Escin inhibits lipopolysaccharide-induced inflammation in human periodontal ligament cells

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Received March 25, 2012; Accepted July 31, 2012

DOI: 10.3892/mmr.2012.1031

Abstract. Periodontitis is a chronic inflammatory disease associated with gram-negative subgingival microflora infection. Accumulating experimental evidence suggests that escin exerts anti-inflammatory and anti-edematous effects. This study was designed to investigate the *in vitro* effects of escin on the inflammatory reaction of human periodontal ligament cells (hPDLs). hPDLs were stimulated with lipopolysaccharide (LPS). The cells were treated with various concentrations of escin. The viability of hPDLs was evaluated using the MTT method. The expression of Toll-like receptor 2 (TLR2) in hPDLs and the levels of IL-1β, TNF-α and IL-6 in the supernatant were measured. Escin significantly attenuated LPS-induced cytotoxicity in a concentration-dependent manner in hPDLs. Treatment with escin partly blocked the expression of TLR2. Escin also lowered the increase of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) induced by LPS. The present findings show that escin exerts a protective effect against LPS-induced inflammation in hPDLs. It was also shown that escin is a promising medicine for the treatment of periodontitis.

Introduction

Periodontitis is a chronic inflammatory disease caused by certain specific subgingival microorganisms, which results in the destruction of supporting tissue around the roots and subsequently leads to a loss of teeth if left untreated. The periodontal destruction involves a complex interaction, which is mediated in part by the individual host immune response to microbial colonization of the periodontal attachment apparatus. The lipopolysaccharide (LPS)-mediated host responses appear to cause certain inflammatory reactions, resulting in the expression of proinflammatory cytokines and amplifying the related host immune response in periodontal diseases (1).

Toll-like receptors (TLRs) play important roles in recognizing the invaded micropathogens in chronic inflammation, such as periodontitis and diabetes mellitus. TLR2 and TLR4 are receptors for the products of major periodontal pathogens, including *Porphyromonas gingivalis* (*P. gingivalis*) (2). *P. gingivalis* LPS stimulated TLR2 rather than TLR4 (3). TLR2 is involved in T helper 2 (Th2) orientation of the immune response and *P. gingivalis* LPS-induced cellular activation (2,4). The subsequent pro-inflammatory cytokine products of TLRs, such as TNF-α, IL-1β and IL-6, modify the occurrence and development of chronic inflammation, including periodontitis. Human periodontal ligament cells (hPDLs), which are capable of differentiating into cells of either cementogenic or osteogenic lineage, play important roles in maintaining homeostasis during the inflammatory response to these cytokines (5).

Escin is the major active component of *Aesculus hippocastanum*. Escin is a natural mixture of triterpene saponins, and has shown clinically significant anti-inflammatory activity postoperatively and in chronic venous insufficiency, haemorroids and traumatic brain edema. It mainly exerts its anti-inflammatory and anti-edematous effects through its anti-histamine and anti-serotoninergic activities, and by reducing the adhesiveness and migration of neutrophils (6). The aim of the present study was to investigate the effect of escin on the inflammation induced by LPS in primary culture of hPDLs.

Materials and methods

Reagents. The escin used in the experiments was sodium aescinate obtained as a lyophilized powder in a 5 mg vial manufactured by Wuhu Lvye Pharmaceutical Co. Ltd. (Wuhu, China). Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), 0.25% trypsin and 1% penicillin/streptomycin were purchased from Gibco (Carlsbad, CA, USA). LPS from *Escherichia coli* 0127:B8 and 3, 4, 5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide was

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Key words: escin, lipopolysaccharide, human periodontal ligament cells, inflammation

purchased from Merck Sharp & Dohme (Darmstadt, Germany). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-1β and IL-6 were from Bender Medsystem (Vienna, Austria). All chemicals and solvents were of analytical grade. Anti-TLR-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) detection reagents and bicinchoninic acid (BCA) protein assay kits were obtained from Beyotime Institute of Biotechnology (Haimen, China). This study was approved by the ethics committee of Binzhou Medical University.

