Ropinirole inhibits inflammatory cytokine production in gingival epithelial cells and suppresses alveolar bone loss in an experimental rat model of periodontitis

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Abstract. The present study explored whether the dopamine 2-like receptor agonist, ropinirole, a drug used for treating Parkinson's disease, suppresses neutrophilic inflammation and alveolar bone loss in an experimental rat model of periodontitis. Periodontitis is a neutrophilic inflammatory disease caused by periodontal pathogens. An excessive T helper (Th)17 immune response is involved in the progression of periodontitis, and interleukin (IL)-17 promotes the exacerbation of inflammation and alveolar bone destruction. Recent evidence has suggested that dopamine signaling plays a key role in Th17 cell differentiation, and that dopamine 2-like receptor agonists suppress cytokine production from Th17 cells. We previously demonstrated that tannic acid, which is a dopamine 2-like receptor agonist, inhibits alveolar bone resorption in an experimental model of periodontitis. The present study used a carrageenan-induced rat model of periodontitis with or without ropinirole. Micro-computed tomography analysis was performed. Cells of the murine gingival epithelial cell line GE1 were stimulated with carrageenan and IL-17A in the presence or absence of ropinirole. The anti-inflammatory effect of ropinirole was analyzed using reverse transcription- quantitative PCR and enzyme-linked immunosorbent assay. Subsequently, in the carrageenan-induced rat model of periodontitis, alveolar bone resorption was observed in the maxillary second molar by micro-computed tomography analysis. Intriguingly, ropinirole suppressed the alveolar bone destruction. The expression levels of C-X-C motif chemokine ligand 1 (CXCL1) and IL-17 receptor A (IL-17RA) in GE1 cells were increased by carrageenan, and CXCL1 expression in GE1 cells was upregulated under IL-17A stimulation. Moreover, ropinirole inhibited CXCL1 and IL-17RA expression in GE1 cells in the presence of IL-17A and carrageenan. Finally, haloperidol promoted CXCL1 expression in GE1 cells in the presence of carrageenan. Overall, these findings suggested that ropinirole suppressed neutrophilic inflammation and alveolar bone destruction in periodontitis by inhibiting CXCL1 expression in gingival epithelial cells through the dopamine 2-like receptor. Thus, ropinirole shows promise as a drug for the treatment of periodontitis.

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Abbreviations: IL, interleukin; CXCL1, C-X-C motif chemokine ligand 1; IL-17RA, IL-17 receptor A; cAMP, cyclic adenosine monophosphate; RT-qPCR, reverse transcription-quantitative PCR; PBS, phosphate-buffered saline; CA, carrageenan; RP, ropinirole; CT, computed tomography

Key words: periodontitis, IL-17, CXCL1, neutrophil inflammation, ropinirole, dopamine receptor

Introduction

Periodontitis is a chronic inflammatory disease characterized by the loss of periodontal attachment and alveolar bone due to bacterial infection. Neutrophils are the key immune cells involved in periodontitis (1). Gingival epithelial cells produce chemokines, such as interleukin (IL)-8 to recruit neutrophils, and periodontal pathogens stimulate dendritic cells via Toll-like receptors, resulting in the induction of the Th1/Th17 subset. Th17 cells are critical mediators of alveolar bone destruction in periodontitis, and gingival epithelial cells possess IL-17 receptor (IL-17R), to which IL-17 binds to induce IL-8 production (2-4). Although rodents lack a direct homologue of IL-8, the C-X-C motif chemokine ligand 1 (CXCL1) is regarded as a functional homologue of IL-8 (5).

The main approach of current periodontal therapy is the physical excision of lesions (6). Scaling and root planing are treatments for removing plaque and calculus from the tooth surface, while periodontal surgery is debridement for excising infected granulation tissue. For deep intrabone defects, it is beneficial to use materials, such as membranes for guided tissue regeneration, enamel matrix derivatives, and fibroblast

growth factor-2 (7,8). Additionally, pharmacological therapies are used as adjunctive treatments (7). Chlorhexidine and sustained-release minocycline are locally applied treatments (9,10). For systemic treatments, antibiotics are often used for short periods of time. However, few reports are available on the development of pharmacological treatments for mitigating inflammation.

