Effects of icariin on the alkline phosphatase activity of human periodontal ligament cells inhibited by lipopolysaccharide

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Abstract. Icariin (ICA), a flavanoid isolated from herbal *Epimedium*, has multiple biological activities. The present study investigated the effects of ICA on the proliferation and alkaline phosphatase (ALP) activity (an index for PDLC differentiation) of human periodontal ligament cells (hPDLCs) inhibited by lipopolysaccharide (LPS). hPDLCs were cultured *in vitro* and stimulated with various concentrations of ICA. The proliferation ability of hPDLCs was detected by an MTT assay. The activity of ALP was determined by the p-Nitrophenyl phosphate method, and the expression of ALP was analyzed by reverse transcription polymerase chain reaction and western blot analysis. ICA exhibited a dose-dependent effect on the proliferation of hPDLCs in a suitable concentration range, from 10^{-6} to 10^{-8} mol/l, and with a mediate optimal concentration (10^{-6} mol/l). The alkaline phosphatase activity was markedly inhibited in 10 μg/ml LPS-treated PDLCs and this inhibition was suppressed in the presence of icariin at a concentration of 10^{-6} mol/L following prolonged treatment (96 h). Therefore, this study provided insight into the use of ICA for periapical tissue regeneration.

Introduction

Periodontitis is one of the most widespread infectious diseases in humans. It is the predominant cause of tooth loss and is associated with a number of systemic diseases, such as diabetes and cardiovascular disease (1). Periodontitis is stimulated by a variety of factors. For example, lipopolysaccharide (LPS), which acts as an endotoxin and elicits strong immune responses, is important in the pathogenesis of periodontitis. LPS directly induces tumor necrosis factor-α release from macrophages, and is the leading stimulus that initiates the host response in the periodontal pocket and activates macrophages to release proinflammatory cytokines (2-5). Moreover, LPS was observed to exhibit a significant cytotoxic effect on periodontal ligament stem cells (PDLSCs), and affect the self-renewal and osteogeneic differentiation potential of PDLSCs (6).

Alkaline phosphatase (ALP) activity in periodontal ligament cells (PDLCs) is an index for osteogeneic differentiation (7). The activity of ALP isoenzymes were observed to be correlated with cementum formation and root development, and mice lacking ALP demonstrated cementum formation inhibition (8-10). In addition, high ALP activity was observed in the periodontal ligament due to the constant renewal of the tissue or pathological conditions. Furthermore, patients with chronic periodontitis exhibited increased ALP activity in the serum (11), which indicated the possible association between ALP activity in PDLCs and periodontitis. Previous studies have demonstrated that LPS diminishes ALP activity in PDLCs, induces the subtype change and inhibits PDLC differentiation (12). Increased ALP activity appeared to be correlated with subclinical recurrent inflammation and further healing or remodeling of the periodontal tissue (13). Thus, improving ALP activity in periodontal ligaments may be applicable for periodontal tissue regeneration and repair.

Icariin (ICA) is the predominant active ingredient of Herba Epimedii, which is a herb used in traditional Chinese and alternative medicine. ICA increases trabecular bone mineral density in ovariectomized rats and stimulates osteoblastic cell proliferation and differentiation (14). Other studies have demonstrated that ICA and its glycosides accelerated osteoblastic but suppressed osteoclastic differentiation (15). In the present study, the effect of ICA on human PDLCs (hPDLCs) inhibited by LPS was investigated, with the aim of identifying a therapeutic agent for the treatment of periapical disease resulting from bacterial infection.

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Abbreviations: hPDLCs, human periodontal ligament cells; ALP, alkaline phosphatase; ICA, icariin; LPS, lipopolysaccharide; RT-PCR, reverse transcription polymerase chain reaction

Key words: icariin, periodontal ligament cells, proliferation, alkaline phosphatase, lipopolysaccharide

