Shikonin inhibits inflammatory cytokine production in human periodontal ligament cells

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Abstract

Shikonin, which is derived from Lithospermum erythrorhizon, a herb used in traditional medicine, has long been considered to be a useful treatment for various diseases in traditional oriental medicine. Shikonin has recently been reported to have several pharmacological properties, e.g., it has anti-microbial, anti-tumor, and anti-inflammatory effects. The aim of this study was to examine whether shikonin is able to influence the production of interleukin (IL)-6, IL-8, and/or chemokine C-C motif ligand (CCL)20, which contribute to the pathogenesis of periodontal disease, in human periodontal ligament cells (HPDLC). The production levels of IL-6, IL-8, and CCL20 in HPDLC were determined using an ELISA. Western blot analysis was used to detect nuclear factor kappa B (NF-κB) pathway activation in HPDLC. Shikonin prevented IL-1β- or tumor necrosis factor (TNF)-α-mediated IL-6, IL-8, and CCL20 production in HPDLC. Moreover, we found that shikonin suppressed the phosphorylation and degradation of inhibitor of kappa B-alpha (IκB-α) in IL-1β- or TNF-α-stimulated HPDLC. These findings suggest that shikonin could have direct beneficial effects against periodontal disease by reducing IL-6, IL-8, and CCL20 production in periodontal lesions.

Introduction

Periodontitis is characterized by gingival inflammation, inflammatory cell infiltration, and alveolar bone loss and is caused by the bacteria in the oral biofilm. Previous studies have revealed that the immune responses that occur in periodontal lesions play a role in the initiation and progression of periodontal disease [1,2]. In particular, cytokines make an important contribution to the pathogenesis of periodontal disease [3, 4].

Interleukin-6 (IL-6) is a cytokine that is involved in various pathological processes, including the response to infection and the progression of inflammation [5-6]. It is produced in a number of different types of cells, such as fibroblasts, osteoblasts, and endothelial cells, in inflammatory lesions [7-9]. IL-6 is known to play an important role in the inflammatory response in periodontal tissues. For example, Naruishi et al. reported that IL-6 enhances vascular endothelial growth factor production in gingival fibroblasts [10]. They also found that treating human gingival fibroblasts with IL-6 and the soluble IL-6 receptor increased their production of cathepsins B and L, which are involved in tissue degradation [11]. We previously reported that IL-6 trans-signaling synergistically enhanced the production of CC chemokine ligand (CCL) 20, which is involved in T helper 17 (Th17) cell migration, in IL-1β-stimulated human periodontal ligament cells [12]. Th17 cells can induce bone destruction in inflammatory lesions, such as those caused by periodontal disease [13] and rheumatoid arthritis [14]. Therefore, IL-6 and CCL20 clearly contribute to the pathogenesis of periodontal disease.

IL-8 also plays a role in the pathogenesis of periodontal disease because it controls the migration of neutrophils in inflammatory tissues, such as those found in periodontitis [15, 16]. Kantarci et al. reported that neutrophils play a major role in the host response to periodontopathogenic bacteria, and neutrophil hyperresponsiveness might enhance tissue damage in the periodontium [17]. The latter report also mentioned that excessive IL-8 production could lead to tissue destruction in periodontal tissues.

Shikonin, which is derived from Lithospermum erythrorhizon, a herb that is used in traditional medicine, has long been used to treat burns, infected crusts, and psoriasis in traditional Chinese medicine [18]. It has been reported that shikonin has several medicinal properties, e.g., it has anti-tumor [19], anti-microbial [20], and anti-inflammatory effects [21]. However, the effects of shikonin on inflammatory cytokine production in periodontal ligament cells (HPDLC) have not been elucidated.

The aim of this study was to examine the effects of shikonin on IL-6, IL-8, and CCL20 production in IL-1β- or tumor necrosis factor (TNF)-α-stimulated HPDLC, which are resident cells in periodontal tissues. Moreover, we investigated whether shikonin treatment influences the activation of the nuclear factor kappa B (NF-κB) pathway in IL-1β- or TNF-α-stimulated HPDLC by examining the phosphorylation of NF-κB p65 and inhibitor of kappa B-alpha (IκB-α), and the degradation of IκB-α.

