Inflammation, Vol. 42, No. 4, August 2019 (© 2019)
DOI: 10.1007/s10753-019-01007-z

ORIGINAL ARTICLE

Sudachitin Inhibits Matrix Metalloproteinase-1 and -3 Production in Tumor Necrosis Factor-α-Stimulated Human Periodontal Ligament Cells

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Abstract— Sudachitin, a polymethoxylated flavonoid found in the skin of *Citrus sudachi*, is a biologically active substance. The aim of this study was to examine whether sudachitin could be used to inhibit the expression of matrix metalloproteinase (MMP)-1 and MMP-3, which are involved in the destruction of periodontal tissues in periodontal lesions, in tumor necrosis factor (TNF)-α-stimulated human periodontal ligament cells (HPDLC). Sudachitin suppressed TNF-α-induced MMP-1 and MMP-3 production in HPDLC. On the other hand, it enhanced tissue inhibitor of metalloproteinase (TIMP)-1 expression. The level of Akt phosphorylation in the TNF-α-stimulated HPDLC was decreased by sudachitin treatment. Moreover, an Akt inhibitor reduced MMP-1 and MMP-3 production and increased TIMP-1 production. These findings indicate that sudachitin reduces MMP-1 and MMP-3 production in TNF-α-stimulated HPDLC by inhibiting the Akt pathway.

KEY WORDS: sudachitin; MMP; Akt; periodontal ligament cells.

INTRODUCTION

Periodontal disease is characterized by the destruction of the connective tissue between dental roots and alveolar bone. Host immune responses to the bacteria residing in dental plaque are involved in the pathogenesis of periodontal disease [1]. It is known that inflammatory mediators, such as matrix metalloproteinases (MMP) [2], cathepsin K [3], and receptor activator of NF-κB ligand (RANKL) [4], in periodontal lesions are involved in the destruction of periodontal tissues. Inflammatory mediators in tissues affected by periodontal disease are produced by not only the resident periodontal cells but also immune cells, such as neutrophils [5], monocytes [6], T cells, and B cells [7].

MMP play important roles in the progression of periodontal disease, because soft tissue destruction is mainly regulated by MMP [2]. Periodontal tissues are mainly formed of type 1 collagen. Therefore, collagenases, such as MMP-1, MMP-8, and MMP-9, are involved in soft tissue destruction in periodontal lesions [2, 8]. MMP-1 is known to be involved in both physiological and pathological tissue remodeling [9]. A recent study showed that patients with periodontitis exhibited higher gingival crevicular fluid MMP-1 levels than subjects with healthy periodontal tissue [10]. So, it is considered that increased

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MMP-1 levels in periodontal lesions promote the destruction of periodontal tissue. MMP-3 is also an essential enzyme because it activates latent MMP, including MMP-1 [11].

Sudachitin is a polymethoxyflavone, which has been isolated from the peel of *Citrus sudachi.* Japanese people use the juice of *C. sudachi* for various purposes, and *C. sudachi* is mainly grown in Tokushima Prefecture in Japan. A few reports have demonstrated that sudachitin has anti-cancer [12] or anti-inflammatory effects [13]. Abe et al. recently reported that sudachitin induced apoptosis in human HaCaT cells [12]. Yuasa et al. demonstrated that sudachitin suppressed tumor necrosis factor (TNF)-α production and inducible nitric oxide synthase expression in lipopolysaccharide-stimulated mouse macrophage-like RAW264 cells [13]. However, it is unclear whether sudachitin is able to regulate MMP expression.

The aim of this study was to examine the effects of sudachitin on MMP-1, MMP-3, and tissue inhibitor of metalloproteinase (TIMP)-1 production in TNF-α-stimulated human periodontal ligament cells (HPDLC), which are one of the types of cells that reside in periodontal tissues, and we previously reported that HPDLC produced MMP-1 and MMP-3. Moreover, we investigated whether sudachitin is able to modulate the activation status of mitogen-activated protein kinase (MAPK), nuclear factor (NF)-κB, and protein kinase B (Akt), which are activated by TNF-α stimulation in HPDLC [14, 15].

MATERIALS AND METHODS

Cell Culture

HPDLC were obtained from Lonza Walkersville, Inc. (Walkersville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (100-units/ml penicillin G and 100-μg/ml streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere. The cells were used between passage numbers 5 and 10.