Material and methods

hPDLC isolation and culture. Human tissue samples were collected from the clinically healthy teeth of 11-14 adolescents who had undergone teeth extraction for orthodontic treatment, no history of periodontal disease and a relatively healthy periodontium. The periodontal ligament tissues were obtained as remnants or discarded tissues following routine dental procedures at the Second Affiliated Hospital of. All protocols for the handling of human tissue were approved by the Research Ethics Committee of Harbin Medical University (Harbin, China) and written informed consent was obtained from the families of the patients. The isolation and culture of human mesenchymal stem cells from healthy periodontal ligament tissues was performed as previously described (16). Briefly, tissues were treated aseptically and incubated overnight at 4˚C with 2 mg/ml dispase (Sigma-Aldrich, St. Louis, Mo, USA) and 4 mg/ml collagenase IV (Worthington Biochemical, Lakewood, NJ, USA). The dissociated cell suspension was filtered through a 70-μm cell strainer (Falcon; BD Biosciences, Franklin Lakes, NJ, USA), plated on nontreated 10-cm petri dishes (VWR International, West Sussex, UK) with complete α-minimal essential medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 20% fetal bovine serum (Clontech Laboratories Inc., Mountain View, CA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Technologies), 2 M L-glutamine, 100 mM nonessential amino acid and 550 μM 2-mercaptoethanol (Sigma-Aldrich). The suspension was cultured at 37˚C in a humidified tissue culture incubator with 5% CO_2 and 95% O_2. After 72 h, the nonadherent cells were removed. The plastic-adherent confluent cells were passaged with 0.05% trypsin containing 1 mM EDTA and continuously subcultured and maintained in complete growth medium. Cells from the fourth to sixth passages were used in the experiments. Cells from the third passage were fixed by 10% formaldehyde solution and stained with vimentin and keratin according to standard immunohistochemical methods.

MTT proliferation assay. hPDLCs were seeded in a 96-well plate ($2x10^3$ cells/well) with varied doses of ICA (0, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/l). After 96 h, 20 μl of MTT was added and the cells were incubated for 4 h. Following the addition of 150 μl dimethylsulfoxide, the cells were agitated for 10 min, and the concentration was analyzed by measuring the absorbance at 490 nm with an iMark microplate reader (Bio-Rad, Hercules, CA, USA).

ALP activity assay. An ALP staining kit (Sigma-Aldrich) was used. Subsequent to fixation with 70% ethanol, cells were incubated with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Red Violet LB Base dissolved in 0.1 M Tris buffer (pH 9.3). The ALP activity assay was conducted according to the manufacturer's instructions and normalized on the basis of protein concentrations.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from cultured cells undergoing osteogenic differentiation using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Adipocyte- and osteocyte-specific genes were amplified using the One-Step RT-PCR kit (Qiagen). The specific primers used were as follows: Forward, 5'-AGGGCTGTAAGGACATCG-'3 and reverse, 5'-GGAGTGCTTGTATCTCGGTT-3' for ALP; and forward, 5'-CATTGCCGACAGGATGCA-3' and reverse, 5-'CATCTGCTGGAAGGTGGACAG-3' for β-actin.

Western blot analysis. Cells were lysed with buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 10 mM sodium fluoride, 20 mM 2-ME, 250 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor mixture (Sigma-Aldrich), and were incubated at 4˚C for 1 h. The lysates were ultrasonicated (AIS92-IIDL ultrasonicator; Aismir, Beijing, China) and centrifuged at 12,000 x g for 10 min. Protein concentrations were determined using the bicinchoninic acid method. Proteins (50-100 μg) were separated on 8-10% polyacrylamide-sodium dodecyl sulfate gels and electroblotted onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia, Picastaway, NJ, USA). Subsequent to blocking with Tris-buffered saline and 5% nonfat dry milk for 2 h, the membrane was incubated overnight at 4˚C with antibodies against human ALP (mouse ant-human; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) followed by incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-mouse; 1:2,000; Pierce, Rockford, IL, USA) for 45 min at room temperature, and the signals were visualized by enhanced chemiluminescence detection. As a loading control, the blots were reprobed with a specific antibody against human β-actin (mouse anti-human; dilution, 1:5,000; Santa Cruz Biotechnology, Inc.).

Statistical analysis. Statistical significance was assessed by two-tailed Student's t-test or analysis of variance. $P<0.05$ and $P<0.01$ were considered to indicate a statistically significant difference.

Results

Isolation of hPDLCs. Following primary culture for 4-10 days, fibroblast-like cells with a long fusiform shape emerged beside the tissue block (Fig. 1A). When subcultured to the fourth generation, cells adhered to the bottom of the culture plate and were observed to exhibit a star- or long fusiform-like shape under the microscope. Cell cytoplasm was plump with round or oval nuclei. Cell arrangement was observed to be in a gyrate or radial shape (Fig. 1B). To confirm the origin of cultured cells, various types of stains were applied. Hematoxylin and eosin-stained cell bodies showed long fusiform- or star-like shapes. Nuclei were rounded or oval-shaped and located in the center of the cell body. Cytoplasm stained with anti-vimentin polyclonal antibody appeared brown, while keratin was not stained (Fig. 1D and E). These results suggested that isolated cells had an interstitial opposed to an epithelial origin.