Materials and Methods

Cell culture

HPDLC were obtained from TaKaRa Biotechnology Co., Ltd. (Otsu, Shiga, Japan) and grown in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 1 mmol/L sodium pyruvate (Gibco), and antibiotics (penicillin G, 100 units/ml; streptomycin, 100 μg/ml; Gibco) at 37°C in a humidified air with 5% CO_2. When the cells reached subconfluence, they were harvested and subcultured. The cells were used between passage numbers 5 and 10.

IL-6, IL-8, and CCL20 production in HPDLC

The HPDLC were stimulated with recombinant human IL-1β (Peprotech, Rocky Hill, NJ, USA) or TNF-α (Peprotech) for 24 hours. The supernatants of the HPDLC were collected, and their IL-6, IL-8, and CCL20 concentrations were measured in triplicate using enzyme-linked immunosorbent assays (ELISA). Duoset ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to obtain these measurements. All assays were performed according to the manufacturer's instructions, and cytokine levels were determined using the standard curve prepared for each assay. In selected experiments, the HPDLC were cultured for 1 hour in the presence or absence of shikonin (0.25, 0.5, 1, or 2 μM; Nagara Science Co., Ltd., Gifu, Japan) prior to their incubation with IL-1β or TNF-α.

Western blot analysis

Western blot analysis was performed to detect the IL-1β- or TNF-α-induced phosphorylation of signal transduction molecules. HPDLC that had or had not been pretreated with shikonin (1 μM) for 1 hour before being stimulated with IL-1β (1 ng/ml) or TNF-α (10 ng/ml) were

washed once with cold phosphate-buffered saline and then incubated on ice for 30 min with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease inhibitor cocktail (Sigma). After the removal of debris by centrifugation, the protein concentrations of the lysates were quantified with the Bradford protein assay using IgG as a standard. Twenty-μg protein samples were loaded onto 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, before being electrotransferred to polyvinylidene difluoride membranes. The membranes were then blocked with 1% non-fat dried milk for 1 hour and reacted with anti-phospho-NF-κB p65 rabbit monoclonal antibody (Cell Signaling Technology), anti-phospho-IκB-α mouse monoclonal antibody (Cell Signaling Technology), anti-NF-κB p65 rabbit monoclonal antibody (Cell Signaling Technology), anti-κB-α mouse monoclonal antibody (Cell Signaling Technology), or anti-GAPDH rabbit monoclonal antibody (Cell Signaling Technology) overnight. The membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (Sigma) for 1 hour at room temperature, and then detection was performed using the ECL prime Western blotting detection system (GE Healthcare, Uppsala, Sweden).

Statistical analysis

Statistical significance was analyzed using the Student's *t* test. *P* values of <0.05 were considered to be significant.

Results

Effects of shikonin on IL-6, IL-8, and CCL20 production in HPDLC

Previous reports have suggested that IL-1β induces the production of large amounts of IL-6, IL-8, and CCL20 in HPDLC [12, 22]. Therefore, we first investigated whether shikonin is able to inhibit IL-6, IL-8, and CCL20 expression. As shown in Fig. 1, shikonin inhibited IL-6, IL-8, and CCL20 production in IL-1β-stimulated HPDLC in a dose-dependent manner. The administration of 2 μg/ml shikonin almost completely abrogated IL-6, IL-8, and CCL20 production in these cells.

Effects of shikonin on IL-6, IL-8, and CCL20 production in TNF-α-stimulated HPDLC

Next, we investigated the effects of shikonin on IL-6, IL-8, and CCL20 production in TNF-α-treated HPDLC because it is known that TNF-α induces IL-6 and IL-8 production in HPDLC [23, 24]. Figs. 2A and B show that shikonin clearly inhibited IL-6 and IL-8 production in TNF-α-stimulated HPDLC in a dose-dependent manner. Fig. 2C shows that TNF-α induced CCL20 production, and shikonin suppressed the CCL20 production induced by TNF-α stimulation.