We have previously demonstrated that dopamine signaling plays a crucial role in Th17 cell differentiation (11,12). There are five subtypes of dopamine receptors, D1 to D5, and these subtypes are classified into two groups: D1-like receptors and D2-like receptors. D1-like receptors, *i.e.,* D1 and D5, induce an increase in intracellular cyclic adenosine monophosphate (cAMP), whereas D2-like receptors, *i.e.,* D2, D3, and D4, induce a decrease in intracellular cAMP (13). In our previous studies, D1-like receptor antagonists inhibited Th17 differentiation, and attenuated neutrophilic inflammation caused by diseases in animal models, such as experimental autoimmune encephalomyelitis, autoimmune diabetes, and nephrotoxic serum nephritis models (11,14,15). It has been reported that Th17 cells markedly produced IL-8, and that the IL-8 production from activated T cells was suppressed by D2-like receptor agonists (16,17), indicating that D2-like receptor agonists can suppress neutrophilic inflammation that has already developed. Moreover, Parrado *et al* demonstrated that dopamine and D2-like receptor agonists upregulated the expression of IL-8 in keratinocytes (18). Dopamine signaling promotes IL-8 production by stimulating both Th17 cells and epithelial cells via the dopamine receptor.

We also showed previously that tannic acid is a D2-like receptor agonist, and that tannic acid suppressed IL-17 production in chemically induced colitis models (19). More recently, we determined that tannic acid inhibited alveolar bone resorption in a carrageenan-induced rat model of periodontitis (20). In the present study, we examined the effect of the D2-like receptor agonist ropinirole, which is used as a drug for treating Parkinson's disease, on periodontitis *in vivo*. To also analyze the action *in vitro*, we evaluated whether ropinirole inhibits cytokine production in a murine gingival epithelial cell line.

Materials and methods

Reagents. We used l-carrageenan (carrageenan; WAKO Pure Chemical Industries Ltd., Osaka, Japan), ropinirole (Sigma-Aldrich Japan, Tokyo, Japan) as a D2-like receptor agonist, haloperidol (WAKO Pure Chemical Industries Ltd.) as D2-like receptor antagonist, and carrier-free recombinant mouse IL-17A (rmIL-17A; R&D Systems, Guthrie, MN, USA). In the preliminary *in vitro* experiments, we have found that 10 mg/ml is more effective than 1 mg/ml (data not shown). It is enough to conduct the *in vivo* experiment at one dose (20). According to a paper by Hashimoto *et al* (15), the endogenous IL-17A concentration is approximately 1.0 to 1.5 ng/ml, and we used this concentration as a reference for deciding on the dose of IL-17A to use in this experiment. We decided on the ropinirole concentrations by referring to previous reports (17,21).

Cell culture. Cells of the murine gingival epithelial cell line GE1 (RCB1709; RIKEN BioResource Center Cell Bank, Japan) were cultured in SFM101 medium (Nissui, Tokyo, Japan) supplemented with 1% fetal bovine serum (Sigma-Aldrich Japan), 100 U/ml penicillin G (Sigma-Aldrich Japan), 100 μg/ml streptomycin (Sigma-Aldrich Japan), and 10 ng/ml epithelial growth factor (Sigma-Aldrich Japan) (22). The cells were incubated in a humidified atmosphere of 5% CO_2 at 33˚C. For the *in vitro* assay, we seeded GE1 cells into 24-well plates at $30x10^4$ cells per well. We added carrageenan at the concentration of 50 or 100 μg/ml and/or rmIL-17A at the concentration of 0.5 or 2 ng/ml to the wells. Ropinirole was added to the wells at the concentration of 1, 10, or 50 μg/ml prior to the addition of carrageenan and/or rmIL-17A. After 24 h of incubation, the cells and supernatants were harvested.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. The harvested cells were rinsed with ice-cold phosphate-buffered saline (PBS). QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) was added to the samples, and the total RNA was extracted. The reverse-transcription reaction was performed with a High Capacity cDNA Reverse Transcription kit (Thermo Scientific). TaqMan Gene Expression Assays for CXCL1 (assay identification number: Mm04207460_m1), IL17-RA (assay identification number: Mm00434214_m1), and glyceraldehyde-3-phosphate dehydrogenase (assay identification number: Mm99999915_g1), which was used as an endogenous control, were obtained from Thermo Scientific. As Taqman probes do not provide the sequence information, we cannot show the sequence information. All experiments were performed in quadruplicate, *i.e.,* each reaction was performed in quadruplicate on four individual samples. Values were normalized to those of glyceraldehyde-3-phosphate dehydrogenase using the $2^{-\Delta\Delta Ct}$ method. This experiment was repeated at least three times.