Effects of icariin on hPDLC proliferation. As shown in Table I, icariin accelerated the proliferation of hPDLCs when the concentration was between 10^{-5} and 10^{-6} mol/l ($P<0.01$ compared with the control group). The effect appeared to be concentration-dependent within this concentration range. Lower icariin concentrations exhibited no significant effect on

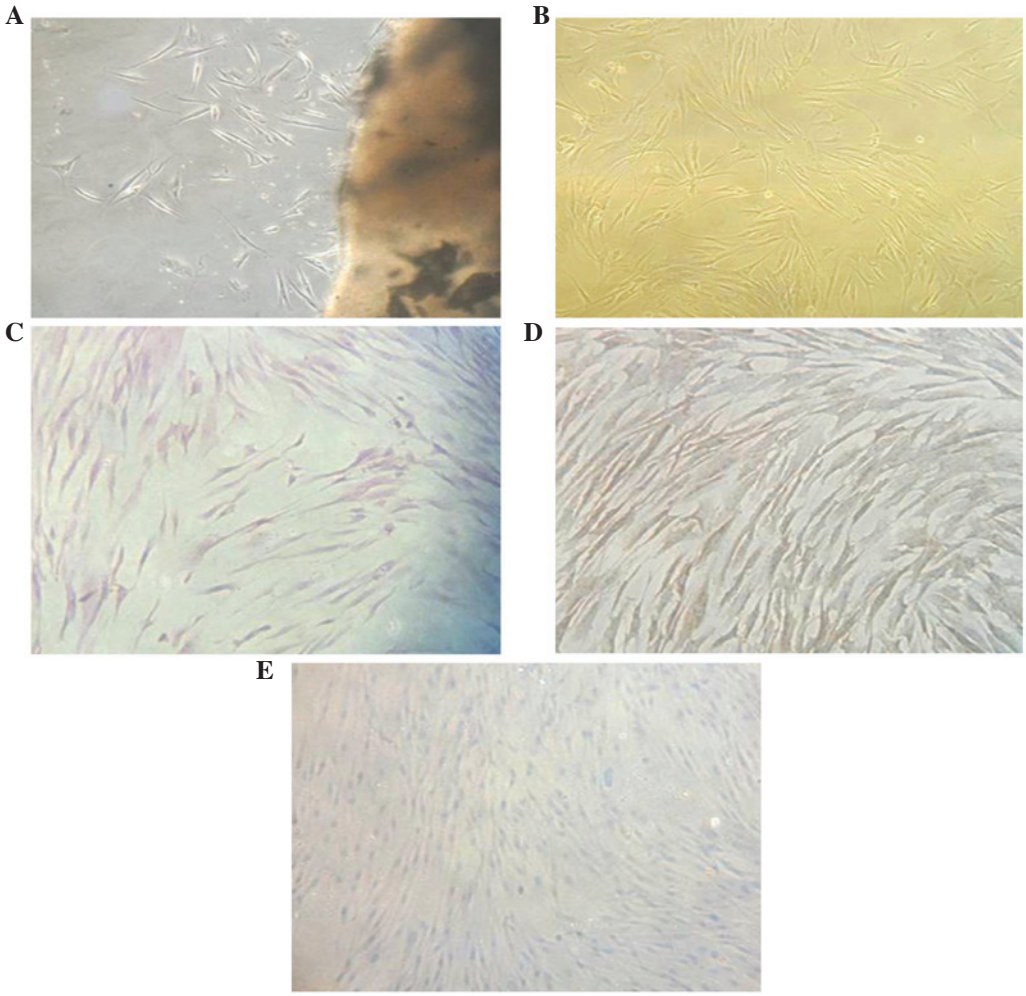

Figure 1. Isolation and identification of hPDLCs. (A) Primary cultured cells. (B) Fourth-passage subcultured cells. (C) Fourth-passage cells stained by hematoxylin and eosin. (D) Third-passage cells stained by vimentin antibodies. (E) Third-passage cells stained by keratin antibodies but appeared marginally colored. hPDLCs, human periodontal ligament cells. (A and B) Magnification, x100; (C-E) magnification, x400.

