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ORIGINAL ARTICLE

Carnosic Acid Inhibits CXCR3 Ligands Production in IL-27-Stimulated Human Oral Epithelial Cells

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Abstract— Carnosic acid, which is a bioactive compound isolated from rosemary, has various pharmacological effects. However, the anti-inflammatory effect of carnosic acid on periodontitis is still unknown. The aim of this study was to investigate the effect of carnosic acid on CXC chemokine receptor 3 (CXCR3) ligands, which are involved in Th1 cells migration and accumulation, production in interleukin (IL)-27-stimulated human oral epithelial cells (TR146 cells). Carnosic acid decreased CXC chemokine ligand (CXCL)9, CXCL10, and CXCL11 production in IL-27-stimulated TR146 cells in a dose-dependent fashion. Moreover, we disclosed that carnosic acid could suppress signal transducer and activator of transcription (STAT)1, STAT3, and protein kinase B (Akt) phosphorylation in IL-27-stimulated TR146 cells. Furthermore, STAT1, STAT3, and Akt inhibitors could suppress CXCR3 ligands production in IL-27-treated TR146 cells. In summary, carnosic acid could reduce CXCR3 ligands production in human oral epithelial cell by inhibiting STAT1, STAT3, and Akt activation.

KEY WORDS: carnosic acid; anti-inflammatory effect; oral epithelial cells.

INTRODUCTION

Periodontal disease is the most common infectious disease in oral cavity, and inflammation in gum tissues is caused by oral bacteria. Excessive inflammation in periodontal tissues could destroy periodontal soft tissues and alveolar bone [1]. There are several reports that Th1 cells in periodontal lesion are involved in alveolar bone resorption [2–4], and CXCR3 ligands are related with Th1 cells accumulation [5]. We recently reported that IL-27 could induce CXCR3 ligands (CXCL9, CXCL10, and CXCL11) production in human oral epithelial cells (TR146 cells) [6]. Next, we would like to find the compound that could suppress CXCR3 ligands production in periodontal lesion for periodontal disease treatment.

Rosemary is an herb whose leaves are used to flavor foods all over the world. Carnosic acid (CA), which is a phenolic diterpene isolated from rosemary (*Rosmarinus officinalis*), has been reported to have various biological properties such as antioxidant [7], antimicrobial [8], anticancer [9], or anti-adipogenic activities [10]. However, there is not the trial that is going to use rosemary for periodontal disease treatment. Moreover, the anti-inflammatory effect of CA on periodontal resident cells is still uncertain.

We hypothesized CA could inhibit inflammatory cytokine production in periodontal lesion. The aim of this study was to examine the effect of CA on CXCR3 ligand production in IL-27-stimulated human oral epithelial cells. We used TR146 cells in this experiment

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because TR146 cells show some morphological similarities to the human oral epithelial cells [11]. Moreover, we examined whether CA treatment inhibit the activation of signal transducer and activator of transcription (STAT)1, STAT3, or protein kinase B (Akt) pathway in IL-27-stimulated TR146 cells.

MATERIALS AND METHODS

Cell Culture

TR146 cells, which are a human oral epithelial cell line, were kindly provided by Dr. Mark Herzberg (University of Minnesota, MN, USA). TR146 cells were cultured in Ham's F12 medium (Nakarai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 1 mmol/L sodium pyruvate (Gibco, Grand Island, MI, USA), 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.

CXCL9, CXCL10, and CXCL11 Production in TR146 Cells

We previously reported that IL-27 (10 ng/mL) significantly induce CXCR3 ligands in TR146 cells [6]. Therefore, TR146 cells were stimulated with recombinant human IL-27 (10 ng/mL; PeproTech, Rocky Hill, NJ, USA) for 24 h in this experiment. The supernatants of TR146 cells were collected and stored − 80 °C until use, and CXCL9, CXCL10, and CXCL11 concentration was measured in triplicate using enzyme-linked immunosorbent assays (ELISA). DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to obtain these measurements. All assays were performed according to the manufacturer's instructions, and CXCL9, CXCL10, and CXCL11 levels were determined using the standard curve prepared for each assay. In selected experiments, the TR146 cells were cultured for 1 h in the presence or absence of CA (3.125, 6.25, 12.5, 25, or 50 μM; Abcam, Cambridge, UK), fludarabine (50 μM; Cayman Chemical, Ann Arbor, MI, USA), WP1066 (5 μM; Santa Cruz Biotechnology, Dallas, TX, USA), or 10-DEBC hydrochloride (1 μM; Santa Cruz Biotechnology), prior to the incubation with IL-27.