The Release of MMP-1, MMP-3, and TIMP-1 from HPDLC

The HPDLC were stimulated with recombinant human TNF-α (10 ng/ml; PeproTech, Rocky Hill, NJ, USA) for 24 h. The supernatants from the HPDLC were collected, and the MMP-1, MMP-3, and TIMP-1 concentrations of the culture supernatants were measured in triplicate with enzyme-linked immunosorbent assays (ELISA), which were created using the DuoSet system (R&D Systems, Minneapolis, MN, USA). All assays were performed according to the manufacturer's instructions, and MMP levels were determined using the standard curve prepared for each assay. In selected experiments, the HPDLC were cultured for 1 h in the presence or absence of sudachitin (6.25, 12.5, 25, or 50 μg/ml; Wako Pure Chemical Corporation, Osaka, Japan) or 10-[4′-(*N*,*N*-diethylamino)butyl]-2-chlorophenoxazine (10DEBC) hydrochloride (1 μM; Santa Cruz Biotechnology, Santa Cruz, CA, USA), before being incubated with TNF-α (10 ng/ml).

Western Blot Analysis

To confirm that TNF-α induced the phosphorylation of signal transduction molecules, Western blot analysis was performed. In this experiment, some of the HPDLC were pretreated with sudachitin (25 or 50 μg/ml) for 1 h, whereas others were not. Then, the HPDLC were stimulated with TNF-α (10 ng/ml) and washed once with cold phosphate-buffered saline, before being incubated on ice for 30 min with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). After the removal of debris *via* centrifugation, the protein concentrations of the lysates were quantified *via* the Bradford protein assay using IgG as a standard. Twenty-μg protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4–20% gel, before being transferred to polyvinylidene difluoride membranes. After being blocked with 1% skimmed milk, the membranes were probed with phospho-p38 MAPK rabbit monoclonal antibody (Cell Signaling Technology), phospho-extracellular signal-regulated kinase (ERK) rabbit monoclonal antibody (Cell Signaling Technology), phospho-c-Jun N-terminal kinase (JNK) rabbit monoclonal antibody (Cell Signaling Technology), phospho-NF-κB p65 rabbit monoclonal antibody (Cell Signaling Technology), phospho-Akt rabbit monoclonal antibody (Cell Signaling Technology), p38 MAPK rabbit monoclonal antibody (Cell Signaling Technology), ERK rabbit monoclonal antibody (Cell Signaling Technology), JNK rabbit monoclonal antibody (Cell Signaling Technology), NF-κB p65 rabbit monoclonal antibody (Cell Signaling Technology), IκB-α mouse monoclonal antibody (Cell Signaling Technology), Akt mouse monoclonal antibody (Cell Signaling Technology), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit monoclonal antibody (Cell Signaling Technology). The immunoreactive bands were visualized by incubating them with the horseradish peroxidase-conjugated

secondary antibody (Sigma) and then detected using the ECL system (GE Healthcare, Uppsala, Sweden).

Statistical Analysis

Statistical significance was analyzed using the Student's *t* test. *P* values of < 0.05 were considered significant in the analyses shown in Figs. 1 and 5.

RESULTS

The Effects of Sudachitin on MMP-1, MMP-3, and TIMP-1 Production in TNF-α-Stimulated HPDLC

We examined the effects of sudachitin on MMP production because MMP are important for the initiation and progression of periodontal disease. Figure 1 shows that sudachitin inhibited MMP-1 and MMP-3 production in TNF-α-stimulated HPDLC in a dose-dependent manner. Moreover, we found that 50 μM of sudachitin significantly enhanced TIMP-1 production.

Effects of Sudachitin on MAPK Activation in TNF-α-Stimulated HPDLC

We previously reported that the p38 MAPK, ERK, and JNK pathways are involved in MMP-1 and MMP-3 production in TNF-α-stimulated HPDLC [14]. Therefore, we examined whether sudachitin modulated MAPK phosphorylation in TNF-α-stimulated HPDLC. Figure 2 demonstrates that sudachitin did not affect the levels of MAPK phosphorylation in TNF-α-stimulated HPDLC, which was contrary to our expectations.