Effects of shikonin on NF-κB p65 and IκB-α phosphorylation and IκB-α degradation in IL-1β-stimulated HPDLC

Moreover, we examined the effects of shikonin on NF-κB pathway activation in IL-1β-stimulated HPDLC because it was reported that IL-1β activates the NF-κB pathway in HPDLC [25]. Fig. 3 shows that shikonin inhibited IκB-α phosphorylation and degradation. On the other hand, shikonin did not modulate NF-κB p65 phosphorylation in IL-1β-stimulated HPDLC.

Effects of shikonin on NF-κB p65 and IκB-α phosphorylation and IκB-α degradation in TNF-α-stimulated HPDLC

Finally, we examined the effects of shikonin on NF-κB pathway activation in TNF-α-stimulated HPDLC. It is known that TNF-α activates the NF-κB pathway in HPDLC [23]. Fig. 4 shows that shikonin downregulated IκB-α phosphorylation and degradation. Conversely, shikonin did not inhibit NF-κB p65 phosphorylation in TNF-α-stimulated HPDLC.

Discussion

In this study, we demonstrated for the first time that shikonin suppressed IL-6, IL-8, and CCL20 production in IL-1β- or TNF-α-stimulated HPDLC. Some researchers have found that shikonin inhibits inflammatory reactions in animal models. Bai et al. reported that shikonin attenuated lipopolysaccharide (LPS)-induced acute lung injuries in mice [26]. Specifically, they demonstrated that shikonin pretreatment significantly inhibited LPS-induced pulmonary histopathological changes and neutrophil accumulation. Moreover, they showed that shikonin decreased the levels of IL-1β and TNF-α in the bronchoalveolar lavage fluid collected from the mice [26]. Xiong et al. also reported that shikonin ameliorated cerulean-induced acute pancreatitis in mice [27]. In addition, they found that shikonin decreased the serum TNF-α, IL-1β, and IL-6 levels of mice.

Previous reports have also shown that the injection of shikonin into lesions reduced inflammation; however, they did not elucidate the mechanism responsible for these effects.

Our *in vitro* study clearly demonstrated that shikonin inhibited inflammatory cytokine production in HPDLC, which are resident periodontal cells. Therefore, as a next step we intend to perform an *in vivo* study using a periodontitis model.

Various researchers have examined the effects of shikonin on cytokine production in different types of cells. The effects of shikonin on NF-κB activation have also been investigated. Yang et al. reported that shikonin inhibited the release of high mobility group box 1 from LPS-treated murine macrophage-like RAW264.7 cells [28]. They also found that shikonin decreased the nuclear to cytoplasm ratio of NF-κB protein expression. It is known that IκB-α prevents NF-κB p65 translocation to the nucleus. Therefore, the findings of the latter report agree with those obtained in the present study. Moreover, Min et al. reported that shikonin inhibited matrix metallopeptidase 9 expression in human highly metastatic adenoid cystic carcinoma (ACC-M) cells [29]. They also demonstrated that shikonin treatment suppressed the levels of phospho-IκB-α in ACC-M cells. These results are also similar to ours. Our data and those obtained in previous reports, indicate that shikonin suppresses inflammatory mediator production by inhibiting the activity of IκB-α. We propose that shikonin might inhibit inflammatory mediator production in cytokine-stimulated HPDLC because the NF-κB pathway is involved in the expression of many kinds of proteins in human cells. Further comprehensive analyses of the effects of shikonin are required.

Mitogen-activated protein kinase (MAPK) pathways are important for cytokine production in HPDLC. So, we examined the effects of shikonin on MAPK phosphorylation in IL-1β or

TNF-α–stimulated HPDLC. As a result, we found that shikonin (1 μM) enhanced p38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun N terminal kinase (JNK) phosphorylation in HPDLC (data not shown). However, in this study we found that shikonin suppressed cytokine production in HPDLC; therefore, we consider that the MAPK activation induced by shikonin has less influence on cytokine production in HPDLC than its inhibition of the NF-κB pathway.

