGACCAATACTTGGTGCTTCC-3'; expected product size 351 bp); GAPDH (GenBank Accession No. XM0033260; forward primer: 5'-GCTCTCCAGAACATCTACC-3'; reverse primer: 5'-GTGTCGCTGTTGAAGTCAG-3'; expected product size 266 bp). PCR conditions were initial denaturation at 94˚C for 2 minutes, then denaturation at 94˚C for 30 s, annealing at a appropriate temperature for each primer pair (OPG: 55˚C, RANKL: 59˚C, and GAPDH: 60˚C) for 30 s, and extension at 72˚C for 30 s, all for 30 cycles, a final extension at 72˚C for 10 minutes. PCR products were subjected to electrophoresis on 1.5% agarose gels and photographed under ultraviolet light after ethidium bromide staining. Band intensities were measured with the use of NIH Image software. The mRNA levels of OPG and RANKL were estimated semi-quantitatively by comparison with GAPDH mRNA level.

Enzyme-linked immunosorbent assay (ELISA)

Cells of the fifth passage were seeded into 24-well plates until confluent. Then the cells were cultured in the absence or presence of 25 ng/mL IL-10 as control group and test group. The culture medium was harvested at 0, 2, 4, 6, and 8 hours for both groups. OPG secretion in the culture supernatants was measured using enzyme-linked immunosorbent assay. Cells of the fifth passage were seeded into 96-well plates until confluent. Then the cells were treated with 25 ng/mL IL-10 with or without PKA inhibitors: 5 mmol/L cAMP-dependent protein kinase peptide inhibitor (c-AMP inh, Promega, Madison, WI, USA) or 112 nmol/L KT5720 (Calbiochem, San Diego, CA, USA) or PKC inhibitors: 10 μmol/L Myristoylated protein kinase C peptide inhibitor (Myr, Promega) or 1 μmol/L Go6983 (Calbiochem). After 24 hours of stimulation, OPG secretion in the culture supernatants was measured using enzyme-linked immunosorbent assay. OPG in the culture supernatants was measured using commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Statistical analysis

All experiments were performed at least three times. Data are expressed as means with standard deviations (SD). Data were subjected to t-test and one-way analysis of variance (ANOVA) with SPSS 10.0 software. The least significant difference (LSD) test was used for the post hoc comparison of specific groups. A p-value less than 0.05 was accepted as statistically significant.

Results

Immunocytochemistry for IL-10

IL-10 was positively immunostained in HDFCs (Fig. 1A), which was in contrast to the negative control (Fig. 1B).

Effect of IL-10 on gene expression of OPG and RANKL

IL-10 enhanced OPG gene expression ($p<0.05$), with maximal expression at 25 ng/mL IL-10 (Figs. 2 and 3A). Conversely, IL-10 suppressed RANKL gene expression ($p<0.05$), with maximal reduction at 25 ng/mL IL-10 (Figs. 2 and 3B).

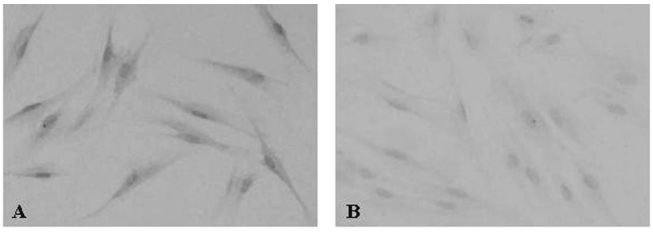

Fig. 1A Fig. 1B

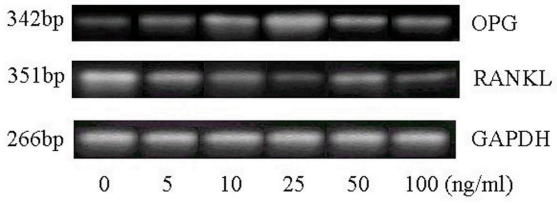

Fig. 2

Fig. 1. Positive immunostaining of IL-10 in HDFCs (A) in contrast to the negative control (B), x400.
Fig. 2. RT-PCR products for OPG and RANKL in HDFCs treated with IL-10 in a concentration-dependent study.