Western Blot Analysis

To determine the IL-27-induced phosphorylation of signal transduction molecules, western blot analysis was performed. TR146 cells stimulated by IL-27 (10 ng/mL) with or without CA (25 μM) pretreatment for 1 h were washed once with cold PBS, before being incubated on ice for 10 min with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease inhibitors cocktail (Sigma, St. Louis, MO, USA). After removal of debris by centrifugation, the protein concentrations of the lysates were quantified with the Bradford protein assay using IgG as a standard. A 20-μg protein sample was loaded onto a 4–20% SDS-PAGE gel, before being electroblotted onto a PVDF membrane. The phosphorylation of STAT1, STAT3, and Akt was assessed using phospho-STAT1 rabbit monoclonal antibody (Cell Signaling Technology), phospho-STAT3 rabbit monoclonal antibody (Cell Signaling Technology), phospho-Akt rabbit monoclonal antibody (Cell Signaling Technology), STAT1 rabbit monoclonal antibody (Cell Signaling Technology), STAT3 mouse monoclonal antibody (Cell Signaling Technology), or Akt mouse monoclonal antibody (Cell Signaling Technology) according to the manufacturer's instructions. Protein bands were visualized by incubation with the HRP-conjugated secondary antibody (Sigma), followed by detection using the ECL Prime Western Blotting Detection System (GE Healthcare, Uppsala, Sweden). Luminescence of the membranes was quantified by ImageJ (version 1.44) software.

Statistical Analysis

Statistical significance was analyzed using the Student's *t* test. P values < 0.05 were considered significant in Figs. 1 and 3.

RESULTS

Effects of CA on CXCR3 Ligand Production in IL-27-Stimulated TR146 Cells

At first, we examined if CA could decrease CXCR3 ligands production in TR146 cells because CXCR3 ligands are important chemokine in the