Cell culture. The primary culture of hPDLs was obtained from healthy 15-22-year-old patients undergoing routine extraction of their first or second premolars in accordance to the method of Somerman *et al* (7), with minor modifications. All patients were, on an individual basis, informed in detail of the aim and procedure of this study, and they signed consent forms. Periodontal ligament tissues were scaled and isolated from the middle third of the roots. Tissue pieces were placed in a 6-well plate containing DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Plates were cultured in humidified air containing 5% CO_2 at 37˚C for approximately 14 days until cells migrated out of the tissue and reach confluence. The tissue pieces were then carefully removed and the cells were collected by trypsinization and cultured in 150 cm^2 culturing flasks. Cells of passage 3 to 9 were used in the subsequent steps.

Cell treatment. hPDLs were subjected to 1 μg/ml LPS stimulation (8) for 6, 12, 24, 48 and 72 h, respectively, for the preliminary investigation of the effect of LPS on hPDL viability. The hPDLs were then subjected to 1 μg/ml LPS and escin (2.5, 5.0, 10.0, 20.0 and 40.0 μg/ml) treatment (9) for 24 h at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The cultured hPDLs undergoing neither LPS nor escin treatment served as controls.

Cell viability assay. hPDL viability was determined using the MTT dye method. Briefly, hPDLs were seeded in 96-well culture plates at an initial density of $1x10^4$ cells per well in 100 μl medium, and allowed to grow to confluence. After treatment as described above, 10 μl MTT solution (5 mg/ml) was added into each well and the culture plates were incubated at 37˚C for 4 h. The supernatant was then discarded and 100 μl dimethyl sulfoxide was added followed by 5 min incubation on a shaker. The absorbance was measured at 620 nm using an automated ELISA reader (Synergy HT, Winooski, VT, USA). All samples were tested in duplicate for each experiment.

Measurement of TNF-α, IL-β and IL-6 levels. hPDLs were subjected to 1 μg/ml LPS and escin at various concentrations (5.0, 10.0 and 20.0 μg/ml) for 24 h. The cell culture supernatant was harvested at the end of treatment and used for the TNF-α, IL-1β and IL-6 assay according to the manufacturer's instructions. Absorbance values were measured at 450 nm using an ELISA plate reader (Synergy HT). All samples were measured in duplicate.

Western blot analysis for TLR-2. hPDLs were detached by 0.02% EDTA and washed three times with PBS. Whole cell lysates were isolated by lysing in 20 mmol/l Tris-HCl, pH 7.5, 2% SDS (w/v), 2 mmol/l benzamidine and 0.2 mmol/l phenyl-methanesulphonyl fluoride. Protein concentrations were determined using a BCA protein assay kit. Samples were heated at 100˚C for 5 min prior to gel loading and were then subjected to SDS-PAGE (8% sodium dodecyl sulfate/polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride membranes using an electrophoretic transfer system. The membranes were blocked in Tris-buffered saline mixed with Tween-20 (TBST) containing 5% skimmed milk for 60 min. Membranes were then incubated overnight at 4˚C with primary antibody against TLR-2 (diluted 1:1000). After washing with TBST, the membranes were incubated with secondary antibody (diluted 1:2000) for 1 h at 37˚C. After washing with TBST, the binding was detected by ECL detection reagents. For the quantitative analysis of the density of the immunoblot bands, densitometry was performed with the Gel-Pro Analyzer Version 3.0. The ratio of the integrated optical density of immunoblot bands of the detected protein to that of β-actin was used for statistical analysis.

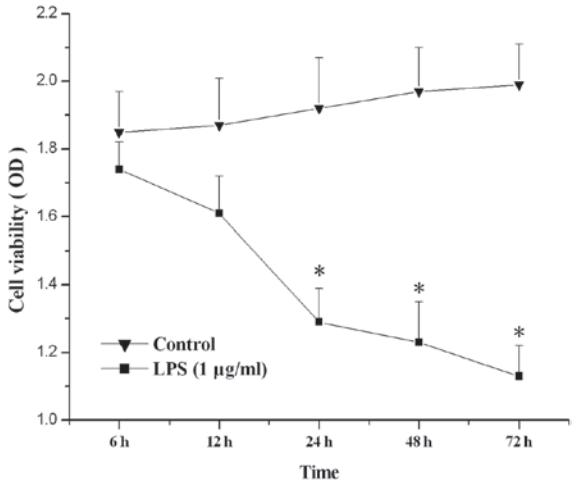

Figure 1. Analysis of hPDL viability after stimulation with 1 μg/ml LPS. Viability was determined by MTT assay after stimulation with 1 μg/ml LPS for 6, 12, 24, 48 and 72 h respectively. Data were expressed as the means ± SD in this study. One-way ANOVA was used to analyze the significant differences within the groups. The comparison between two groups was determined by the post hoc test. *P<0.05 as compared with control group. hPDLs, human periodontal ligament cells; LPS, lipopolysaccharide; OD, optical density.