Measurement of CXCL1 in the culture supernatants. CXCL1 protein in the harvested supernatants was measured using a mouse CXCL1 ELISA kit (Proteintech, Rosemont, IL USA) according to the manufacturer's instructions. All experiments were performed in quadruplicate, *i.e.,* each reaction was performed in quadruplicate on four individual samples. This experiment was repeated at least three times.

Carrageenan-induced rat model of periodontitis. The bilateral maxillary bone including the second molar of two rats were used in each group. We used 6 rats in total. The experiments were approved by the Animal Research Committee of Saitama Medical University (approval number: 2877), and conducted according to the institutional guidelines for ethical animal experiments. The humane endpoint is 20% body-weight reduction and severe suffering. All rats were housed in appropriate animal care facilities at Saitama Medical University, Japan. Five-week-old male Wistar rats (200 to 300 g in weight) were obtained from Japan SLC (Shizuoka, Japan). The rats had access to food and water *ad libitum,* and were maintained on a 12-h light/dark cycle at 23±1˚C with 60±10% humidity. We used a mixture of medetomidine hydrochloride (0.15 mg/kg; Nippon Zenyaku Kogyo, Fukushima, Japan), midazolam (2 mg/kg; Astellas Pharma, Tokyo, Japan), and butorphanol tartrate (2.5 mg/kg; Meiji Seika Pharma, Tokyo, Japan) as an anesthetic mixture, which was administered intraperitoneally (23-25). These drugs do not affect

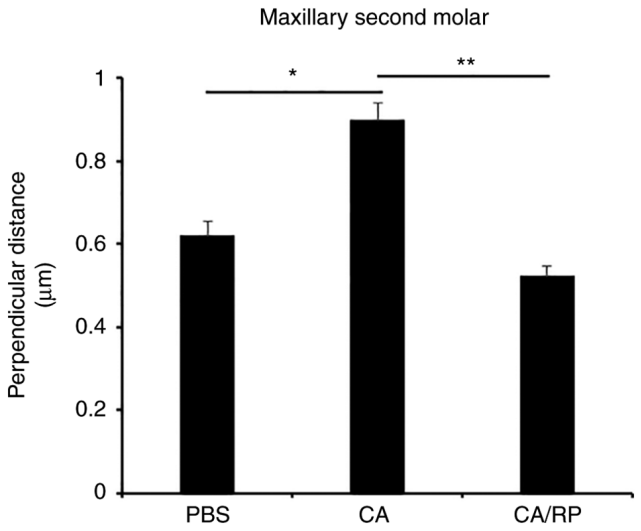

Figure 1. Ropinirole inhibits bone loss in a CA-induced rat model of periodontitis. The bilateral maxillary second molars were calculated. The measurement of the perpendicular distance. *P<0.05 and **P<0.001. CA, carrageenan; RP, ropinirole; PBS, phosphate buffered saline.