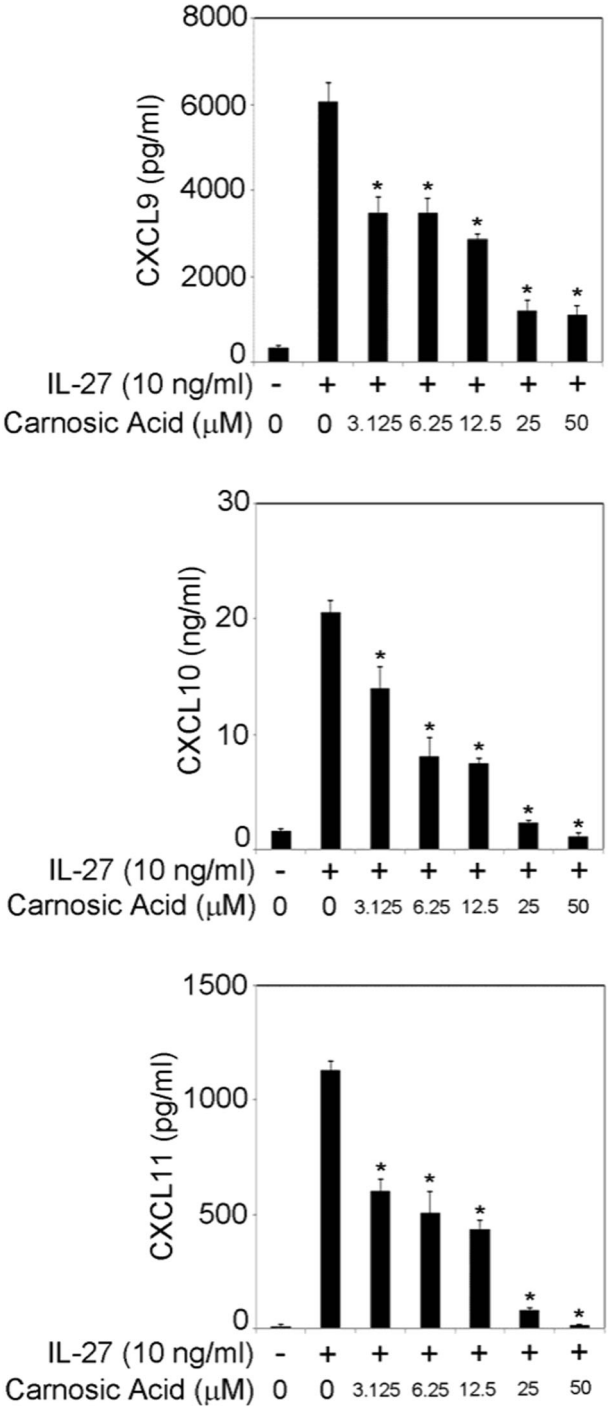

Fig. 1. Effects of carnosic acid on CXCL9, CXCL10, and CXCL11 production in IL-27-stimulated TR146 cells. TR146 cells were prestimulated with carnosic acid (3.125, 6.25, 12.5, 25, or 50 μM) for 1 h, and then TR146 cells were treated with IL-27 (10 ng/mL), and the supernatants were collected after 24 h. The production levels of CXCL9, CXCL10, and CXCL11 in the supernatants were measured by ELISA. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show the SD of the values. $^*P < 0.05$, significantly different from the IL-27-stimulated TR146 cells without carnosic acid.

