The effects of berteroin on inflammatory mediators and antioxidant enzymes expression in human periodontal ligament cells

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Abstract

Berteroin is a bioactive substance classified as an isothiocyanate found in cruciferous vegetables such as cabbage, arugula, and salad leaves. In this study, we aimed to determine whether berteroin exerts anti-inflammatory effects on human periodontal ligament cells (HPDLCs), a resident cells of periodontal tissue. Berteroin suppressed interleukin (IL)-1β or tumor necrosis factor (TNF)-α-induced chemokines (C-C motif chemokine ligand (CCL)2, CCL20, C-X-C motif chemokine ligand (CXCL)10, IL-8, and IL-6) production and intercellular adhesion molecule (ICAM)-1 expression in HPDLCs. In addition, berteroin inhibited phosphorylation of IκB kinase (IKK)-α/β, nuclear factor (NF)-κB p65, and IκB-α and degradation of IκB-α in the NF-κB pathway induced by IL-1β or TNF-α stimulation. Moreover, berteroin could inhibit signal transducer and activator of transcription (STAT)3 phosphorylation in TNF-α-stimulated HPDLC. Furthermore, berteroin increased the expression of the antioxidant enzymes, heme oxygenase (HO)-1 and NAD(P)H quinone dehydrogenase (NQO)1, in IL-1β or TNF-α-stimulated HPDLCs. These results suggest that berteroin may decrease the production of inflammatory mediators in HPDLCs by suppressing the NF-κB pathway, and may also decrease the local reactive oxygen species (ROS) production in periodontal lesions by increasing the production of antioxidant enzymes.

Introduction

Periodontitis is an inflammatory disease that affects many people worldwide, and it has been shown that an excessive immune response in periodontal lesion is involved in the destruction of periodontal tissue (Cavalla et al., 2021). Currently, antibiotics are widely used for the treatment of periodontitis, and new anti-inflammatory agents are expected to be used due to the problem of bacterial resistance. (Ng et al., 2023)

Berteroin is a natural analog of sulforaphane, which is known to be found in cruciferous vegetables. Several reports have been published on berteroin with regard to its bioactive effects, such as anticancer and anti-inflammatory effects. In terms of anticancer activity, berteroin has been shown to inhibit the proliferation of human colon cancer cell lines (Kim et al., 2010). As for the anti-inflammatory effect, it has been reported that berteroin can inhibit the production of inflammatory mediators in mouse macrophages stimulated with LPS (Jung et al., 2014). However, very few reports have investigated the bioactive effects of berteroin, and there have been no attempts to use berteroin in the treatment of periodontitis.

The purpose of this study was to determine whether berteroin has an anti-inflammatory effect on HPDLCs as a basic study for the use of berteroin in the treatment of periodontitis. Our previous studies revealed that HPDLCs could produce various kinds of chemokines and adherent molecules (Hosokawa et al., 2021; Hosokawa et al., 2020). Therefore, we sought to determine the effects of berteroin on the production of chemokines (CCL2, CCL20, CXCL10, IL-8, and IL-6) and adhesion molecules (ICAM-1) that are involved in inflammatory cell infiltration and retention in HPDLCs stimulated by the inflammatory cytokines, IL-1β and TNF-α. We also examined the effect of berteroin on NF-κB and STAT3 activation, which is related to the production of these inflammatory mediators. We also investigated the effect of berteroin on the
expression of HO-1 and NQO1, antioxidant enzymes that are known to exhibit anti-inflammatory effects at the site of periodontitis lesions (Trivedi and Lal, 2017; Waddington et al., 2000).

Materials and Methods

Cell Culture

HPDLCs were obtained from Lonza Walkersville, Inc. (Walkersville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and antibiotics (Gibco; 100-units/ml penicillin G and 100-µg/ml streptomycin) at 37°C in a humidified 5% CO₂ atmosphere. The cells were taken for subculture when they were subconfluent using a 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco). The cells were used between passage numbers 5 and 10.

Cytotoxic Assay

Cell Count Reagent SF (Nakarai Tesque, Kyoto, Japan) was used to test cell viability. HPDLCs were seeded in 96-well cell culture plates, and cultured for 2 days. The media was withdrawn after 2 days, and 90 µL of DMEM containing various doses of berteroin (Cayman Chemical, Ann Arbor, MI, USA) was added. The cells were then cultured for an additional 24 hours. We added 10 µL of Cell Count Reagent SF, allowed the cells to remain for 2 hours, and used a microplate reader to detect the absorbance at 450 nm.