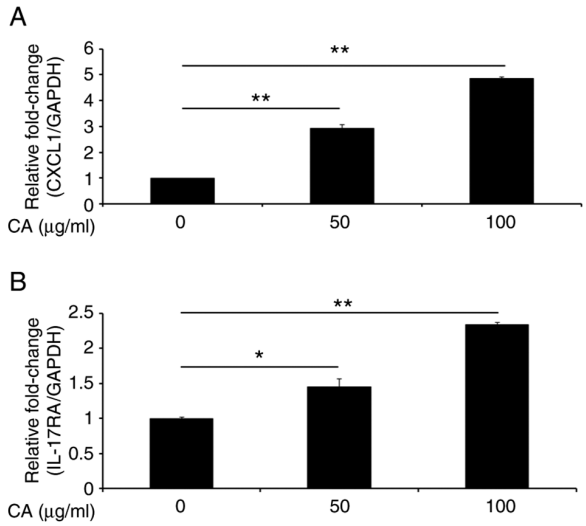

Figure 2. CA upregulates the expression of both CXCL1 and IL-17RA in GE1 cells. (A) The expression of *CXCL1* mRNA in GE1 cells treated with CA (50 or 100 μg/ml) for 24 h as estimated by RT-qPCR analysis. (B) The expression of *IL-17RA* mRNA in GE1 cells treated with CA (50 or 100 μg/ml) for 24 h as estimated by RT-qPCR analysis. Data were calculated from three repeated experiments. *P<0.05 and **P<0.001. CA, carrageenan; CXCL1, C-X-C motif chemokine ligand 1; RT-qPCR, reverse transcription-quantitative PCR; IL-17RA, IL-17 receptor A.

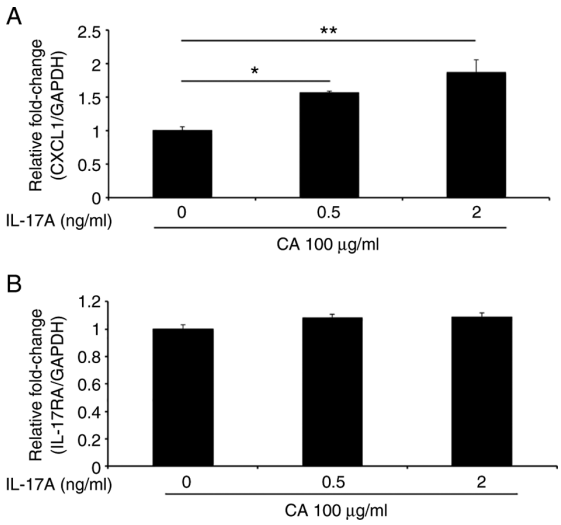

Figure 3. IL-17A upregulates CXCL1 expression in GE1 cells in the presence of CA. (A) The expression of *CXCL1* mRNA in GE1 cells treated with IL-17A (0.5 or 2 ng/ml) in the presence of CA (100 μg/ml) for 24 h as estimated by RT-qPCR analysis. (B) The expression of *IL-17RA* mRNA in GE1 cells treated with IL-17A (0.5 or 2 ng/ml) in the presence of CA (100 μg/ml) for 24 h as estimated by RT-qPCR analysis. Data were calculated from three repeated experiments. *P<0.05 and **P<0.001. CA, carrageenan; CXCL1, C-X-C motif chemokine ligand 1; IL-17RA, IL-17 receptor A; RT-qPCR, reverse transcription-quantitative PCR.

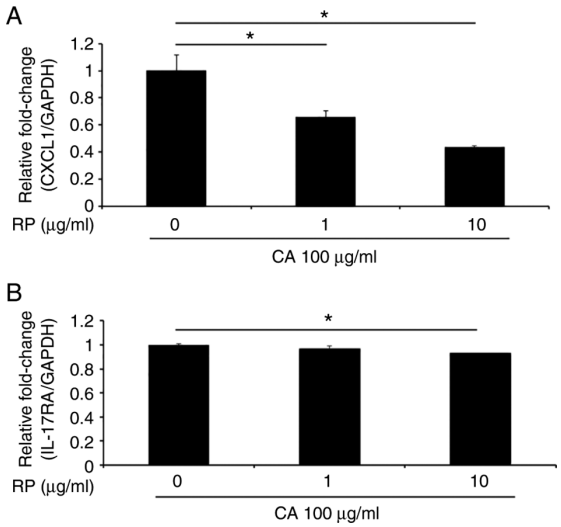

Figure 4. Upregulation of both CA-modulated CXCL1 and IL-17RA are inhibited by RP. (A) The expression of *CXCL1* mRNA in GE1 cells treated with RP (1 or 10 μg/ml) in the presence of CA (100 μg/ml) for 24 h as estimated by RT-qPCR analysis. (B) The expression of *IL-17RA* mRNA in GE1 cells treated with RP (1 or 10 μg/ml) in the presence of CA (100 μg/ml) for 24 h as estimated by RT-qPCR analysis. Data were calculated from three repeated experiments. *P<0.05. CXCL1, C-X-C motif chemokine ligand 1; IL-17RA, IL-17 receptor A; CA, carrageenan; RP, ropinirole; RT-qPCR, reverse transcription-quantitative PCR.