There have been several reports about the effects of shikonin on MAPK activation. Huang et al. reported that shikonin treatment inhibited ERK phosphorylation, while it activated p38 MAPK and JNK phosphorylation in human lens epithelial cells [30]. In addition, Chen et al. found that shikonin activated the p38 MAPK, ERK, and JNK pathways in prostate cancer cells (PC-3 and DU145 cells) [31]. These findings regarding MAPK activation are similar to those obtained in the present study. We consider that the effects of shikonin on MAPK activation differ among cell types.

In summary, the current study demonstrated that shikonin is able to suppress IL-1β- or TNF-α-induced IL-6, IL-8, and CCL20 production in HPDLC. In addition, it revealed that shikonin inhibited IκB-α phosphorylation and degradation in IL-1β- or TNF-α-stimulated HPDLC. These results suggest that shikonin could be used to treat periodontal disease; i.e., as an inhibitor of proinflammatory cytokine expression in periodontal lesions.

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Conflicts of interest

The authors confirm that they have no conflicts of interest.

Figure legends

Fig. 1. Effects of shikonin on IL-6, IL-8, and CCL20 production in IL-1β-stimulated HPDLC HPDLC were incubated with shikonin (0.25, 0.5, 1, or 2 μM) for 1 hour, before being stimulated with IL-1β (1 ng/ml) for 24 hours, and then their supernatants were collected. The concentrations of IL-6 (A), IL-8 (B), and CCL20 (C) in the supernatants were measured using ELISA. The results are shown as the mean and SD values of a representative experiment performed in triplicate. The error bars represent the SD. * = $P<0.05$, significantly different from the IL-1β-stimulated HPDLC that were not pretreated with shikonin

Fig. 2. Effects of shikonin on IL-6, IL-8, and CCL20 production in TNF-α-stimulated HPDLC HPDLC were incubated with shikonin (0.25, 0.5, 1, or 2 μM) for 1 hour and then stimulated with TNF-α (10 ng/ml). Their supernatants were collected after 24 hours. The concentrations of IL-6 (A), IL-8 (B), and CCL20 (C) in the supernatants were measured using ELISA. The results are shown as the mean and SD of a representative experiment performed in triplicate. The error bars indicate the SD. * = $P<0.05$, significantly different from the TNF-α-stimulated HPDLC that were not pretreated with shikonin

Fig. 3. Effects of shikonin on IL-1β-induced NF-κB p65 and IκB-α phosphorylation and

IκB-α degradation The cultured cells were pretreated with shikonin (1 μM) for 60 min and then stimulated with 1 ng/ml IL-1β for 15, 30, or 60 min. The cells were lysed in lysis buffer containing protease inhibitors, and the phosphorylation of NF-κB p65 and IκB-α was assessed using Western blot analysis. A representative Western blot that indicates the phospho-NF-κB p65, total NF-κB p65, phospho-IκB-α, total IκB-α, and GAPDH levels detected in the HPDLC during three independent experiments is shown.

Fig. 4. Effects of shikonin on TNF-α-induced NF-κB p65 and IκB-α phosphorylation and IκB-α degradation The cultured cells were pretreated with shikonin (1 μM) for 60 min and then stimulated with 10 ng/ml TNF-α for 15, 30, or 60 min. The cells were lysed in lysis buffer containing protease inhibitors, and the phosphorylation of NF-κB p65 and IκB-α was assessed using Western blot analysis. A representative Western blot that indicates the phospho-NF-κB p65, total NF-κB p65, phospho-IκB-α, total IκB-α, and GAPDH levels detected in the HPDLC during three independent experiments is shown.