Enzyme-linked immunosorbent assay (ELISA)

HPDLCs were seeded into 24-well culture plates. After HPDLCs were subconfluent, IL-1β (10 ng/ml; Peprotech, Rocky Hill, NJ, USA) or TNF-α (10 ng/mL: Peprotech) with or without berteroin (1.5625, 3.125, 6.25, or 12.5 µM) was applied to HPDLCs for 24 hours. The concentrations of cytokines (CXCL10, CCL2, CCL20, IL-6, and IL-8) in HPDLCs culture supernatant were measured using DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN, USA) as directed by the manufacturer.

Protein extraction and immunoblot analysis

HPDLCs were cultured in 12-well cell culture plates, and total protein was collected in cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) after IL-1β (10 ng/ml) or TNF-α (10 ng/mL) stimulation for 15, 30, or 60 minutes with or without berteroin (6.25 or 12.5 µM) pretreatment for 1 hour, or IL-1β (10 ng/ml) or TNF-α (10 ng/mL) stimulation for 24 hours with or without berteroin (3.125, 6.25, or 12.5 µM). The protein concentrations in the lysates were determined using the BCA Protein Assay Kit (TaKaRa, Shiga, Japan). A similar quantity of protein was loaded onto a 4–20% SDS-polyacrylamide gel electrophoresis (PAGE) gel and electrotransferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hour at room temperature with 1% skim milk before being incubated with primary antibodies against Phospho-IKK-α/β (1/1000 dilution, Cell Signaling Technology), IKK-β (1/1000 dilution, Cell Signaling Technology), Phospho-NF-κB p65 (1/1000 dilution, Cell Signaling Technology), NF-κB p65 (1/1000 dilution, Cell Signaling Technology), Phospho-IκB-α (1/1000 dilution,
Cell Signaling Technology), IkB-α (1/1000 dilution, Cell Signaling Technology), phospho-STAT3 (1/2000 dilution, Cell Signaling Technology), STAT3 (1/2000 dilution, Cell Signaling Technology), ICAM-1 (1/1000 dilution, Cell Signaling Technology), HO-1 (1/1000 dilution, Cell Signaling Technology), NQO1 (1/1000 dilution, Cell Signaling Technology), or Glyceraldehyde-3-phosphate dehydrogenase (1/8000 dilution, Cell Signaling Technology) at 4°C overnight. The membranes were then washed and treated with a secondary antibody conjugated with horse radish peroxidase (HRP) (1/10000 dilution, Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 hour. Protein bands on X-ray images were detected using the ECL Prime Western-blotting detection system (Cytiva, Tokyo, Japan).

**Statistical Analysis**

In order to determine statistical significance, we performed one-way analysis of variance (ANOVA) followed by a post hoc Tukey-Kramer test, and p values less than 0.05 were considered significant.

**Results**

**Cell cytotoxicity of berteroin on HPDLCs**

We first examined the cytotoxic effects of berteroin on HPDLCs. The results showed that 25 µM of berteroin was cytotoxic, but concentrations of berteroin below 12.5 µM did not show cytotoxic effects (Fig. 1). Therefore, we decided to use berteroin at concentrations of 12.5 µM or less in this study.

**The effects of berteroin on cytokine production in IL-1β or TNF-α-stimulated HPDLCs**

We have previously reported that HPDLCs produce various cytokines and chemokines upon IL-1β or TNF-α stimulation (Hosokawa et al., 2021; Hosokawa et al., 2020). Therefore, we decided to investigate whether or not berteroin can suppress the production of these inflammatory cytokines. We found that CCL2, CCL20, CXCL10, IL-6, and IL-8 production induced by IL-1β or TNF-α stimulation was significantly suppressed in the presence of berteroin (Figs. 2 and 3).

**The effect of berteroin on ICAM-1 expression in IL-1β or TNF-α-stimulated HPDLCs**

Previous studies have shown that IL-1β and TNF-α stimulation induces ICAM-1 expression in HPDLCs (Joe et al., 2001; Lee et al., 2006). Therefore, we investigated whether berteroin can suppress ICAM-1 expression in HPDLCs. We found that IL-1β and TNF-α-induced ICAM-1 expression was clearly suppressed in the presence of berteroin (Fig. 4).

**The effect of berteroin on NF-κB activation in IL-1β or TNF-α-stimulated HPDLCs**
Our previous studies have shown that IL-1β or TNF-α stimulation activate the NF-κB pathway in HPDLCs (Hosokawa et al., 2021; Hosokawa et al., 2020). We have also shown that activation of the NF-κB pathway is involved in the production of various inflammatory mediators, including proinflammatory cytokines and adhesion molecules (Hosokawa et al., 2021; Hosokawa et al., 2020). Therefore, we investigated whether berteroin treatment affects IL-1β or TNF-α-induced activation of the NF-κB pathway in HPDLCs using western blot analysis. We found that 12.5 µM berteroin clearly inhibited the phosphorylation of IKK-α/β, NF-κB p65 and IκB-α, and the degradation of IκB-α in IL-1β or TNF-α-stimulated HPDLCs (Figs. 5 and 6).