dopamine signaling (26). For euthanasia, sodium pentobarbital (200 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) was administered intraperitoneally. We verified cardiac and respiratory arrest. We used a previously reported carrageenan-induced rat model of periodontitis (27). While the rats were under anesthesia, buccal and lingual gingiva were exfoliated using an explorer. We divided the rats into three groups: a PBS group, a carrageenan (CA) group, and a carrageenan and ropinirole (CA/RP) group. For the PBS group, silk ligature, cut to the length of the mesiodistal distance of the mandibular second molar, was immersed in PBS, which is the solvent for carrageenan. For the CA group, the silk ligature was immersed in 1% carrageenan. For the CA/RP group, the silk ligature was immersed in 1% carrageenan and 10 μg/ml ropinirole. These ligatures were inserted into the periodontal pocket once a week for 4 consecutive weeks. The ligatures were inserted once a week and removed until the animal was sacrificed. The ligatures did not affect the animals' quality of life because 20% body-weight

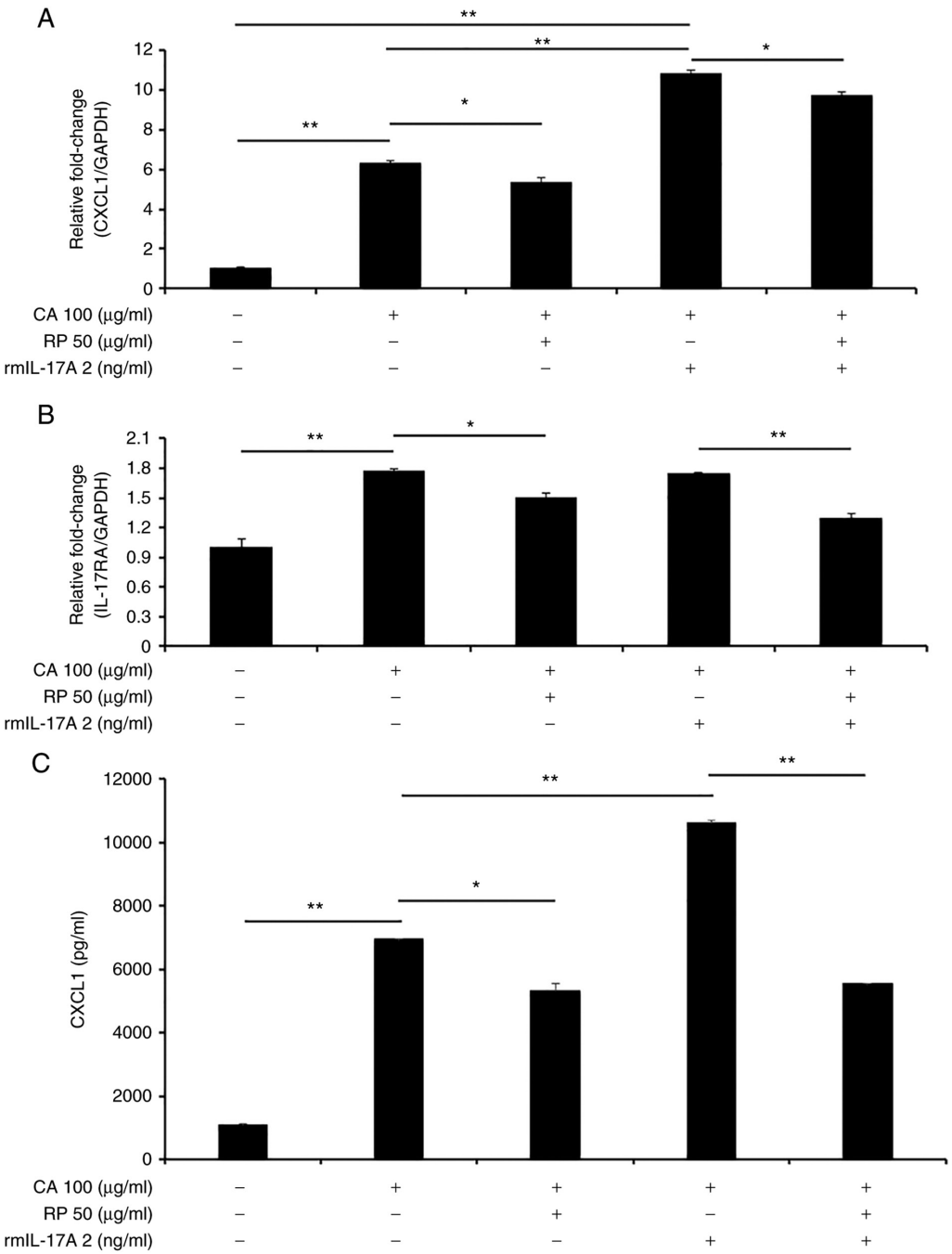

Figure 5. Upregulation of both IL-17A- and CA-modulated CXCL1 and IL-17RA are inhibited by RP. (A) Expression of *CXCL1* mRNA in GE1 cells treated with RP (50 μg/ml) in the presence or absence of CA (100 μg/ml) with or without of rmIL-17A (2 ng/ml) for 24 h as estimated by RT-qPCR analysis. (B) Expression of *IL-17RA* mRNA in GE1 cells treated with RP (50 μg/ml) in the presence or absence of CA (100 μg/ml) with or without of rmIL-17A (2 ng/ml) for 24 h as estimated by RT-qPCR analysis. (C) Protein level of CXCL1 in GE1 cells treated with RP (50 μg/ml) in the presence or absence of CA (100 μg/ml) with or without of rmIL-17A (2 ng/ml) for 24 h as estimated by ELISA assay. Data were calculated from three repeated experiments. *P<0.05 and **P<0.001. CXCL1, C-X-C motif chemokine ligand 1; IL-17RA, IL-17 receptor A; CA, carrageenan; RP, ropinirole; RT-qPCR, reverse transcription-quantitative PCR; rmIL-17A, recombinant mouse IL-17A.