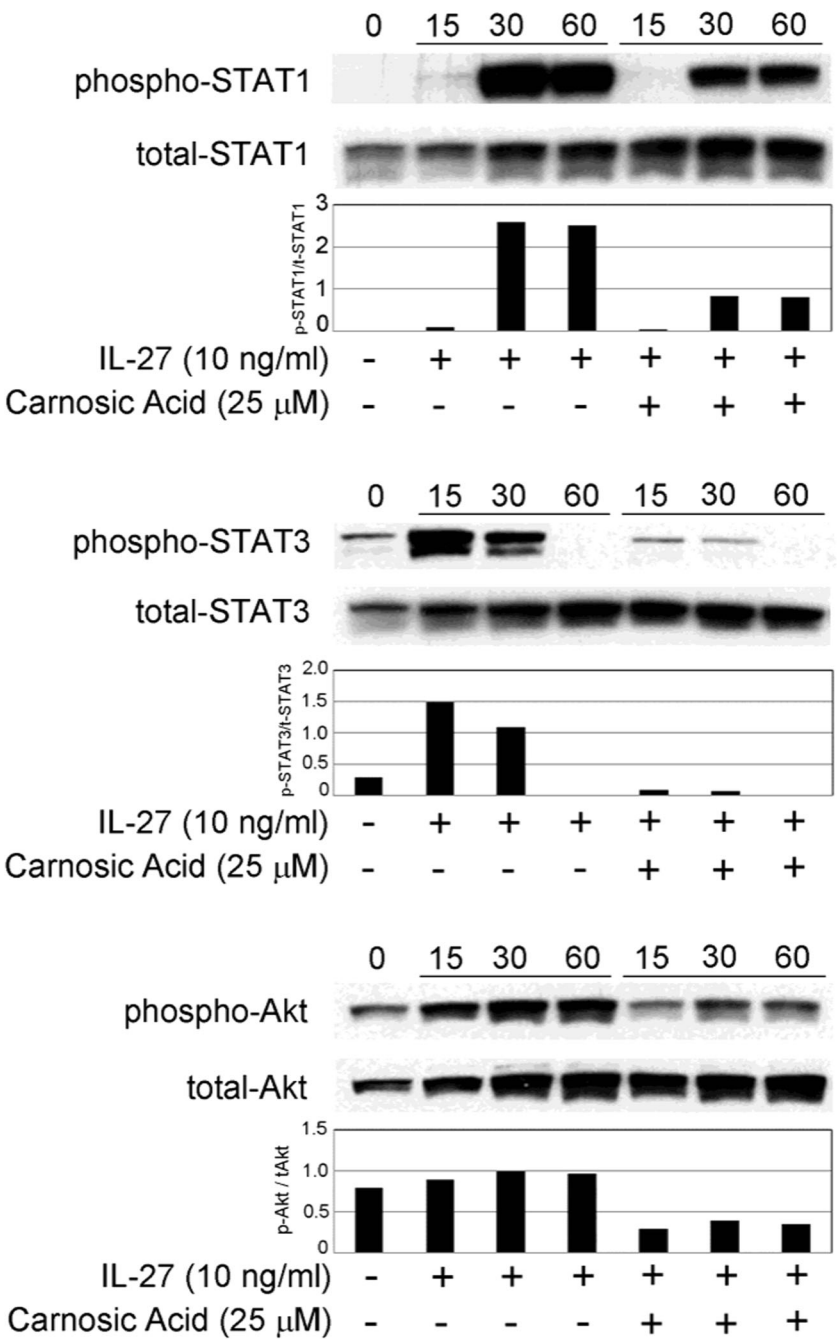

Fig. 2. Effects of carnosic acid on STAT1, STAT3, and Akt phosphorylation in IL-27-stimulated TR146 cells. TR146 cells were pretreated with carnosic acid (25 μM) for 1 h, and then the TR146 cells were stimulated with IL-27 (10 ng/mL), and proteins were collected after 15, 30, or 60 min. Expressions of phospho-STAT1, total STAT1, phospho-STAT3, total STAT3, phospho-Akt, or total-Akt were determined by western blot analysis. We show a representative data from three independent experiments.

pathogenesis of periodontal disease [12]. Figure 1 clearly shows that CA reduced CXCL9, CXCL10, and CXCL11 production in IL-27-stimulated TR146 in a dose dependent manner.

Effects of CA on STAT1, STAT3, and Akt Activations in IL-27-Stimulated TR146 Cells

We previously revealed IL-27 could activate STAT1, STAT3, and Akt pathways in TR146 cells [6]. Therefore, we examined the effect of CA on phosphorylation of STAT1, STAT3, and Akt in IL-27-stimulated TR146 cells. Figure 2 shows CA (25 μM) decreased the level of phosphorylation of STAT1, STAT3, and Akt in IL-27-treated TR146 cells.

Effects of STAT1, STAT3, and Akt Inhibitors on CXCL9 Production in IL-27-Stimulated TR146 Cells

We previously reported that STAT1, STAT3, and Akt pathways are involved in CXCL10 and CXCL11 production in IL-27-stimulated TR146 cells [13]. However, it is uncertain if these signal transduction pathways are related to CXCL9 production. Figure 3 shows that pretreatment with fludarabine (a STAT1 inhibitor), WP1066 (a STAT3 inhibitor), and 10-DEBC hydrochloride (an Akt inhibitor) significantly suppressed CXCL9 production in IL-27-treated TR146 cells. Judging from our previous report and this report, STAT1, STAT3, and Akt pathways are important for CXCR3 ligands production in IL-27-stimulated TR146 cells.