Table I. Effect of ICA concentration on hPDLC proliferation.

ICA concentration (mol/l)	Proliferation ability (OD490)
0	0.36±0.04
10^{-5}	0.32±0.04[a]
10^{-6}	0.49±0.04[b]
10^{-7}	0.40±0.05[a]
10^{-8}	0.36±0.02
10^{-9}	0.36±0.03

[a]P<0.05 and [b]P<0.01 compared with 0 mol/l group. Data are presented as the mean ± SD (n=8). ICA, icariin; hPDLC, human periodontal ligament cell.

Table II. ALP activity of different groups after 96 h incubation..

Groups	ALP activity (U/μl/μg protein)
N	3.55±0.41
LPS	1.18±0.38
ICA+LPS	2.1±0.44

Data are presented as the mean ± SD (n=6). ALP, alkaline phosphatase. N, control group; LPS, lipopolysaccharide-treated group; ICA+LPS, icariin- and LPS-treated group.

hPDLC proliferation while higher concentrations inhibited the division of hPDLCs (P<0.05).

Icariin promotes differentiation of hPDLCs inhibited by LPS. High ALP activity is a well-known index for hPDLC ossification. Previous studies have shown that LPS inhibited hPDLC differentiation and this process was associated with ALP activity decreases. When icariin (10^{-5} mol/l) was added to media containing LPS, the ALP activity was not changed at ~24 h; however, following incubation for 96 h, hPDLCs exhibited high ALP activity (Table II).

To identify at which level ALP activity returned to normal levels, RT-PCR and western blot analysis were used to measure the gene expression and protein levels

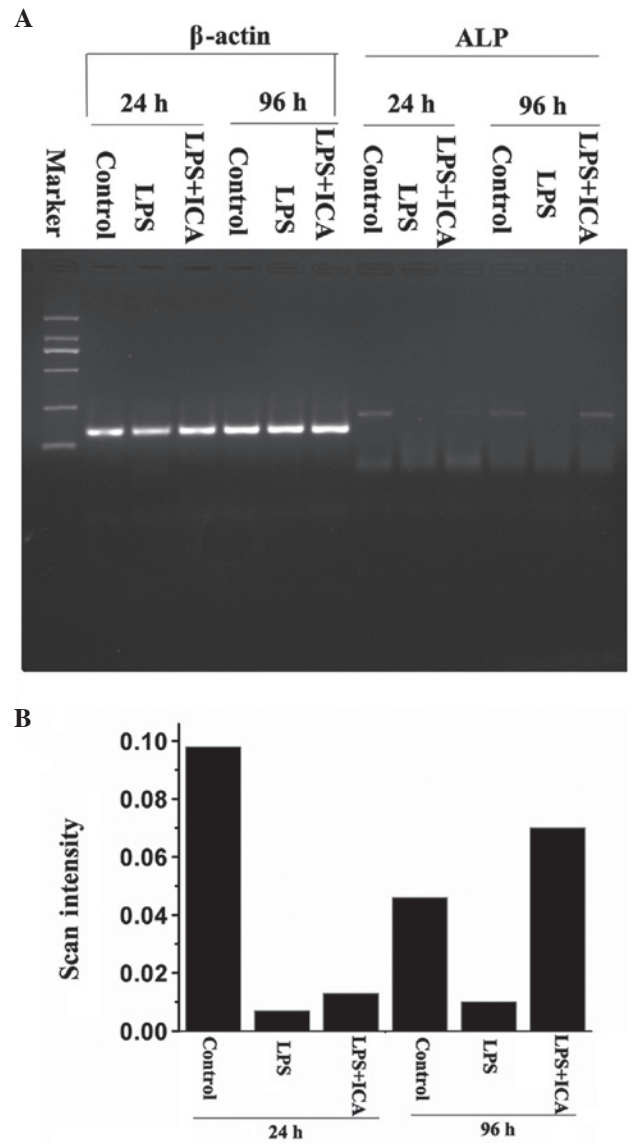

Figure 2. Reverse transcription polymerase chain reaction (RT-PCR) detection of alkaline phosphatase (ALP) gene expression. (A) Periodontal ligament cells (PDLCs) were treated with lipopolysaccharide (LPS) or LPS and icariin (ICA). Cells were harvested following treatment for 24 or 96 h, RNA was isolated and RT-PCR was conducted using β-actin as a reference. (B) DNA agarose gels were run and band intensities were scanned. The experiment was replicated. Actin served as the internal control.