reduction has not been observed. The experimental protocol is shown in Fig. S1.

Micro-computed tomography (CT) analysis. The rats were euthanized 4 weeks after the first operation. The maxillary bone was dissected, and subsequently fixed with 70% ethanol for analysis on a micro-CT 35 (SCANCO Medical, Brüttisellen, Switzerland). After preparing three-dimensional images (Fig. S2), the perpendicular distance between the cemento-enamel junction and the alveolar margin on the palatal side of the maxillary second molar was measured as shown in Fig. S3 (26). This experiment was repeated at least three times.

Statistical analysis. Differences between more than three groups were analyzed by one-way analysis of variance with Tukey's post-hoc tests. P-values <0.05 were considered to indicate statistical significance. All data are presented as

the mean ± standard error of the mean. The analyses were performed using EZR software version 1.52 which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) (28). These experiments were repeated at least three times.

Results

Ropinirole suppresses alveolar bone loss in an experimental rat model of periodontitis. Previously, we demonstrated that tannic acid, which is a D2-like receptor agonist, inhibited alveolar bone resorption in a carrageenan-induced rat model of periodontitis (20). In the present study, we used the same model to analyze the function of ropinirole, and conducted micro-CT analysis. The results revealed that the perpendicular distance was increased in the CA group when compared to the PBS group, and the perpendicular distance of the CA/RP group was significantly shorter than that of the CA group (Fig. 1).