The effect of berteroin on STAT3 phosphorylation in TNF-α-stimulated HPDLCs

We previously showed STAT3 activation is related to chemokines production in TNF-α-stimulated HPDLCs (Hosokawa et al., 2020). Therefore, we examined the effect of berteroin on STAT3 phosphorylation. Figure 7 clearly shows that berteroin treatment decreased the level of STAT3 phosphorylation in TNF-α-stimulated HPDLCs.

The effect of berteroin on HO-1 and NQO1 expression in HPDLCs

Antioxidant enzymes are known to play an important role in reducing inflammation in periodontitis (Trivedi and Lal, 2017). Therefore, we investigated whether berteroin can induce the expression of the antioxidant enzymes HO-1 and NQO1 in HPDLCs. The results showed that HO-1 and NQO1 expression in HPDLCs increased in a concentration-dependent manner upon berteroin treatment in IL-1β or TNF-α-stimulated HPDLCs (Fig. 8).

Discussion

Periodontal disease is an inflammatory disease associated with periodontopathogenic bacteria, and excessive immune responses have been reported to be related to periodontal tissue destruction (Cavalla et al., 2021). It has also been shown that certain leukocyte subsets are particularly involved in tissue destruction. The cellular inflammatory infiltration of T cells, B cells, macrophages, and neutrophils inside gingival connective tissue increases, as does inflammatory mediator production. These inflammatory cells also communicate with stromal cells including osteoblasts, periodontal ligament cells, and gingival fibroblasts (Hienz et al., 2015). Therefore, we focused on the expression of chemokines and adhesion molecules that are actively involved in leucocytes infiltration and retention. We found that berteroin decreased the expression of ICAM-1, one of the adhesion molecules, and some kinds of chemokines produced by HPDLCs. It has already been reported that berteroin suppresses IL-1β and TNF-α production in mouse macrophages stimulated by LPS (Jung et al., 2014). Since macrophages are also present at the site of periodontitis lesions (Yin et al., 2022), we hypothesize that administration of berteroin in periodontal lesions may rapidly attenuate inflammation in periodontitis lesions by exerting an anti-
inflammatory effect on both periodontal resident cells and inflammatory cells. Further investigations are necessary to prove this hypothesis.

We revealed that berteroin suppresses the activation of the NF-κB pathway by attenuating IKK-α/β, NF-κB p65, and IκB-α phosphorylation and IκB-α degradation in this study. Previous reports have shown that berteroin has the ability to inhibit the NF-κB pathway activation. Namely, Jung et al. reported that berteroin could inhibit translocation of NF-κB p65 to the nucleus (Jung et al., 2014). Judging from our report and previous reports, we believe that the anti-inflammatory effect of berteroin may be mainly due to its ability to inhibit the activation of the NF-κB pathway.

The breakdown of homeostatic equilibrium between ROS and antioxidant enzymes causes oxidative stress, which is known to worsen periodontal diseases as well as numerous inflammatory diseases (Trivedi and Lal, 2017; Waddington et al., 2000). Therefore, it is important in the treatment of periodontitis that antioxidant enzyme expression, such as HO-1 and NQO1, is elevated in the localized periodontitis lesions. In this study we found that berteroin upregulates HO-1 and NQO1 expression in HPDLCs. This result suggests that berteroin treatment might decrease ROS expression in periodontal lesions. In addition, since HO-1 has been reported to suppress NF-κB activation (Wang and He, 2022), the increase in HO-1 expression by berteroin may be involved in the suppression of NF-κB activation in HPDLCs.

Conclusions

In conclusion, this study demonstrated that berteroin had anti-inflammatory effects on HPDLCs. As a result, it is crucial to investigate if berteroin has the same effect on other constituent cells of periodontal tissue. Furthermore, in vivo investigations using animal models are thought to be required.

Declarations

Ethical Approval

not applicable.

Competing interests

The authors declare no conflicts of interest.

Author’s Contributions

Yoshitaka Hosokawa performed the experiments, analysed the data, and wrote the paper. Ikuko Hosokawa performed parts of the experiments and provided valuable suggestions for this study. Masahiro Shimoyama and Risa Okamoto performed parts of experiments. Kazumi Ozaki and Keiichi Hosaka contributed to the study conception and design. All authors read and approved the final manuscript.
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**Availability of data and materials**

The data of this study are available from the corresponding author upon reasonable request.