Statistical analysis. Data were expressed as the means ± standard deviation (SD) in this study. One-way ANOVA was used to analyze the significant differences within the groups. The comparison between two groups was determined by the post hoc test. A value of P<0.05 was considered to indicate a statistically significant difference between groups.

Results

The effect of LPS on the viability of hPDLs. Compared with control, LPS (1 μg/ml) treatment for 24, 48 or 72 h significantly lowered the viability of hPDLs (P<0.05; Fig. 1).

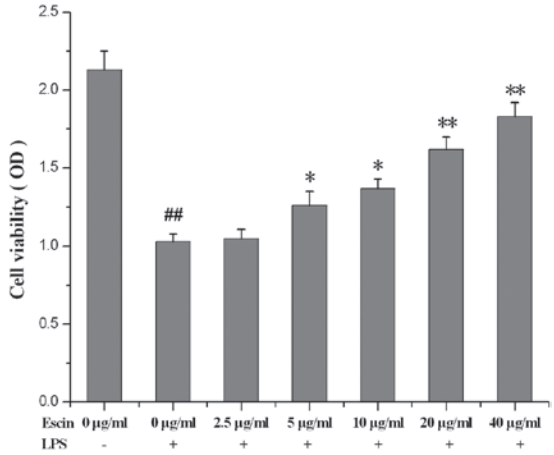

Figure 2. Analysis of hPDL viability after treatment with escin (0, 2.5, 5, 10, 20 and 40 μg/ml) and 1 μg/ml LPS for 24 h. Data were expressed as the means ± SD. One-way ANOVA was used to analyze the significant differences within the groups. The comparison between two groups was determined by the post hoc test. *P<0.05; **P<0.01 compared with control group. hPDLs, human periodontal ligament cells; LPS, lipopolysaccharide; OD, optical density.

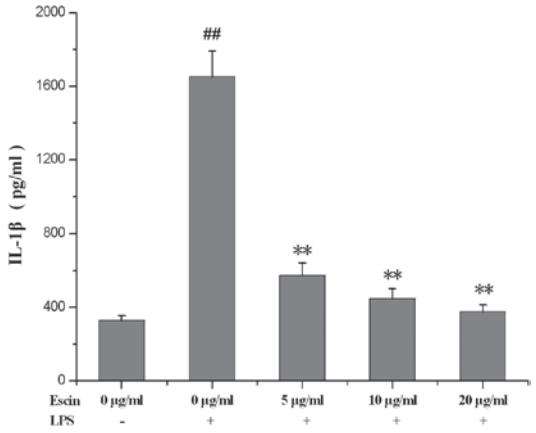

Figure 4. IL-1β level in the supernatant of hPDLs culture stimulated with 1 μg/ml LPS and treated with escin (0, 5, 10 and 20 μg/ml) for 24 h. IL-1β level was tested by enzyme-linked immunosorbent assay. Data were expressed as the means ± SD. One-way ANOVA was used to analyze the significant differences within the groups. The comparison between two groups was determined by the post hoc test. $^{\#\#}$P<0.01 compared with control group; **P<0.01 compared with LPS group. hPDLs, human periodontal ligament cells; LPS, lipopolysaccharide.