Carrageenan induces CXCL1 expression in gingival epithelial cells in association with IL-17 modulation. As an inflammatory cytokine, IL-8 plays a key role for neutrophil migration in human inflammatory diseases (29). Since carrageenan induces IL-8 secretion in human colonic epithelial cells (30), we examined whether carrageenan modulates CXCL1 expression in murine gingival epithelial cells. As shown in Fig. 2A, *CXCL1* expression in GE1 cells was enhanced by the addition of carrageenan in a dose-dependent manner. As the level of IL-17 is significantly higher in gingival crevicular fluid from patients with periodontitis than that from healthy participants (31), we evaluated the influence of carrageenan on *IL-17RA* mRNA expression in gingival epithelial cells. We found that carrageenan induced *IL-17RA* expression in GE1 cells (Fig. 2B).

We next examined whether IL-17A, which is the prototypical member of the IL-17 family, modulates *CXCL1* and *IL-17RA* mRNA expression in the presence of carrageenan. As shown in Fig. 3A, *CXCL1* expression was increased by carrageenan in a dose-dependent manner. In contrast, *IL-17RA* expression was not affected by the addition of carrageenan (Fig. 3B). These results suggest that carrageenan modulates IL-17A-mediated CXCL1 expression in gingival epithelial cells.

Ropinirole inhibits CXCL1 and IL-17RA expression in gingival epithelial cells in the presence of IL-17A and carrageenan. We next examined whether ropinirole, which is a D2-like receptor agonist, inhibits *CXCL1* and *IL-17A* mRNA expression in GE1 cells in the presence of carrageenan *in vitro*. As expected, *CXCL1* expression was significantly suppressed by the addition of ropinirole in a dose-dependent manner (Fig. 4A). In addition, *IL-17A* expression was significantly suppressed by the addition of ropinirole at a dose of 10 μg/ml (Fig. 4B).

Furthermore, we examined whether ropinirole at a high dose modulates *CXCL1* and *IL-17RA* mRNA expression in the presence or absence of IL-17A with or without carrageenan. Ropinirole at 50 μg/ml in the presence of carrageenan significantly inhibited the upregulation of *CXCL1* and *IL-17RA* (Fig. 5A and B). In addition, we confirmed that the CXCL1 protein level was also suppressed by the addition of ropinirole in the presence of carrageenan and rmIL-17A (Fig. 5C). These results indicated that ropinirole inhibits the IL-17A-mediated CXCL1 expression induced by carrageenan in gingival epithelial cells. We speculate that carrageenan binds to unknown receptor and subsequently activates IL-17RA expression, resulting in CXCL1 upregulation (Fig. S4). Thus, carrageenan modulates IL-17RA as a stimulator.

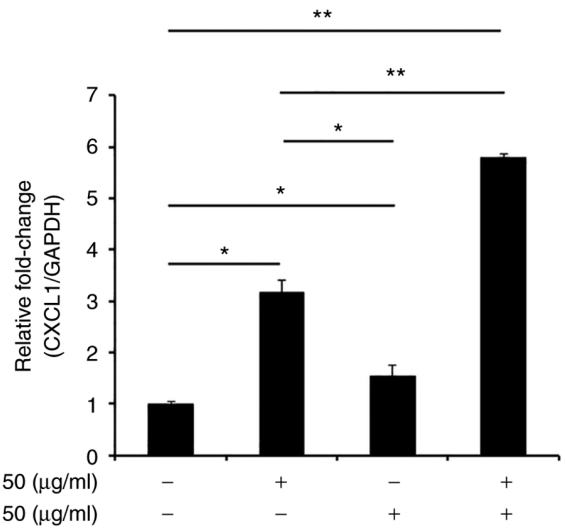

Figure 6. Haloperidol promotes CXCL1 upregulation in GE1 cells. The expression of *CXCL1* mRNA in GE1 cells treated with haloperidol (50 μg/ml) in the presence or absence of CA (50 μg/ml) for 24 h as estimated by reverse transcription-quantitative PCR. Data were calculated from three repeated experiments. *P<0.05 and **P<0.001. CA, carrageenan; HP, haloperidol; CXCL1, C-X-C motif chemokine ligand 1.