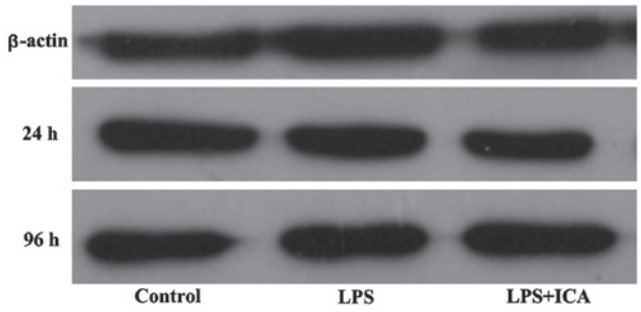

Figure 3. Western blot analysis of alkaline phosphatase (ALP) expression. Cells were harvested and total proteins were extracted. Equal quantities of protein were loaded and electrophoresis was carried out. ALP protein levels were analyzed following treatment for 24 and 96 h. LPS, lipopolysaccharide-treated group; LPS+ICA, LPS and icariin co-treatment group.

of ALP, respectively. The RT-PCR results showed that no significant *ALP* expression difference was detected after 24 h. Differentiation emerged between the LPS group and icarrin-treated group after 96 h. The ALP activity of the icariin group was greater than that observed in the control group ($P<0.01$) (Fig. 2).

These results suggested that the *ALP* gene expression level that had been inhibited by LPS was recovered by icariin following prolonged exposure. These results were similar in the western blot analysis (Fig. 3). LPS suppressed ALP expression at 24 and 96 h, while icariin accelerated this inhibition at 24 h, but markedly increased the expression at 96 h, exceeding the level of the control group ($P<0.01$). Thus, the ALP activity fluctuation was directly correlated with the protein level of the enzyme, which was controlled by the ALP expression.

Discussion

The apex dentis lesion of the un-develped permanent teeth of teenagers could be medically induced to apical foramen occlusion. Yang *et al* (17) observed that the occlusion was hindered by the accumulation of dentin, osteoid dentin, cementum and mineralization, which differentiated from parodontium connective tissue. Parodontium predominantly consists of PDLCs and collagenous fiber (18). PDLCs are heterogenic multipotential stem cells that possess important biological functions. PDLCs proliferate and differentiate into osteoblasts, which synthesize periodontal ligament, alveolar bone and cementum, thus repairing and regenerating periapical tissue (19).

Herba epimedii is a traditional Chinese herb that has multiple medical effects. Modern pharmacological studies have suggested that icariin extracted from herba epimedii inhibited osteoclast function and accelerated the proliferation and differentiation of osteoblasts (20). Icariin also decreased the cytotoxicity resulting from LPS; however, the detailed mechanism of this action remains to be elucidated. In the present study, the effects of icariin on PDLC proliferation in an *in vitro* culture were investigated. The results suggested that icariin significantly accelerated PDLC proliferation at a concentration range between 10^{-7} and 10^{-6} mol/l in a concentration-dependent manner. A concentration of 10^{-6} mol/l exhibited the strongest effect.

ALP activity is one of the most important markers for ossification (7). ALP conducts dephosphorylation, destroys inhibitors of mineralization and acts as a calcium binding protein or phosphate transporter to promote ossification. ALP activity in PDLCs indicates ossification transformation. LPS, a pathogenic sugar residue isolated specifically on Gram negative bacteria, is a toxic antigen to cells in the periapical tissue (21). LPS is important in the development of periapical diseases (7). In hPDLCs, LPS was observed to decrease the ALP activity, leading to a loss of potential to differentiate into osteoblasts and cementoblasts. Thus, increasing the ALP activity in inhibited PDLCs is crucial for PDLC regeneration and differentiation. The addition of icariin to LPS-inhibited PDLCs increased ALP activity following exposure for >96 h, while a short exposure period (~24 h) did not show a marked increase. The molecular mechanism underlying the

antagonism was suggested to be mediated by icariin increasing ALP gene and protein expression levels, which promotes ALP activity. Improved ALP activity induced PDLC renewal and the potential for differentiation. This provided insight into the use of icariin as a treatment for periapical disease resulting from LPS.

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