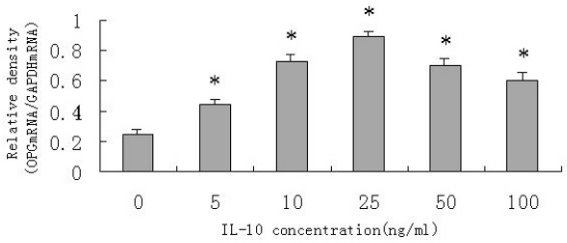

Fig. 3A

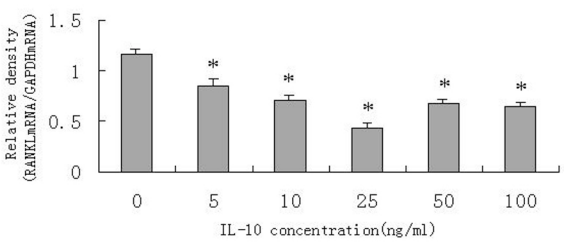

Fig. 3B

Fig. 3. Ratio between OPG and GAPDH in HDFCs treated with IL-10 (A). Ratio between RANKL and GAPDH in HDFCs treated with IL-10 (B). An asterisk (*) represents a statistically significant change from the control value treated without IL-10 ($p<0.05$).

Effect of IL-10 on OPG secretion

IL-10-induced OPG secretion (25 ng/mL) was increased after 4 hours of stimulation ($p<0.05$, Fig. 4).

Effect of PKA inhibitors on IL-10-induced OPG secretion

PKA inhibitors, c-AMP Inh and KT5720, did not significantly decreased IL-10-induced OPG secretion in HDFCs ($p>0.05$, Fig. 5A).

Effect of PKC inhibitors on IL-10-induced OPG secretion

PKC inhibitors, Myr and Go6983, significantly decreased IL-10-induced OPG secretion in HDFCs ($p<0.05$, Fig. 5B).

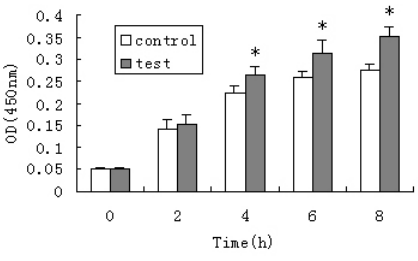

Fig. 4

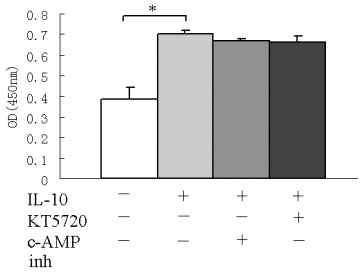

Fig. 5A

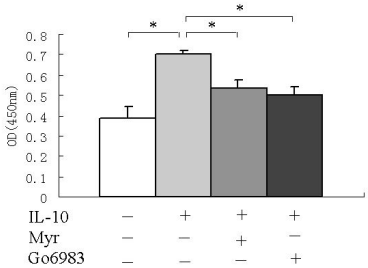

Fig. 5B

Fig. 4. OPG secretion in HDFCs treated with IL-10 in a time-dependent study. An asterisk (*) represents statistically significant OPG secretion of the test group compared with that of the control group ($p<0.05$).
Fig. 5. Effect of PKA inhibitors, c-AMP inh and KT5720 on IL-10-induced OPG secretion in HDFCs (A). Effect of PKC inhibitors, Myr and Go6983 on IL-10-induced OPG secretion in HDFCs (B). An asterisk (*) represents statistical significance ($p<0.05$).

Discussion

Wise et al.[21] found that the eruption time of the rat first mandibular molar was significantly delayed by 1 day or more as a result of OPG injection. Kong et al.[22] have demonstrated that in null mice devoid of RANKL gene, the teeth do not erupt. Tomotaka et al.[23] have reported that osteoclastogenesis during mouse tooth germ

development is mediated by RANK-RANKL pathway. All of these studies indicate that OPG and RANKL are key regulators in osteoclastogenesis needed for alveolar bone resorption during tooth eruption.

The present study has confirmed the expression of IL-10 at protein level in HDFCs. Liu et al.[17] has shown that rat dental follicle cells express IL-10 gene in vitro and it is also expressed in vivo in rat dental follicles from postnatal day 1 to day 9 during tooth eruption, suggesting that IL-10 is involved in the tooth eruption process. IL-10 knockout mice have accelerated alveolar bone loss.[24] IL-10 may inhibit alveolar bone resorption in the tooth eruption process.