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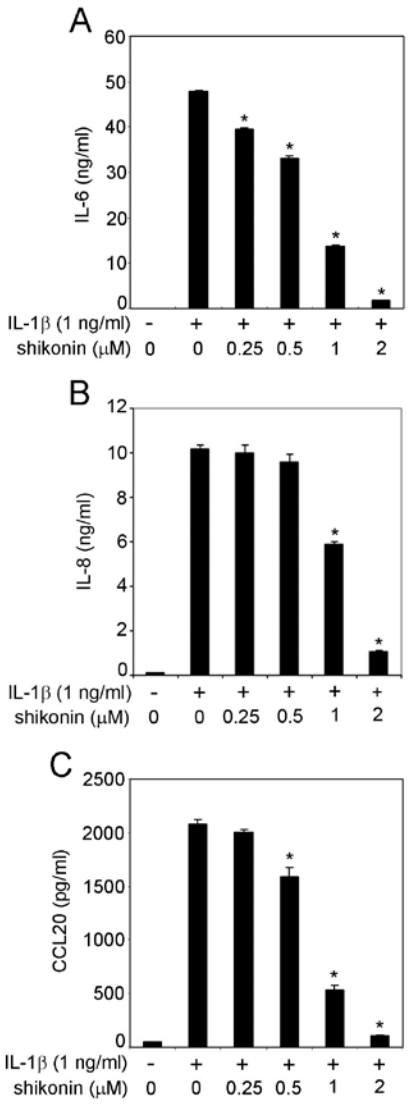
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Fig. 1

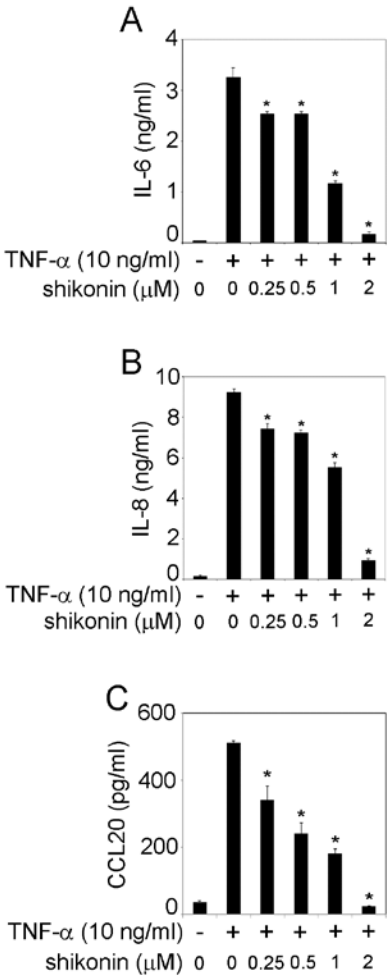
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Fig. 2

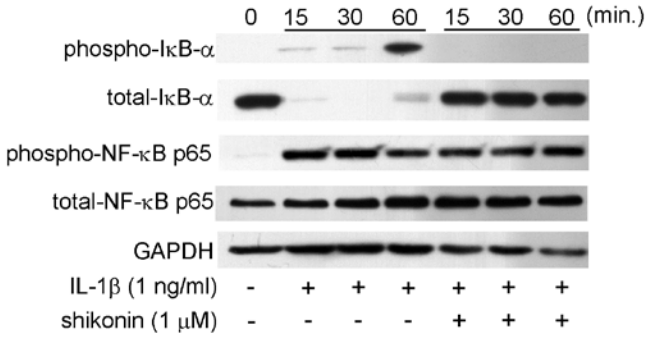
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Fig. 3

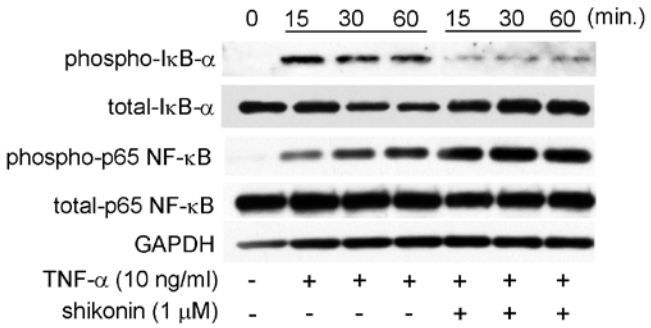
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Fig. 4