Effects of Sudachitin on NF-κB Activation in TNF-α-Stimulated HPDLC

Some previous reports have found that the NF-κB pathway is involved in MMP-1 and MMP-3 production in HPDLC [16, 17]. Therefore, we considered that we should examine the effects of sudachitin on NF-κB activation in TNF-α-stimulated HPDLC. In particular, we investigated NF-κB p65 phosphorylation and IκB-α degradation in this experiment. Figure 3 shows that sudachitin did not modulate NF-κB p65 phosphorylation or IκB-α degradation in TNF-α-stimulated HPDLC.

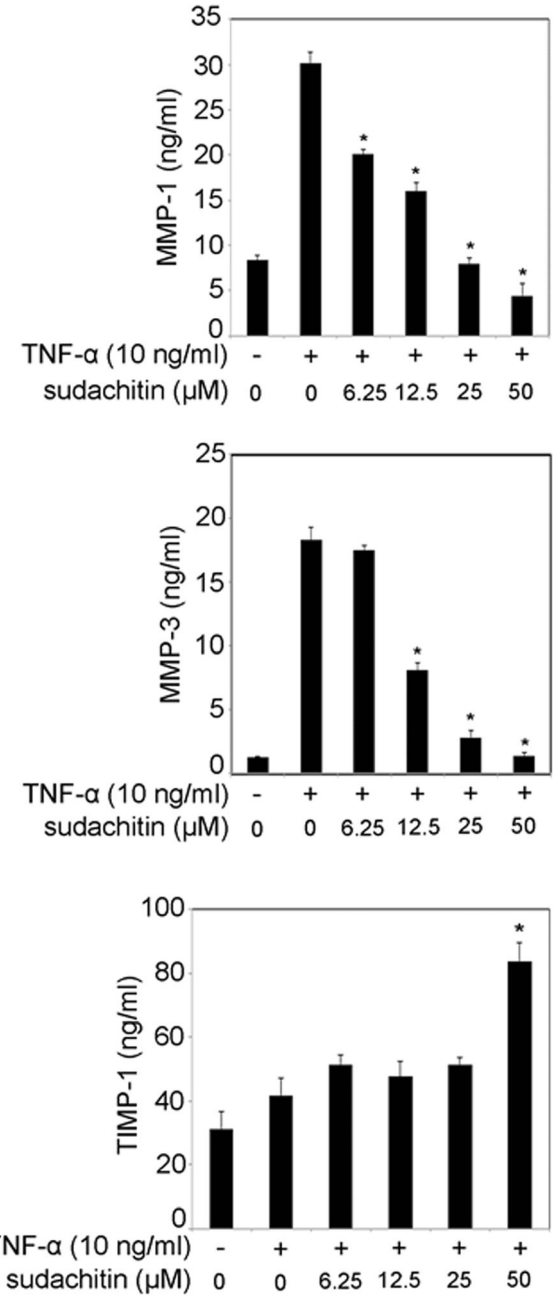

Fig. 1. Effects of sudachitin on MMP-1, MMP-3, and TIMP-1 production in TNF-α-stimulated HPDLC. HPDLC were pretreated with sudachitin (6.25, 12.5, 25, or 50 μM) for 1 h, before being stimulated with TNF-α (10 ng/ml). The supernatants were collected after 24 h. The expression levels of MMP-1, MMP-3, and TIMP-1 in the supernatants were measured using ELISA. The results are shown as the mean and standard deviation (SD) of one representative experiment performed in triplicate. The error bars represent SD. **P* < 0.01 significantly different from the result for the TNF-α-stimulated HPDLC that were not treated with sudachitin.