Haloperidol promotes CXCL1 expression in gingival epithelial cells in the presence of carrageenan. To examine whether the modulation of CXCL1 production by carrageenan is dependent on D2-like receptors, we lastly explored the effect of haloperidol, which is a D2-like receptor antagonist, on CXCL1 production. As anticipated, haloperidol significantly promoted *CXCL1* expression (Fig. 6). This result confirmed that carrageenan enhances CXCL1 production via D2-like receptors.

Discussion

Accumulating evidence has suggested that dopamine, which is produced by dendritic cells, acts on T lymphocytes (32). We and other researchers have previously reported the efficacy of agents that target dopamine receptors via Th17-mediated responses in animal models of various neutrophilic inflammatory diseases, including experimental autoimmune encephalomyelitis, type 1 diabetes, nephrotoxic serum nephritis, colitis, neutrophilic airway inflammation, and rheumatoid arthritis models (11,14,15,33-35). Th17-dependent neutrophilic inflammation processes occur in periodontitis (36). Namely, Th17 cells not only promote IL-8 production in gingival epithelial cells, but they also play a critical role for bone

destruction in periodontitis (2,37). In the present study, the inflammatory stimulant IL-17A induced the production of CXCL1 in gingival epithelial cells, and ropinirole inhibited the IL-17A-induced CXCL1 production.

Ropinirole has an agonistic effect on D2 receptor. It is indicated for use in the treatment of early and late Parkinson's disease (38). The three cardinal motor signs of Parkinson's disease are akinesia, rigidity and low-frequency rest tremor, and these symptoms interfere with the hand movements that control tooth brushing. According to prior research on periodontitis in patients with Parkinson's disease, the morbidity of periodontitis is high in patients with Parkinson's disease, because these patients cannot brush their teeth well (39-42). However, little information is available on the prevalence of periodontitis in patients with Parkinson's disease who are treated with different medications. For the treatment of Parkinson's disease, L-dopa and D2 receptor agonists are most commonly prescribed. Common adverse effects in elderly patients treated with D2 receptor agonists are delusion and hallucinations. Therefore, L-dopa is often selected as the first-line drug (43). To clarify the morbidity of periodontitis in Parkinson's disease, it would be of value to compare patients who were prescribed L-dopa to those who were prescribed D2 receptor agonists. We speculate that the application of a local drug delivery system may be suitable for the treatment of periodontal tissues and alveolar bone defects.

Two major models for mimicking periodontitis are the ligature model and the oral gavage with periodontopathogens model. In the ligature model, ligature induces bacterial colonization and the accumulation of plaque, leading to epithelial migration and tissue destruction. In contrast, the oral gavage with periodontopathogens model involves the inoculation of human bacterial strains. De Molon *et al* showed that the ligature model was more useful than the oral gavage with periodontopathogens model for long-term experiments (44). However, the ligature model is technically quite difficult to perform. In contrast, the carrageenan-induced model, which was proposed by Yamamoto *et al,* is technically simple to perform (27). Moreover, the carrageenan-induced model enables the induction of neutrophilic inflammation, because carrageenan induces acute inflammation *in vivo*, and IL-8 production *in vitro* (30,45).

The present study has several limitations. First, experiments with the ligature model without carrageenan should be performed to verify whether ropinirole is effective for periodontitis. Second, we only tested murine gingival cells *in vitro*, and in the future, it will be necessary to perform such experiments in human gingival epithelial cells to determine whether similar results can be obtained with human cells. Finally, it remains unknown whether dopamine receptors are expressed in the gingival tissues of patients with periodontitis. The significant issue facing current research is examining dopamine receptors in human tissues. In addition, it is needed to explore the receptors which binds to carrageenan.

In conclusion, the results of the present study suggest that ropinirole suppresses bone destruction in a rat model of periodontitis. We believe this new finding indicates that further studies are warranted.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author contributions

TS and SM confirm the authenticity of all the raw data. TS, MU and SM conceived and designed the study. YI, MK and RT acquired the data. KI and MU contributed to the interpretation of the results. YI, TS and SM drafted the manuscript. KI, MK and MU critically revised the manuscript for important intellectual content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the Animal Research Committee of Saitama Medical University (Saitama, Japan; approval no. 2877).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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