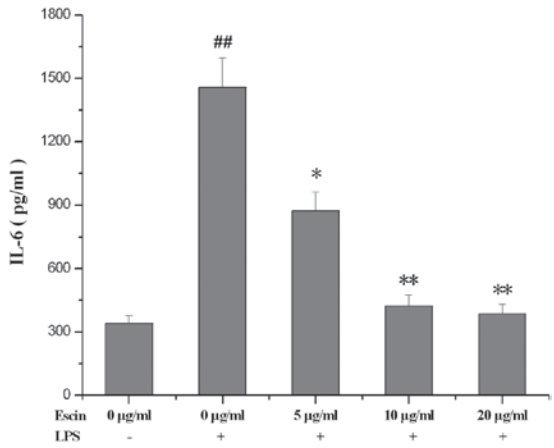

Figure 3. IL-6 level in the supernatant of hPDL culture stimulated with 1 μg/ml LPS and treated with escin (0, 5, 10 and 20 μg/ml) for 24 h. The IL-6 level was tested by enzyme-linked immunosorbent assay. Data were expressed as the means ± SD. One-way ANOVA was used to analyze the significant differences within the groups. The comparison between two groups was determined by the post hoc test. $^{\#\#}$P<0.01 compared with control group; *P<0.05, **P<0.01 compared with LPS group. hPDLs, human periodontal ligament cells; LPS, lipopolysaccharide.

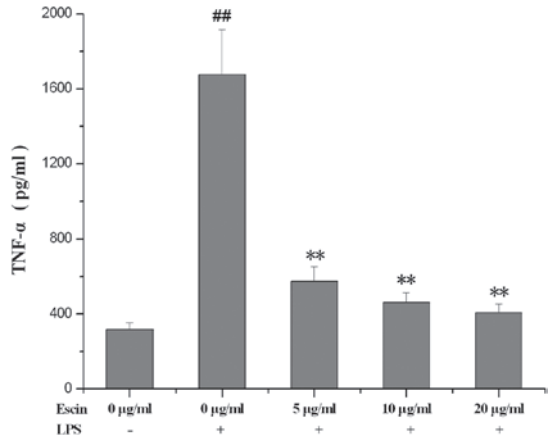

Figure 5. TNF-α level in the supernatant of hPDL culture stimulated with 1 μg/ml LPS and treated with escin (0, 5, 10 and 20 μg/ml) for 24 h. TNF-α level was tested by enzyme-linked immunosorbent assay. Data were expressed as the means ± SD. One-way ANOVA was used to analyze the significant differences within the groups. The comparison between two groups was determined by the post hoc test. $^{\#\#}$P<0.01 compared with control group; **P<0.01 compared with LPS group. hPDLs, human periodontal ligament cells; LPS, lipopolysaccharide.

The effect of escin on the viability of LPS-stimulated hPDLs. Escin (5, 10, 20 and 40 μg/ml) attenuated the decreased viability induced by LPS in hPDLs in a dose-dependent manner (Fig. 2).

The effect of escin on the IL-6 level in supernatant. Compared with the control, the IL-6 level in the supernatant of LPS-treated hPDL culture was significantly increased. Treatment with escin for 24 h reduced the IL-6 level in a dose-dependent manner (Fig. 3).

The effect of escin on the IL-1β level in supernatant. The IL-1β level was increased in the supernatant of LPS-treated hPDL culture. Following treatment with escin for 24 h, the IL-1β level in the supernatant significantly decreased (Fig. 4).

The effect of escin on the TNF-α level in supernatant. The TNF-α level in the supernatant of LPS-treated hPDL culture was increased compared to that of the control. Escin treatment for 24 h significantly lowered the TNF-α level (Fig. 5).

The effect of escin on TLR2 expression in LPS-treated hPDLs. LPS led to an increase in TLR2 expression. Escin treatment partially blocked the increase of TLR2 expression induced by LPS (Fig. 6).