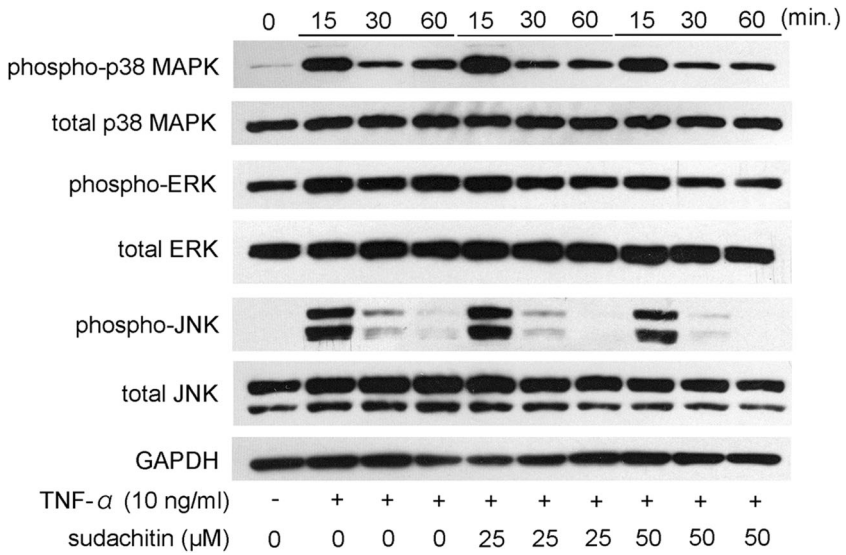

Fig. 2. Effects of sudachitin on TNF-α-induced p38 MAPK, ERK, and JNK phosphorylation in HPDLC. The cultured cells were pretreated with sudachitin (25 or 50 μM) for 1 h and then stimulated with 10 ng/ml of TNF-α for 15, 30, or 60 min. The cell extracts were subjected to SDS-PAGE followed by Western blotting analysis with antibodies against phospho-specific p38 MAPK, p38 MAPK, phospho-specific ERK, ERK, phospho-specific JNK, JNK, and GAPDH. Each photograph is representative of the results of three separate experiments.

The Effects of Sudachitin on Akt Activation in TNF-α-Stimulated HPDLC

There are no previous reports about the role of Akt activation in MMP-1 or MMP-3 production in HPDLC. However, it has been reported that the Akt pathway positively regulates MMP-1 and MMP-3 expression in human dermal fibroblasts [18]. Therefore, we expected that sudachitin might modulate Akt activation in TNF-α-stimulated HPDLC. Figure 4 clearly shows that sudachitin treatment reduced the level of Akt phosphorylation.

The Effects of an Akt Inhibitor on MMP-1, MMP-3, and TIMP-1 Expression in TNF-α-Stimulated HPDLC

We found that sudachitin reduced Akt phosphorylation in TNF-α-stimulated HPDLC. However, it is unclear whether Akt activation contributes to MMP-1, MMP-3, or TIMP-1 production. So, we used an Akt inhibitor to examine the role of Akt phosphorylation in MMP-1, MMP-3, and TIMP-1 production. Figure 5 shows that the Akt inhibitor significantly inhibited MMP-1 and MMP-3 production, whereas it enhanced TIMP-1 production. This

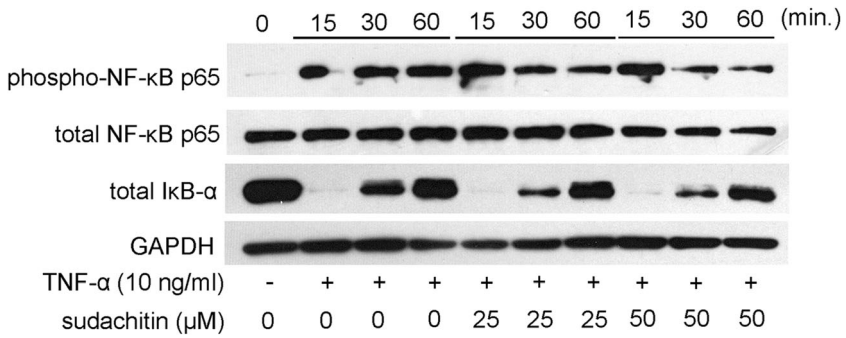

Fig. 3. Effects of sudachitin on TNF-α-induced NF-κB p65 phosphorylation and IκB-α degradation in HPDLC. The cultured cells were pretreated with sudachitin (25 or 50 μM) for 1 h and then stimulated with 10 ng/ml of TNF-α for 15, 30, or 60 min. The cell extracts were subjected to SDS-PAGE followed by Western blotting analysis with antibodies against phospho-specific NF-κB p65, NF-κB p65, IκB-α, and GAPDH. Each photograph is representative of the results of three separate experiments.