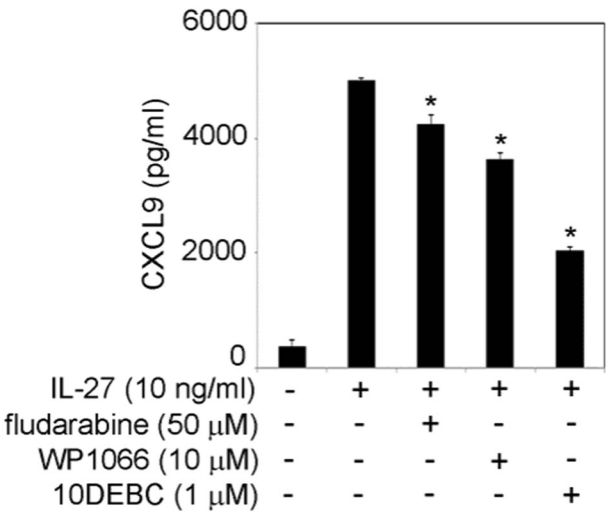

Fig. 3. Effects of a STAT1 inhibitor, a STAT3 inhibitor, and an Akt inhibitor on CXCL9 production from IL-27-stimulated TR146. TR146 cells were pretreated with fludarabine (a STAT1 inhibitor, 50 μM), WP1066 (a STAT3 inhibitor, 10 μM), or 10-DEBC hydrochloride (an Akt inhibitor, 1 μM) for 1 h, and then TR146 cells were stimulated with IL-27 (10 ng/mL), and the supernatants were collected after 24 h. The production level of CXCL9 in the supernatants was measured by ELISA. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show the SD of the values. $*P < 0.05$, significantly different from the IL-27-stimulated TR146 cells without chemical inhibitors.

DISCUSSION

Th1 cells could promote alveolar bone loss in periodontal lesion because Th1 cells could induce proinflammatory mediators and RANKL activation [4]. So, it is important to decrease the number of Th1 cells in periodontally diseased tissues. CXCR3 ligands (CXCL9, CXCL10, and CXCL11) are involved in migration of Th1 cells in inflammatory lesion [5], and we previously reported that periodontal resident cells could produce CXCR3 ligands. Proinflammatory cytokines, such as TNF-α, IFN-γ, oncostatin M, could induce CXCL10 production in human gingival fibroblasts [14–16]. We recently showed IL-27 could induce CXCL10 and CXCL11 production in TR146 cells [13]. In this report, we found CA could decrease CXCL9, CXCL10, and CXCL11 production. This result might explain that topical application of CA might inhibit alveolar bone resorption in periodontal lesion because the number of Th1 cells might be decreased. We should use animal model to prove this hypothesis.

Other researchers examined the anti-inflammatory effects of CA. Liu et.al recently reported that CA could inhibit TNF-α, IL-1β, IL-6, IL-8, and IL-17 production in human fibroblast-like synoviocytes [17]. It has been reported that human gingival fibroblasts and human periodontal ligament cells could produce high amount of proinflammatory cytokines, such as IL-6 and IL-8 [18, 19]. It is known that proinflammatory cytokines produced from these cells worsen periodontal disease [1]. CA might inhibit proinflammatory cytokines in gingival fibroblasts and periodontal ligament cells because CA decreased the production of several cytokines in synoviocytes. We think further examinations using gingival fibroblast and periodontal ligament cells are important because CA could contact gingival fibroblast and periodontal ligament cells as well as epithelial cells when we apply CA in periodontal pockets.

We reported STAT1, STAT3, and Akt pathways could control CXCL10 and CXCL11 production in IL-27-stimulated TR146 cells [13]. We had to examine the effect of CA on STAT1, STAT3, and Akt activation in this experiment. It has been reported that CA could inhibit the activation of STAT3 in renal and colon cancer cells [20]. Moreover, CA could decrease the phosphorylation level of Akt in human prostate cancer cell [21]. So, our reports about STAT3 and Akt are

similar with previous reports. However, we could not find the report that examined the effect of CA on STAT1 activation. So, this report is the first report that CA could inhibit STAT1 activation. It is certain that Th1-type cytokine, such as IFN-γ, could activate STAT1 pathway. So, CA might effectively reduce the level of inflammation in Th1-type disease.

In summary, CA could suppress CXCL9, CXCL10, and CXCL11 production in IL-27-treated human oral epithelial cells (TR146 cells) by inhibiting the activation of STAT1, STAT3, and Akt pathways. So, topical application of CA might reduce the accumulation of Th1 cells in periodontal lesion.

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COMPLIANCE WITH ETHICAL STANDARDS

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

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