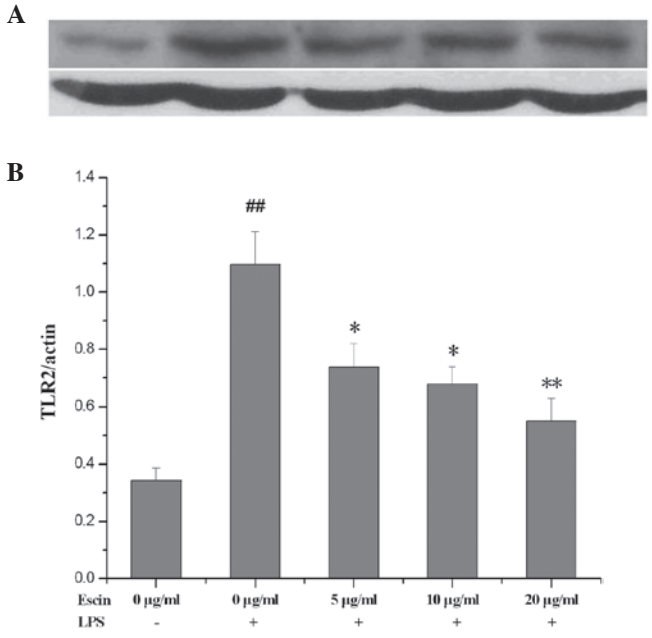

Figure 6. Effect of escin on the expression of TLR2 in LPS-induced hPDLs. (A) Representative western blotting images. Lane 1, LPS(-)/escin(-); Lane 2, LPS (1 μg/ml)/escin(-); Lane 3, LPS (1 μg/ml)/escin (5 μg/ml); Lane 4, LPS (1 μg/ml)/escin (10 μg/ml); Lane 5, LPS (1 μg/ml)/escin (20 μg/ml). Equal loading of proteins was illustrated by actin bands. (B) The mean ± SD values of the densitometric analysis for 3 different observations. $^{\#\#}$P<0.01 compared with control group; *P<0.05, **P<0.01 compared with LPS group. TLR2, Toll-like receptor 2; hPDLs, human periodontal ligament cells; LPS, lipopolysaccharide.

Discussion

Escin, a natural mixture of triterpenoid asponin isolated from the seed of the horse chestnut, possesses anti-cancerous, anti-oedematous and anti-inflammatory effects (10). In this study, escin increased the viability of hPDLs pre-treated by LPS in a dose-dependent manner, and the findings suggest that escin may protect the hPDLs from injury induced by LPS.

Periodontal bacteria stimulate, via TLR2 and TLR4, the production of proinflammatory cytokines in the host, such as IL-1β and TNF, which induce alveolar bone resorption (11). TLR2 is responsible for the predominantly Th2-oriented immune response observed in progressive periodontitis (4). TLR2 was associated with exacerbation of *P. gingivalis*-induced periodontal bone loss due to TLR2-induced TNF-α-dependent osteoclastogenesis (12).

TLR2 is important in *P. gingivalis*-induced Th2 oriented immune responses. The reduction of the TLR-2 level in LPS-induced hPDLs treated by escin indicates that escin may play a role in the Th2 oriented immune response in periodontitis.

Extensive studies have shown that IL-6, TNF-α and IL-1β (the products of the TLR2 signaling pathway) are the major products of inflammatory cells and contribute to the progression of inflammation (13,14). The present results showed that escin treatment attenuated the inflammatory cytokine responses of hPDLs in response to *P. gingivalis* LPS (Figs. 3-5).

TNF-α and IL-1β are considered to be pivotal cytokines in periodontal disease, since they are expressed in higher levels in the gingival crevicular fluid and inflamed periodontal tissues, and cause alveolar bone resorption (15,16). It was also proven that the inflammatory response and bone loss in ligature-induced periodontitis in monkeys were reduced by local inhibition of the IL-1β pathway in periodontal tissues (17).

IL-6 is a major mediator of the host response to injury and infections by inducing the differentiation of activated B lymphocytes into antibody-secreting cells (18). The diseased gingiva of periodontitis patients contained higher levels of IL-6, which is capable of stimulating bone resorption (19). However, IL-6 secretion was upregulated by *P. gingivalis* LPS, the presence of escin reduced IL-6 secretion in hPDLs, indicating that escin may prevent periodontal tissue destruction, including alveolar bone resorption. The possible mechanism by which escin affects cytokine production is likely to be due to an altered sensitivity of the isolated hPDLs induced by the LPS of *P. gingivalis* (14).

In conclusion, escin has an anti-inflammatory effect in hPDLs. The molecular mechanism of the anti-inflammatory effect of escin in periodontitis is related to the downregulation of TNF-α, IL-1β and IL-6 through the TLR2 signaling pathway in the Th2-oriented immune response, which requires further study.

Acknowledgements

This research was supported by the 'Taishan Scholars' project of the Government of Shandong Province, China.

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