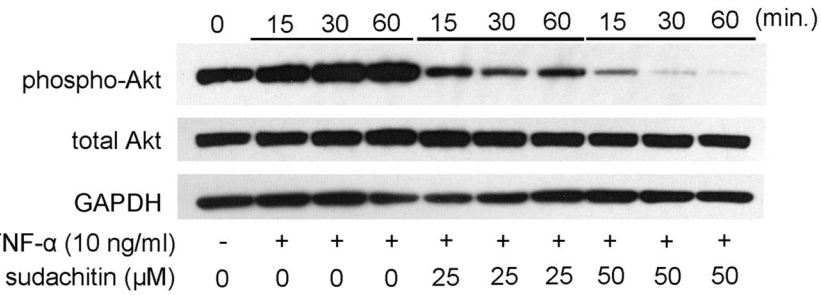

Fig. 4. Effects of sudachitin on TNF-α-induced Akt phosphorylation in HPDLC. The cultured cells were pretreated with sudachitin (25 or 50 μM) for 1 h and then stimulated with 10 ng/ml of TNF-α for 15, 30, or 60 min. The cell extracts were subjected to SDS-PAGE followed by Western blotting analysis with antibodies against phospho-specific Akt, Akt, and GAPDH. Each photograph is representative of the results of three separate experiments.

result indicates that the Akt pathway positively regulates MMP-1 and MMP-3 expression and negatively regulates TIMP-1 expression in TNF-α-stimulated HPDLC. Judging from the data shown in Figs. 4 and 5, we consider that the inhibition of Akt activation by sudachitin in TNF-α-stimulated HPDLC involves increases in MMP-1 and MMP-3 production and a reduction in TIMP-1 production.

DISCUSSION

In this study, we found that sudachitin inhibited MMP-1 and MMP-3 production in TNF-α-stimulated HPDLC. MMP-1 and MMP-3 are able to break down type 1 collagen. It is known that MMP-1 and MMP-3 are involved in the destruction of periodontal soft tissues in periodontal lesions [1, 11], and MMP-1 and MMP-3 expression is enhanced in diseased periodontal tissue [19, 20]. So, the inhibition of MMP-1 and MMP-3 expression in periodontal lesions is important for preventing the initiation and progression of periodontal disease. Ohyama et al. recently reported that sudachitin inhibited osteoclast formation in cultures of isolated osteoblasts and osteoclast precursors [21]. This suggests that sudachitin could be used to inhibit alveolar bone resorption in periodontal lesions. Judging from our findings and those obtained in Ohyama's study, the topical application of sudachitin to periodontal lesions might help to prevent the breakdown of both alveolar bone and periodontal soft tissue by inhibiting osteoclast differentiation and MMP production.

In the current study, we demonstrated that MMP-1 and MMP-3 play important roles in the destruction of periodontal tissues. In addition, MMP-8 and MMP-9 are also involved in the progression of periodontal disease. MMP-8 and MMP-9 are secreted by neutrophils and monocytes in inflammatory lesions and induce connective tissue destruction in periodontitis [8]. We should examine the effects of sudachitin on MMP-8 and MMP-9 production in inflammatory cells in our next study.

We also found that sudachitin inhibited Akt phosphorylation in TNF-α-stimulated HPDLC. This is the first study to examine the effects of sudachitin on Akt activation. However, some previous studies investigated the effects of polymethoxylated flavonoids from other citrus fruits on Akt activation. For example, nobiletin from *Citrus depressa* and *Citrus reticulate* inhibited Akt activation in human ovarian cancer cells [22]. Furthermore, tangeretin from *Citrus tangerine* or sweet oranges inhibited Akt activation in platelet-derived growth factor-BB-treated rat aortic smooth muscle cells [23]. We hypothesize that polymethoxylated flavonoids from citrus fruits generally have inhibitory effects on Akt activation. In a future study, we will examine the effects of various polymethoxylated flavonoids to further investigate our hypothesis.

We demonstrated that Akt inhibition reduced MMP-1 and MMP-3 expression but enhanced TIMP-1 expression. A previous study showed that Akt inhibitors reduced MMP-1 and MMP-3 secretion in ultraviolet B-stimulated human dermal fibroblasts [18]. Moreover, Zhou et al. reported that Akt inhibition enhanced TIMP-1 production in interleukin 13 and transforming growth factor-β1-stimulated human airway fibroblasts [24]. Our findings are similar to these results. However, we consider that this is the first study to simultaneously examine the effects of Akt inhibition on MMP and TIMP expression. So, we consider that the Akt pathway has a marked influence on the MMP/TIMP ratio in inflammatory lesions.