The tooth eruption process in rat has been reported as follows: The eruption time of the rat first mandibular molar is on postnatal day 3 and day 10.[10,25-27] Furthermore, RANKL gene expression is significantly increased on postnatal days 9-11 compared with the earlier postnatal days,[27] while OPG gene expression is decreased on postnatal day 3.[10] On postnatal day 3, the major burst of osteoclastogenesis for tooth eruption occurs.[25,26] The absence of up-regulation of RANKL emphasizes the importance of down-regulation of OPG at this time. A steady-state output of RANKL, coupled with the decline in OPG, increases the RANKL/OPG ratio to favor osteoclastogenesis at this time.[27] The osteoclasts then erode the alveolar bone to form the eruption pathway,[28] so the tooth eruption occurs. After this day, OPG is increased,[10] whereas RANKL remains at a low level, resulting in the decline of RANKL/OPG ratio to inhibit osteoclastogenesis.[27] On postnatal day 10, there is a minor burst of osteoclastogenesis.[25,26] The RANKL/OPG ratio is again increased to favor osteoclastogenesis. In summary, the RANKL/OPG ratio promoting osteoclastogenesis may be greater on postnatal day 3 than day 10.[27] We conclude that the normal tooth eruption in rat occurs at the specific time (postnatal day 3 and day 10) when osteoclastogenesis arises, resulting in tooth eruption, and there also exists specific period (days except postnatal day 3 and day 10) in the whole tooth eruption process when the osteoclastogenesis is inhibited and the teeth remain unerupted. This may be critical to keep the normal morphology of the alveolar bone. It is postulated that the phenomenon may be similar in the normal tooth eruption process in humans. Thus, IL-10 may play an important role in the inhibition of osteoclastogenesis needed for alveolar bone resorption by enhancement of OPG gene expression and suppression of RANKL gene expression in the tooth eruption process.

Human periodontal ligament cells secrete IL-10[19] and OPG[29] and express both OPG and RANKL gene.[30] After the dental follicle has developed into the periodontal ligament of the adult, it would be necessary to have limited osteoclastic activity so that the attachment of the periodontal ligament to the alveolar bone would not be disrupted.[12] So it is possible that IL-10 may inhibit osteoclastogenisis by up-regulating OPG gene expression and down-regulating RANKL gene expression in human periodontal ligament cells after tooth eruption.

The regulation of OPG by cytokines and hormones has been studied in different cell lines. Kondo et al.[31] have reported that OPG gene expression in murine marrow stromal cells stimulated with PTH is regulated by PKA and PKC activation. Villa et al.[32] have demanstrated that CGRP inhibits OPG secretion in human osteoblast-like cells via cAMP/PKA-dependent pathway. These reports suggest that IL-10-induced OPG secretion might be regulated by PKA or PKC activity in HDFCs. So we used specific PKA and PKC inhibitors in order to find out whether PKA or PKC pathway is involved in IL-10-induced OPG secretion.

In the present study, we found that the PKA inhibitors, c-AMP Inh and KT5720, did not decrease IL-10-induced OPG secretion, indicating that PKA pathway is not involved in IL-10-induced OPG secretion in HDFCs. Wise et al.[33] have indicated that PKA pathway is not involved in OPG gene expression in rat dental follicle cells. On the contrary, the PKC inhibitors, Myr and Go6983, decreased IL-10-induced OPG secretion,

suggesting that IL-10-induced OPG secretion is dependent on PKC pathway. The PKC-alpha isoform may be critical in OPG regulation.[33] Other studies also used PKC activators to demonstrate the involvement of PKC activity in OPG gene expression or secretion. Wise et al.[33] have demonstrated that PMA, an activator of PKC activity, enhances OPG gene expression in rat dental follicle cells. Takami et al.[34] have reported that PMA stimulates OPG gene expression in mouse primary osteoblasts, and PKC inhibitors, such as calphostin and staurosporin, suppress OPG gene expression. Brandstrom et al.[35] have shown that in human bone marrow stromal cells, PdBU, an activator of PKC activity, increases OPG secretion. Given the above, PKC activity is a very important signal transduction pathway in the regulation of OPG gene expression or secretion in some cell lines.

In conclusion, the molecular basis for interleukin-10 on the inhibition of osteoclastogenesis is by enhancement of OPG gene expression and suppression of RANKL gene expression in HDFCs. Furthermore, IL-10-induced OPG secretion is dependent on PKC pathway. IL-10 signaling may play an important role in the tooth eruption process. Our research suggests further studies on other regulatory molecules and their pathways to elucidate the mechanisms of tooth eruption.

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