**References**


**Figures**

**Figure 1**

**The effect of berteroin on cell viability of HPDLCs.**

HPDLCs were seeded on 96-well cell culture plates, grown for 2 days, and then treated for 24 hours with berteroin (1.5625-25 mM). Cell Count Reagent SF was used to measure the viability of cells. The data is provided as the mean standard deviation of four separate experiments. * = \( P<0.05 \), significantly different from HPDLCs not treated with berteroin.
Figure 2

The effect of berteroin on IL-1β-induced production of cytokines.

HPDLCs were cultured in 24-well cell culture plates for 24 hours in the presence of IL-1β (10 ng/ml) with or without berteroin (1.5625-12.5 mM). The levels of CCL2, CCL20, CXCL10, IL-6, or IL-8 in the supernatant were measured using the ELISA kits indicated in the Materials and Methods section. The data are
presented as the mean SD of three independent experiments. * = P<0.05, significantly different from IL-1b-stimulated HPDLCs without berteroin.

Figure 3

The effect of berteroin on TNF-α-induced production of cytokines.
HPDLCs were cultured in 24-well cell culture plates for 24 hours in the presence of TNF-a (10 ng/ml) with or without berteroin (1.5625-12.5 mM). The levels of CCL2, CCL20, CXCL10, IL-6, or IL-8 in the supernatant were measured using the ELISA kits indicated in the Materials and Methods section. The data are presented as the mean SD of three independent experiments. * = P<0.05, significantly different from TNF-a-stimulated HPDLCs without berteroin.

![A. ICAM-1 and GAPDH expression](image1)

**B. ICAM-1 and GAPDH expression**

![B. ICAM-1 and GAPDH expression](image2)

**Figure 4**

**The effect of berteroin on ICAM-1 expression in IL-1b or TNF-a-stimulated HPDLCs** HPDLCs were grown in 12-well cell culture plates for 24 hours in the presence of IL-1b (10 ng/ml) or TNF-a (10 ng/ml) with or without berteroin (3.125, 6.25 or 12.5 mM). The lysates were extracted, and the expression of ICAM-1 was examined using Western blot analysis. (A) Fig.4A shows representative western blot image of the expression of ICAM-1 and GAPDH in IL-1b-stimulated HPDLCs. (B) Fig.4B shows representative western blot image of the expression of ICAM-1 and GAPDH in TNF-a-stimulated HPDLCs.
Figure 5

The effect of berteroin on the activation the NF-kB pathway in IL-1b-stimulated HPDLCs

HPDLCs were pretreated with or without berteroin (6.25 or 12.5 mM) for 1 hour before being stimulated with IL-1b (10 ng/ml) for 15, 30, or 60 minutes, and the phosphorylation of IKK-a/b, NF-kB p65 and IkB-α and the degradation of IkB-α were evaluated using western immunoblotting. Fig.5 shows that representative western blot images of the expression of phospho-IKK-α/β, total IKK-β, phospho-NF-κB p65, total NF-κB p65, phospho-IκB-α, IκB-α, and GAPDH.
Figure 6

The effect of berteroin on the activation the NF-kB pathway in TNF-a-stimulated HPDLCs

HPDLCs were pretreated with or without berteroin (6.25 or 12.5 mM) for 1 hour before being stimulated with TNF-a (10 ng/ml) for 15, 30, or 60 minutes, and the phosphorylation of IKK-a/b, NF-kB p65 and IKB-aand the degradation of IKB-a were evaluated using western immunoblotting. Fig.6 shows representative western blot images of the expression of phospho-IKK-a/b, total IKK-b, phospho-NF-kB p65, total NF-kB p65, phospho-IkB-a, IkB-a, and GAPDH.
Figure 7

**The effect of berteroin on the phosphorylation of STAT3 in TNF-α-stimulated HPDLCs**

HPDLCs were pretreated with or without berteroin (6.25 or 12.5 mM) for 1 hour before being stimulated with TNF-α (10 ng/ml) for 15, 30, or 60 minutes, and the phosphorylation of STAT3 was evaluated using western immunoblotting. Fig. 7 shows representative western blot images of the expression of phospho-STAT3, total STAT3, and GAPDH.
Figure 8

Effects of berteroin on HO-1 and NQO1 expression in IL-1b or TNF-a-stimulated HPDLCs

HPDLCs were grown for 24 hours in the presence of IL-1b (10 ng/ml) or TNF-a (10 ng/ml) with or without berteroin (3.125, 6.25 or 12.5 mM). The lysates were extracted, and the expression of HO-1 and NQO1 was investigated using Western blot analysis. (A) Fig.7A shows representative western blot image of the expression of HO-1, NQO1, and GAPDH in IL-1b-stimulated HPDLCs. (B) Fig.7B shows representative western blot image of the expression of HO-1, NQO1, and GAPDH in TNF-a-stimulated HPDLCs.