We demonstrate that sudachitin treatment did not modulate MAPK or NF-κB activation in TNF-α-stimulated HPDLC. A previous study showed that sudachitin treatment inhibited JNK, ERK, and IκB-α phosphorylation in RANKL-stimulated mouse bone marrow

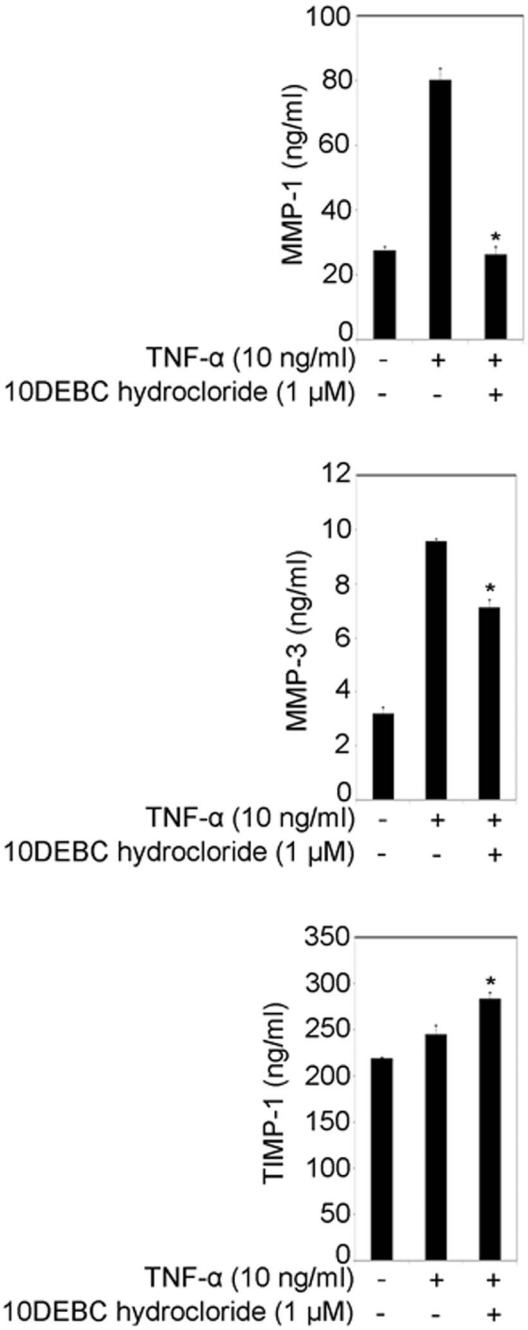

Fig. 5. Effects of an Akt inhibitor on TNF-α-stimulated MMP-1, MMP-3, or TIMP-1 production in HPDLC. The cells were preincubated with 10DEBC hydrochloride (1 μM) for 1 h and then incubated with TNF-α (10 ng/ml). After 24 h' incubation, the supernatants were collected, and MMP-1, MMP-3, and TIMP-1 production was measured by ELISA. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars represent SD. *$P < 0.01$ significantly different from the result for the TNF-α-stimulated HPDLC that were not treated with the Akt inhibitor.

cells [21]. We consider that the discrepancies between our findings and those of the abovementioned study were caused by differences in the cells, stimulation time, and sudachitin concentration used.

In summary, we demonstrated that sudachitin inhibited MMP-1 and MMP-3 production in TNF-α-stimulated HPDLC. People in Japan use the juice of *C. sudachi* for various purposes, but the peel of *C. sudachi* is usually thrown away. Sudachitin is present in the peel of *C. sudachi* [25]; therefore, it could be obtained from both the juice and peel of *C. sudachi*. Further investigations, including an *in vivo* study, are needed to examine the use of sudachitin as a treatment for periodontal disease.

FUNDING INFORMATION

This study was supported by JSPS KAKENHI (grant numbers: 16K11834 and 18K09600).

COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of Interest. The authors declare that they have no conflicts of interest.

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